CTRP6 is an endogenous complement regulator that can effectively treat induced arthritis


The complement system is important for the host defence against infection as well as for the development of inflammatory diseases. Here we show that C1q/TNF-related protein 6 (CTRP6; gene symbol C1qtnf6) expression is elevated in mouse rheumatoid arthritis (RA) models. C1qtnf6−/− mice are highly susceptible to induced arthritis due to enhanced complement activation, whereas C1qtnf6-transgenic mice are refractory. The Arthus reaction and the development of experimental autoimmune encephalomyelitis are also enhanced in C1qtnf6−/− mice and C1qtnf6−/− embryos are semi-lethal. We find that CTRP6 specifically suppresses the alternative pathway of the complement system by competing with factor B for C3(H2O) binding. Furthermore, treatment of arthritis-induced mice with intra-articular injection of recombinant human CTRP6 cures the arthritis. CTRP6 is expressed in human synoviocytes, and CTRP6 levels are increased in RA patients. These results indicate that CTRP6 is an endogenous complement regulator and could be used for the treatment of complement-mediated diseases.
The complement system is a component of the innate immune system, and plays important roles in host defence against microbial infection. The complement system is also suggested to be involved in the pathogenesis of a group of inflammatory diseases. Rheumatoid arthritis (RA) is one of such diseases in which serum concentrations of complement active fragments (C3a and C5a) are elevated. The development of arthritis is suppressed by the deficiency of the complement system in RA models. The importance of complement activation is also suggested in other inflammatory diseases such as multiple sclerosis (MS) and glomerulonephritis.

Complement activation is a proteolytic cascade consisting of three distinct pathways. The classical pathway (CP) is initiated by C1q binding to immune complexes. The lectin pathway (LP) is triggered by binding of mannose-binding lectin or ficolins to pathogen-associated carbohydrate motifs. In the alternative pathway (AP), C3(H2O) is initially produced by spontaneous hydrolysis of the internal thioester bond in C3 (Supplementary Fig. 1). Then, factor B binds C3(H2O), and the complex is activated by factor D to generate fluid-phase C3 convertase (C3(H2O)Bb) (tick-over). On the surface of pathogens, C3b, factor B and factor P form C3 convertase (C3bBbP), which amplifies the reaction (amplification loop). C3b, in combination with Bb, cleaves C5, generating anaphylatoxin C5a and C5b, which form the membrane attack complex (MAC). C3a and C5a have chemotactic and anaphylactic activities; they also suggested to be involved in the pathogenesis of a group of inflammatory diseases such as multiple sclerosis (MS) and glomerulonephritis.

In this study, by generating C1qtnf6−/− mice, we demonstrated that CTRP6 is a novel regulator of the complement AP and plays important roles in the maintenance of pregnancy and the pathogenesis of disease models such as collagen-induced arthritis (CIA) and anti-collagen antibody-induced arthritis (CAIA), experimental autoimmune encephalomyelitis (EAE), and Arthus reaction. Because CTRP6 levels are also increased in the serum of RA patients and CTRP6 exerts a potent therapeutic effect on CIA, we suggest that CTRP6 could be used clinically to treat RA and other complement-mediated diseases.

**Results**

**Generation of C1qtnf6−/− mice.** Because C1qtnf6 is highly expressed in mouse RA models (Supplementary Fig. 2b), we generated C1qtnf6−/− mice to elucidate the pathological roles of CTRP6 in the development of arthritis (Supplementary Fig. 2c−e). C1qtnf6−/− mice were fertile and showed no obvious abnormalities before 6 months of age. However, the frequency of obtaining homozygotes from the matings of (129P2/Ola × C57BL/6) F1 background mice was less than the expected Mendelian ratio (wild type (WT):heterozygote:homozygote = 159 (31.7%):245 (48.9%):97 (19.4%), P = 0.021). We used mice after backcrossing with C57BL/6 mice by the high-speed congenic strategy, in which C57BL/6-specific alleles were confirmed in 84 microsatellite markers, throughout the experiments. Aged C1qtnf6−/− mice produced significantly higher levels of autoantibodies, such as antibodies to nuclear antigens (ANAs), IgG- and IgM-type rheumatoid factor (RF) (Supplementary Fig. 3a), although cell populations of lymphoid tissues (Supplementary Fig. 3b−d) and antibody production against thymus-dependent and -independent antigens were normal (Supplementary Fig. 3e−h). Renal function was also normal in these C1qtnf6−/− mice (Supplementary Fig. 3i).

**C1qtnf6−/− mice are susceptible to CIA.** We next examined the development of CIA in C1qtnf6−/− mice. Mice were immunized with chicken type-II collagen (IIC) emulsified in complete Freund’s adjuvant (CFA) containing a low concentration of *Mycobacterium tuberculosis* (1.65 mg ml−1) to induce mild arthritis. In WT mice under this regimen, the induced arthritis was mild. By contrast, in C1qtnf6−/− mice, the onset of arthritis was earlier, and the severity score was higher than in WT mice, although the incidence of arthritis was similar between the two strains (Fig. 1a). Histology of the joints of WT mice revealed much more severe changes, including proliferation of synovial lining cells, infiltration of inflammatory cells and bone destruction associated with pannus formation (Fig. 1b). The number and percentage of CD4+ T cells in lymph node (LN) were similar between C1qtnf6−/− and WT mice, whereas the B220+B-cell population was significantly expanded in C1qtnf6−/− mice (Fig. 1c). Moreover, IIC-specific IgG levels after IIC immunization were significantly higher in C1qtnf6−/− than in WT mice (Fig. 1d). The proliferative response to IIC of LN cells in C1qtnf6−/− mice was similar to that of WT mice, suggesting that T-cell priming was normal (Fig. 1e). These results suggest that C1qtnf6−/− mice are more susceptible to CIA than WT mice.

We also analysed susceptibility of C1qtnf6−/− mice to CAIA, in which antibodies against IIC were directly injected to induce arthritis. We found that C1qtnf6−/− mice were much susceptible to CAIA than WT mice (Fig. 1f). Because CAIA is independent from antibody production, these results suggest that the effect of CTRP6 on the autoantibody-induced inflammation is important rather than on the production of antibodies.

**C1qtnf6 Tg mice are refractory to the development of CIA.** Next, we generated C1qtnf6 Tg mice carrying mouse C1qtnf6 under the control of the CAG promoter to examine the effects of excess CTRP6. Serum CTRP6 levels were ~2.5 times higher in C1qtnf6 Tg mice than in non-Tg littermates (Supplementary Fig. 4a–d). Heterozygous C1qtnf6 Tg mice were born in the expected Mendelian ratio (WT:C1qtnf6 Tg = 290 (53.5%):252 (46.5%), P = 0.273), were fertile and showed no obvious abnormalities including the renal function (Supplementary Fig. 4e).
We examined the susceptibility of C1qtnf6 Tg mice to CIA, using a higher concentration of M. tuberculosis (2.5 mg ml

−1) in CFA to induce severe arthritis. The arthritic severity score was significantly lower in C1qtnf6 Tg mice than in WT mice, although the incidence was similar (Fig. 2a). Histology of the joints of C1qtnf6 Tg mice exhibited milder pathological changes than that of WT mice (Fig. 2b,c). Serum IIC-specific IgG levels were also decreased in these C1qtnf6 Tg mice (Fig. 2d). Furthermore, C1qtnf6 Tg mice were refractory against the induction of CAIA compared with WT mice (Fig. 2e). These results suggest that high concentration of CTRP6 can suppress the development of arthritis.

CTRP6 specifically inhibits complement AP. Interestingly, we found that the concentrations of C3a and C5a in the plasma of IIC-immunized mice were increased in C1qtnf6−/− mice compared with WT mice (Fig. 3a). Furthermore, the deposition of C3b in the joints was increased in C1qtnf6−/− mice (Fig. 3b,c). In contrast, the deposition of C3b in the joints was lower in C1qtnf6 Tg mice than in WT mice (Fig. 3d,e). These results suggest that CTRP6 regulates complement activation.

Then, we examined the effects of CTRP6 deficiency on three complement pathways in vitro. Plates coated with the ovalbumin (OVA)/anti-OVA immune complex, mannann and lipopolysaccharide (LPS) were used to activate the CP, LP and AP, respectively. Sera were diluted with GVB+ buffer containing Ca2+ for CP and LP assays, while sera for determination of AP activity were diluted with GVB/Mg2+ EGTA to eliminate Ca2+,

because the AP is Ca2+ independent. We found that AP activity was specifically enhanced in C1qtnf6−/− sera compared with WT sera (Fig. 4a). Furthermore, MAC formation under AP activation conditions was enhanced in C1qtnf6−/− mouse sera (Supplementary Fig. 5a). In contrast, C1qtnf6 Tg mouse serum specifically suppressed AP activation (Fig. 4b; Supplementary Fig. 5b). C3 and factor B levels were comparable in WT, C1qtnf6−/− and C1qtnf6 Tg mouse sera (Supplementary Fig. 5c). These results suggest that CTRP6 specifically suppresses AP activation.

Then, we directly analysed the effect of CTRP6 on AP activation in vitro. Because C1q domain of human and mouse CTRP6 are highly homologous and CTRP6 mainly forms oligomers in the serum (Supplementary Fig. 5d), we analysed the effect of monomeric exogenous recombinant human CTRP6 (rhCTRP6) on the complement activity of mouse sera. C1qtnf6−/− sera were used to avoid possible influence of endogenous CTRP6. Results showed that rhCTRP6 specifically inhibited AP activation in a dose-dependent manner (Fig. 4c; Supplementary Fig. 5f).

On the basis of these findings, we investigated in further detail the effects of CTRP6 on AP activation. Upon activation of the AP, spontaneously generated C3(H2O) forms a complex with factor B, followed by cleavage of factor B by factor D to generate C3(H2O)Bb, a C3 convertase, and Ba. C3 is cleaved to C3a and C3b (consisting of C3a and C3β); C3β is cleaved to C3a and C3b (consisting of C3a and C3b) by C3 convertase (Supplementary Fig. 6). After incubation of human C3, factor B and factor D in the presence of various concentrations of rhCTRP6 in vitro, the reaction mixture was
subjected to SDS–polyacrylamide gel electrophoresis (PAGE) and visualized by Coomassie brilliant blue (CBB) staining (Fig. 4d; Supplementary Fig. 5g). The result showed that rhCTRP6 inhibited proteolytic activation of factor B in a dose-dependent manner, indicating that CTRP6 suppressed C3 convertase formation. Next, we examined the effect of CTRP6 on the formation of the C3(H2O)–factor B complex, the first step of C3 tick-over. After incubating a mixture of human C3(H2O), human factor B and rhCTRP6, C3-containing protein complexes were immunoprecipitated using anti-C3 antibodies. Co-immunoprecipitation of factor B decreased as a function of rhCTRP6 dose (Fig. 4e; Supplementary Fig. 5h), suggesting that CTRP6 competitively inhibits factor B binding to C3(H2O). Furthermore, exogenously added rhCTRP6 co-precipitated with C3 from C1qtnf6−/− serum (Fig. 4f; Supplementary Fig. 5i,j). Direct binding of rhCTRP6 to human C3(H2O), but not to C3b, was shown by surface plasmon resonance (SPR) analysis (KD = 1.1 × 10−9) (Fig. 4g; Supplementary Fig. 5k). Furthermore, we examined the association between CTRP6 and factor H, because factor H also binds C3(H2O)28 and adiponectin, one of the CTRP family associated with factor H to modulate the AP activation29. However, CTRP6 did not bind factor H nor influence its activity (Supplementary Fig. 5l,m). These results demonstrated that CTRP6 regulates AP activation by binding to C3(H2O).

**C1qtnf6 deficiency enhances the Arthus reaction and EAE.** The Arthus reaction is an immune complex-mediated type-III hypersensitivity that depends on complement activation30. To confirm that CTRP6 is a complement inhibitor, we examined the sensitivity of C1qtnf6−/− mice to an IgG-mediated reverse passive Arthus (RPA) reaction. We found that vascular permeability was significantly enhanced in C1qtnf6−/− mice compared with WT mice (Supplementary Fig. 7a). These results support the notion that CTRP6 is a regulator of the complement system.

MS is an autoimmune disease of the central nervous system. Studies of EAE, an experimental model of MS, have suggested that the AP, but not the CP or LP, is important for the pathogenesis30,31. Another recent study has also suggested the involvement of complement activation in MS pathogenesis8. Therefore, we investigated whether C1qtnf6 deficiency affected the development of EAE. The severity score was much higher in C1qtnf6−/− mice than in WT mice, although the incidence of disease was similar between the two strains (Supplementary Fig. 7b). These results provide further support for the notion that CTRP6 is a novel regulator of the complement system.

**CTRP6 is effective for the treatment of CIA.** Next, we examined whether CTRP6 can treat established arthritis. We immunized DBA/1J mice with IIC/CFA (M. tuberculosis 0.5 mg ml−1), followed by a booster injection with IIC/CFA on day 21. Starting on day 28, we performed daily injections of rhCTRP6 into the articular cavity of the knee joints of affected mice (rhCTRP6 into left leg; PBS or rhCTRP9 into right as a control), and evaluated the swelling of the ankle joints. As shown in Fig. 5a and Supplementary Fig. 8, ankle joint swelling was promptly ameliorated by the injection of rhCTRP6 to the knee joint, whereas the ankle joints of the PBS- or rhCTRP9-injected side still remained swollen. In the rhCTRP6-treated joints of the WT mice, messenger RNA (mRNA) expression levels of Il1b, Tnfa and F4/80 were decreased, whereas Il10 mRNA level did not change (Fig. 5b). Histology of joints revealed that PBS-injected joints were destroyed, but rhCTRP6-injected joints were normal.
immunization were stained with anti-C3b antibody and DAPI (nucleus). The photomicrographs were taken with fluorescence and DIC optics. Scale bar, 20 μm. One of representative histologies is shown. The relative fluorescence intensity (RFI) of C3b (WT, KO: n = 8 each). ***P < 0.001. Student’s t-test. The data were reproduced in another independent experiment with similar results. (b,c) Cryostat sections of the WT and C1qtnf6−/− ankle joints at day 42 after primary immunization were stained with anti-C3b antibody and 4’,6-diamidino-2-phenylindole (DAPI) (nucleus). The photomicrographs were taken with fluorescence and differential interference contrast (DIC) optics. Scale bar, 20 μm. One of representative histologies is shown. The relative fluorescence intensity (RFI) of C3b (WT, KO: n = 6 each). ***P < 0.001. Student’s t-test. (d,e) Cryostat sections of WT and C1qtnf6 Tg ankle joints at day 42 after primary immunization were stained with anti-C3b antibody and DAPI (nucleus). The photomicrographs were taken with fluorescence and DIC optics. Scale bar, 50 μm. One of representative histologies is shown. The relative fluorescence intensity of C3b (WT, Tg: n = 7 each). ***P < 0.001. Student’s t-test. Average and s.e.m. are shown.

Figure 3 | Complement activation in CIA-induced mice. (a) Plasma samples before (pre) and 7 days after (IIC) IIC immunization were collected, and C3a (upper) and C5a (lower) levels were measured by ELISA (WT, KO: n = 8 each). *P < 0.05. Student’s t-test. The data were reproduced in another independent experiment with similar results. (b) Cryostat sections of the WT and C1qtnf6−/− ankle joints at day 42 after primary immunization were stained with anti-C3b antibody and 4’,6-diamidino-2-phenylindole (DAPI) (nucleus). The photomicrographs were taken with fluorescence and differential interference contrast (DIC) optics. Scale bar, 20 μm. One of representative histologies is shown. The relative fluorescence intensity (RFI) of C3b (WT, KO: n = 6 each). ***P < 0.001. Student’s t-test. (d) Cryostat sections of WT and C1qtnf6 Tg ankle joints at day 42 after primary immunization were stained with anti-C3b antibody and DAPI (nucleus). The photomicrographs were taken with fluorescence and DIC optics. Scale bar, 50 μm. One of representative histologies is shown. The relative fluorescence intensity of C3b (WT, Tg: n = 7 each). ***P < 0.001. Student’s t-test. Average and s.e.m. are shown.

CTRP6 is expressed in synovial cells of RA patients. Finally, we investigated CTRP6 expression in RA patients. We found that CTRP6 concentration was increased in sera of RA patients compared with that of healthy controls (Fig. 6a). CTRP6 was detected in synovial lining cells of RA patients (Fig. 6b). Double-immunostaining using RA synovial tissues showed that CTRP6 expression was localized to Hsp47+ synovial cells32 (Fig. 6c–f; Supplementary Fig. 9). Furthermore, we found that C1qtnf6 expression in primary fibroblast-like synoviocytes was enhanced by IL-1β stimulation (Fig. 6g). These results indicate that CTRP6 expression is elevated in synovial cells in RA patients.

Discussion

Because C3a and C5a have chemotactic, anaphylatoxic and IgG production-promoting activity, and the MAC has strong cytolytic activity, complement activity must be strictly controlled under physiological conditions. When this control fails, excess or inappropriate activation of a complement pathway can cause multiple human diseases1,8,33. Thus, complement regulatory factors are important for the homeostasis of the complement system. In this report, we have shown that CTRP6 is a novel endogenous regulator of the AP and the AP was excessively activated in C1qtnf6−/− mice. CTRP6 efficiently suppresses the AP ‘tick-over’ by inhibiting factor B binding to C3(H2O) in a competitive manner. It is known that AP activation is also regulated by factor I and factor H; both regulate the ‘AP amplification loop’ (Supplementary Fig. 1), and factor H also blocks the tick-over reaction. Factor I, in cooperation with factor H, cleaves C3b to generate inactive C3b (iC3b). Factor H also accelerates decay of C3 convertase (C3bBb) by dissociating Bb34. Factor H-deficient (Cfh−/−) mice spontaneously develop membrane-proliferative glomerulonephritis due to C3 dysregulation, but factor I-deficient (Cfi−/−) mice do not develop glomerulonephritis, in spite of C3 dysregulation9,10. C1-inhibitor-deficient (C1inh−/−) mice show increased vascular permeability35. Furthermore, deficient mice of the complement receptor 1-related gene/protein-γ (Cry1) gene, which regulates CP and AP activation, are 100% embryonic lethal due to spontaneous complement activation36.

It is reported that factor H also interacts with C3(H2O) via the residues Glu-744 and Glu-747 of C3 (ref. 37). However, factor B–C3(H2O) interaction, which is inhibited by CTRP6, occurs at different residues from the factor-H-binding site38. Furthermore,
CTRP6 did not bind factor H nor influence its activity (Supplementary Fig. 5i,m). Thus, the inhibitory binding of CTRP6 is different from that of factor H. Although the binding affinity between C3(H2O) and CTRP6 was relatively low (K_D = 1.1 x 10^{-5}), the real binding affinity may be greater than this because CTRP6 forms oligomers in the serum (Supplementary Fig. 5d). Because the binding of factor B and C3(H2O) initiates AP activation, the regulation of this step should be critically important for the AP activation. Actually, we found that the frequency of homozygotes from the mating between C1qtnf6<sup>F1</sup> heterozygotes on the (129P2/Ola x C57BL/6) background was less than that expected by the Mendelian ratio, partially resembling Crry<sup>-/-</sup> mice<sup>16</sup>. This observation suggests a beneficial role of CTRP6 in the development of fetus, consistent with the high levels of expression in the placenta. Furthermore, aged C1qtnf6<sup>-/-</sup> mice produced significantly higher levels of autoantibodies, probably reflecting the increase of C3a and C5a, which can promote IgG production<sup>1</sup>, although we did not observe any renal failure caused by immune complexes. Thus, CTRP6 plays important roles in the regulation of the complement system under the physiological conditions. Although adiponectin, another CTRP family member, also suppresses the activation of AP, this occurs through the activation of factor H<sup>29</sup>, a different mechanism from CTRP6. AP activation is implicated in the development of RA and other inflammatory diseases<sup>5-8,39</sup>. IgG in immune complexes with agalactosylated complex type N-glycans, which are increased in sera of RA patients<sup>40</sup> as well as in RA models<sup>41</sup>, can activate the AP<sup>42</sup>. In this report, we showed that the development of CIA, in which complement activation plays a deleterious role<sup>39</sup>, was greatly exacerbated in C1qtnf6<sup>-/-</sup> mice. This observation suggests that CTRP6 is involved in the regulation of arthritis development by suppressing the AP. Although we observed changes of anti-IgG levels in C1qtnf6<sup>-/-</sup> mice and C1qtnf6 Tg mice upon induction of CIA, these are secondary effects of AP activation/suppression and are not responsible for their severity score changes, because the severity scores in CAIA model, in which antibody production is not necessary, also changed in C1qtnf6<sup>-/-</sup> mice and in C1qtnf6 Tg mice.
The involvement of the AP in the development of RA was also suggested in other RA models. The development of arthritis in K/BxN serum-transferred mice, a model in which autoantibodies against glucose-6-phosphate isomerase play an important role, is suppressed by the deficiency of factor B, C3, C5 or C5a, but not by C1q or MBP-A, suggesting the involvement of the AP in the pathogenesis. The development of arthritis in SKG mice, which carry a mutation in the T-cell receptor component ZAP70, is suppressed by the deficiency of C5aR. In that report, the authors showed that C5a signalling induces the development of Th17 cells.

Figure 5 | Treatment of CIA with rhCTRP6. (a,b) RhCTRP6 or PBS as a control was daily injected into the articular cavity of the left (rhCTRP6) or right (rhCTRP9) knee joint of CIA-induced DBA/1J mice from day 28 (n = 11). (a) Incidence (left) and severity score (right) of CIA. *P < 0.05, χ²-test and Mann–Whitney U-test. (b) Messenger RNA expression in the joints was measured by semi-quantitative PCR analysis. *P < 0.05, **P < 0.01, and ***P < 0.001. Student’s t-test. (c) Haematoxylin and eosin. Tibia, talus and navicular are represented as Tib, Tal and Nav, respectively. Scale bar, 300 μm. One of representative histologies is shown (n = 6). **P < 0.01. Student’s t-test. (d) Cryostat sections of the joints were stained with anti-C3b antibody, isotype IgG and 4',6-diamidino-2-phenylindole (nucleus) (left). One of representative histologies is shown. The photomicrographs were taken with fluorescence and differential interference contrast (DIC) optics. The relative fluorescence intensity of C3b (right) was determined by ImageJ (n = 4). Scale bar, 50 μm. ***P < 0.001. Student’s t-test. (e,f) RhCTRP6 or rhCTRP9 as a control was daily injected into the articular cavity of the left (rhCTRP6) or right (rhCTRP9) knee joint of CIA-induced C3⁻/⁻ mice from day 21 (n = 10). (e) Incidence (left) and severity score (right) of CIA in C3⁻/⁻ mice. χ²-test and Mann–Whitney U-test. (f) Messenger RNA expression in the joints of C3⁻/⁻ mice after treatment with rhCTRP6 are shown relative to those of control-treated mice were measured by semi-quantitative PCR analysis. *P < 0.05. Student’s t-test. Average and s.e.m. are shown.
Figure 6 | CTRP6 is expressed in synoviocytes of arthritic joints of RA patients. (a) The CTRP6 levels in the serum of healthy controls (HC) and RA patients (RA) were measured by ELISA (HC: n = 22, RA n = 30). P < 0.05. Student’s t-test. (b) Synovium from a RA patient was stained with anti-CTRП6 antibody or isotype IgG using dianaminobenzidine tetrahydrochloride staining. Scale bar, 50 μm. (c–f) Cryostat sections of synovia from RA patients were stained with anti-CTRП6 antibody and 4’6-diamidino-2-phenylindole (nucleus), and either anti-CD3 antibody (c), or anti-CD20 antibody (d), or anti-CD68 antibody (e), or anti-Hsp47 antibody (f). Scale bar, 20 μm. (g) Expression of C1qtnf6 in primary fibroblast-like synoviocytes stimulated with IL-1α (n = 3) is shown. P < 0.05. Student’s t-test. Average and s.e.m. are shown.

Methods
Reverse transcription and real-time PCR. Total RNA was extracted using Sepasol RNA I Super (Nacalai Tesque, Japan). RNA was reverse transcribed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA). Reverse transcription (RT)-PCR using Taq (TaKaRa, Japan) and iCycler System (Bio-Rad, USA) was performed with the sets of primers described in

IIC. Furthermore, it was recently reported that absence of C3α and C5α signalling can promote Treg cell differentiation by inducing TGF-β from T cells48, implying that excess AP activation may suppress the development of Treg cells. Therefore, excess activation of the complement system not only directly induces inflammation by recruiting macrophages and neutrophils but also induces autoimmunity and inflammation by enhancing Th17 cell differentiation, suppressing Treg cell differentiation and enhancing antibody production. Thus, CTRP6 may be an important regulator of inflammation by preventing excess complement activation under pathological conditions.

We have shown that CTRP6 injection is effective for the treatment of CIA. When rhCTRP6 was injected into the articular cavity of the left knee, clear suppressive effect on the ankle swelling of the same leg was observed, indicating that knee-joint-injected rhCTRP6 diffuses into the ipsilateral ankle joint. We used a contralateral leg as the control, which was injected with PBS or CTRP9 to the knee joint. Although the effect of rhCTRP6 to the contralateral leg was only marginal, the severity score of the control ankle joints could be lower than those of rhCTRP6-un-treated mice due to the possible contribution of systemic effects. C3−/− mice were refractory against the CTRP6 treatment, indicating that the inhibition of the AP is responsible for the therapeutic effect. We observed that rhCTRP6 enhanced IL-10 production from WT and C1qtnf6−/− bone marrow (BM)-derived macrophages (Supplementary Fig. 10), consistent with a report that shows IL-10 induction by CTRP6 from Raw264.7 macrophages45. Importantly, Il10 mRNA expression was still elevated in the CTRP6-treated joints of C3−/− mice (Fig. 5e). These observations suggest that the therapeutic effect of CTRP6 on CIA mainly depends on its complement inhibitory activity, but not on the IL-10-inducing activity. In support for this notion, it was reported that the disease activity of RA and circulating levels of IL-10 are not correlated46.

CTRP family including adiponectin is known as an adipokine. The CTRP family regulates systemic energy homeostasis via adipor1, adipor2 and unknown receptors47. And we have already reported that CTRP3 is important for the development of autoimmune disease but is not a complement regulator19,21. Thus, it remains a possibility that CTRP6 has complement-independent functions.

Suppression of the complement system is an attractive strategy for the treatment of inflammatory diseases involving excess complement activation. Indeed, an antibody against C5α (eculizumab) has been successfully used for the treatment of paroxysmal nocturnal hemoglobinuria48. The involvement of the complement system is also suggested in other diseases. We have shown that EAE, in which the involvement of the AP has been implicated8, is significantly enhanced in C1qtnf6−/− mice. Furthermore, C1QTNF6 locus is a susceptibility locus associated with autoimmune diseases including RA and type-1 diabetes49–51. Together, these observations suggest that CTRP6 may play important roles not only in RA but also in other inflammatory diseases that involve AP activation, such as MS, type-1 diabetes52, age-related macular degeneration, systemic lupus erythematosus and glucuronidonephritis13,53. Thus, CTRP6 is a promising candidate of the medicine for the treatment of RA and other inflammatory diseases in humans.
Supplementary Table 1. Quantitative real-time RT-PCRs were performed using SYBR Premix Ex Taq (TaKaRa) and an iCycler System (Bio-Rad) with the primers described in Supplementary Table 1.

Mice. Clqtnf6+/− mice were generated using E14.1 embryonic stem (ES) cells, and Clqtnf6 Tg mice were generated by pronuclear microinjection into C57BL/6J mouse embryos and were used as heterozygotes as described in the following sections. C3−/− mice (C57BL/6J background) were obtained from Jackson laboratories. Mice were kept under specific pathogen-free conditions in the clean rooms by the Center for Experimental Medicine and Systems Biology, Institute of Medical Science, University of Tokyo, and the Research Institute for Biomedical Sciences, Tokyo University of Science. All experiments were approved by the committee of Life Science Research Ethics and Safety of the University of Tokyo and the Animal Care and Use Committee of Tokyo University of Science, and were conducted according to the institutional ethical and safety guidelines for gene manipulation experiments.

Generation of Clqtnf6+/− mice. The Clqtnf6+/− allele was created by homologous recombination in E14.1 ES cells. Homology arms of the Clqtnf6 gene-targeting vector were amplified from genomic DNA of E14.1 ES cell by PCR methods using the primer set 1 and 2 for the 5′ arm and 3′ arm, respectively (Supplementary Table 2). The lengths of the 5′ and 3′ arms are 3.5 and 6.9 kb, respectively. The targeting vector was constructed by replacing the genomic locus containing the third exon of the Clqtnf6 gene, which encodes the C1q domain, with a neomycin-resistance gene (NeoR) under the control of a PGK1 promoter for positive selection. A diphtheria toxin A gene under the MC1 promoter was ligated to the end of the targeting vector for negative selection. In addition, the stop codon and HindIII sequence for screening were introduced into exon 2 in the 5′ homology arm of the targeting vector. The targeting vector was electroporated into ES cells and selected in G418 (Nacalai Tesque). We screened 223 neomycin-resistant ES clones by PCR and Southern blotting. The first screening was performed using the primer set 3 (Supplementary Table 2). For positive clones from the PCR screening, we confirmed correct homologous recombination by Southern blotting using 5′ and 3′ flanking probes. Genomic DNA of the targeting clones was digested using HindIII and EcoRV, respectively, for detection by the 5′ and 3′ probe. The DNA fragment for the 3′ probe was amplified using the primer set 4 and 5 for 5′ and 3′ fragments, respectively (Supplementary Table 2). As a result of this screening, we obtained one targeted clone (clone ID: 3C2) (targeting efficiency: 0.5%). Using this clone, chimeric mice were generated by an aggregation method modified from previous reports.54,55. The contribution of ES cells to chimeric mice was checked by coat colour. To generate heterozygous offspring, male chimeric mice were mated with C57BL/6J female mice, and (1292P/Ola x C57BL/6) F1 progeny were obtained. In this report, we used mice of the C57BL/6 background. Mice were backcrossed with C57BL/6J mice for five generations using the high-speed congeneric strategy.55. The proportion of C57BL/6J homozygous loci in male mice of the N5 generation was 100% (84 markers). The genotyping of C57BL/6J mice (C57BL/6) F1 background at 8 weeks of age) were immunized intraperitoneally with 100 μl of 100 μg TNP(29)-KLH (Biosearch Technologies) emulsified with Inulm Alum (Thermo Scientific). On day 21, mice were given an intraperitoneal booster injection with the same amount of TNP-KLH/Inulm Alum. On days 0, 14 and 30, the serum was collected and TNP-specific IgG1 and IgM were determined by ELISA using 20 μg ml−1 TNP(30)-bovine serum albumin (BSA)-coated plates and alkaline phosphatase-conjugated polyclonal rabbit antibodies to mouse IgG1 and IgM (Zymed, 61–0122, 1:1,000 dilution and 62–6822, 1:1,000 dilution).

To elucidate the antibody production against thymus-independent antigens, mice (female C57BL/6J background at 8 weeks of age) were immunized intraperitoneally with 100 μl of 100 μg TNP(65)-Aminoethylcarboxymethyl-FICOLL (Biosearch Technologies). On days 0 and 7, the serum was collected and TNP-specific IgG3 and IgM were determined by ELISA using 20 μg ml−1 TNP(30)-BSA-coated plates and alkaline phosphatase-conjugated polyclonal rabbit antibodies to mouse IgG3 and IgM (Zymed, 1100–04, 1:1,000 dilution and 62–6822, 1:1,000 dilution).

Titrations of autoantibodies. We collected serum from aged mice (male 129P2_Ola x C57BL/6J F1 background at 1 year of age) and titrated autoantibodies, such as ANA and anti-Beta2Glycoprotein-1 (B2GPI), by the line-linked immunosorbent assay (ELISA) with plates coated with nuclear antigens and HRP-conjugated polyclonal goat antibodies to mouse IgG using the Autoimmune ELISA kit (Alpha Diagnostic, USA, 5210). To measure RF, we performed ELISA with plates coated with heat-denatured rabbit IgG (Santa Cruz, USA, sc-2027, 1:8 dilution) and alkaline phosphatase-conjugated polyclonal rabbit antibodies to mouse IgG (Zymed, USA, 62–6622, 1:1,000 dilution) and IgM (Santa Cruz, sc-2070, 1:1,000 dilution).

Flow cytometric analysis. For flow cytometry, we harvested thymus, spleen and LN from mice (male 129P2_Ola x C57BL/6J F1 background at 8 weeks of age) and stained with a 100-fold dilution of phycoerythrin-Cy7-, fluorescein isothiocyanate-Cy5-, Pacific Blue-, APC- and phycoerythrin-conjugated monoclonal antibodies (mAbs). We purchased rat or hamster monoclonal antibodies to mouse CD4 (GK.1.5), CD3 (145–2C11), B220 (RA3–6B2) and CD11b (M1/70) from Biologend (USA) and CD8 (53–6–7) from eBioscience (USA) and CD11c (HL3) from BD Pharmingen (USA). We performed cell-surface staining according to standard techniques, and we analysed stained cells using a FACS Canto II cytometer and either CellQuest (BectonDickinson, USA) or FlowJo software (Tree Star, USA).

Thymus-dependent and -independent antibody production. We used 2,4,6-trinitrophenyl (TNP)-keyhole limpet haemocyanin (KLH) (Biosearch Technologies, USA) and TNP-Aminoethylcarboxymethyl-FICOLL (Biosearch Technologies). TNP-specific IgG3 and IgM were determined by ELISA using 20 μg ml−1 TNP(30)-bovine serum albumin (BSA)-coated plates and alkaline phosphatase-conjugated polyclonal rabbit antibodies to mouse IgG1 and IgM (Zymed, 61–0122, 1:1,000 dilution and 62–6822, 1:1,000 dilution).

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Titrations of protein concentration in urine. Urine was collected from mice (male C57BL/6J background at 1 year of age) and total protein levels were measured using a bicinchoninic acid assay kit (Thermo Scientific).

Collagen-induced arthritis. On day 0, mice (female C57BL/6J background at 6–8 weeks of age) were immunized intradermally near the base of the tail with 100 μl of 2 mg ml−1 chicken IIC (Sigma, USA) emulsified with CFA composed of IFA (Thermo Scientific) plus M. tuberculosis H37Ra (Difco, USA; 1.65 or 2.5 mg ml−1)12. On day 21, mice were given an intradermal booster injection near the previous injection sites with the same amount of IIC/CFA. We judged the development of arthritis in C57BL/6J mice by macroscopic evaluation. Arthritis development in each paw was graded as follows: 0, no change; 1, mild swelling; 2, obvious joint swelling; 3, severe joint swelling and ankylosis changes (maximum of 12 points for individual mice)52.

To examine the therapeutic effect of CTRP6 for autoimmune arthritis, we immunized mice with CTRP6 to arthritic mice. DBA/1J mice (female, 6–8 weeks of age) were immunized with 100 μl of 2 mg ml−1 IIC emulsified with CFA (Difco: 0.5 mg ml−1 M. tuberculosis H37Ra) on day 0 and on day 21 as described above. Then, 300 ng/30 μl rhCTRP6 (Aviscera Bioscience, USA) or control (30 μl PBS or 300 ng/30 μl rhTRP; Aviscera Bioscience) was injected every day into the articular cavity of the left (CTR6) or right (control) knee joint from day 28. We judged the development of arthritis in DBA/1J mice by macroscopic evaluation. Arthritis development in each paw was graded as follows: 0, no change; 1, erythema and mild swelling confined to the tarsal joints; 2, erythema and mild swelling extending from the tarsal joint to digit; 3, erythema and moderate swelling extending from metatarsal joining to metatarsa; and 4, swelling encompassing the ankle, foot and digits, or ankylosis of the limb (maximum four points for one leg of individual mice)37. WT and C3−/− mice (C57BL/6J background, female, 6–8 weeks of age) were immunized with 200 μl of 2 mg ml−1 IIC emulsified with CFA (Thermo Scientific) plus M. tuberculosis H37Ra (Difco, 0.5 mg ml−1) on day 14 as described above. WT and C3−/− mice were immunized with either rhCTRP6 (Aviscera Bioscience) or rhCTR6 (Aviscera Bioscience) (300 ng/30 μl for each) as a control was injected every day into the articular cavity of the left (CTR6) or right

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control) knee joint from day 21. We checked possible aggregation of rhCTRP6, which is free from any aggregation. We judged the development of arthritis by macroscopic evaluation as described above56.

Histopathology. Mice were killed under ether anaesthesia, and their hindlimbs were processed for histopathology. Whole-ankle joints were fixed in 10% formalin in 1mM phosphate buffer, pH 7.2, decalcified in 10% formic acid and embedded in paraffin. Serial sections of 2–3μm thicknesses were taken sagittally through the tibia and stained with haematoxylin and eosin for examination by light microscopy. The lesions, including the calcaneus bone and anterior and posterior synovial tissues at the ankle joints, were evaluated histopathologically. Histological severity of each joint was graded as follows: 0, normal; 1, thickening and proliferation of the synovial lining, with slightly inflammatory cell infiltration; 2, grade 1 changes plus granulomatous lesions in the synovial sublining tissue; 3, grade 2 changes plus pannus formation and bone destruction. Arthritis index of the ankle joint was estimated from the average grade of talus and around bones including tibia and calcaneum of each mouse.

For immunostaining, limbs were frozen and embedded in special cryomedium SCEM (Leica Microsystems, Japan) and cryosectioned (5μm thickness) onto a cryofilm (Leica Microsystems). The sections were fixed in cold acetone for 5 min and blocked with 20% goat serum (Wako, Japan)/1% BSA (Sigma) in PBS at room temperature for 1 h. Rat anti-C3 antibody (Abcam, ab11862, 1,000 dilution), rat anti-factor H, IgG (BD Biosciences, USA, 344701, 1,000 dilution) and Alexa Fluor 488 anti-rat IgG (Invitrogen, USA, A-11006, 1,000 dilution) were used for immunostaining. Nuclei were stained with 4′,6-diamidino-2-phenylindole. Relative fluorescence intensity of C3b was determined by ImageJ. The slides were analyzed on a Nikon A1Rsi confocal microscope (Nikon, Japan) operated by NIS-Elements software (Nikon).

ILC-specific antibody titration. For titration of ILC-specific antibodies in CIA, we collected serum samples from mice before primary immunization (pre) and on days 14, 28 and 42 after immunization and diluted them 1:100. We performed ELISA using 20μg/ml ILC-coated plates and alkaline phosphatase-conjugated polyclonal rabbit antibodies to mouse IgM and IgG (Zymed, 62–6822, 1:1,000 dilution) or rabbit polyclonal antibodies to mouse IgM and IgG (Abcam, ab157711) and factor B and 100 ng factor D with/without 100 ng rhCTRP6 and/or 1 μg factor H (CompTech) in 15 μl of reaction buffer were incubated at 37°C for 1 h, and the reaction mixtures were subjected to SDS–PAGE and visualized by CBB staining. The inhibitory effect of CTRP6 on AP C3 convertase activity was calculated from the difference in AP activity using ImageJ. To investigate the association between CTRP6 and factor H, 1 μg C3, 1 μg factor B and 100 ng factor D with/without 100 ng rhCTRP6 and/or 1 μg factor H (CompTech) in 15 μl of reaction buffer were incubated at 37°C for 1 h, and the reaction mixtures were subjected to SDS–PAGE and visualized by CBB staining.

Titration of C3 and factor B. C3 and factor B levels in sera were measured using an ELISA kit (C3: Abcam, ab57771 and factor B: USCN Life Science, USA, 4E9211).

Competitive binding assay. For measurement of competition between human CTRP6 and human factor B for binding to C3(H2O), a mixture of 20 μg C3(H2O); 20 μg factor B (provided by T. Fujita); 0, 20 or 100 ng rhCTRP6 (Aviscera Bioscience); 1.5 μg anti-C3 antibody (Abcam, ab11871); and 20 μl Protein A/G PLUS-Agarose (Santa Cruz) in 0.5 ml of a reaction buffer (2 mM CaCl2, 0.5 mM MgCl2, 20 mM Tris-HCl (pH 7.6)) was incubated at 37°C for 2 h. After washing with PBS/0.05% Tween-20, samples were subjected to SDS–PAGE and visualized by CBB staining. Immunoprecipitated factor B was semi-quantified by the band intensity of factor B, which is normalized to the band intensity of C3b using ImageJ.

Immunoprecipitation and western blotting. We collected C3gfnf6 −/− mouse serum and pre-cleaned with Protein A/G Plus-Agarose (Santa Cruz). To immunoprecipitate CTRP6-associated proteins, 200 ng CTRP6 (BioVendor) or 10 μM acetic acid (control) was added to 1 ml pre-cleared C3gfnf6 −/− mouse serum, and immunoprecipitated with 2 μg rabbit polyclonal antibody (Anaspec, 54561) to CTRP6. Immunoprecipitates were washed five times with PBS-1% Tween-20 and dissolved in SDS–PAGE running buffer (0.04 M Tris-HCL: 0.22 M glycine; 1% SDS; SDS, pH 8.3). Aliquots (10 μl) of the samples were resolved in SDS–PAGE on 12.5% gels. Resolved proteins were transferred onto polyvinylidene difluoride membranes. Membranes were blocked with 5% skim milk/Tris-buffered saline containing 0.1% Tween-20 (TBST) at room temperature for 1 h, followed by incubation overnight at 4°C with rat mAb against C3 (Abcam, ab11862, 1,000 dilution) or rabbit polyclonal antibody against CTRP6 (Anaspec, 54562, 1,000 dilution). The membranes were then washed three times with TBST, followed by 1 h incubation at room temperature with HRP-conjugated goat polyclonal antibody against rat IgG (Zymed, 81–9520, 1,000 dilution). The membranes were washed six times with TBST and visualized using the ECL Prime Western Blotting Detection System (GE Healthcare, Japan).

SPR-based interaction assay. Each human recombinant protein (CTRP6, Aviscera Bioscience; C3b, Alpha Diagnostic) was linked to CMS chips via amino coupling. The coupling was performed according to the standard N-ethyl-N′-(3-dimethylaminopropyl)-carbodiimide hydrochloride/N-hydroxysuccinimide procedure. In brief, the CM5 matrix was activated by injection of 70 μl of a 1:1 mixture of 400 mM N-ethyl-N′-(3-dimethylaminopropyl)-carbodiimide hydrochloride and 100 mM N-hydroxysuccinimide at a flow rate of 20 μl/min, followed by application of the appropriate recombinant protein solution. Activated functional groups on the sensor chip not saturated by proteins were blocked with 1 M ethanolamine. Binding of human CTRP6 was stopped when the desired level of about 100 resonance units was reached. Next, the indicated analyte (CTRP6, factor H, Sigma; C3b, provided by T. Fujita) was injected. C3b(H2O) was generated by five freeze/thawing cycles of C3 (ref. 62). Regeneration of the sensor chip was achieved by injecting the buffer described below for 3 min. The SPR assay and kinetics analysis was performed using a BLAcore 2000. The experiments were performed in 10 mM HEPES (pH 7.4) buffer containing 150 mM NaCl, 3 mM EDTA and 0.005% surfactant P20 at a flow rate of 20 μl/min.

Reverse passive Arthus (RPA) reaction. For IgG-mediated cutaneous RPA reaction, mice were intradermally injected with 10 μg anti-OVA Ab (Millipore, AB1225), and then intravenously with 500 μg OVA1% Evans Blue (OVA: Sigma and Evans Blue; Wako) in PBS (pH 7.4). Skin was harvested 4 h later and incubated with dimethylformamide; eluted Evans blue in the supernatants was quantified by measuring the absorbance at 570 nm (ref. 63).

Experimental autoimmune encephalomyelitis (EAE). The MOG35-55 peptide (MEYGVYRSPFRSVHLRNYGK) was synthesized and purified by HPLC at our institute (Dr. S. Imajoh-Ohmi, Division of Medical Proteomics Laboratory, Institute of Medical Science, University of Tokyo). Mice were immunized subcutaneously in flanks on day 0 with 300 μg MOG35-55 peptide emulsified in CFA (1:1), which consisted of IFA with 5 mg/ml M. tuberculosis (Difco). On day 7, mice were given a booster injection subcutaneously in flanks with the same amount of MOG35-55. We judged the development of the severity of EAE by macroscopic evaluation. The severity score was graded as follows: 0, no change; 0.5, partially limping; 1, paralysed tail; 2, hind limb paraparesis; 2.5, one hindlimb paralysed; 3, both...
hindlimbs paralysed; 3.5, hindlimbs paralysed and weakness in forelimbs; 4, forelimbs paralysed64.

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Author contributions
M.A.M. was the primary contributor to this work and wrote the manuscript. S.K. and S.S. gave advice and technical support. A.I., N.U. and T.S. performed all experiments using human samples. T.Y. and R.K. carried out western blotting and SPR-based interaction assays. H.I. generated the targeting construct for generation of C1qtnf6−/− mice, and T.T., S.K., N.S. and Y.L. generated KO and Tg mice. R.Y., S.I., T.M. and A.S. assisted with gel chromatography, flow cytometric analysis, immunohistochemistry and EAE, respectively. H.-H.C. and Y.H. helped with qPCR analysis. K.T. and M.M. performed C3 convertase activity assays. T.F. provided recombinant complement components and M.H. gave advice. Y.I. organized and supervised the project and wrote the manuscript.

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