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Self *in vivo* production of a synthetic biological drug CTLA4Ig using a minicircle vector

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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 *in vivo*. We inserted the sequence of abatacept into the minicircle vector, and by successful *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$^+$ 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. 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[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)[3,4]. CTLA4 is a homologue to CD28 but binds with a higher affinity and terminates T cell activation[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 ~10 mg kg^{-1} 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[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[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 *in vivo* delivery[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[10]. 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[11,12]. 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)[13]. The ΦC31 integrase induces a recombination between the attB and attP site, which eventually

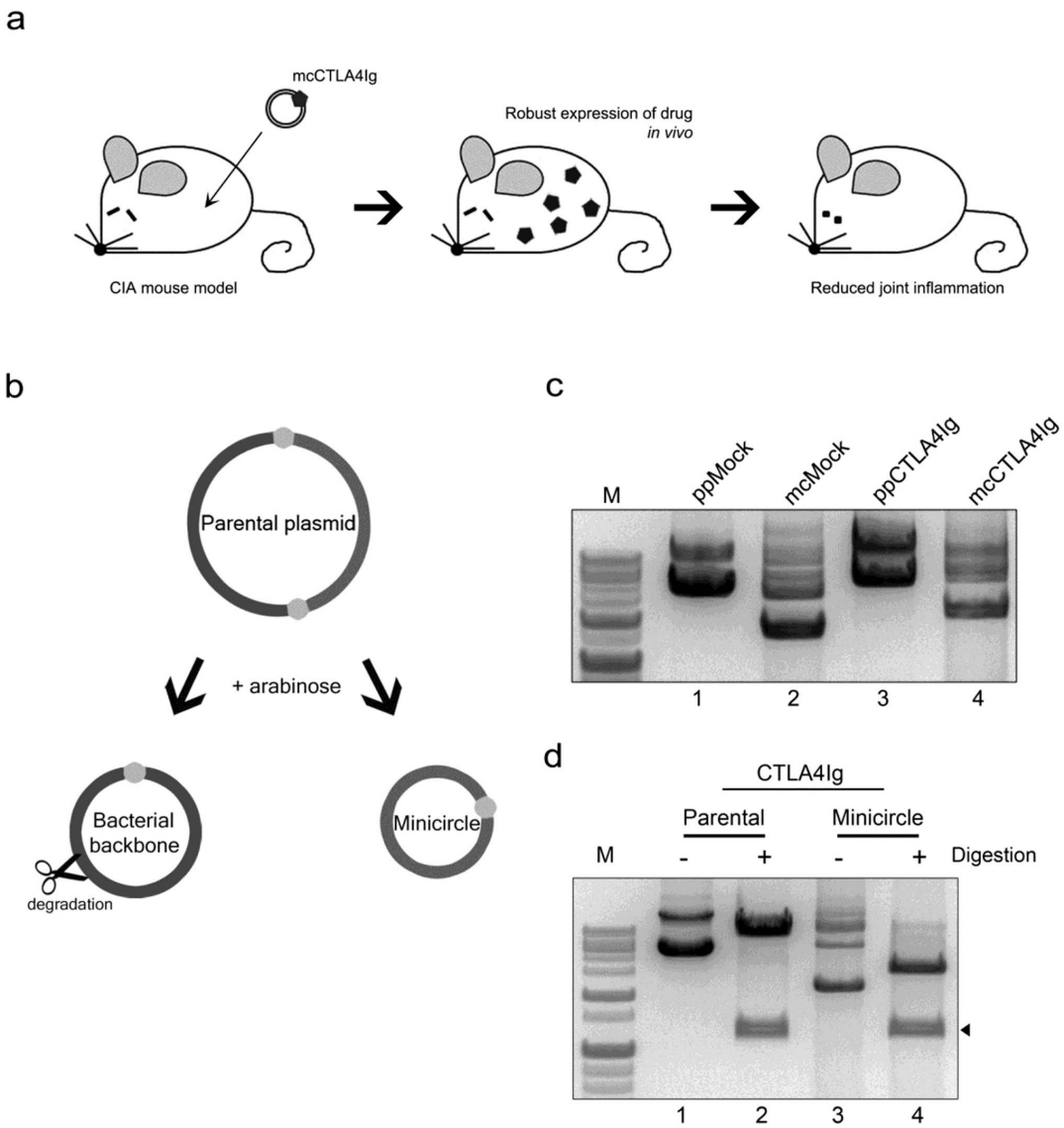

Figure 1 | Scheme of experimental concept and generation of minicircles encoding mcCTLA4Ig. (a) Our concept is to make a self-production system to deliver a protein drug *in vivo* by injecting the plasmid encoding the drug sequence. The injected mcCTLA4Ig will induce robust expression of the protein 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.

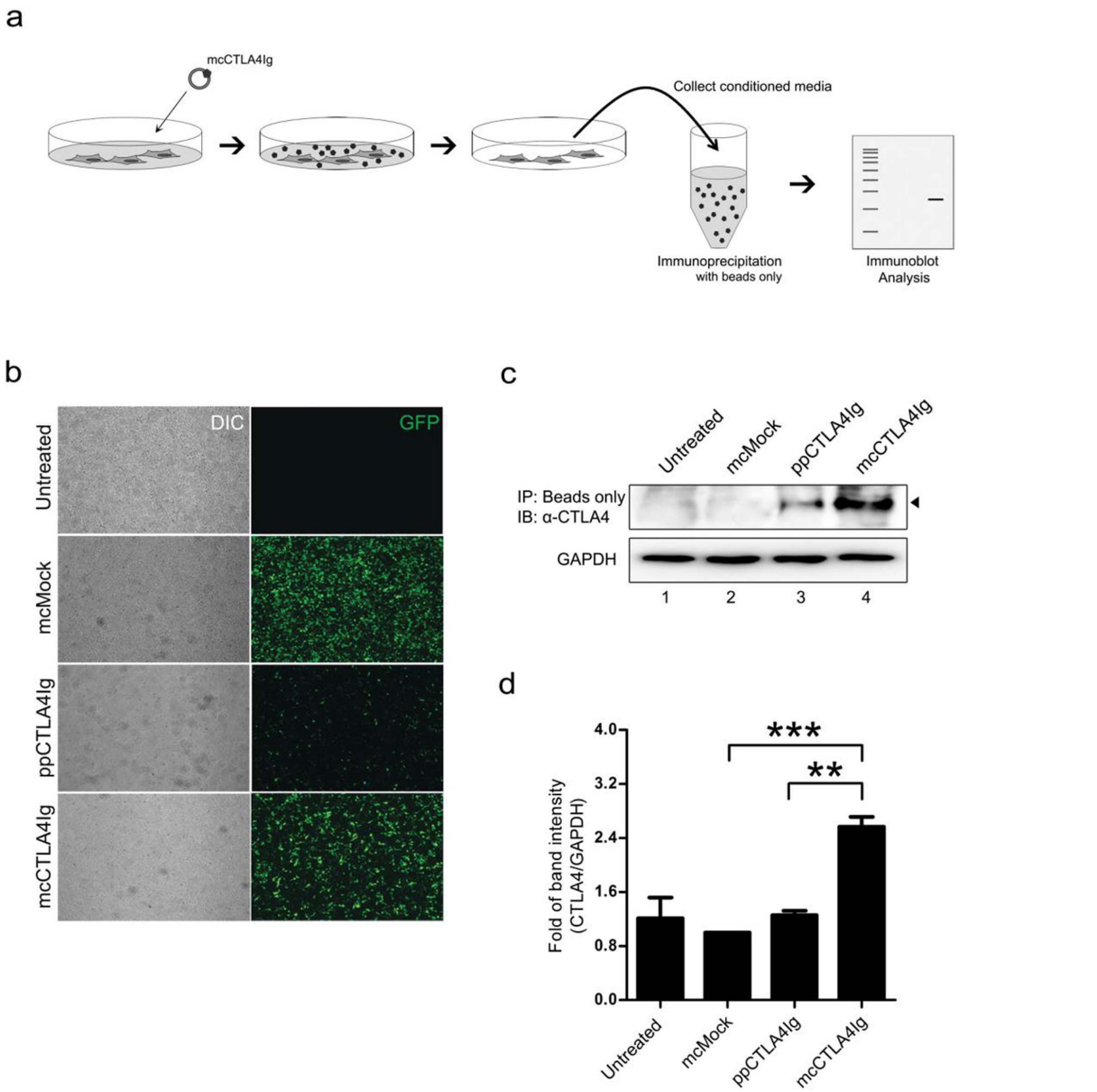

Figure 2 | Transfection assay of mcCTLA4Ig in HEK293T cells and CTLA4Ig protein *in vitro* detection. (a) This figure shows a brief review of the detection system about spontaneous production of target protein drug. HEK293T cells were transfected with mcCTLA4Ig and other plasmids. The cells were cultured for 48 hours to accumulate the protein drug in the media. Conditioned media was collected and immunoprecipitated with CTLA4 antibody. The spontaneously produced protein was detected by immunoblotting. (b) GFP expression was measured 24 hours after HEK293T cells were transfected with each plasmid. mcMock- and mcCTLA4Ig-treated cells showed a similarly high expression of GFP. (c) The immunoblotting 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.

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 *Bam*HI and *Xba*I, 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 in culture media. CTLA4Ig was not detected in mcMock-transfected cell supernatant (Fig. 2c). The secreted proteins in the conditioned media were both expressed by mcCTLA4Ig and ppCTLA4Ig. The expression however was higher in mcCTLA4Ig than ppCTLA4Ig-transfected cells. This indicates that the transfection efficiency of mcCTLA4Ig was higher than that of ppCTLA4Ig (Fig. 2d). In conclusion, mcCTLA4Ig was able to express and secrete the CTLA4Ig protein drug in vitro.

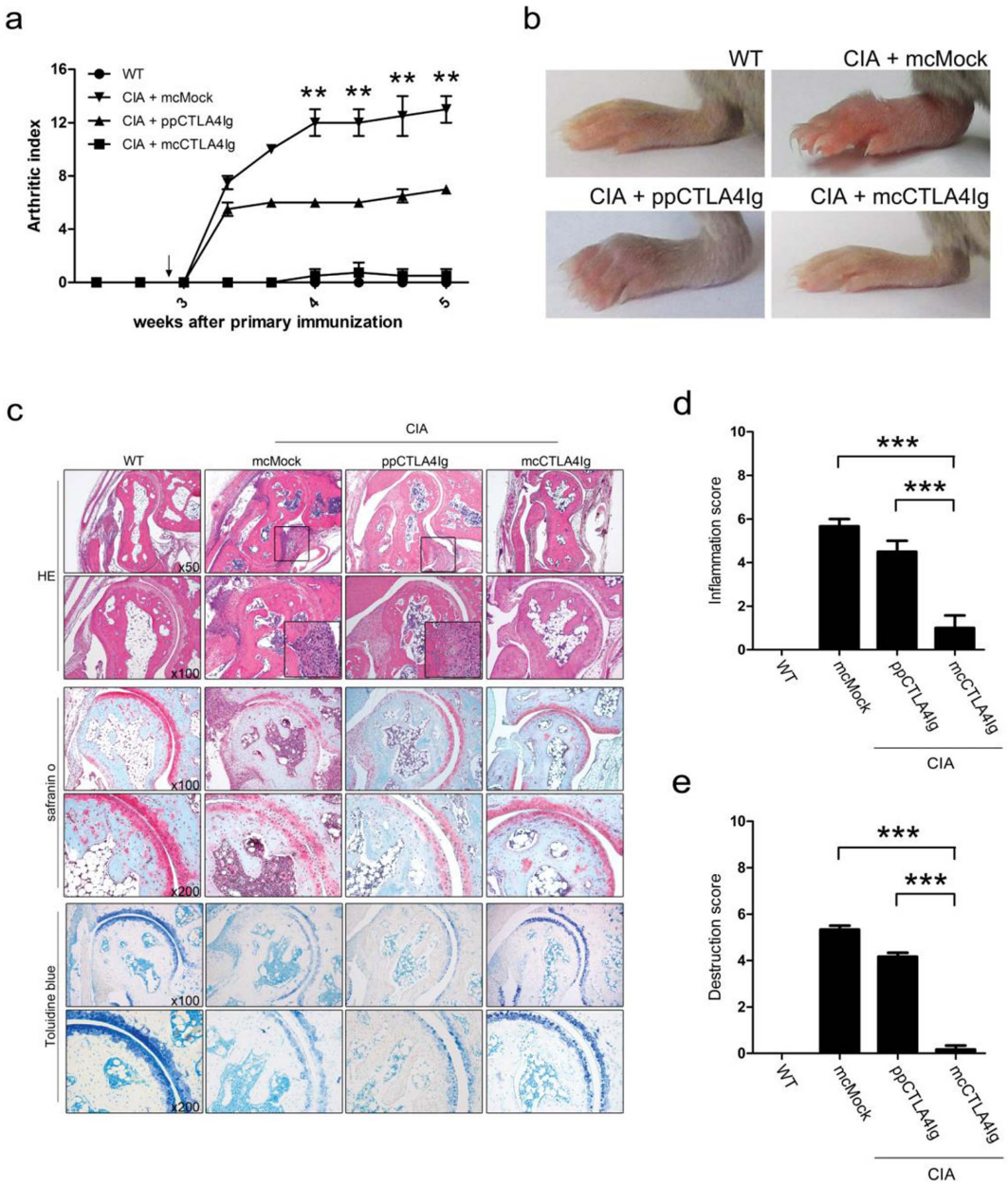

Figure 3 | Systemic intravenous delivery of mcCTLA4Ig in CIA mice. (a) Arthritic symptoms were scored after primary immunization. Plasmid DNA was injected intravenously on day 19. On day 21, a second immunization was performed in each group ($n = 5$ mice in each groups). The arthritis score decreased 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. (** $P < 0.005$, *** $P < 0.001$).

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

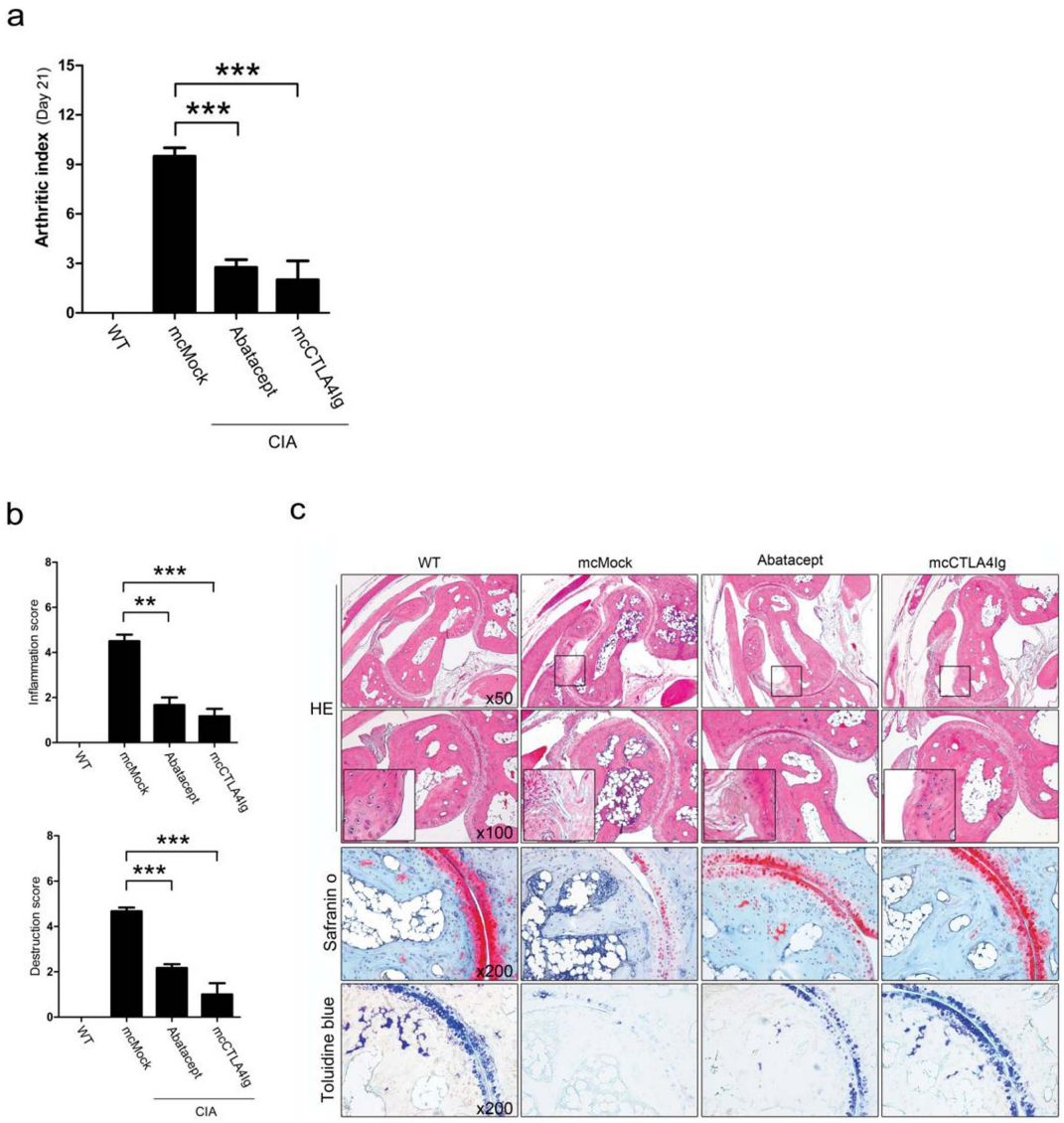

Figure 4 | Comparing mcCTLA4Ig with abatacept. (a) The arthritis score was lower in both groups. Both groups also showed a lower score in the inflammation score and destruction score compared to the score of mcMock treated group. (b) Histological analysis of cartilage and bone erosion. Joints were stained with H&E, safranin O and toluidine blue. (** $P < 0.005$, *** $P < 0.001$).

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 attenuation of arthritis by mcCTLA4Ig was confirmed by the inflammation and destruction scores (Fig. 3d and 3e). The mcMock group showed the highest score while the inflammation severity decreased in mcCTLA4Ig-injected group (Fig. 3d). Also, the destruction severity was the lowest in mcCTLA4Ig-injected group while mcMock-injected group showed a relatively high score (Fig. 3e).

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

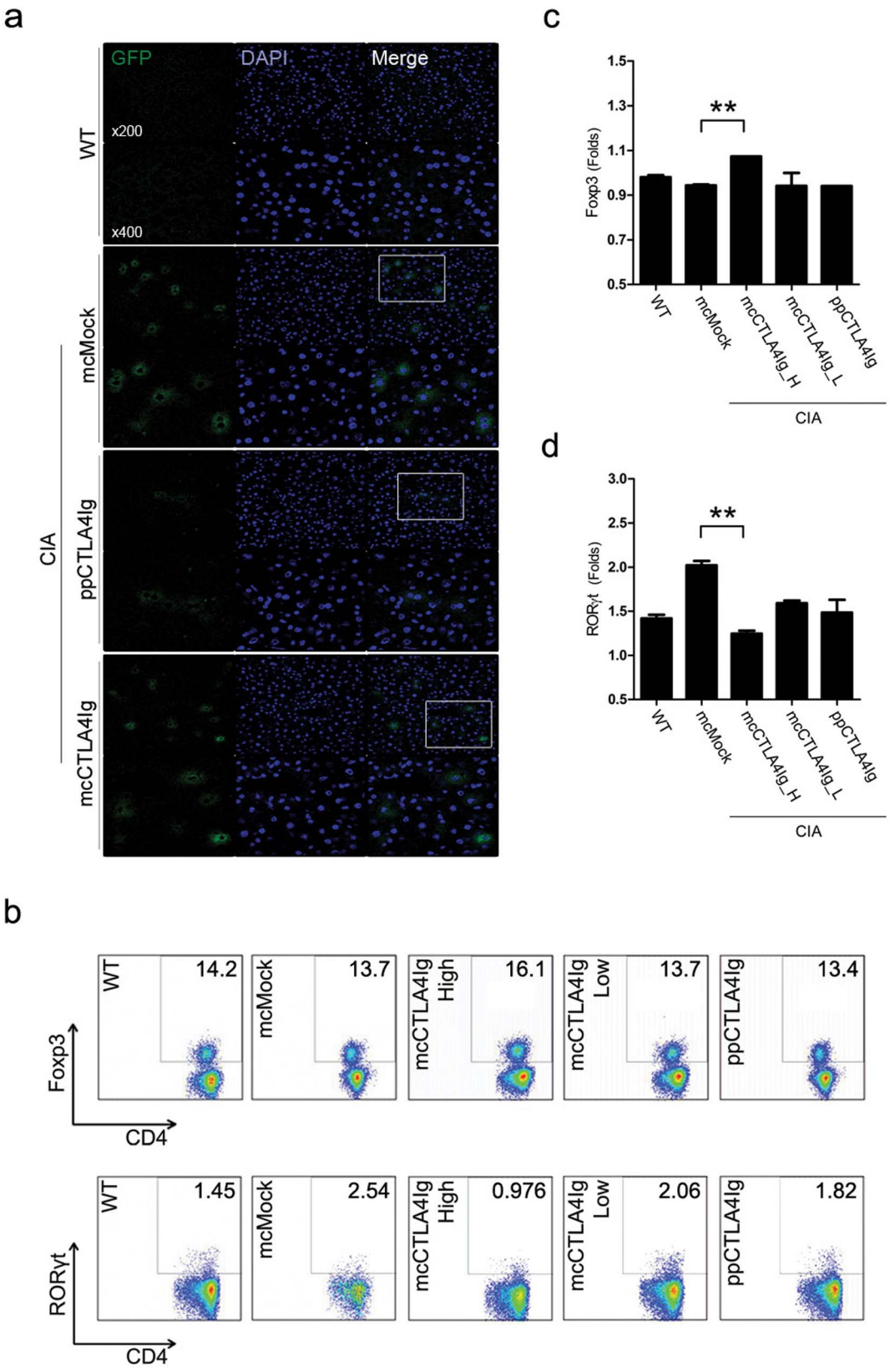

Figure 5 | Successful transfection of mcCTLA4Ig into hepatocytes. (a) GFP expression of mcMock plasmids in CIA mice liver is shown as positive fluorescence. The minicircular form showed a relatively high positive expression in hepatocytes compared to the parental form. (b) Splenocytes of CIA were analyzed by flow cytometry. (c) mcCTLA4Ig-treated mice had more $Foxp3^+$ cells compared with mice given a low dose of mcCTLA4Ig or ppCTLA4Ig. (d) Fewer $ROR\gamma t^+$ cells were seen in mice given mcCTLA4Ig compared with those given mcMock, low-dose mcCTLA4Ig, or ppCTLA4Ig. (** $P < 0.005$).

role in the onset, development and recovery of RA. Among them, Foxp3 (Forkhead box P3) positive T cells and RORγt (RAR-related orphan receptor gamma t) positive Th17 cells are the main focus in the pathophysiology of RA. Foxp3+ T cells are reported to be deficient in RA, but Th17 cells are upregulated in established RA. Abatacept is known to increase the $Foxp3^+$ proportion within the T cell population and to regulate Th17 cells[18,19]. Gating was done on CD4+ cells for expression of Foxp3 and RORγt. The population of $Foxp3^+$ T cells slightly increased in the low dose mcCTLA4Ig-injected group compared to the mcMock-injected group. However, when given in a higher dose, the population of $Foxp3^+$ T cells showed a more significant increase (Fig. 5c). ppCTLA4Ig and mcMock did not change the $Foxp3^+$ T cell population showing a lower population compared with that of the wild type. Splenocytes from mice treated with the higher concentration of mcCTLA4Ig also showed a marked decrease in the Th17 population (Fig. 5d). By contrast, splenocytes of CIA mice treated with mcMock had an increased population of Th17 cells, suggesting that the immune response was still active. These findings suggest that mcCTLA4Ig altered the populations of $Foxp3^+$ T cells and Th17 cells.

mcCTLA4Ig inhibited osteoclast formation. The common feature in CIA mice and human rheumatoid arthritis is the bone destruction caused by the osteoclast activation[20]. 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[21]. 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 antiosteoclastogenic 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[22]. There is evidence that CTLA4Ig can bind directly to osteoclast precursor cells and inhibit the differentiation process[23]. Other studies have reported that CTLA4Ig can prevent inflammation, cartilage damage, pannus formation, and bone resorption, and can thereby protect the bone architecture[24,25].

The gene delivery of CTLA4Ig has been attempted several times using viral vector systems. Previous studies have shown an improvement in the survival of heterogenetic skin grafts on burn wounds with an adenovirus vector Ad–CTLA4Ig[26]. Coexpression of CTLA4Ig with an adenoviral vector had a potent effect in the Gunn rat model of Crigler–Najjar syndrome[27]. However, viral-based delivery has obstacles such as genome integration, mutation, and infection-associated inflammatory responses[28]. 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*[29]. 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[30]. 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 transfected 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[31,32]. It has also been shown that the immune response to DNA is similar to that in viral infection[33]. 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.

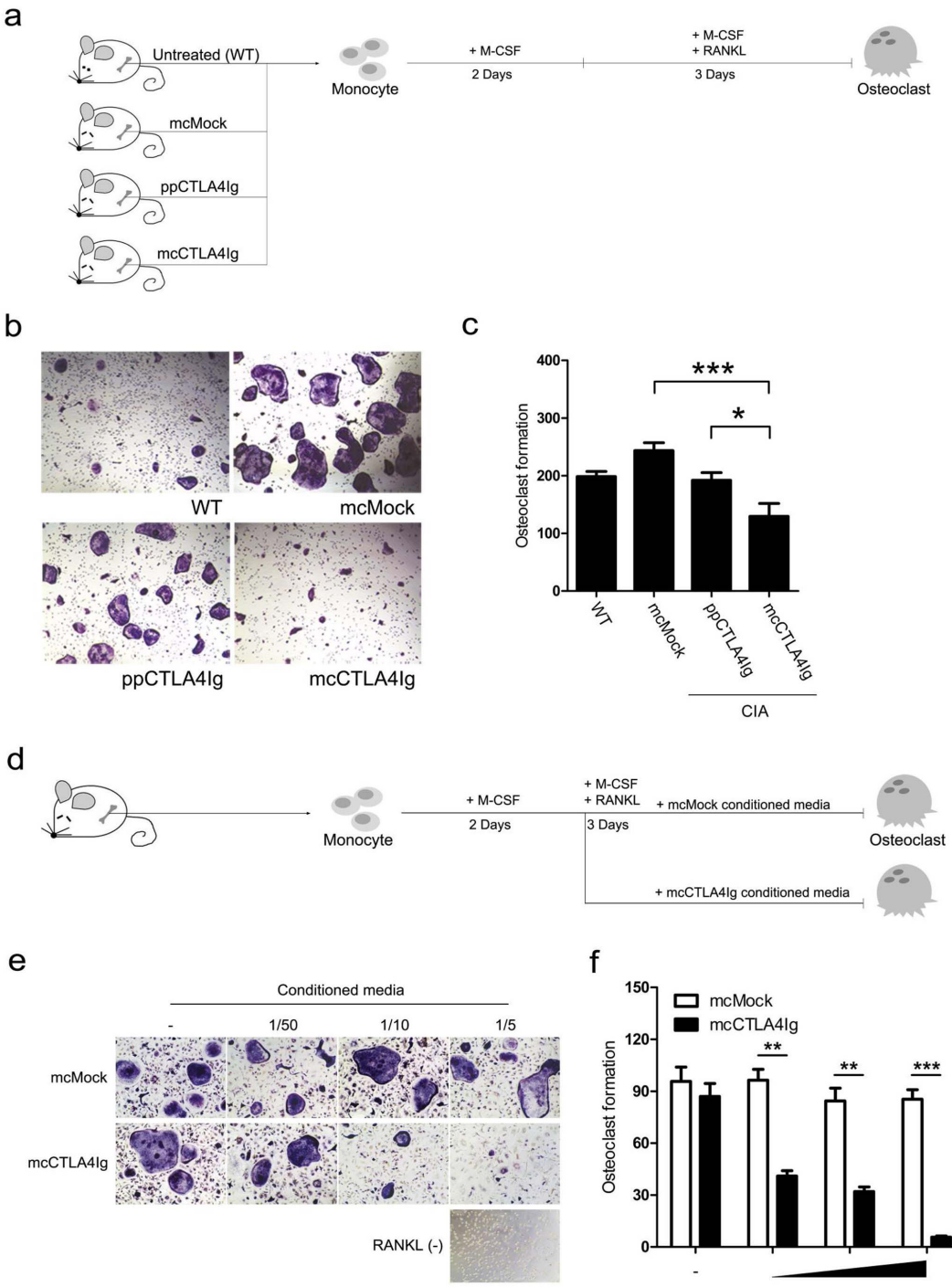

Figure 6 | The effect of mcCTLA4Ig on osteoclast differentiation *in vivo* and *in vitro*. (a) Schematic presentation of osteoclastogenesis in vivo. The image of osteoclasts which were differentiated from bone marrow macrophage of each mice treated with the plasmid of mcMock, mcCTLA4Ig, and ppCTLA4Ig. (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. (* $P < 0.01$, ** $P < 0.05$, *** $P < 0.001$).

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

minicircle DNA *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[15]. 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[17]. 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[14,17]. 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. Nanoparticular PEGylated carriers may be useful as an alternative method for delivering mcCTLA4Ig *in vivo*.

In summary, we report a novel self-containing drug delivery system of CTLA4Ig with a minicircle. Synthetic protein drugs were produced *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 *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 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 *Xba*I and *Bam*HI 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*[11]. 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 L-arabinose was added to the overnight cultures, 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 *Nde*I, and the resultant linearized DNA was removed with Plasmid-Safe™ 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^{-1} penicillin, and 100 μg ml^{-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-agarose 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^{-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 vivo* delivery of DNA vectors and abatacept treatment.** Two days before the second immunization, the mice were treated with the prepared plasmids as described by Ebina *et al*[37]. The plasmid DNA vectors were delivered by hydrodynamic delivery using intravenous injection into the tail vein. Mice were injected with 20 μg of plasmids according to the previous study done by Gracey Maniar et al[10]. 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[38,39].

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 measured using the procedure of Huckel *et al.* by three individual researchers[40]. 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 *ex vivo*. To detect Th17 cells in the mouse spleen, splenocytes were isolated from each group. The cells were incubated with allophycocyanin (APC)-conjugated rat anti-mouse CD4 antibody (catalogue number, 561091; BD Biosciences, San Jose, CA, USA), and the cells were permeabilized using a Foxp3 staining buffer set (00-5523; eBioscience, San Diego, CA, USA). The Th17-specific marker RORγt was detected by anti-human/mouse RORγt antibody conjugated with phycoerythrin (PE) (eBioscience, 12-6988). After the overall cellular staining process, the cell populations were identified and estimated using an LSRFortessa cell analyzer (BD Biosciences). The acquired data were analyzed using FlowJo version 7.6.5 software (TreeStar Inc. Ashland, OR, USA). Cells were first gated for CD4+ proportion and analyzed for the expression of RORγt.

Regulatory T cells phenotyping. Splenocytes were isolated from mice and stained with APC-conjugated anti-mouse CD4 (561091; BD Biosciences). A Foxp3/Transcription Factor Staining Buffer Set (00-5523; eBioscience) was used for permeabilization and fluorescein isothiocyanate (FITC)-conjugated anti-mouse Foxp3 (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+ and subsequently analyzed for the expression of Foxp3.

***In 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 (Welgene, Daegu, Korea) supplemented with 10% FBS. The next day, cells were counted to $3 \times 10^{\wedge}5$ 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 $3 \times 10^{\wedge}5$ 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. Conditioned media of mcMock and mcCTLA4Ig was added in several ratios, 0, 1/50, 1/10 and 1/5. After 72 hours, differentiated osteoclasts were stained with TRAP staining followed by the manufacturers guide. The stained cells were counted.

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Author contributions

Y.A.R. designed and performed the study, analyzed the data, and wrote the manuscript. J.K. performed cell culture. Y.A.R. and N.P. produced and purified minicircle vectors. H.J. obtained tissues and stained them. Y.A.R., Y.K., H.Y., S.M.J. and S.H.P. analyzed the data. J.H.J. designed and supervised the study, analyzed the data, and wrote the manuscript.

Additional information

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