Self \textit{in vivo} production of a synthetic biological drug CTLA4Ig using a minicircle vector

Yeri Alice Rim\textsuperscript{1}, Hyoju Yi\textsuperscript{1}, Youngkyun Kim\textsuperscript{1}, Narae Park\textsuperscript{1}, Hyerin Jung\textsuperscript{1}, Juryun Kim\textsuperscript{1}, Seung Min Jung\textsuperscript{1,2}, Sung-Hwan Park\textsuperscript{2} & Ji Hyeon Ju\textsuperscript{1,2}

\textsuperscript{1}CiSTEM laboratory, Convergent Research Consortium for Immunologic Disease, Seoul St. Mary’s Hospital, College of Medicine, The Catholic University of Korea, Seoul, 137-701, South Korea, \textsuperscript{2}Division of Rheumatology, Department of Internal Medicine, Seoul St. Mary’s Hospital, College of Medicine, The Catholic University of Korea, Seoul, 137-701, Republic of Korea.

Cytotoxic T lymphocyte-associated antigen 4 immunoglobulin fusion protein (CTLA4Ig, abatacept) is a B7/CD28 costimulation inhibitor that can ward off the immune response by preventing the activation of naïve T cells. This therapeutic agent is administered to patients with autoimmune diseases such as rheumatoid arthritis. Its antiarthritic efficacy is satisfactory, but the limitations are the necessity for frequent injection and high cost. Minicircles can robustly express the target molecule and excrete it outside the cell as an indirect method to produce the protein of interest \textit{in vivo}. We inserted the sequence of abatacept into the minicircle vector, and by successful \textit{in vivo} injection the host was able to produce the synthetic protein drug. Intravenous infusion of the minicircle induced spontaneous production of CTLA4Ig in mice with collagen-induced arthritis. Self-produced CTLA4Ig significantly decreased the symptoms of arthritis. Injection of minicircle CTLA4Ig regulated Foxp3\textsuperscript{+} T cells and Th17 cells. Parental and mock vectors did not ameliorate arthritis or modify the T cell population. We have developed a new concept of spontaneous protein drug delivery using a minicircle vector. \textit{Self in vivo} production of a synthetic protein drug may be useful when biological drugs cannot be injected because of manufacturing or practical problems.

Rheumatoid arthritis (RA) is a severe autoimmune disease characterized by persistent inflammation in the joint that can lead to permanent structural damage. RA can be induced by several types of cells. The regulation of activated T cells however, is decisive in preventing the onset and maintenance of RA\textsuperscript{1,2}. The costimulatory signal of CD28 and CD80/86 (B7) is the major trigger that can activate T cells, and this signal can be inhibited by cytotoxic T lymphocyte-associated antigen 4 (CTLA4)\textsuperscript{3,4}. CTLA4 is a homologue to CD28 but binds with a higher affinity and terminates T cell activation\textsuperscript{5,6}. Therefore, there have been many efforts to clone an artificial CTLA4 molecule for use as a regulator of the immune response in autoimmune diseases. The CTLA4–immunoglobulin fusion protein (CTLA4Ig, abatacept) has been produced based on this concept. Abatacept is clinically successful as a B7/CD28-inhibitor which can turn off the immune response by preventing the activation of naïve T cells.

Commercial abatacept is given intravenously to many RA patients. Despite its significant efficacy, it has several limitations. First, infusion of abatacept should be performed on a monthly basis for a long period. The injection is conducted by a 30-minute intravenous infusion of a dose of \(\sim 10 \ \text{mg kg}^{-1} \ \text{body weight}\). Fifteen days after the first injection, the second injection is required, and the third injection is required around day 30. Thereafter the patient should receive continued therapy every 4 weeks\textsuperscript{7}. Secondly, abatacept is an expensive drug due to the sophisticated manufacturing process and the fact that it can be infused only by health professionals in special clinics. The production and distribution of a well-purified monoclonal antibody requires properly equipped facilities and a drug delivery system. Therefore, this treatment is time consuming and costly\textsuperscript{8}.

Gene therapy is not yet a practicable alternative to conventional treatment such as the use of monoclonal antibodies. However it is valued by scientists as an indirect method for delivering therapeutic molecules. Gene therapy can be useful from the economical and practical viewpoints if the strategy can overcome the current hurdles. A minicircle vector is a small episomal vector that is a circular expression cassette without a bacterial plasmid backbone. The bacterial backbone can induce immune responses in mammalian cells, which makes the minicircle an attractive tool for \textit{in vivo} delivery\textsuperscript{9}. Also by eliminating the bacterial backbone, minicircles obtain their small size characteristics, which increase the delivery and uptake possibilities by the cell. This can lead to
long-term expression that is 10 to 1000 times higher than that of other original plasmids. One study has reported that minicircles can induce a constant and strong expression of the gene because of the unique methylation patterns and the ability to avoid gene silencing. Therefore, minicircle vectors are an ideal vehicle to transfer a gene both in vitro and in vivo, and these vectors have been used in preclinical gene therapy research for years. Using this technique, it is conceptually feasible that the in vivo transfection of vectors carrying the full sequence of a protein drug can induce the host to produce the protein drug.

In this study, we inserted the full sequence of abatacept into minicircle vectors and introduced it into mice, and the host was able to produce the synthetic protein drug. Self in vivo production of a synthetic protein drug may be useful when biological drugs cannot be injected properly because of manufacturing difficulties or practical clinical problems.

Results

Construction of CTLA4Ig minicircles (mcCTLA4Ig). We injected minicircle vectors encoding CTLA4Ig into mice with collagen-induced arthritis (CIA) to induce self in vivo production. The overall scheme of this experiment is shown in Figure 1A. The protein drug will be robustly expressed in vivo. Once the plasmid is injected, the protein drug will be robustly express at a high level in vivo, and the arthritis symptoms diminish in CIA mice (Fig. 1a). To prove the possibility of this concept, we prepared the drug-containing minicircle vector. mcCTLA4Ig was created by treating the parental plasmid with arabinose, which removes the bacterial backbone and downsizes the plasmid by inducing the phage ΦC31 integrase to perform a site-specific intramolecular recombination of the sequences (Fig. 1b). The ΦC31 integrase induces a recombination between the attB and attP site, which eventually

Figure 1 | Scheme of experimental concept and generation of minicircles encoding mcCTLA4Ig. (a) Our concept is to make a self-production system to deliver a protein drug in vivo by injecting the plasmid encoding the drug sequence. The injected mcCTLA4Ig will induce robust expression of the protein drug and eventually attenuate arthritis in collagen-induced arthritis mice. The mice were drawn by the 1st author of this article, Ms Yeri Alice Rim. (b) Minicircles can be induced by removing the bacterial backbone from the parental plasmid with arabinose. (c) mcCTLA4Ig was compared to ppCTLA4Ig and ppMock. Gel electrophoresis of the parental plasmids and minicircle plasmids of mock and CTLA4Ig-inserted mock plasmids. The mock minicircle has a size of 3 kb and that mcCTLA4Ig has a size of ~6 kb. The increased size of ppCTLA4Ig and mcCTLA4Ig, compared to ppMock and mcMock verified that the drug sequence was inserted. (d) Digestion of mcCTLA4Ig with BamHI and XbaI revealed the existence of inserted sequence and successful cloning. The arrow indicates mcCTLA4Ig. Y.A.R. created this figure.
splits the plasmid into two circular DNAs, the minicircle (mcCTLA4Ig) and the bacterial backbone.

Comparing the size of ppMock, ppCTLA4Ig and the minicircles that were induced from the two plasmids showed that the CTLA4Ig sequence was inserted by the increased size (Fig. 1c). By cutting the plasmid with BamHI and XbaI, we confirmed that the cloned CTLA4Ig sequence was inserted properly (Fig. 1d).

In vitro expression of mcCTLA4Ig. A process combining immunoprecipitation and immunoblot assay was used to detect the CTLA4Ig protein in vitro. The transfected cells robustly expressed the CTLA4Ig protein, and the CTLA4Ig protein accumulated in the culture media since the drug is secreted in a soluble form. The method to capture the protein drug is briefly shown in Fig. 2a. Successful transfection of the minicircle vectors was confirmed by green fluorescent protein (GFP) expression in HEK293T cells (Fig. 2b). The immunoblotting data of each transfected cells showed the existence of CTLA4Ig protein. The protein expression was seen in ppCTLA4Ig and mcCTLA4Ig, but not in mcMock. The mcCTLA4Ig-transfected cells supernatant showed the highest expression. (d) The band intensity was measured on the basis of the result shown in figure 2C. DIC: Differential interference contrast.
mcCTLA4Ig injection suppressed arthritis development in CIA mice. CIA mice were injected with plasmid DNAs by hydrodynamic tail vein injection to confirm the effects of mcCTLA4Ig in vivo. The mcMock-injected CIA mice developed severe arthritis, whereas CIA mice injected with mcCTLA4Ig showed marked attenuation of arthritis development (Fig. 3a). ppCTLA4Ig-injected CIA mice showed a slight reduction in the arthritis score, but this reduction was less than the effect of the mcCTLA4Ig-injected group. The same pattern of differences between the groups was also observed at the thickness of the inflamed hind paws (Fig. 3b). Body weight of each group was also measured since animals in pain tend to lose more weight than healthy ones. The severely arthritic mice injected with mcMock lost weight but the other groups gained weight. Improvement in arthritis and reduction of inflammatory stress were accompanied by greater body weight gain in mcCTLA4Ig mice compared to ppCTLA4Ig and mcMock-injected groups (Supplementary Fig. 3). The main characteristics of joint arthritis are massive synovial hypertrophy with cellular infiltration, damaged cartilage and erosive bone. Hematoxylin and eosin (H&E) staining showed the synovial hypertrophy with diffuse infiltration with various immune cells, and invasion of pannus into adjacent structures at the joints of each group. Minimal

Figure 3 | Systemic intravenous delivery of mcCTLA4Ig in CIA mice. (a) Arthritic symptoms were scored after primary immunization. Plasmid DNA was injected intravenously on day 19. On day 21, a second immunization was performed in each group (n = 5 mice in each groups). The arthritis score decreased in the mcCTLA4-treated group and was similar to that in the normal wild-type (WT) mice. ppCTLA4Ig also reduced the arthritis score, but to a lesser extent than in mcCTLA4Ig. (b) The amount of swelling differed between the groups, and paw swelling was related to the arthritis score. The paws of mcCTLA4Ig-treated mice had minimal swelling. (c) Histological analysis of cartilage and bone erosion. Joint tissues from WT mice and CIA mice which were treated with mcMock, ppCTLA4Ig, or mcCTLA4Ig were stained with H&E, safranin O and toluidine blue staining. The ×100 data is a magnified image of the ×50 data. The boxes show a magnified image of the pannus surrounding the cartilage. (d) Inflammation score of the histological data. The score was based on the extent of synovial hyperplasia and infiltration of cells. (e) Joint destruction scores were based on the cartilage loss estimated in samples stained by safranin O and toluidine blue, and the extent of pannus formation. (** P < 0.005, *** P < 0.001).
inflammation and joint destruction were observed in CIA mice which were treated with mcCTLA4Ig. To examine the loss of cartilage in the joints, we stained the cartilage using safranin O and toluidine blue. Cartilage destruction was minimal in CIA mice treated with mcCTLA4Ig (Fig. 3c), but was extensive in CIA mice injected with mcMock. The mcCTLA4Ig-injected mice showed almost the same amount of cartilage compared with the wild type. The group injected with ppCTLA4Ig showed slight recovery but not to the same extent as in the group injected with mcCTLA4Ig. These results suggested that the CTLA4Ig drug secreted by the plasmid DNA was effective in ameliorating the experimental arthritis.

The effect of CTLA4Ig was compared with the actual protein drug, abatacept (Fig. 4). Arthritic score showed that abatacept and mcCTLA4Ig both suppressed the progress of arthritis. Inflammation severity and destruction severity were also lower in the two groups. However in every rate, mcCTLA4Ig showed a lower rate compared to abatacept. These results indicate that mcCTLA4Ig can suppress RA and has a similar or better ability compared to the actual abatacept.

**In vivo expression of mcCTLA4Ig upregulated Foxp3⁺ T cells and downregulated Th17 cells.** As the plasmids were injected into CIA mice, vectors are thought to be transfected in hepatocytes in vivo (Fig. 5a). Previous studies done by several groups showed that a large proportion of plasmid DNA remains bounded to the plasma membrane in the liver because it is a large organ that contains a lot of blood vessels 14–17, which is thought to be responsible for the high transfection and expression efficiency. The expression of GFP was not detected in wild type since no vector was injected. The ppCTLA4Ig group showed a low expression which is the result of low transfection caused by the bigger size of the vector. However, mcMock and mcCTLA4Ig both showed a high GFP expression in vivo. We assumed that the regulation of Foxp3⁺ regulatory T cells and RORγt⁺ Th17 cells would accompany the suppression of arthritis by mcCTLA4Ig and isolated splenocytes to survey the population ex vivo (Fig. 5b). Various types of T cells play a critical role in the development and maintenance of RA, and the regulation of Foxp3⁺ T cells and RORγt⁺ Th17 cells is essential for the suppression of arthritis.
Figure 5 | Successful transfection of mcCTLA4Ig into hepatocytes. (a) GFP expression of mcMock plasmids in CIA mice liver is shown as positive fluorescence. The minicircular form showed a relatively high positive expression in hepatocytes compared to the parental form. (b) Splenocytes of CIA were analyzed by flow cytometry. (c) mcCTLA4Ig-treated mice had more Foxp3<sup>+</sup> cells compared with mice given a low dose of mcCTLA4Ig or ppCTLA4Ig. (d) Fewer ROR<sub>c</sub><sup>+</sup> cells were seen in mice given mcCTLA4Ig compared with those given mcMock, low-dose mcCTLA4Ig, or ppCTLA4Ig. (** P < 0.005).
Abatacept is known to increase the Foxp3+ cells deficient in RA, but Th17 cells are upregulated in established RA. Abatacept is known to negatively affect the differentiation of osteoclast from monocytes in vivo. Previous reports show that the immune environment in vivo can affect the osteoclast differentiation in vitro. Therefore, we have attempted two different methods to observe the inhibitory effect of mcCTLA4Ig. First, we performed the osteoclast differentiation in vivo with the plasmid treated mice monocytes. We also assumed that the in vitro differentiation of CIA mice monocytes treated with conditioned media would show a similar outcome. As shown in schematic figure, osteoclasts were differentiated from the monocytes of CIA which were treated mcMock, ppCTLA4Ig, and mcCTLA4Ig (Fig. 6a). As a result, the monocytes of mcCTLA4Ig-treated group showed a lower differentiation rate. The size of the osteoclasts was relatively smaller than that of mcMock- or ppCTLA4Ig-treated group (Fig. 6b). ppCTLA4Ig seemed to have a slight suppressive effect on osteoclast formation, but this effect was not significant. mcCTLA4Ig had a significantly stronger antosteoclastogenic effect compared with both the mcMock and ppCTLA4Ig (Fig. 6c). Next, we differentiated osteoclast from the monocytes derived from the same CIA mice and treated the mcCTLA4Ig and mcMock conditioned media at the RANKL stimulation stage (Fig. 6d). The mcMock conditioned media treated monocytes eventually developed into numerous osteoclasts. However the mcCTLA4Ig conditioned media showed an inhibitory effect on the differentiation of osteoclast in a dose dependent manner (Fig. 6e, f). These data indicate that mcCTLA4Ig can effectively suppress the osteoclast formation.

Discussion
The present study suggests that mcCTLA4Ig can be used as a novel drug delivery system that can treat RA effectively. To our knowledge, it is the first to show an effective treatment that suppress arthritis by transferring a synthetic biological drug into the body using a small DNA vector that encodes the sequence of the CTLA4Ig protein drug. The injected vectors secreted the bioactive form of CTLA4Ig and eased the symptoms of arthritis.

The commercial protein CTLA4Ig is a potent immune modulator agonist used to treat various diseases such as cardiac diseases, immune diseases, and transplant rejection. In the case of RA, it is known that the CTLA4Ig protein alters the T cell population. CTLA4Ig increases the Foxp3+ Treg cell population and regulates Th17 cells. CTLA4Ig also affects the phenotype and functions of monocytes and has an inhibitory effect on osteoclasts. There is evidence that CTLA4Ig can bind directly to osteoclast precursor cells and inhibit the differentiation process. Other studies have reported that CTLA4Ig can prevent inflammation, cartilage damage, pannus formation, and bone resorption, and can thereby protect the bone architecture.

The gene delivery of CTLA4Ig has been attempted several times using viral vector systems. Previous studies have shown an improvement in the survival of heterograft skin grafts on burn wounds with an adeno virus vector Ad–CTLA4Ig. Coexpression of CTLA4Ig with an adeno viral vector had a potent effect in the Gunn rat model of Crigler–Najjar syndrome. However, viral-based delivery has obstacles such as genome integration, mutation, and infection-associated inflammatory responses. It is clinically more relevant to develop an effective but safe method for transferring the gene into eukaryotic cells. Therefore, we have developed a nonviral method to treat arthritis with gene therapy.

Minicircle vectors are small, circular expression cassettes that exclude the bacterial backbone. The minicircle vector can be obtained easily from the parental plasmid directly from the culture medium. The small size is achieved by cutting off the bacterial backbone. The bacterial backbone is useless after the bacterial propagation state and induces immune response in vivo. The minicircle induction process linearizes and degrades the remaining junk DNAs into small pieces, which can be removed using a standard purification process. As a result, the minicircle plasmid achieves a relatively high expression—up to 1000 times higher than for other vectors.

In this study, we successfully developed a minicircle plasmid containing the sequence of CTLA4Ig and showed that this minicircle technology application has potential in the treatment of RA. As expected, our mcCTLA4Ig vector reduced the arthritic conditions in CIA mice (Fig. 3a), as was shown by previous studies of commercial CTLA4Ig. mcCTLA4Ig increased the Foxp3+ T cell population, regulated the Th17 cell population, and suppressed osteoclast differentiation ex vivo.

To support the in vivo antiarthritic outcomes, we verified the expression of mcCTLA4Ig in vitro. However, because of the properties of the system, there were some limitations detecting the expression in vivo. The expression capacity of the plasmid is difficult to estimate or control and we therefore measured the amount of secreted proteins that were expressed in vitro. The in vitro expression system using HEK293T cells has the possibility of accumulating the proteins at a sufficiently high level to be detected. By contrast, we could not measure the concentration of the proteins in blood, partly because it was difficult to concentrate the proteins that exist in blood, which is also a source that was hard to obtain in a sufficient volume from mice. Therefore, as an indirect way to show mcCTLA4Ig expression, GFP-expressing cells were detected in the liver (Fig. 5a). GFP positivity confirmed that the protein drug was synthesized in vivo by mcCTLA4Ig expression in hepatocytes.

We tried to improve the expression efficiency of mcCTLA4Ig. The larger size of mcCTLA4Ig compared with mcMock may have decreased the expression rate. We transsected the plasmids into HEK293T cells to monitor and compare GFP expression. The expression of GFP was highest in mcMock, which was expected because it has the smallest size, ~3 kb. However, despite the larger size of ~6 kb, mcCTLA4Ig had a relatively high expression of GFP in vitro (Fig. 2c). This confirmed that the doubled size only minimally affected the competency of expression.

Excessive expression of a protein usually requires a higher concentration of DNA. However, injections involving a high concentration of DNA can induce immune responses. It has also been shown that the immune response to DNA is similar to that in viral infection. Therefore, further studies are needed to identify other options instead of increasing the concentration of mcCTLA4Ig. Modification of the vector structure (e.g., using a superior promoter) is a feasible substitute. Combinations with other drugs may also be able to improve the outcome of mcCTLA4Ig.
The low protein expression level may have also resulted from the relatively low purity of the plasmid DNA, which can sometimes be caused by remnants of the mixture of parental or bacterial DNAs. Despite the purification process, the isolated plasmid DNA might have included a small amount of bacterial backbone. Another cause can be the incomplete induction process for the minicircles. This impurity may disturb the function of mcCTLA4Ig itself. However, previous studies have shown that bacterial or other DNA sequences must bind to the plasmid covalently in cis to cause gene silencing. The DNA fragments existing in trans do not show any silencing effect on the gene expression of the plasmid of interest.34–36 Still, advanced purification and induction processes are still required for clinical application.

Another limitation of our treatment is the delivery method for the vector. We used hydrodynamic tail vein injection to deliver the naked

Figure 6 | The effect of mcCTLA4Ig on osteoclast differentiation in vivo and in vitro. (a) Schematic presentation of osteoclastogenesis in vivo. The image of osteoclasts which were differentiated from bone marrow macrophage of each mice treated with the plasmid of mcMock, mcCTLA4Ig, and ppCTLA4Ig. (b) Low grade osteoclastogenesis was shown in mcCTLA4Ig-treated group. (c) Osteoclasts were counted regardless of the nucleus number. Three individual trials were done in osteoclast number counting. (d) Schematic presentation of osteoclastogenesis in vitro. The image of osteoclasts were differentiated from CIA mice and conditioned media was treated in vitro. (e) Low grade of osteoclastogenesis was shown in mcCTLA4Ig-treated group in vitro which is similar to the in vivo results. (f) Osteoclasts were counted in the same protocol used in the in vivo method. (a) P < 0.01, ** P < 0.05, *** P < 0.001).
minicircle DNA \textit{in vivo}. Hydrodynamic gene delivery is a simple and effective delivery method. Rapid infusion of large volume of DNA-mixed solution creates a relatively high pressure, which leads to the direct cytosolic delivery through the pores formed in the plasma membrane, increasing the penetration into the cells\textsuperscript{14}. A high penetration rate is an advantage of the hydrodynamic delivery system against other nonhydrodynamic injection methods. Other injection methods for naked DNA have a higher risk of escape or degradation by lysosomes\textsuperscript{15}. However, high pressure tends to localize the DNA directly into the organs with a large network of vessels such as the liver. Several studies have reported the effective uptake of DNA vectors into hepatocytes. Liver-oriented and pressure-dependent hydrodynamic delivery has several limitations. Hydrodynamic delivery can lead to destruction of hepatocytes and elevate liver enzymes\textsuperscript{14,15}. Therefore, there are some limitations to using hydrodynamic vector delivery into a host with liver problems, and a less aggressive method is required for clinical use. Nanoparticulate PEGylated carriers may be useful as an alternative method for delivering mcCTLA4Ig \textit{in vivo}

In summary, we report a novel self-containing drug delivery system of CTLA4Ig with a minicircle. Synthetic protein drugs were produced \textit{in vivo} by mcCTLA4Ig. Spontaneously produced CTLA4Ig attenuated the symptoms of arthritis in arthritis animal model, similar to the action of commercial CTLA4Ig (abatacept). The self \textit{in vivo} production system of synthetic protein drugs by minicircles is now an emerging concept and may be promising for its cost effectiveness and convenience. Further studies are needed to widen the application of mcCTLA4Ig. Enhanced vector architecture of nanotech-applied delivery systems is a feasible way to increase the application of mcCTLA4Ig. Further modifications may make the self \textit{in vivo} production system advantageous in clinical treatment of several immune-related diseases including RA.

**Methods**

**CTLA4Ig minicircle production.** To construct the therapeutic minicircle, CTLA4Ig sequence was subcloned into the mock parental plasmid pMC.CMV–MCS–EF1–GFP–SV40–PolyA, which was purchased from SBI (System Biosciences, Mountain View, CA, USA). CTLA4Ig is constructed by 3647 base pairs, and its detailed sequence is shown in Supplementary data 1. The plasmid contains XbaI and BamHI restriction sites, and the cloning outcome was confirmed by double digesting the two sites. The minicircles were produced using a method based on the method described by Kay et al\textsuperscript{16}. A single colony was grown for 8 h in 2 ml of Luria–Bertani broth with kanamycin. Four hundred microliters of the culture was inoculated into 800 ml of Terrific Broth with kanamycin, and the mixture was incubated for 15 h. The next day, induction medium supplemented with 1-arabinose was added to the overnight culture, and the cultures were incubated at 30 °C for 5 h. Plasmid DNA was extracted from the bacteria using NucleoBond Xtra plasmid purification kits (Macherey-Nagel, Duren, Germany). Purified DNA was digested with NdeI, and the resultant linearized DNA was removed with Plasmid-Safe\textsuperscript{17} ATP-dependent DNase (Epicentre, Madison, WI, USA). The scheme of this process is shown in Supplementary data 2.

**Cell culture and transfection.** HEK293T cells were maintained in Dulbecco’s modified Eagle medium (Gibco Life Technology, Gaithersburg, MD, USA) supplemented with 7.5% FBS (Fetal bovine serum, Gibco), 100 U ml\textsuperscript{-1} penicillin, and 100 μg ml\textsuperscript{-1} streptomycin (Gibco). Cells were transfected with the plasmids using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer’s instructions. The expression of GFP in cells was measured by fluorescence microscopy at 24 h after the transfection process.

**Detection of CTLA4Ig.** 48 hours after transfection, the conditioned media of the transfected HEK293T cells were collected. The media was incubated with protein A/G PLUS-agarse beads (Santa cruz, CA, USA). The captured protein was detached from the bead and detected by western blotting with the CTLA4 antibody (Abcam, Cambridge, MA).

**Animals.** Six-week-old female DBA/1J mice were purchased from OrientBio (Seongnam, Korea) and maintained in the pathogen-free animal facilities of the Catholic University of Korea.

**Ethics.** All procedures involving animals were in accordance with the Laboratory Animals Welfare Act, the Guide for the Care and Use of Laboratory Animals, and the Guidelines and Policies for Rodent Experimentation provided by the Institutional Animal Care and Use Committee (IACUC) of the school of medicine of the Catholic University of Korea. This study protocol was approved by the institutional review board of The Catholic University of Korea (CUMC-2011-0010-03).

**Induction and assessment of CIA.** Mice were immunized intradermally with bovine type II collagen (CII, Chondrex, Redmond, WA, USA) emulsified in Freund’s complete adjuvant containing 2 mg ml\textsuperscript{-1} of heat-killed Mycobacterium tuberculosis (Chondrex). On day 21, mice received a “booster” second injection by the same method. Each limb of the arthritic mice was given a clinical score every 2 days for 2 weeks after the secondary immunization. The arthritis was scored on a 0–4 scale, where 0 = normal, 1 = slight swelling, 2 = pronounced swelling, 3 = severe swelling without joint deformation, and 4 = joint deformation accompanied by ankylosis. The scores for the four limbs were totaled, giving a maximum of 16 points per animal. Each score was determined by two independent examiners.

**In \textit{vivo} delivery of DNA vectors and abatacept treatment.** Two days before the secondary immunization, the mice were treated with the prepared plasmids as described by Ebina et al\textsuperscript{18}. The plasmid DNA vectors were delivered by hydrodynamic delivery using intravenous injection into the tail vein. Mice were injected with 20 μg of plasmid DNA according to the previous study done by Gracey Maniar et al\textsuperscript{18}. The DNA was delivered in a total volume of almost 10% of body weight within 5–7 s. Abatacept was injected i.p. once weekly at a dose of 20 mg/kg after CIA induction. Previous studies have shown that the anti-abatacept responses are suppressed in mice\textsuperscript{19,20}.

**Histological evaluation of arthritis.** The hind limb of each mouse was removed and fixed in 10% formalin and decalcified in 10% (w/v) EDTA, and the samples were embedded in paraffin. Four-micrometer-thick sections were stained with H&E, safranin O, or toluidine blue. The inflammation score and joint destruction score were determined using the method of Hackel et al\textsuperscript{21} by three individual researchers\textsuperscript{22}. The inflammation score of each group was measured by the severity of infiltration and pannus formation. The destruction score was measured based on the cartilage and bone loss.

**Th17 population \textit{ex vivo}.** To detect Th17 cells in the mouse spleen, splenocytes were isolated from each group. The cells were incubated with allopurinol (APC) conjugated rat anti-mouse CD4 antibody (catalogue number, 561091; BD Biosciences, San Jose, CA, USA), and the cells were permeabilized using a Fixp3 staining buffer set (00-5523; ebioscience, San Diego, CA, USA). The Th17-specific marker ROR\textsuperscript{+}\textit{γ} was detected by anti-human/mouse ROR\textsuperscript{+}\textit{γ} antibody conjugated with phycocyanin (PE) (ebioscience, 12 4988). After the overall cellular staining process, the cell populations were identified and estimated using an LSRFortessa cell analyzer (BD Biosciences). The data were acquired using FlowJo version 7.6.5 software (TreeStar Inc. Ashland, OR, USA). Cells were first gated for CD4\textsuperscript{+} proportion and analyzed for the expression of ROR\textsuperscript{+}\textit{γ}.

**Regulatory T cells phenotyping.** Splenocytes were isolated from mice and stained with APC-conjugated anti-mouse CD4 (561091; BD Biosciences). A Fixp3/Transcription Factor Staining Buffer Set (00-5523; ebioscience) was used for permeabilization and fluorescein isothiocyanate (FITC)-conjugated anti-mouse Fixp3 (11-5773; ebioscience) was used for intracellular staining. The stained cells were identified and their population estimated using an LSRFortessa cell analyzer (BD Biosciences), and the data were analyzed using FlowJo software. Cells were first gated for CD4\textsuperscript{+} and subsequently analyzed for the expression of Fixp3.

**In \textit{vivo} Osteoclast differentiation.** Monocytes were isolated from mice bone marrow. ACK buffer was treated for the elimination of red blood cells. Cells were washed with PBS and filtered with a strainer. Cells were seeded and maintained in alpha-MEM (Weglene, Daegu, Korea) supplemented with 10% FBS. The next day, cells were counted to 3 × 10\textsuperscript{5} cells and stimulated with 10 ng/ml M-CSF (Peprotech, Oak park, CA) for 48 hours. Then, second stimulation was given with 30 ng/ml RANKL (Peprotech) and M-CSF. After 72 hours, differentiated osteoclasts were stained with TRAP staining followed by the manufacturers guide. The stained cells were counted.

**In vitro Osteoclast differentiation.** Monocytes were isolated from mice bone marrow. ACK buffer was treated for the elimination of red blood cells. Cells were washed with PBS and filtered with a strainer. Cells were seeded and maintained in alpha-MEM supplemented with 10% FBS. The next day, cells were counted to 3 × 10\textsuperscript{5} cells and stimulated with 10 ng/ml M-CSF (Peprotech, Oak park, CA) for 48 hours. Then, second stimulation was given with 30 ng/ml RANKL (Peprotech) and M-CSF. After 72 hours, differentiated osteoclasts were stained with TRAP staining followed by the manufacturers guide. The stained cells were counted.

1. Cutolo, M. & Nadler, S. G. Advances in CTLA-4-Ig-mediated modulation of inflammatory cell and immune response activation in rheumatoid arthritis. *Autoimmun Rev.* \textbf{12}, 758–767 (2013).
2. Vital, E. M. & Emery, P. Abatacept in the treatment of rheumatoid arthritis. *Ther Clin Risk Manag.* \textbf{2}, 365–375 (2006).
3. Podolsj, J. R. & Miller, S. D. Targeting the B7 family of co-stimulatory molecules: successes and challenges. *BioDrugs*. 27, 1–13 (2013).

4. McCoy, K. D. & Le Gros, G. The role of CTLA-4 in the regulation of T cell immune responses. *Immunol Cell Biol.* 77, 1–10 (1999).

5. Linsley, P. S. et al. CTLA-4 is a second receptor for the B cell activation antigen B7. *J. Exp Med.* 174, 561–569 (1991).

6. Engelhardt, J. J., Sullivan, T. J. & Allison, J. P. CTLA-4 overexpression inhibits T cell responses through a CD28-B7-dependent mechanism. *J. Immunol.* 177, 1052–1061 (2006).

7. Genovese, M. C. et al. Subcutaneous abatacept versus intravenous abatacept: a phase IIIb noninferiority study in patients with an inadequate response to methotrexate. *Arthritis Rheum.* 63, 2854–2864 (2011).

8. Soini, E. J., Leussu, M. & Hallinen, T. Administration costs of intravenous biologic drugs for rheumatoid arthritis. *Springerplus*. 2, 531 (2013).

9. Chen, Z. Y., He, C. Y., Ehrhardt, A. & Kay, M. A. Minicircle DNA vectors devoid of bacterial DNA result in persistent and high-level transgene expression in vivo. *Mol Ther.* 8, 495–500 (2003).

10. Gracey Maniar, L. E. et al. Minicircle DNA vectors achieve sustained expression reflected by active chromatin and transcriptional level. *Mol Ther.* 21, 131–138 (2013).

11. Kay, M. A., He, C. Y. & Chen, Z. Y. A robust system for production of minicircle DNA vectors. *Nat Biotechnol.* 28, 1287–1289.

12. Gill, D. R., Pringle, I. A. & Hyde, S. C. Progress and prospects: the design and production of plasmid vaccines. *Gene Ther.* 16, 165–179 (2009).

13. Huang, M. et al. Novel minicircle vector for gene therapy in murine myocardial infarction. *Circulation*. 120, S230–237 (2009).

14. Sebestyen, M. G. et al. Mechanism of plasmid delivery by hydrodynamic tail vein injection. I. Hepatocyte uptake of various molecules. *J. Gene Med.* 8, 852–873 (2006).

15. Andrianazfo, F., Leccoz, M., Wattiaux-De Coninck, S., Wattiaux, R. & Jadot, M. Hydrodynamics-based transfection of the liver: entrance into hepatocytes of DNA that causes expression takes place very early after injection. *J. Gene Med.* 6, 877–883 (2004).

16. Budker, V. et al. Hypothesis: naked plasmid DNA is taken up by cells in vivo by a receptor-mediated pathway. *J. Gene Med.* 2, 76–88 (2000).

17. Kobayashi, N., Nishikawa, M., Hirata, K. & Takakura, Y. Hydrodynamics-based procedure involves transient hyperpermeability in the hepatic cellular membrane: implication of a non-specific process in efficient intracellular gene delivery. *J. Gene Med.* 1, 584–592 (2004).

18. Razmara, M., Hilliard, B., Ziarani, A. K., Chen, Y. H. & Tykocinski, M. L. CTLA-4 x Ig converts naive CD4+ CD25+ T cells into CD4+ CD25+ regulatory T cells. *Int. Immunol.* 20, 471–483 (2008).

19. Ying, H. et al. Cutting edge: CTLA-4–B7 interaction suppresses TH1 cell differentiation. *J. Immunol.* 185, 1375–1378 (2010).

20. De Klerck, B. et al. Enhanced osteoclast development in collagen-induced arthritis in interferon-gamma receptor knock-out mice as related to increased splenic CD11b+ myelopoiesis. *Arthritis Res Ther.* 6, R220–231 (2004).

21. Alnæsli, M., Penninger, J. M. & Teng, Y. T. Immune interactions with CD4+ T cells promote the development of functional osteoclasts from murine CD11c+ dendritic cells. *J. Immunol.* 177, 3314–3326 (2006).

22. Zais, M. M. et al. Regulatory T cells protect from local and systemic bone destruction in arthritis. *J. Immunol.* 184, 7238–7246 (2010).

23. Axmann, R. et al. CTLA-4 directly inhibits osteoclast formation. *Ann Rheum Dis.* 67, 1603–1609 (2008).

24. Yue, D. et al. CTLA-4Ig blocks the development and progression of citrullinated fibrinogen-induced arthritis in DR4-transgenic mice. *Arthritis Rheum.* 62, 2941–2952 (2010).

25. Ijima, K. et al. Successful gene therapy via intraarticular injection of adenovirus vector containing CTLA4Ig in a murine model of type II collagen-induced arthritis. *Hum Gene Ther.* 12, 1063–1077 (2001).

26. Luo, G. et al. CTLA4Ig introduced by adenovirus vector locally to prolong the survival of xenogenic skin grafts on rat burn wounds. *J. Trauma*. 59, 1209–1215 (2005).

27. Thummala, N. R. et al. A non-immunogenic adenoviral vector, coexpressing CTLA4Ig and bilirubin-uridine-diphosphoglucuronosyltransferase permits long-term, repeatable transgene expression in the Gunn rat model of Crigler-Najjar syndrome. *Gene Ther.* 9, 981–990 (2002).

28. Marshall, E. Gene therapy death prompts review of adenovirus vector. *Science* 286, 2244–2245 (1999).

29. Yamamoto, M., Harigaya, T., Ichikawa, T., Hoshino, K. & Nakashima, K. Reconstituent mouse prolactin: expression in Escherichia coli, purification and biological activity. *J. Mol Endocrinol.* 8, 165–172 (1992).

30. Knoerzer, D. B., Karr, R. W., Schwartz, B. D. & Mengle-Gaw, L. J. Collagen-induced arthritis in the BB rat. Prevention of disease by treatment with CTLA-4-Ig. *J. Clin Invest.* 96, 987–993 (1995).

31. Shedlock, D. J. & Weiner, D. B. DNA vaccination: antigen presentation and the induction of immunity. *J. Leukoc Biol.* 68, 793–806 (2000).

32. Hassett, D. E., Slifa, M. K., Zhang, J. & Whitton, J. L. Direct ex vivo kinetic and phenotypic analyses of CD6(+) T-cell responses induced by DNA immunization. *J. Virol.* 74, 8286–8291 (2000).

33. Hassett, D. E., Zhang, J., Slifa, M. & Whitton, J. L. Immune responses following neonatal DNA vaccination are long-lived, abundant, and qualitatively similar to those induced by conventional immunization. *J. Virol.* 74, 2620–2627 (2000).

34. Osborn, M. J. et al. Minicircle DNA-based gene therapy coupled with immune modulation permits long-term expression of alpha-L-iduronidase in mice with mucopolysaccharidosis type I. *Mol Ther.* 19, 450–460 (2011).

35. Chen, Z. Y., Riu, E., He, C. Y., Xu, H. & Kay, M. A. Silencing of episomal transgene expression in liver by plasmid bacterial backbone DNA is independent of CpG methylation. *Mol Ther.* 16, 548–556 (2008).

36. Chen, Z. Y., He, C. Y., Meuse, L. & Kay, M. A. Silencing of episomal transgene expression by plasmid bacterial DNA elements in vivo. *Gene Ther.* 11, 856–864 (2004).

37. Ebina, K. et al. Adenovirus-mediated gene transfer of adipopectin reduces the severity of collagen-induced arthritis in mice. *Biochem Biophys Res Commun.* 378, 186–191 (2009).

38. Tepper, M. A., Linsley, P. S., Tritesdler, D. & Esselstyn, J. M. Tolerance induction by soluble CTLA4 in a mouse skin transplant model. *Transplant Proc.* 26, 3151–3154 (1994).

39. Haggerty, H. G., Bigbee, C., Gonchoroff, D. G. & Flynn, J. L. Abatacept treatment does not impair host resistance to chronic mycobacterium tuberculosis infection in mice. *Ann Rheum Dis.* 65, 319–319 (2006).

40. Huckel, M. et al. Attenuation of murine antigen-induced arthritis by treatment with a decoy oligodeoxynucleotide inhibiting signal transducer and activator of transcription-1 (STAT-1). *Arthritis Res Ther.* 8, R17 (2006).