CTRP3 plays an important role in the development of collagen-induced arthritis in mice

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A B S T R A C T

Rheumatoid arthritis (RA) is an autoimmune inflammatory disease exhibited most commonly in joints. We found that the expression of $C1qtnf3$, which encodes C1q/TNF-related protein 3 (CTRP3), was highly increased in two mouse RA models with different etiology. To elucidate the pathogenic roles of CTRP3 in the development of arthritis, we generated $C1qtnf3^{-/-}$ mice and examined the development of collagen-induced arthritis in these mice. We found that the incidence and severity score was higher in $C1qtnf3^{-/-}$ mice compared with wild-type (WT) mice. Histopathology of the joints was also more severe in $C1qtnf3^{-/-}$ mice. The levels of antibodies against type II collagen and pro-inflammatory cytokine mRNAs in $C1qtnf3^{-/-}$ mice were higher than WT mice. These observations indicate that CTRP3 plays an important role in the development of autoimmune arthritis, suggesting CTRP3 as a possible medicine to treat RA.

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1. Introduction

Rheumatoid arthritis (RA) is an autoimmune disease that causes inflammation and bone destruction most commonly in joints. Inflammatory cytokines such as IL-1, IL-6, TNF-α, and IL-17 play important roles for the development of RA, and antibodies and inhibitors against these cytokines are successfully used for the treatment of RA [1,2]. As high titers of autoantibodies are detected in the serum of RA patients, antibodies against B cells are also useful for the treatment of RA [3]. These approaches have significantly improved the efficacy of RA treatment. However, because some patients are refractory to these treatments or become refractory during these treatments, the development of new therapeutics is still awaited.

We previously generated two RA models; one is human T-cell leukemia virus type I (HTLV-I) transgenic (Tg) mice and the other is IL-1 receptor antagonist (IL-1Ra) deficient (KO) mice, both of which spontaneously develop autoimmune arthritis [4,5]. Because multiple genes are implicated in the development of autoimmune diseases, we searched for novel disease-related genes using DNA microarray techniques. As a result of comprehensive gene expression analysis between RA models and wild-type (WT) mice, we identified 554 genes of which expression changed more than 3 times in both RA models compared with WT mice [6]. The $C1qtnf3$ gene, which encodes CTRP3 (also named CORS-26, cartducin and cartnectin), is one of such genes and is highly expressed in both models.

CTRP3 is a soluble secreted protein consisted of a short N-terminal variable region, collagen domain and C-terminal C1q domain. CTRP3 belongs to a member of C1q/TNF-related protein (CTRP) family, having a crystal structure resembling TNF and complement C1q [7]. The C1q domain may be important for the binding to the receptor, although the receptor has not been identified yet, because the C1q domain of complement C1q and adiponectin, one of CTRP family members, is important for the recognition and binding to their receptors. CTRP family members are involved in host defense, inflammation and glucose metabolism [7], and CTRP3 is

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identified as a growth factor, and promotes proliferation of chondrogenic precursors and chondrocytes [8]. Recently, it was reported that CTRP3 inhibits LPS-induced inflammatory cytokine production from human adipocytes, monocytes and fibroblasts [9–11]. However, the physiological roles of CTRP3 *in vivo* still remain to be elucidated. In this study, we have generated *C1qtnf3*$^{-/-}$ mice, and investigated the effect of CTRP3 deficiency on the development of autoimmune arthritis.

2. Materials and methods

2.1. Generation of C1qtnf3$^{-/-}$ mice

Table 1 Genomic DNA containing *C1qtnf3* gene was isolated from EGR-101 ES cells derived from C57BL/6 embryos [12]. The targeting vector was constructed by replacing a 170 bp genomic fragment containing the exon 4 of the *C1qtnf3* gene, which encodes the C1q domain, with the 1.7 Kb DNA fragment containing *neomycin resistance gene* (*Neo*^{r}) under the PGK1 promoter which was flanked by the loxP sequences. For the negative selection, a *diphtheria toxin* (*DT*) *A* gene under the MC1 promoter was ligated to the 3′ end of the targeting vector. The targeting vector was electroporated into ES cells and G418-resistant clones were selected (Nacalai Tesque, Japan). Homologous recombinant ES clones were screened by PCR and Southern blotting using primers and probes as indicated in Fig. 1. Chimeric mice were generated by an aggregation method [13]. The genotyping of *C1qtnf3*-deficient mice was carried out using the following PCR primers: primer 1, 5′-GATGCAGAGCAA-TATCACACAG-3′; primer 2, 5′-GTTGATTCTTGCATCTCACCTG-3′; primer 3, 5′-GCTCGGTACCCATCAAGCTTAT-3′. Primer 1 and 2 were used to detect WT-allele (336 bp), and primer 1 and 3 were used to detect mutant-allele (195 bp). The lack of *C1qtnf3* transcripts was confirmed by RT-PCR using the following PCR primers: 5′-CTTCAGCATGTACAGCTATG-3′ and 5′-GTTGCCCATTCTTAGCCAG-ACT-3′.

We used 8–10 week-old C57BL/6 background mice of the same sex in all experiments. Mice were kept under SPF conditions in the clean rooms at 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 institutional animal use committees and were conducted according to the institutional ethical guidelines for animal experiments and safety guidelines for gene manipulation experiments.

2.2. In vitro complement activation assay

Plates (Nunc, Denmark) were coated with OVA/anti-OVA immune complex (OVA: Sigma, USA, and anti-OVA Ab: Millipore, Germany), 50 μg/ml mannans (Sigma, USA), or 200 μg/ml LPS (Sigma, USA) for the assay of complement activation of the classical pathway (CP), lectin pathway (LP) and alternative pathway (AP), respectively [14,15]. Serum was diluted with GVB^{++} buffer for the assay of the CP and LP activity and with GVB/Mg^{2+} EGTA buffer for the AP activity. 10% serum was incubated on plates at 37 °C

for 1 h and the reaction was stopped by cold 20 mM EDTA/PBS. We detected the deposition of C3b by rat monoclonal antibody against mouse C3 (Abcam, UK).

2.3. Collagen-induced arthritis (CIA)

We immunized female mice with 100 μl of 2 mg/ml type II collagen (IIC) (Sigma, USA) emulsified with complete Freund's adjuvant (CFA). CFA consisted of incomplete Freund's adjuvant (Thermo Scientific, USA) and 1.65 mg/ml heat-killed *Mycobacterium tuberculosis* (H37Ra; Difco, USA), and was injected intradermally at three sites near the base of the tail on day 0. On day 21, mice were given booster injections near the former injection sites with same amount of IIC/CFA intradermally. We judged the development of arthritis 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 ankylotic changes [16].

2.4. Histopathology

Mice were sacrificed under ether anesthesia and ankle joints of hind limbs were removed, fixed, decalcified and paraffin embedded for histopathology. Serial sections were stained with H&E. The lesions, including the calcaneus bone and anterior and posterior synovial tissues of the ankle joints, were evaluated histopathologically. Each joint was graded on a scale of 0–3, where 0 = normal, 1 = thickening and proliferation of the synovial lining, with slight inflammatory cell infiltration, 2 = grade 1 changes plus granulomatous lesions in the synovial sublining tissue, and 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 [16].

2.5. C3a and C5a titration

C3a and C5a levels in plasma were measured by ELISA with the capture antibody–coated plates and detection antibodies against C3a or C5a (BD Pharmingen, USA), using 10 mM EDTA-chelated sera from female mice (8–10 weeks of age).

2.6. Synoviocyte culture

The primary synoviocytes were harvested from synovium of knee and ankle and were cultured in DMEM medium (Gibco) containing 10% FBS and 1% penicillin-streptomycin. Cells were stimulated with IL-1α (0, 1, 5 and 10 pg/ml) for 24 h in the absence or presence of 1 ng/ml human recombinant CTRP3 (Adipobioscience, USA). IL-6 levels in the culture supernatants after 24 h were measured with Mouse IL-6 ELISA MAX™ Standard (BioLegend, USA).

2.7. Flow cytometric analysis

For flow cytometry, cells were stained with Pacific-Blue-, FITC-, and APC-conjugated monoclonal antibodies (mAbs), as previously described [17]. We purchased rat or hamster mAbs to mouse CD3 (145-2C11) and B220 (RA3-6B2) from Biolegend (USA) and CD11c (HL3) from BD Pharmingen (USA). Cells were stained according to standard techniques, and analyzed by a FACS Canto II cytometer and either CellQuest (BectonDickinson, USA) or FlowJo software (Tree Star, USA).

Table 1
Primers for quantitative real-time PCR.

| Gene name	Forward primer	Reverse primer
C1qtnf3	CTTCAGCATGTACAGCTATG	GTTGCCCATTCTTAGCCAGACT
Gapdh	TTCACCACCATGGAGAAGGC	GGCATGGACTGTGGTCATGA
Tnf	GCCTCCCTCTCATCAGTTCT	CACTTGGTGGTTTGCTACGA
Il1b	CAACCAACAAGTGATATTCTCCATG	GATCCACACTCTCCAGCTGCA
Il6	ACAATTGCCAATCTGCGAA	CAAGCCTCGCGACCATTCTTGA
Il10	GCTCTTACTGACTGGCATGAG	CGCAGCTCTAGGAGCATGTG
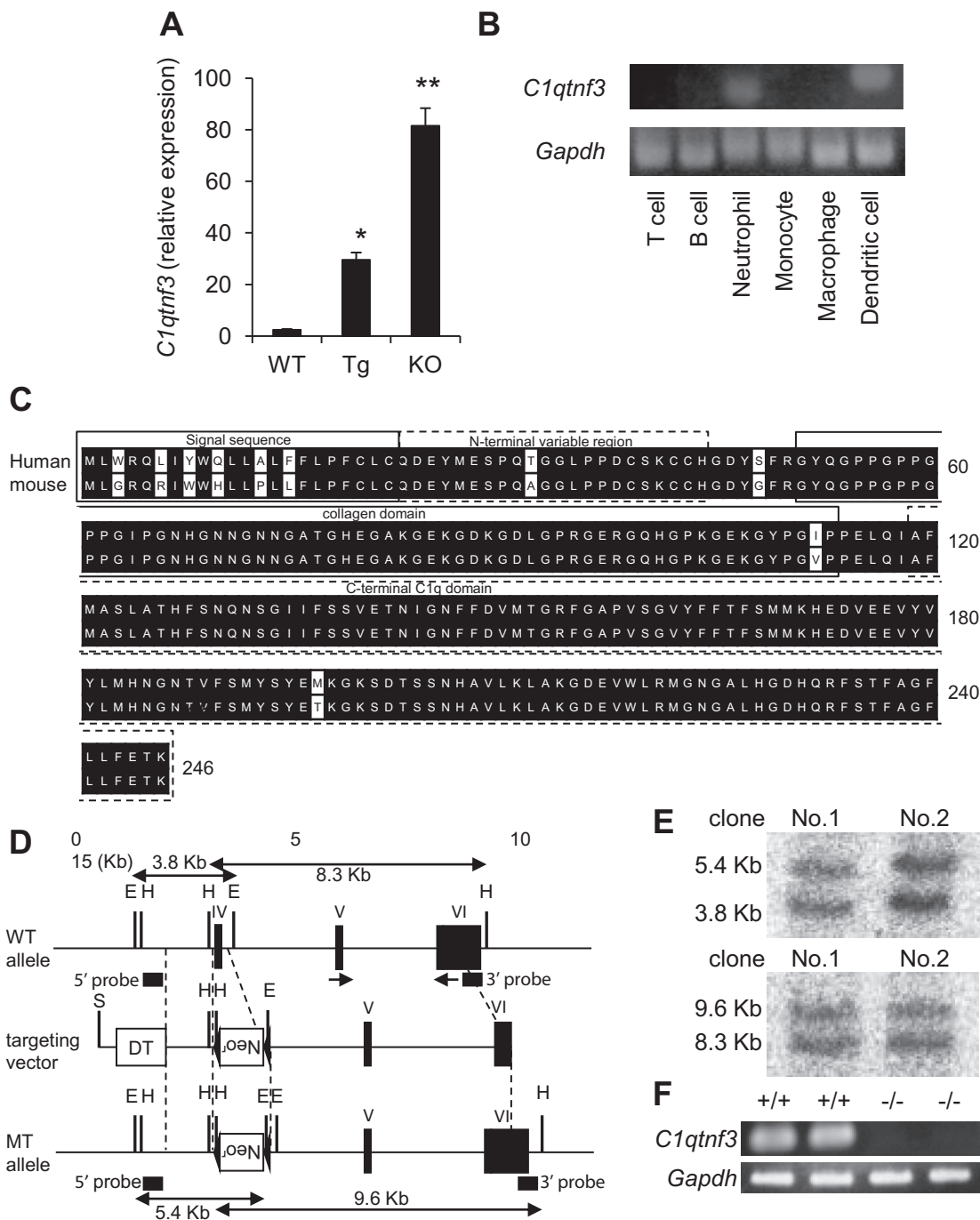
Fig. 1. Generation of *C1qtnf3*$^{-/-}$ mice. (A) The expression of *C1qtnf3* mRNA in the joints of WT mice (WT), HTLV-I Tg mice (Tg), and IL-1 Ra KO mice (KO) were determined by quantitative RT-PCR. (WT: $n = 6$, Tg: $n = 6$, and KO: $n = 7$). Average and SEM are shown. $^*P < 0.01$, $^{**}P < 0.001$. (B) The expression of *C1qtnf3* mRNA in immune cells was determined by RT-PCR. The data were reproduced in another independent experiment with similar results. (C) ClustalW alignment of the C1q domain of human and mouse CTRP3 amino acid sequences. The Gene ID of human and mouse *C1qnf3* are 114899 and 81799, respectively. Identical amino acids are shown as white text on black background. The amino acid identity of signal sequence, N-terminal variable region, collagen domain and C1q domain between human and mouse is 72% (16/22), 95% (21/22), 99% (68/69) and 99% (127/128), respectively. (D) Structure of mouse *C1qtnf3* locus (WT allele), the *C1qtnf3* targeting construct (Targeting vector), and the predicted mutated *C1qtnf3* gene (MT allele). Exons are represented by black boxes. For negative selection, a *DT* gene was attached to the 5′ end of the genomic fragment. *Sac*II (S) in the targeting vector was used for linearization. (E) The homologous recombination of the *C1qtnf3* locus of ES cells was examined in the *Eco*RI (E)- or *Hind*III (H)-digested genome by Southern blotting using the 5′ probe (upper) or 3′ probe (lower), respectively. The clone 1 was used for the generation of the *C1qtnf3*$^{-/-}$ mice. (F) The lack of *C1qtnf3* transcripts from kidney was confirmed by RT-PCR, using the primers in the figure (-> <-).

2.8. IIC-specific lymph node (LN) cell response

LN cells were harvested from mice at day 7 after IIC-immunization. LN cells were cultured in the absence or presence of 100 or 200 μg/ml denatured IIC for 72 h, followed by incorporation of [³H] thymidine (0.25 μCi/ml) (Amersham, UK) for 6 h. Then cells were harvested with Micro 96 cell harvester (Skatron, Norway) and radioactivity was measured with Micro Beta (Pharmacia

Biotech, USA). IFN-γ levels in the culture supernatants from the proliferation assay after 72 h were measured with Mouse IFN-gamma DuoSet (R&D Systems, USA).

2.9. IIC-specific antibody titration

IIC-specific IgG levels were measured in sera from mice on day 42 after IIC/CFA-immunization by ELISA using 20 µg/ml IIC-coated plates and alkaline phosphatase-conjugated polyclonal rabbit antibodies to mouse IgG (Zymed, USA).

2.10. In vitro B cell proliferation assay

Splenic B cells were purified by anti-mouse B220 microbeads, according to manufacture's instructions (Miltenyi Biotec, Germany). B220$^+$ cells were cultured with anti-mouse IgM F(ab)'$_2$ fragment (0, 1, 5 and 10 µg/ml) (Jackson ImmunoResearch, USA) for 72 h, followed by incorporation of [^{3}H] thymidine (0.25 µCi/ml) (Amersham, UK) for 6 h. Then, the radioactivity in harvested cells was measured.

2.11. In vitro neutrophil activation

Neutrophils were purified from bone marrow by anti-mouse Ly-6G microbeads (Miltenyi Biotec, Germany). Neutrophils were cultured with C5a (0, 0, 1 and 1 µg/ml) (R&D Systems, USA) for 1 h. Then, IL-1β levels in the supernatants were measured by Mouse IL-1β ELISA MAX™ Standard (Biolegend, USA).

2.12. Statics

Incidence of CIA was evaluated by the χ^2 test, and the severity score by the Mann–Whitney U-test. Two-sided Student's t-test was used for all other statistical evaluations.

3. Results

3.1. Generation of C1qtnf3$^{-/-}$ mice

We previously identified C1qtnf3 as a candidate for autoimmune related gene based on the comprehensive gene expression analysis using DNA microarrays of two RA models; HTLV-I Tg mice and IL-1Ra KO mice [6]. We confirmed the augmentation of C1qtnf3 expression in the joints of RA models using qPCR techniques. We also found that the C1qtnf3 mRNA was expressed in neutrophils and dendritic cells (DCs) (Fig. 1A and B).

Then, to elucidate the pathogenic roles of CTRP3 in the development of autoimmune arthritis, we have generated C1qtnf3$^{-/-}$ mice. C1q domain is highly conserved between humans and mice (Fig. 1C), Thus, we generated C1qtnf3$^{-/-}$ mice by deletion the C1q domain-encoding exon 4 (Fig. 1D). We confirmed correct targeting of the C1qtnf3 locus and the lack of C1qtnf3 transcript (Fig. 1E and F). C1qtnf3$^{-/-}$ mice were fertile, and were born in the expected Mendelian ratios, and showed no obvious abnormalities before 1 year of age.

3.2. C1qtnf3$^{-/-}$ mice are susceptible to CIA

To assess the role of CTRP3 in the development of autoimmune arthritis, we next examined the development of CIA in C1qtnf3$^{-/-}$
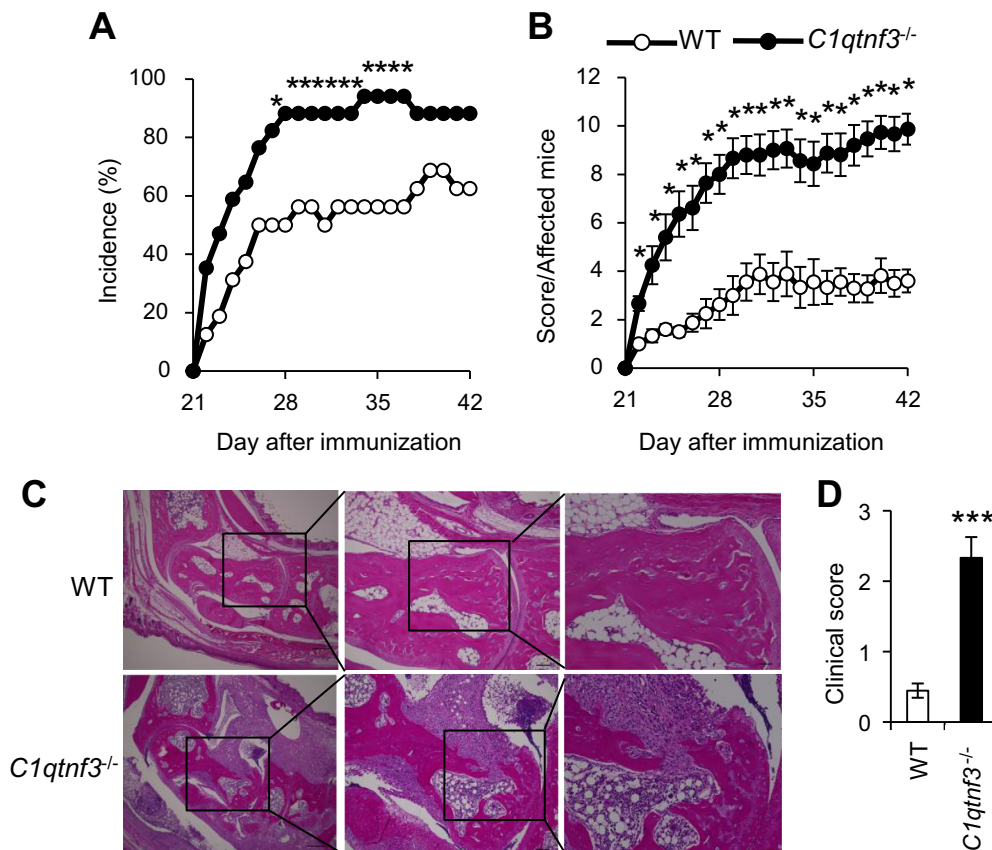

Fig. 2. CIA is exacerbated in C1qtnf3$^{-/-}$ mice. (A, B) Incidence (A) and severity score (B) of CIA. These data were combined from two independent experiments (WT: n = 16 and C1qtnf3$^{-/-}$ n = 17). (C, D) Histopathology (C) and histological score (D) of the joints at day 42 after IIC/CFA immunization (WT, C1qtnf3$^{-/-}$: n = 6 each). One of representative histologies (n = 6) is shown in (C). H&E staining. Tibia, talus and navicular bone are represented as Tib, Tal, and Nav, respectively. Scale bars: 300 µm (left), 100 µm (middle), and 30 µm (right). *P < 0.05, ***P < 0.001. Average and SEM are shown.

mice. The incidence and the clinical score of arthritis of $C1qtnf3^{-/-}$ mice was markedly increased compared to WT mice (Fig. 2A and B). Also, histological examination of the ankle joints of WT mice at day 42 after first IIC/CFA-immunization under this regimen showed mild pathological changes. In contrast, that of $C1qtnf3^{-/-}$ mice showed much severe changes, including proliferation of synovial lining cells, infiltration of inflammatory cells, and bone destruction associated with pannus formation (Fig. 2C and D). These results suggested that CTRP3 suppresses the development of CIA.

3.3. Inflammation is enhanced in the joints of C1qtnf3⁻/⁻ mice

Because CTRP3 has C1q domain, we examined the possibility that CTRP3 is involved in the complement system. We measured the levels of complement active product C3a and C5a in plasma at day 7 after IIC/CFA-immunization. The result showed C3a and C5a levels in $C1qtnf3^{-/-}$ mice were similar to that in $C1qtnf3^{+/+}$ mice (Fig. 3A and B). Furthermore, we found that CTRP3 deficiency did not influence complement activation *in vitro* (Fig. 3C). These observations indicate that CTRP3 is not complement regulator.

Next, we examined the expression of inflammatory cytokines in the joints at day 42 after IIC/CFA-immunization. The result showed that cytokine production was enhanced in $C1qtnf3^{-/-}$ mice than WT mice (Fig. 3D). Because these cytokines are supported to be released from synoviocytes, we examined whether CTRP3 deficiency directly affected inflammatory cytokine production from these cells. We cultured synoviocytes from joints, and after stimulation with IL-1α, IL-6 levels in the supernatants of the culture were measured by ELISA. The result showed that IL-6 from synoviocyte of $C1qtnf3^{-/-}$ mice was similar to that of WT mice, and exogenous CTRP3 did not suppress IL-6 release from synoviocytes (Fig. 3D).

These observations suggest that infiltrated immune cells in the joints are responsible for the suppression of inflammation in $C1qtnf3^{-/-}$ mice.

3.4. IIC-specific B cell response is enhanced in C1qtnf3⁻/⁻ mice

Infiltration of T cells and B cells into synovial lining and periarticular space is commonly observed in RA patients as well as RA models. We found that T cell and B cell was increased in LNs of $C1qtnf3^{-/-}$ mice than that of WT mice at day 42 after the IIC/CFA-immunization, but the cell population was unchanged (Fig. 4A and B). Then, LN cells were harvested from mice at day 7 after IIC/CFA-immunization and the proliferative response against IIC was examined. We found that recall T cell proliferation response was comparable between $C1qtnf3^{-/-}$ mice and WT mice (Fig. 4C). IFN-γ production after IIC re-stimulation was also comparable between $C1qtnf3^{-/-}$ and WT LN cell cultures (Fig. 4D). These results suggest that T cell priming in $C1qtnf3^{-/-}$ mice is normal.

Next, we examined production of IIC-specific IgG in $C1qtnf3^{-/-}$ mice at day 42 after IIC/CFA-immunization. The results showed that IIC-specific IgG levels in sera from $C1qtnf3^{-/-}$ mice were significantly higher than that of WT mice (Fig. 4E). Then, we examined the effect of CTRP3 on B cell proliferation *in vitro*. However, the B cell proliferation induced by anti-IgM was comparable between $C1qtnf3^{-/-}$ B cells and WT B cells, and exogenous CTRP3 did not suppress B cell proliferation (Fig. 4F).

Furthermore, we examined the effect of CTRP3 on neutrophil activation because *C1qtnf3* was expressed in neutrophils (Fig. 1B). Neutrophils from bone marrows were stimulated with C5a, and IL-1β levels in the culture supernatants were measured by ELISA. The results showed that IL-1β production from $C1qtnf3^{-/-}$ neutrophils was similar to that of WT mice, and exogenous CTRP3 did not
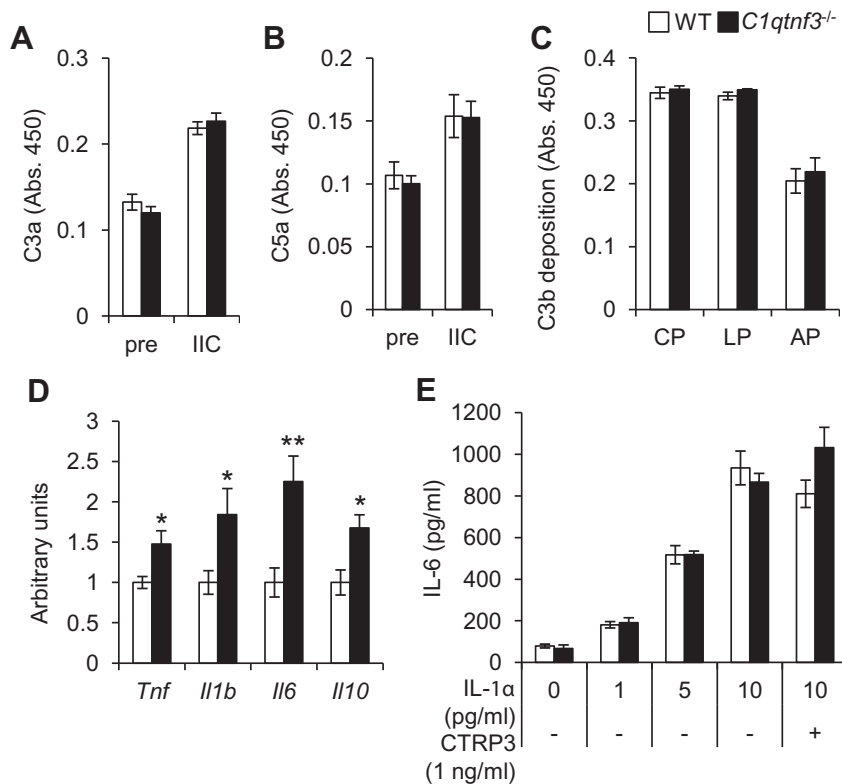

Fig. 3. Inflammation is augmented in $C1qtnf3^{-/-}$ mice. (A, B) C3a (A) and C5a (B) levels in plasma at day 7 after IIC/CFA-immunization were measured by ELISA (WT, $C1qtnf3^{-/-}$: n = 6 each). (C) The complement activation of the CP, LP and AP was determined by C3b deposition (WT, $C1qtnf3^{-/-}$: n = 8 each). (D) Messenger RNA expression in the joints at day 42 after IIC/CFA-immunization was measured by semi-quantitative PCR (WT, $C1qtnf3^{-/-}$: n = 9 each). (E) IL-6 production from synoviocytes after IL-1α (0–10 pg/ml) stimulation in the absence or presence of 1 ng/ml CTRP3 was measured by ELISA (WT, $C1qtnf3^{-/-}$: n = 3 each). All data were reproduced in another independent experiment with similar result. *P < 0.05, **P < 0.01. Average and SEM are shown.
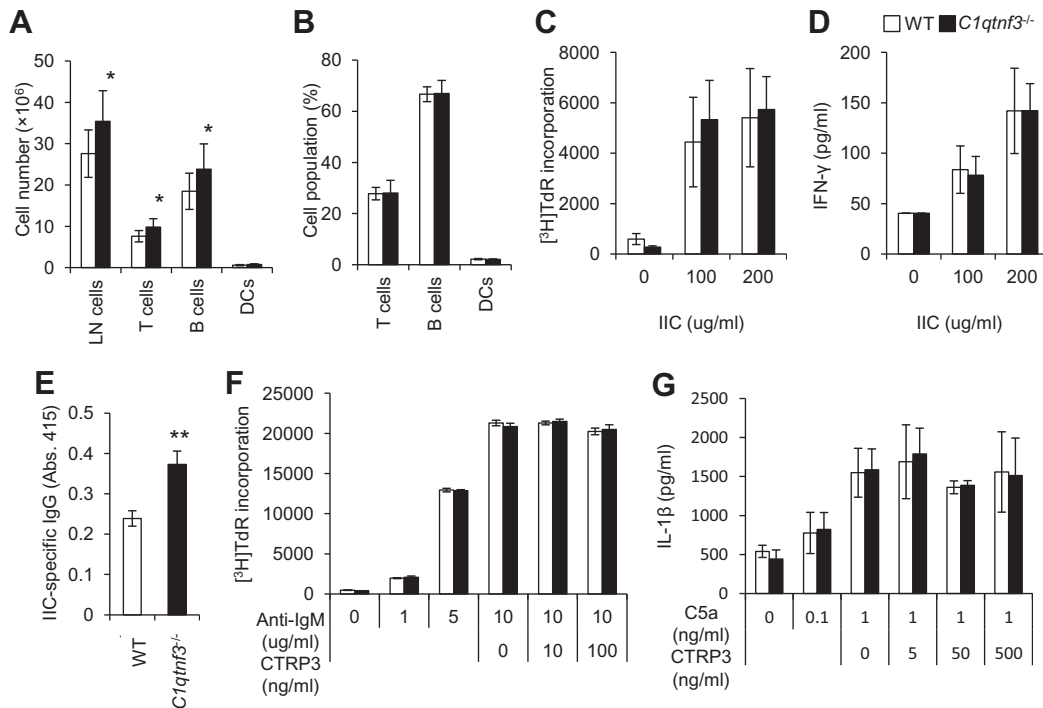
Fig. 4. IIC-specific antibody production was enhanced in $C1qtnf3^{-/-}$ mice. (A, B) At day 42 after IIC/CFA immunization, the number of total cells, T cells, B cells and DCs (A) and their relative contents (B) in inguinal LNs were analyzed using CD4-, B220- and CD11c-specific antibodies by flow cytometry (WT: $n = 9$, $C1qtnf3^{-/-}$: $n = 11$). Similar results were obtained in another independent experiment. (C, D) Seven days after IIC/CFA immunization, inguinal LN cells were removed and cultures with IIC (0, 100, 200 mg/ml). Then, IIC-specific proliferative response was measured by [³H] TdR incorporation (C). IFN-γ concentration in the culture supernatant was determined (WT, $C1qtnf3^{-/-}$: $n = 4$ each) (D). Similar results were obtained in another independent experiment. (E) At day 42 after IIC/CFA immunization, the sera were collected and IIC-specific IgG levels were determined by ELISA. The data from two independent experiments are combined and shown (WT: $n = 16$ and $C1qtnf3^{-/-}$: $n = 17$). (F) B cells were incubated with anti-IgM Ab (0–10 mg/ml) and CTRP3 (0, 10, 100 ng/ml). Then, the proliferative response was measured by [³H] TdR incorporation (WT, $C1qtnf3^{-/-}$: $n = 3$ each). (G) IL-1β production from neutrophils after C5a stimulation (0–1 ng/ml) in the presence of CTRP3 (0–500 ng/ml) was measured by ELISA (WT, $C1qtnf3^{-/-}$: $n = 3$ each). All data were reproducible in another independent experiment. $^*P < 0.05$, $^{**}P < 0.01$. Average and SEM are shown.

suppress IL-1β release from neutrophils (Fig. 4F). These observations suggest that CTRP3 plays an important role in the development of autoimmune arthritis by regulating antibody production.

4. Discussion

In this report, we have generated $C1qtnf3^{-/-}$ mice and examined the development of CIA in these mice, because $C1qtnf3$ expression is highly enhanced in the joints of three RA models; HTLV-I Tg mice, $Il1rn^{-/-}$ mice and K/BxN mice [18]. We showed that the development of CIA was greatly exacerbated in $C1qtnf3^{-/-}$ mice, suggesting a regulatory role for CTRP3 in the development of autoimmune arthritis. We found that antibody production against IIC was significantly increased in $C1qtnf3^{-/-}$ mice after induction of CIA. Because antibody production against IIC is crucial for the development of arthritis, this deficiency is suggested to be responsible for the suppression of CIA in these mutant mice.

However, we detected no abnormality in recall proliferative response against IIC in T cells from mutant mice. B cell intrinsic function was also normal, because B cell proliferative response was normal after stimulation with anti-IgM. Furthermore, cytokine production after neutrophil activation with complement component C5a was normal, suggesting that neutrophils, one of high CTRP3 producers, are not responsible for the elevated inflammation in joints. Although CTRP3 is reported to suppress inflammatory cytokine release in human monocytes [10], CTRP3 did not suppress the cytokine production in synovial resident/infiltrated cells from arthritic joints.

CTRP3 is also implicated in chondrogenesis, because the proliferation of chondrogenic precursor cells and chondrocytes was enhanced by this protein *in vitro* [8]. The involvement in the development of mandibular condylar cartilage is also suggested [19]. However, we detected no obvious skeletal abnormalities in $C1qtnf3^{-/-}$ mice; they were fertile and were born in the expected Mendelian ratios without any obvious skeletal deformity. It is possible that the effects of CTRP3-deficiency are compensated by other CTRP family members, because other CTRP family members, such as adiponectin, are also involved in the regulation of chondrogenesis [20]. Involvement of CTRP family members in the regulation of the complement system is also suggested, because adiponectin is involved in the regulation of the complement CP by activating C1q [21] and of the AP in collaboration with complement factor H [22]. However, we detected no abnormalities of the complement system in $C1qtnf3^{-/-}$ mice, suggesting that CTRP3 is not involved in the complement system.

Thus, probably excessive proliferation of T cells and B cells found in $C1qtnf3^{-/-}$ mice may be responsible for the increased antibody production and exacerbation of arthritis in these mice. However, it is difficult to elucidate the exact roles of CTRP3 in antibody production, because the receptor for CTRP3 as well as the receptor expressing cells has not been identified yet. Clearly, it remains to be elucidated how CTRP3 regulates antibody production. Nonetheless, our observations clearly demonstrate that CTRP3 can alleviate the development of autoimmune arthritis, suggesting that CTRP3 is a possible candidate of the medicine for the treatment of RA and other autoimmune diseases.

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