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Lulu Cao, …, Xiaoxin Zhu, Lu Wang

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**Graphical abstract**

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CTRP4/interleukin-6 receptor signaling ameliorates autoimmune encephalomyelitis by suppressing Th17 cell differentiation

Lulu Cao\textsuperscript{1,2,3}, Jinhai Deng\textsuperscript{2,3}, Wei Chen\textsuperscript{2,3}, Minwei He\textsuperscript{2,3}, Ning Zhao\textsuperscript{2,3}, He Huang\textsuperscript{2,3}, Lu Ling\textsuperscript{4}, Qi Li\textsuperscript{5}, Xiaoxin Zhu\textsuperscript{5,*}, Lu Wang\textsuperscript{2,3,*}

1. Department of Rheumatology and Immunology, Peking University People’s Hospital & Beijing Key Laboratory for Rheumatism Mechanism and Immune Diagnosis (BZ0135), Beijing, PR China
2. Department of Immunology, School of Basic Medical Sciences, Health Science Center, Peking University, Beijing 100191, PR China
3. Key Laboratory of Medical Immunology, Ministry of Health, School of Basic Medical Science, Peking University, Beijing 100191, PR China
4. Department of Clinical Laboratory, Beijing Chaoyang Hospital, Capital Medical University, Beijing 100020, PR China
5. Institute of Chinese Materia Medica, China Academy of Chinese Medical Sciences, Beijing, 100700, PR China

*Corresponding authors

Address correspondence to: Lu Wang, Department of Immunology, School of Basic Medical Sciences, Health Science Center, Peking University, 38 Xueyuan Road, Haidian District, Beijing, PR China. Phone:010-82802846; Email: wanglu@bjmu.edu.cn. Xiaoxin Zhu, Institute of Chinese Materia Medica, China Academy of Chinese Medical Sciences, Beijing, 100700, PR China. Phone:010-82801149; Email: zhuxiaoxin@icmm.ac.cn.

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**Abstract**

C1q/TNF related protein 4 (CTRP4) is generally thought to be released extracellularly and plays a critical role in energy metabolism and protecting against sepsis. However, its physiological functions in autoimmune diseases have not been thoroughly explored. In this study, we demonstrated that Th17 cell-associated experimental autoimmune encephalomyelitis was greatly exacerbated in Ctrp4−/− mice compared to WT mice due to increased Th17 cell infiltration. The absence of Ctrp4 promoted the differentiation of naïve CD4+ T cells into Th17 cells in vitro. Mechanistically, CTRP4 interfered with the interaction between IL-6 and IL-6R by directly competing to bind with IL-6R leading to suppression of IL-6-induced activation of STAT3 pathway. Furthermore, the administration of recombinant CTRP4 protein ameliorated the disease symptoms. In conclusion, our results indicated that CTRP4, as an endogenous regulator of the IL-6 receptor signaling pathway, may be a potential therapeutic intervention for Th17 driven-autoimmune diseases.
Introduction

Multiple sclerosis (MS) is an inflammatory disorder of the central nervous system (CNS) identified by chronic demyelination and axonal damage. Upon activation in the peripheral lymphoid organs, the autoimmune T cells enter the CNS through the blood-brain barrier and become reactivated, resulting in the enrichment of leukocytes, and disseminated inflammation, demyelination, and symptoms of severe disease (1, 2). Experimental autoimmune encephalomyelitis (EAE) has been reported to be one of the most frequently used animal models of MS. The initiation of EAE is associated with peripheral priming of myelin-specific dysregulated Th1 and Th17 cells (2), which contribute to the pathogenesis of MS and highly correlate with disease severity and relapse frequency (3). Th17 cells categorized as pathogenic or non-pathogenic subtypes depending on the cytokine milieu display considerable plasticity (4). TGFβ and IL-6 are the main factors to drive classical Th17 cell differentiation (5), while IL-6, IL-23 and IL-1β cytokines trigger Th17 to develop pathogenic functions with tissue-destructive properties (6).

Interleukin-6 (IL-6) is an integral cytokine responsible for the transcriptional programming of Th17 cells. In addition to its key role in Th17 cell induction, IL-6 is necessary to retain the transcriptional and functional identity of Th17 (7), which is indispensable for the EAE disease process. There appears to be multiple benefits associated with interfering with the IL-6-STAT3 signaling axis in the treatment of autoimmune diseases (8). Assembly of a complex between IL-6 and different forms of
IL-6R is required for the transmission of different IL-6 signaling, including classical signaling, trans-signaling and trans-presentation signaling. Classic signaling is triggered by the binding of IL-6 to the membrane form of IL-6R, followed by the activation of gp130 and the sequential recruitment of STAT3. Although gp130 is ubiquitously expressed, membrane IL-6R expression is restricted to limited cells. Soluble IL-6R (sIL-6R) is critical to supplement for the activation of IL-6 signaling in cells not expressing IL-6R on the surface through a process termed trans-signaling. Recently, an additional mode termed trans-presentation has been identified: DC expressed IL-6R binds to IL-6 and then forms a complex, followed by interacting with gp130-expressing T cells, leading to the differentiation of pathogenic Th17 cells.

CTRP4 featuring two highly conserved complement C1q domains is a classical secreted protein. As a metabolic regulator, CTRP4 secreted from the brain modulates food intake and body weight(9, 10). Furthermore, its role in the CNS has been extended, and found that the deletion of CTRP4 impaired hippocampal-dependent learning and memory of mice (11). Other studies have highlighted a potential role for CTRP4 in the immune system. For instance, CTRP4 inhibited the progression of colorectal cancer (12) and the absence of CTRP4 in sepsis model was also associated with exacerbated activation of macrophages with TLR4 internalization, leading to inflammatory cytokines release(13). In addition, exome sequencing of systemic lupus erythematosus patients identified a rare mutation of CTRP4/C1QTNF4 (H198Q) that inhibited TNF-mediated NF-κB activation and cell death (14), implying the potential role of CTRP4 in autoimmune diseases. In spite of this, there are conflicting reports until now: proinflammatory role
of CTRP4 have been observed in cancer-related inflammation, while anti-inflammatory properties have been observed in other inflammatory setting. Although nucleolin (a shuttling protein) has been identified as a cell surface docking protein binding to CTRP4 on monocytes and dead cells (15), this paradox cannot be explained. Therefore, other potential receptors may exist and need to be investigated for deeper understanding of the precise molecular mechanisms of CTRP4 in physiological and pathological settings.

In this study, we evaluated the role of CTRP4 in Th17 cells homing, priming and differentiation during induction and progression of EAE. Our results found that CTRP4 deficiency exacerbated symptoms in a T cell-intrinsic manner, possibly owing to its role in preferential Th17 differentiation. Mechanistically, we identified a previously unreported interaction between CTRP4 and IL-6R and subsequently inhibited IL-6-IL-6R binding, thereby suppressing STAT3 activation. Thus, our findings highlight the possible biological relevant ligand for IL-6R, which is of great importance to fill out the current knowledge of IL-6/IL-6R/gp130 buffer system, and provide a potential inhibitor for clinical application in autoimmune diseases.
**Results:**

1. CTRP4 deficiency impairs peripheral T cell homeostasis

The previous observation of preferential expression of Ctrp4 raised the possibility that CTRP4 is functionally expressed in T cells (13). In this work, we firstly examined the distribution of the four major thymic populations and found the percentage of CD4+CD8- DN, CD4+CD8+DP, CD4+ and CD8+SP thymocytes were comparable between Ctrp4-/- and control mice (Figure 1A). Furthermore, analysis of DN subsets revealed no detectable differences in the percentage of DN1, DN2, DN3 and DN4 in Ctrp4-/- mice, suggesting that CTRP4 were dispensable for T cell development in the thymus (Figure 1B). In homeostatic conditions, peripheral T cell pool is primarily composed of naïve T cells, but with increasing age, the pool remains fairly constant and begins to expand in memory-like cells characterized by CD44hi CD62Llo markers as a result of homeostatic proliferation induced by self-peptides/ MHC ligands. The increase in memory-like CD4+ T cells in Ctrp4-/- mice was observed along with a compensatory reduction in naïve CD4+ T cells (Figure 1C), while the proportion of memory-like CD8+ T showed no discrepancy in the spleens (Figure 1D).

Subsequently, we examined the effector T cell subsets in the periphery. Among the CD44hi memory T cell population in the spleen, Th17 cell was increased in the Ctrp4-/- mice (Figure 1E), while the percentage of Th1 and Th2 cells remained unchanged compared with the counterparts in the WT mice (Figure 1E, Supplemental Figure 1A). Moreover, the frequency of Treg cells was similar between WT and Ctrp4-/- mice.
To further confirm which CD4+ subset was more closely involved, we measured the expression levels of various Th cell signature genes. The results indicated that the levels of Th17 cell lineage-specific genes (Il17a, Il17f and Rorc) were upregulated in Ctrp4−/− mice (Figure 1F), whereas the levels of genes (Tbx21, Gata3, and Foxp3) remained unaltered (Supplemental Figure 1C). Of note, the mRNA level of Ctrp4 in CD4+T cells activated with anti-CD3 and anti-CD28 antibodies was not significantly upregulated relative to naïve CD4+T cells. During TCR activation in a particular cytokine milieu, naïve CD4+ T cells could differentiate into a variety of different lineages of Th cells. The results showed that the expression of Ctrp4 was limited in Th1 and Treg cells but significantly increased in both Th17 and Th2 cells (Supplemental Figure 1D). In line with the change of Ctrp4 transcriptional level, the increased protein level in Th17 cells supernatants was observed (Supplemental Figure 1E). Thus, we inferred that CTRP4 was involved in regulating the peripheral T cell homeostasis, particularly the Th17 cells of effector CD4+T cells.

2. CTRP4 production by CD4+T cells alleviates EAE symptom

To gain further insight into the pathophysiological roles of CTRP4 in the T cell-mediated autoimmune disease, we studied disease progression in a EAE model to mimic human MS. After EAE induction, Ctrp4−/− mice developed disease earlier, lost more body weight, and the clinical scores gradually reached a high peak at day17. Due to slower remission, a higher average disease score was observed in Ctrp4−/− mice (Figure 2A). Histological staining demonstrated increased immune cell infiltration and
demyelination in the spinal cord of $Ctrp4^{-/-}$ mice (Figure 2B), suggesting a role for CTRP4 in alleviating EAE progression and occurrence.

We then examined the composition of recruited immune cells in the CNS. Compared with WT mice, immunophenotyping combined with intracellular cytokine staining showed a higher number of CD4$^+$ T cells in $Ctrp4^{-/-}$ mice (Figure 2C, Supplemental Figure 2A). In addition, the increased number of CNS-infiltrating active macrophages (CD45$^+$F4/80$^+$) were present in $Ctrp4^{-/-}$ mice during the peak phase of EAE (Figure 2D). The exacerbated disease observed in $Ctrp4^{-/-}$ mice was associated with a significant increase of CD4$^+$IL17A$^+$ and CD4$^+$IL17A$^+$ IFNγ$^+$ T cells (Figure 2, E-F). Moreover, no detectable differences were observed in the percentage of CD4$^+$IFNγ$^+$ and CD4$^+$CD25$^+$Foxp3$^+$ cells derived from WT and CTRP4-deficient mice (Figure 2, E-F).

Subsequently, we investigated the abundance of CD4$^+$ T subsets in peripheral lymphoid organs. The total number of CD4$^+$ T cells was comparable in control and $Ctrp4^{-/-}$ mice (Supplemental Figure 2B). However, with respect to the subpopulations of CD4$^+$, the number of IL17A$^+$ and IL17A$^+$ IFNγ$^+$ CD4$^+$ T cells were significantly increased in the LN and spleen of $Ctrp4^{-/-}$ mice (Supplemental Figure 2, C-F). Based on our findings, we inferred that increased peripheral Th17 cells were responsible for the enrichment of CNS-infiltrating CD4$^+$ cells especially Th17 cells.

To evaluate the potential of Ctrp4 in antigen-specific expansion of CD4$^+$T cells, we accessed the recall response of MOG$_{35-55}$-specific T cells isolated at the early effector phase of EAE progression. Upon re-stimulation with MOG peptides, the antigen-
specific T cells from Ctrp4-deficient mice substantially enhanced proliferative activity
(Figure 2G) and remarkably produced more IL-17A and IFN-γ, which positively
correlated with the clinical symptoms (Figure 2H). By gating CFSE<sup>low</sup>CD4<sup>+</sup> T cells,
MOG<sub>35-55</sub>-peptide-specific Th17 cells was observed in CTRP4-deficient mice (Figure
2I).

Next, we examined whether CTRP4 executed its protective function primarily
through immune cells by generating bone marrow chimeric mice. Notably, the
irradiated WT mice reconstituted with WT bone marrow cells were protected from EAE
and exhibited delayed disease onset compared to WT recipients transplanted with
Ctrp4<sup>-/-</sup> bone marrow. Irradiated Ctrp4<sup>-/-</sup> recipient mice transplanted with WT bone
marrow were more resistant to EAE induction than those transferred with Ctrp4<sup>-/-</sup> bone
marrow (Figure 3A). Histopathologic examination of affected spinal cord was further
used to validate the disease severity (Figure 3B). Likewise, Ctrp4<sup>-/-</sup> mice transplanted
with Ctrp4<sup>-/-</sup>bone marrow had a significant increase in CD4<sup>+</sup> T cell and Th17 cells
infiltration in the CNS than Ctrp4<sup>-/-</sup> mice transplanted with WT bone marrow,
suggesting that CTRP4 regulated inflammation primarily by controlling the recruitment
of Th17 cell (Figure3,C-D). Collectively, these results supported the essential roles of
CTRP4 in the immune cells rather than non-hematopoietic compartments.

To investigate the T-cell intrinsic effect of CTRP4, we generated T cell conditional
CTRP4 KO mice (Supplementary Figure3, A-B). When compared with littermate
control, CTRP4<sup>flox/flox</sup>CD4-cre mice developed a significantly more severe EAE
progression (Figure 3F). The increased immune cells infiltration and demyelination in spinal cord sections of CTRP4^floxflox^CD4-cre mice were observed (Figure 3G), confirming the T cell-intrinsic role of CTRP4 in driving EAE-associated pathogenesis. Therefore, it might be interpreted that CTRP4 exerted neuroprotective effects through T cell-intrinsic mechanism.

3. CTRP4 suppresses IL-6-driven Th17 cell differentiation

In order to provide further insight into the mechanisms, we next investigated whether CTRP4 influenced the ability of naïve CD4^+^T cells towards Th17 differentiation, or the ability of Th17 cells to expand, survive or infiltrate into CNS, which are required for EAE onset and progression. Naïve CD4^+^ T cells were cultured and analyzed under different Th17 cell-polarizing condition. In non-pathogenic conditions, loss of Ctrp4 greatly increased the number of IL-17A-producing cells and consequently the production of IL-17A(Figure 4A). The mRNA expression of transcription factor also supported the findings (Figure 4B). Moreover, we assessed whether CTRP4 deficiency affected the differentiation of pathogenic Th17 cells. When cultured with IL-1β+IL-6+IL-23, a condition required for the acquisition of the pathogenic Th17 cell phenotype, Ctrp4^−/−^ mice possessed a higher frequency of Th17 cells, leading to much higher levels of IL-17A production (Figure 4A). Consistent with the phenotypic data, Ctrp4^−/−^ CD4^+^ T cells significantly upregulated Th17-associated gene signatures including Rorc, Il17a, and Il17f, indicating that elevated IL-17 secretion was partially attributed to the altered transcriptional regulation. Of note, Ctrp4 also affected the pathogenic capacity of Th17,
as evidenced by the increased mRNA expression of *Il23r* and *Ifng* in *Ctrp4*−/− CD4+ T cells, which transcribed into cytokines essential for Th17 cell stability and pathogenicity (Figure 4C).

Next, we evaluated whether *Ctrp4* deficiency had an impact on T cell proliferative capacity. By flow cytometry analysis of CFSE dilution, we found *Ctrp4*−/− CD4+ T cells showed a similar proliferative capacity when stimulated with TCR activation or in the presence of Th17 cell-polarizing cytokines (Figure 4, D-E). Additionally, CTRP4-deficient CD4+ T cells had no effect on apoptotic induction (Figure 4, F-G). Encephalitogenic T cells express high level of chemokine receptors to mediate the initial rolling and adhesion steps of transmigration and facilitate their recruitment to the CNS (16). Of note, the absence of *Ctrp4* in CD4+ T cells exhibited no alteration regarding CCR6, CCR2, CD49d or CD29 expression, suggesting that *Ctrp4* did not alter the ability of Th17 cells to migrate to inflammation sites (Supplemental Figure 4, A-B). Consistent with this finding, the spinal cords of *Ctrp4*-deficient mice expressed similar mRNA levels of various chemokines mediating immune-cell recruitment compared with the levels of WT cohort (Supplemental Figure 4C). Additionally, the absence of *Ctrp4* in Th17 cells had no effect on the expression of the key activation markers CD25, CD44, CD69, or CD103 (Supplemental Figure 4D). Therefore, it can be concluded that *Ctrp4* directed CD4+ T cell fate choice toward differentiation into Th17 cells ultimately leading to severe disease without affecting Th17 cell proliferation, apoptosis, migration, or activation of Th17 cells.
4. **CTRP4 acts as a negative regulator of IL-6R signaling**

Next, we assessed the involvement of IL-6 signaling in mediating the function of CTRP4 on Th17 cell differentiation. To verify this, Jurkat cells were co-transfected with pmCherry-CTRP4 and EGFP-IL-6R plasmids. The colocalized pattern was observed both in the cytoplasm and on the membrane (Figure 5A). Furthermore, the direct interaction was confirmed by co-immunoprecipitating in Jurkat cells (Figure 5B). Membrane-extracted protein from in vitro differentiated Th17 cells was co-immunoprecipitated with anti-IL6R to detect the interactions between CTRP4 and IL-6R under physiological condition. It was similar to what was seen in Jurkat (Figure 5C).

To identify the region on IL-6R required for CTRP4 binding, HEK293T cells were co-transfected with Myc-tagged CTRP4 and different FLAG-tagged IL-6R truncated domains. Of note, IL-6R interaction with CTRP4 was dependent on the D3 domain (Aa214-329) of IL-6R(Figure 5D), which covers most of the IL-6 interface area(17). To better evaluate interactive binding, the equivalent quantities of Jurkat cell membrane extract were incubated with serial dilution of $^{125}$I-CTRP4. Saturation-binding assays demonstrated that the direct binding affinity between CTRP4 and IL-6R was a $K_D$ of 3.941 nM (Figure 5E). Moreover, the abilities of unlabeled CTRP4, IL-6, and OSM (another cytokine of the gp130 family) to replace $^{125}$I-labeled CTRP4 in the competition-binding assays were evaluated. The result demonstrated that unlabeled CTRP4 and IL-6 strongly competed with radio-iodinated CTRP4 to bind with IL-6R with an IC$_{50}$ value of 77.25 nM and 5.233 nM, respectively, while OSM did not exert
a competitive effect (Figure 5F).

To investigate whether CTRP4 disturbed the formation of the IL-6-IL-6R complex, HEK293T cells were transfected with pmCherry-tagged IL-6 and EGFP-tagged IL-6R. Despite of pronounced colocalization between IL-6 and IL-6R in cytoplasm and membrane, the pattern of colocalization was degenerated in the presence of CTRP4 (Figure 5G). Likewise, the results were confirmed by ELISA assays. In the plates precoated with IL-6R protein, CTRP4 bound to IL-6R in a dose-dependent manner (Figure 5H). As expected, the excess of IL-6 inhibited the binding of CTRP4 and IL-6R (Figure 5I). Consistent with this, co-immunoprecipitation further supported that CTRP4 interfered with the binding between IL-6 and IL-6R (Figure 5J). Although the binding affinity between IL-6R and CTRP4 was relatively low compared with the affinity of IL-6 for IL-6R, the circulating CTRP4 level was higher by more than an order of magnitude than IL-6, which afforded more opportunity for CTRP4 to bind with IL-6 under disease states (Figure 5K). Given that nucleolin has been identified as the only known receptor for CTRP4, we assessed whether CTRP4 binding with nucleolin affected Th17 differentiation. After nucleolin expression was reduced with RNA interference, CTRP4 failed to alter the percentage of Th17 cells, excluding the possibility that nucleolin is involved in modulating Th17 differentiation (Figure 5L).

IL-6 also suppresses Treg generation by reducing TGFβ-induced Foxp3 expression(18). When cultured under iTreg cell-polarizing conditions, Ctrp4−/− CD4+ T cells were polarized into Treg cells to the same extent as WT naïve CD4+ T cell
Moreover, $Ctrp4^{-/-}$ Treg cells suppressed effector CD4$^+$ T cell proliferation in vitro with similar efficiency as WT Treg cells (Supplemental Figure 5B). This is consistent with the observation that $Ctrp4^{-/-}$ EAE mice had unchanged percentage of CD4$^+$ CD25$^+$Foxp3$^+$ cells in CNS (Figure 2F). Thus, our data demonstrated that CTRP4 is dispensable for the generation and suppressive capacity of Treg cells in vitro.

5. Binding of CTRP4 to IL-6R suppresses IL-6-mediated STAT3 phosphorylation

IL-6 promotes Th17 cell differentiation through the activation of the STAT3 especially at tyrosine 705(19), which prompted us to evaluate the inhibitory effect of CTRP4 on JAK/STAT3 signaling. First, we found that the expression of IL-6R was comparable between WT and $Ctrp4$-deficient CD4$^+$ T cells upon TCR stimulation (Supplemental Figure 6, A-B). Notably, the increased expression level of pSTAT3 in $Ctrp4^{-/-}$ CD4$^+$ T cells stimulated with physiological concentrations of IL-6 was observed, and the activation effect was observed in a more pronounced fashion after stimulation with IL-6 (Figure 6A). This was supported by the enhanced STAT3 phosphorylation in the $Ctrp4^{-/-}$ CD4$^+$ T cells when exposed to IL-6 compared with that in WT CD4$^+$ T cells by flow cytometry (Figure 6B). In terms of other associated signaling molecules, pJAK2, pERK, and pAkt also showed higher expression in the $Ctrp4^{-/-}$ CD4$^+$ T cells in response to IL-6, suggesting that the IL6-STAT3 signaling pathway was constitutively hyper-activated after the loss of CTRP4 in CD4$^+$ T cells (Figure 6A). To address the molecular mechanisms by which CTRP4 inhibited the
progression of EAE in vivo, we firstly measured the expression of the IL6R and gp130 in EAE model. Flow cytometry analysis showed that the expression levels of IL-6R and gp130 in the CD4+ T cells isolated either from inflammation sites or peripheral lymphoid organs remained comparable between groups (Supplemental Figure 6C). Notably, the deficiency of CTRP4 in CD4+T cells resulted in an increase in Y705-phosphorylated STAT3 in the spinal cord tissue of EAE mice compared to the level of CD4+T cells in the WT mice, while the abundance of phosphorylated STAT1 was equivalent (Figure 6C). In addition, the Ctrp4−/− CD4+cells were treated with IL-27 for indicated periods to verify whether CTRP4 only responded to IL-6 rather than other gp130 family cytokines. The results showed that the JAK-STAT3 signaling pathway was activated in Ctrp4−/− CD4+ T cells, similarly to what was observed in WT cells (Supplemental Figure 7A).

We further verified whether the addition of rhCTRP4 protein impaired Th17 cell differentiation. A decrease in the percentage of Th17 was observed in vitro with rhCTRP4 treatment (Figure 6D). Under Th17 cell-differentiating conditions, CD4+ T cells treated with rhCTRP4 significantly reduced Rorc transcript levels (Figure 6E) and IL17A production in the supernatant (Figure 6F). Furthermore, naïve CD4+ T cells with various concentrations of rhCTRP4 under Th17-polarizing condition were evaluated and found that higher doses of rhCTRP4 inhibited Th17 cell differentiation to a larger extent, suggesting that rhCTRP4 impaired the Th17 differentiation in a dose-dependent manner (Figure 6G). Consistent with the aforementioned results, the pretreatment of naïve CD4+ T cells with rhCTRP4 abrogated STAT3 activation in response to IL-6.
Accordingly, rhCTRP4 also attenuated IL-6-induced phosphorylation of JAK2 and STAT3 to normal levels in a dose-dependent manner (Figure 6I). The IL-6-triggered STAT3 phosphorylation activation was abolished as early as 5 min after exposure to rhCTRP4, and the inhibitory effect was sustained for at least 60 min (Figure 6J). Taken together, these results further confirmed that CTRP4 negatively regulated the IL6/STAT3 signaling.

Previous studies have shown that the IL-6 signaling cascade is initiated by the binding of IL-6 to membrane-bound IL-6R and gp130, which is called the classical signaling (20). Then, we used Ba/F3 cell, an IL-3-dependent mouse pro-B-cell line lacking both endogenous IL-6R and gp130 expression (21), to establish the cell lines with stable expression of both gp130 and IL-6R (Ba/F3-gp130-IL6R) or expression of gp130 only (Ba/F3-gp130) through the lentiviral system (Supplemental Figure 7B-C), and then analyzed their proliferation response to IL-6 signaling. Proliferation of Ba/F3-gp130-IL6R relies on IL-6-mediated classical signaling, while proliferation of Ba/F3-gp130 relies on both IL-6 and IL-6R mediated trans-signaling. First of all, IL-6 alone significantly promoted the proliferation of Ba/F3-gp130-IL-6R cells, whereas the IL-6-mediated proliferation rate was reduced in the presence of rhCTRP4, suggesting that CTRP4 affected classical IL-6 signaling (Figure 7A). Afterwards, we investigated the roles of rhCTRP4 on IL-6 trans-signaling. Ba/F3-gp130 were not responsive to IL-6 and sufficiently restored growth in response to a combination of IL-6 plus siIL-6R. Exogenous CTRP4 significantly suppressed the proliferation of Ba/F3-gp130 cells induced by the combination of IL-6 and IL-6R (Figure 7B), indicating that CTRP4...
retains the ability to respond to IL-6 trans-signaling.

Next, we aimed to get deep insight of the inhibitory effect of CTRP4 on the already formed IL-6-IL-6R complex. Experimentally, Ba/F3-gp130 were pretreated with hyper-IL6, which mimics the pre-assembled IL-6-IL6R complex, in the presence or absence of rhCTRP4. The strong proliferative response induced by hyper-IL6 was not impaired after addition of CTRP4 (Figure 7B). Western blot analysis showed a remarkable increase of phospho-STAT3 in CD4+T cells after treatment with hyper-IL6, whereas the addition of CTRP4 was not able to inhibit the activation of STAT3 induced by hyper-IL6 (Figure 7C). To ascertain whether CTRP4 was functionally important for already formed IL-6-IL-6R complex, naïve CD4+ cells were differentiated under the Th17 cell-polarizing condition with hyper-IL6 or with the combination of IL-6 and IL-6R. The results showed that the frequency of Th17 cells was moderately increased after hyper-IL6 treatment, and the presence of rhCTRP4 inhibited the generation of Th17 under the condition containing the mixture of IL-6 and IL-6R protein. However, the presence of CTRP4 showed no significant effects on inhibiting Th17 differentiation under conditions containing IL6–IL6Ra complex (Figure 7D). Hence, these results indicated that CTRP4 was unable to disrupt the already formed IL6–IL6Ra complex.

6. rhCTRP4 treatment reduces neuroinflammation in EAE

Next, we wondered whether rhCTRP4 was effective in alleviating established EAE disease in WT mice by daily intraperitoneal injection of rhCTRP4 starting from day 9 postimmunization, a time point widely considered to represent the onset of symptoms.
In comparison with BSA treatment, rhCTRP4 treatment demonstrated significantly increased therapeutic efficacy and reduced EAE severity (Figure 8A). The histological analysis revealed that the administration of rhCTRP4 was accompanied by decreased inflammation and demyelination in the affected spinal cord (Figure 8B). In CNS, the number of CD4+ T cells, particularly CD4+IL-17A+ and CD4+IFNγ+IL-17A+ cells, were dramatically reduced by therapeutic rhCTRP4 administration (Figure 8C). Similar results were observed by immunofluorescence staining of IL-17A (Figure 8D). The reduced nuclear translocation of p-STAT3 and STAT3 phosphorylation in EAE mice treated with rhCTRP4 also supported the CTRP4-mediated inhibitory effects on STAT3 activation in vivo (Figure 8, E-F). When T cells from primed mice challenged with the MOG peptide to detect the reactivity towards the antigen, the T cells from EAE mice treated with rhCTRP4 displayed a markedly dampened proliferative response (Figure 8G), along with reduced IL-17A and IFNγ generation (Figure 8H).

Given that CTRP4-deficient mice showed more aggressive pathogenesis, we reasoned whether the addition of rhCTRP4 rescued the severe disease phenotype. After intraperitoneally administration with rhCTRP4, CTRP4-deficient mice developed milder EAE as demonstrated by lower clinical scores (Figure 8I). In summary, therapeutic delivery of rhCTRP4 ameliorated the clinical severity of EAE associated with reduced encephalitogenic effector T cells responses.

7. CTRP4 directly inhibits IL-6 signaling in vivo
To investigate whether CTRP4 directly inhibited IL-6 function in vivo, EAE mice were injected intraperitoneally with rhCTRP4 or vehicle as well as with the neutralizing anti-IL-6R antibody. We found mice treated with anti-IL-6R antibody developed significantly milder disease demonstrated by delayed disease onset, relative to that of control mice injected with IgG, suggesting that IL-6R blockade contributed to the remission of EAE as expected. However, the protective effects of rhCTRP4 were abrogated in the absence of IL-6R (Figure 9A), as evidenced by the similar clinical severity of mice treated with anti-IL-6R antibody in the presence or absence of rhCTRP4. Furthermore, the reduction of CD4+ T cells caused by rhCTRP4 administration was abolished by the addition of anti-IL-6R (Figure 9B). For subsets of CNS infiltrating CD4+ T cells, the decreased tendency of Th17 in CNS infiltrating CD4+ T cells in mice treated with rhCTRP4 disappeared in the presence of anti-IL6R (Figure 9C), confirming the importance of the interaction CTRP4 with IL-6R in vivo.

Next, we investigated whether the suppressive effect of CTRP4 on STAT3 activation contributed to the protection of the host resistant to EAE. To this end, a STAT3 small-molecule inhibitor S3I-201 (22) was used to suppress STAT3 signaling in vivo and found S3I-201 administration improved the severe symptom caused by CTRP4 deficiency (Figure 9D), as indicated by a reduction of immune cells infiltrated into the spinal cord and the decreased demyelination (Figure 9E). Similar to the inhibitory effects of CTRP4 on STAT3 activation in vitro, CTRP4 ameliorating disease by inhibiting STAT3 in vivo.
8. Mice transferred with MOG-reactive T cells that expand in the presence of rhCTRP4 develop mild EAE

To further assess whether CTRP4 impaired the encephalitogenic potential of effector T cells in vivo, we used an adoptive transfer model of EAE. The in vitro polarization of the CD4+ T cells treated with rhCTRP4 showed a significant reduction in the frequency of MOG-specific Th17 cells and IL-17A secretion in the culture supernatants compared to that stimulated with BSA (Figure 10A). With respect to the co-expression of inflammatory cytokine in Th17 cells, rhCTRP4 downregulated the production of GM-CSF and IFNγ (Figure 10B), which were associated with the encephalitogenic potential of the Th17 cells to elicit neuroinflammation. In consistent with this, rhCTRP4 significantly decreased Il17a, Ifng, Il1r, and Il23r mRNA transcripts in MOG-reactive CD4+ T cells (Figure 10C).

The expanded MOG-reactive CD4+ T cells in the presence of rhCTRP4 were transferred into irradiated recipient mice, and the recipient mice exhibited significantly milder symptoms compared to mice receiving MOG-reactive CD4+ T cells stimulated with BSA (Figure 10D). We observed that the numbers of CD45.2+CD4+ host T cells and CD45.1+ CD4+ donor T cells were comparable in CNS between the two groups (Figure 10E). Moreover, the number of Th17 cells among the CD45.1+ donor T cell population from mice receiving donor T cells stimulated with rhCTRP4 were similar to those of the donor T cells stimulated with BSA (Figure 10G). However, we observed a dramatic difference in the pattern of pathogenic cytokines of Th17 cells from the
recipient mice receiving T cells treated with exogenous CTRP4, co-expressing higher GM-CSF and IFNγ (Figure 10, F-G). Collectively, we inferred that pretreatment with rhCTRP4 during the ex vivo expansion of MOG-reactive CD4+ T cells rendered the T cells less encephalitogenic in inducing autoimmune CNS inflammation.
Discussion

Herein, we provided comprehensive evidence to confirm the immunomodulatory properties of CTRP4 in modulating T cell function during the pathophysiology of EAE: (i) the initial priming of T cells in peripheral lymphoid organs, (ii) differentiation of primed T cells towards Th17 cells, (iii) migration of pathogenic T cells into the CNS leading to the onset of symptom. In addition, we found the mechanism of reduction of IL-6 activity by CTRP4 was not through regulating IL-6R or gp130 expression, but through directly bound with IL-6R, leading to the suppression of IL-6-induced activation of STAT3, which is an essential regulator of the lineage commitment to Th17 cells (23). Of note, McGeachy et al. pointed toward a critical role for STAT3 in maintaining the capacity of Th17 cells to produce cytokine in response to antigenic stimuli compared with stimulation with PMA and ionomycin (24). This is in consistent with our results that CD4+ T cells from Ctrp4-deficient mice enhanced cytokines production after re-stimulation with MOG peptides in Figure 2I and showed no change in proliferative capacity when stimulated with TCR activation in Figure 4D. CTRP4 may also play a role in the disease by regulating other cell types, like macrophages infiltrating in CNS (Figure 2D). Additional work is needed for more comprehensive understanding of the universal role of CTRP4 in other IL-6R-expressing cells.

The biological effects of IL-6 are highly complex, as they are mediated via multiple pathways. Notably, Casey Weaver’s findings indicated that the role of IL-6 signaling is beyond the inductive phase of Th17 and is thought to be responsible for Th17 cell
maintenance(25). Our results also showed that CTRP4 added to ex vivo culture was able to suppress Th17 responses and the MOG-reactive CD4+ T cells expanded in the presence of rhCTRP4 induced milder symptoms after adoptively transferred into irradiated recipient. Altogether, our data was consistent with the finding from the Weaver group’s report that IL-6R-deficient TH17 cells rapidly lost their Th17 phenotype. Based on these results, we inferred that CTRP4 disrupted the Th17 generation via both the classical and trans-signaling pathways. For sure, more detailed evidence is needed to be proved.

Although Mufazalov IA et al claimed that IL-6R is the only biological relevant receptor for IL-6 in mice (26), it should be noted that HuaYu et al demonstrated IL-6 is also able to bind to CD5 in B1a independently of IL-6R (27). Of note, CD5 expression was rather restricted to B1a cells, implying IL-6-CD5 module was not common. In addition, IL-6 overexpressing mice developed a lethal immune dysregulation syndrome with massively infiltrated CD11b+ myeloid cells expressing robust IL-6Ra but no CD5. This may partly explain why the interaction between CD5 and IL-6 failed to work. In addition, Wael El-Rifai et al. found that TFF1 interfered with IL-6R and further compromised the formation of IL-6-mediated IL6Ra–gp130 complex, which played a protective role in mucosal against gastric tumorigenesis (28). Furthermore, other antagonists targeting gp130 lead to the disturbance of IL-6/IL-6R/gp130 system. For instance, IL-27p28 is a natural antagonist to block gp130-mediated signaling via interaction at a lower affinity compared to IL-6R-gp130 interaction. Furthermore, tumor-necrosis factor receptor–associated factor5 (TRAF5) has also been reported to
constitutively bind to gp130, antagonizing IL-6-driven activation of STAT3(29). Indeed, our work is also of great importance to fill out the current knowledge of IL-6/IL-6R/gp130 system.

It is well established that the administration of IL-6 receptor antagonist tocilizumab hold promise for treating potentially fatal cytokine release syndrome observed in CAR-T therapy (30) or SARS-CoV-2 infection(31), underscoring the importance to define regulators of IL-6 homeostasis. Different IL-6 signaling modes could be distinguished by circulating form of gp130. IL-6 forms a complex with the soluble IL-6R and gp130 in blood to prolong its half-life. Thus, sIL-6R and sgp130 are thought to comprise a biological buffer system to regulate IL-6 biological effect by capturing free IL-6 and neutralizing rapidly(32). Our study found that CTRP4 could be a part of the broader biological buffer system by competitively binding IL-6R, even though with a lower binding affinity. Further work is required to determine whether at least partial IL-6R’s biological functions are contributed by its ability to interact with CTRP4. Besides, IL-6 blockade strategy failed to show efficacies to all autoimmune diseases, suggesting that new insights into the understanding of IL-6 system could help to promote the development of therapeutic drugs.
Methods

Experimental Mice The generation of Ctrp4^-/- mice had been previously described\textsuperscript{23}. The CTRP4^floxflox mice were from Prof. Li Yingxian’s lab. CD4-Cre and EIIa-Cre mouse strains came from the Jackson Laboratory. B6.SJL mice were purchased from the Jackson Laboratory. Six- to twelve-week-old mice were used for most of the experiments. Age- and sex-matched littermates with the appropriate genotypes were used as controls. All mice were bred and maintained under SPF conditions in an animal facility.

EAE model establishment The MOG\textsubscript{35-55} peptide with the amino acid sequence MEVGWYRPSRVVHLRNGK was synthesized by Synpeptide. Mice were subcutaneously immunized with 200 μg of MOG\textsubscript{35-55} emulsified with incomplete Freund’s adjuvant (Cat#F5506, Sigma) containing 5 mg/mL heat-killed Mycobacterium tuberculosis (Cat#231141, BD Bioscience), followed by a tail vein injection of 200 ng of pertussis toxin (Cat#179B, List Biological Laboratories) on day 0. Intraperitoneal injection of 200 ng of pertussis toxin was administered on day 2. To verify the effect of rhCTRP4 in EAE, WT or Ctrp4^-/- mice were immunized and then administrated intraperitoneally 500 ng/mice rhCTRP4 or vehicle daily starting on day 9 after immunization until sacrificed. The mice were monitored daily for clinical signs of disease on a scale of 1–4 as follows (33): 0, no clinical symptom; 1, limp tail without hind-limb weakness; 1.5, tail paralysis and waddling gait; 2, partial hind limb weakness; 2.5, paralysis of one hind limb; 3, completely paralyzed hind legs; 3.5, complete hind
limb and partial forelimb paralysis; 4, complete paralysis accompanied by urinary or fecal incontinence. For pharmacological inhibition of STAT3, STAT3 inhibitor S3I-201 (Selleck, Cat: S1155) were inject intraperitoneally at 5 mg/kg every two days. For IL-6R blockade, WT mice were intraperitoneally injected with 10 mg/kg of anti-IL-6R (clone15A7; BioXcell) or IgG1 (cloneLTF-2; Bio X cell) on day 0 before EAE induction or day7, 14, 21 postimmunization. rhCTRP4 or BSA was administrated intraperitoneally in mice pretreated with neutralization antibodies every other day, starting on day 1 after immunization.

**Induction of EAE by passive transfer of pathogenic CD4+ T cells** B6.SJL donor mice (CD45.1+) were induced EAE model. Ten days later, draining lymph node cells were isolated and then re-stimulated with 50 μg/mL of MOG peptide and Th17 cell-polarizing factors (20 ng/mL rhIL-6, 20 ng/mL rhIL-23, 20 ng/ml IL-1β and 10 μg/mL anti-IL4 and 10 μg/mL anti-IFN-γ) to generate MOG-specific Th17 cells. After 4 days in culture, the resting cells were sorted with anti-CD4 microbeads (Miltenyi), and the number of CD4+ T cells was calculated and then 1x10^6 CD4+ T cells per mouse were intravenously injected into irradiated C57/BL6J recipient mice (4 Gy). Next, the mice were injected with 200 ng pertussis toxin in PBS on day 0 and 2 days after transfer.

**T cell purification and differentiation** Naïve CD4+ T (CD4+CD44hiCD62LhiCD25−) cells were purified by flow cytometry. IL-6, TGFβ, and IL-6/IL-6R protein chimeras were purchased from R&D Systems. IL-23 and IL-1β were purchased from PeproTech. Naïve T cells were stimulated with plate-bound anti-CD3 (2 μg/mL) and anti-CD28 (2
μg/mL) antibodies in the presence of anti-IL4 (10 μg/ml; 11B11; BioLegend) and anti-IFN-γ (10μg/ml; XMG1.2; BioLegend) to generate Th0 cells; IL-6 (30 ng/ml), TGF-β1 (2 ng/ml), anti-IL4 and anti-IFN-γ antibodies were added to induce Th1 cell differentiation. Pathogenic Th17 cells were generated in the presence of IL-6 (20 ng/ml), IL-1β (20 ng/ml), IL-23 (20 ng/ml), anti-IL4 and anti-IFN-γ. Treg cells were generated in the presence of IL-2 (10 ng/mL) and TGF-β1(5 ng/mL). For the induction of Th1 cells, 25 ng/ml rmIL-12 and 10 ug/ml anti-IL4 were used. Regarding Th2 cells, 10 ng/mL mIL-4 and 10 ug/mL anti-IFNγ were applied. At the end of the culture period of different Th differentiation conditions, we re-stimulated CD4+T cells with PMA (50 ng/mL) and ionomycin (500 ng/mL) in the presence of 2 μM monensin for 4 hr for intracellular staining or only re-stimulated with PMA and ionomycin for 4 hr for the quantification of mRNA expression.

In vitro Treg cell suppression assay Effector T cells (CD4+ CD25-) cells were obtained of WT mice by magnetic separation and subsequently labeled with 5 nM CFSE (Invitrogen) for 10 min at 37°C. CFSE-labeled effector T cells (1X10^5) were then cocultured with Treg (CD4+CD25+) according to the indicated ratio in the presence of anti-CD3 (clone: 145-2C11;BD) and anti-CD28 (clone: 37.51; BD). After 72 hr, the cells were harvested to measure CFSE dilution by flow cytometry.

Flow cytometry and related reagents At scarify, single-cell suspensions were isolated from lymph nodes, spleens, and CNS. Briefly, the brain and spinal cord were obtained, homogenized and then incubated with collagenase D (2.5 mg/mL, Roche Diagnostics)
and DNase I (1 mg/mL, Sigma) for 30 min. Mononuclear cells were enriched by gradient centrifugation at 670 g for 30 min on a 37/70% Percoll gradient without interruption. Before staining, cells were blocked with anti-CD16/CD32 antibodies. The following antibodies were used for the flow cytometry: anti-CD3 (OKT3), anti-CD45 (30-F11), anti-MHCII (M5/114.15.2), anti-CD4 (GK1.5), anti-CD8 (53-6.7), anti-CD25 (PC61.5), anti-CD62L (MEL-14), anti-CD44 (IM7), anti-Foxp3 (FJK-16S), anti-Ly-6G/Gr-1 (1A8-Ly6g), anti-GM-CSF (MP1-22E9), anti-F4/80 (BM8), anti-CCR2 (SA203G11), anti-CCR6 (29-2L17), anti-CD29 (HMβ1-1), anti-CD49d (R1-2), anti-IL-17A (eBio17B7), and anti-IFN-γ (XMG1.2) antibodies were purchased from eBioscience and Biolegend. In addition, anti-pSTAT3 (Y705) (cat:557814), pSTAT1 (Y701) (cat:502069) antibodies were purchased from BD Bioscience. Phosphoflow cytometry analysis was performed using BD Phosflow buffers (554655 and 558050). Stained cells were analyzed by FACSCanto flow cytometer, and were analyzed with FlowJo software.

**Quantitative RT-PCR** Total RNA samples were extracted with Trizol reagent and then reverse-transcribed to cDNA according to the manufacturer’s instructions. Quantitative RT-PCR was performed using SYBR Green (Thermo) with a Roche LightCycler480 system. All the primers used for real-time PCR were listed in supplemental Table 1. The condition for real-time PCR was 40 cycles at 94°C for 15 s followed by 40 cycles at 60°C for 60 s.

**Western blot analysis** Cell lysates were prepared in RIPA buffer containing protease
inhibitor cocktail and phosphatase inhibitor cocktail. Proteins were subjected to PAGE gels and transferred to nitrocellulose membranes and subsequently probed with anti-phosphor-STAT3 (CST, Cat. no. #9145, 1:1000 dilution), anti-STAT3 (CST, Cat.no.#4904,1:1000), anti-phospho-JAK2 (CST, Cat.no.#3771, 1:1000 dilution), anti-phospho-ERK (CST, Cat.no.#4370, 1:1000 dilution), anti-ERK(CST, Cat.no.#4695, 1:1000 dilution), anti-phosphor-Akt (CST, Cat.no.#4060, 1:1000 dilution), anti-Akt (CST, Cat.no.#4691, 1:1000 dilution), anti-IL-6R (Santa Cruz, Cat.no.sc373708, 1:1000 dilution), anti-IL-6 (CST, Cat.no.#12153, 1:1000 dilution), anti-FLAG (sigma-Aldrich; Cat.no.F3165, 1:1000 dilution) and anti-c-Myc (Sigma-Aldrich, Cat.no.C3956, 1:1000 dilution). The membranes were then incubated with appropriate secondary antibodies and developed with Amersham ECL (GE Healthcare).

**Immunoprecipitation** HEK293T cells and Jurkat cells purchased form ATCC were cultured according to the guide. Immunoprecipitation was performed as we previously described (34). Briefly, various cells were lysed in a lysis buffer (20 mM Tris-HCl, pH 7.5; 150 mM NaCl; 0.5 mM EDTA; 1.5 mM MgCl2; 0.1%NP-40; 10% glycerol and protease inhibitor cocktail) at 4 °C for 30 min. Integral membrane protein utilized two-phase partitioning system to efficiently separate (Thermo Mem-PER plus, cat:89842). Equal amounts of protein were immunoprecipitated with anti-IL6R, or anti-CTRP4 antibodies home made as described in previous study(35), bound to protein G Sepharose (GE Healthcare) or anti-FLAG M2 Affinity Gel (Cat#A2220, Sigma). After extensive washing five times with lysis buffer, the indicated proteins were subjected to western blot analysis.
**Radio-ligand binding assay** rhCTRP4 protein was purified from CTRP4-CHO cells as described (13). CTRP4 was labeled with $^{125}$I in 0.01 N PB buffer with chloramine T at 4°C for 30 s, followed by elution with 0.01 N PB buffer in a SEPHADEX-G25 column. For saturation experiments, the equivalent quantity of Jurkat cell membrane extract was incubated with different 2-fold serial dilutions of $[^{125}$I]-CTRP4. Nonspecific binding was measured in the presence of a 500-fold excess of CTRP4 at each concentration of $[^{125}$I]-CTRP4. For competition-binding assays, the equivalent quantity of cell membrane extract and $[^{125}$I]-CTRP4 were incubated with different concentrations of unlabeled CTRP4, OSM, or IL-6. The reaction was incubated for 24 hr at 4°C and 25% PEG was added, followed by counting on a γcounter.

**ELISA** Human IL-6R (R&D) was pre-coated onto plates and maintained overnight at 4°C and then incubated with human IL-6 or rhCTRP4. The plates were blocked with 200 μl of 1% BSA in PBS for 2 hr, followed by incubating with anti-IL-6R primary antibody. The color changes were read at OD450. Under different Th17 differentiation conditions, supernatants from cell cultures were collected and measured for cytokines secreted by IL-17A and IFNγ ELISA kit (R&D SM1700; MIF00). The levels of MOG-specific antibodies and the level of CTRP4 were determined by anti-Mouse MOG antibody Quantitative ELISA Kit (Anaspec) and Mouse Complement C1q tumor necrosis factor-related protein 4 ELISA Kit (Abbexa) according to the manuscript’s instructions.

**Histological assay of spinal cord sections** The spinal cords were dissected and fixed...
with 4% PFA, dehydrated, and embedded in paraffin. For immunofluorescence staining, sections were incubated at 55°C for 30 min for antigen retrieval. The sections were pretreated with a 0.3% solution of H$_2$O$_2$ to block endogenous peroxidase activity. The sections were then incubated with 10% goat serum in PBS-T, followed by incubating with the primary antibodies including anti-IL-17A (Invitrogen, 14-7179-80, 1:100 dilution) and anti-CD4 (Invitrogen, 14-9766-82, 1:100 dilution). Next, the sections were detected with Alexa Fluor 555-labeled streptavidin at room temperature for 1 hr. After staining with DAPI to visualize cell nuclei, the slides were analyzed by fluorescence microscopy.

**Lentiviral infection** The IL-6R, gp130 or CTRP4 cDNA were subcloned into the lentivirus vector TG006. For lentivirus package and production, HEK293T cells were co-transfected with 10 μg of TG006-gp130 transfer vector and 5 μg of psPAX2 and 5 μg of VSVG packing vector in 1.5mL of opti-MEM using Lipofectamine 2000 reagent. 72 hr after transfection, lentivirus particles were harvested, filtered, and added to Ba/F3 cells or Jurkat in the presence of polybrene (8 μg/ml). After 24 hr, the medium containing the viral particles was replaced by the viral particle-free culture medium. The cells were cultured and maintained in culture medium containing 2 μg/ml puromycin to obtain target cells.

**Bone marrow chimeras** The recipient mice were exposed to lethal-dose γ-irradiation (10 Gy) to destroy hematopoietic stem cells. After 2 hr recovery period, bone marrow cells derived from the tibiae and femurs of donor mice aged between 2 and 4 months
were intravenously injected into irradiated recipients (5X10^6/mouse). The chimeric mice were housed for a total of 8 weeks for the complete recovery of the hematopoietic niche and then subjected to EAE induction as described hereafter.

**Statistical analysis** Our data were randomly collected. Experimental results were analyzed for significance using Student’s t-test and Mann-Whitney U test for two groups. Statistical significance also was assessed by one-way ANOVA or two-way ANOVA followed by the Bonferroni or Dunnett’s multiple comparisons test for three or more groups. Statistical analyses were performed using GraphPad Prism (version 9.0).

**Study Approval** The animal studies were conducted according to the guidelines of Institutional Animal Care of Peking University, under approved the Ethical Office of Peking University People's Hospital, project license 2022PHE107.

**Data availability** All supporting data values associated with the main manuscript and supplement material, including values for all date points shown in the graphs and values behind any reported means, are available in the Supporting Data Values.xls file.
Author contributions

C.L.L designed and performed experiments, analyzed results, and wrote the manuscript.

C.W, D.J.H, H.M.W and N.D.X conducted the experiments and analyzed data. L.Q, Z.X.X, and W.L supervised the study and writing of the manuscript.

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Reference


**Figure 1** *Ctrp4* deficiency perturbs T cell homeostasis

(A) Surface staining of CD4 and CD8 on *Ctrp4*−/− or WT thymocytes. Numbers in quadrants indicated the percentage of different stage cells, including CD4−CD8−DN, CD4+CD8−DP, CD8−CD4+ single-positive and CD4−CD8+ single-positive T cells (n=10 animals per group from one representative experiment of three).

(B) Flow cytometry analysis of the transition between the different populations of DN T-cell precursors in the thymus, which were marked by the differential expression of CD44 and CD25. DN1:CD44+CD25−; DN2:CD44+CD25+; DN3:CD44−CD25−; DN4: CD44−CD25−.

(C-D) Representative plots showed the percentage of naïve (CD44lowCD62Lhi) and memory/effector (CD44hiCD62Llow) CD4+ T cells (C) and CD8+ T (D) in the spleens of *Ctrp4*−/− or WT mice.

(E) Flow cytometry analyses of Th1(IFNγ+) and Th17 (IL-17A+) effector T cells in the spleen of *Ctrp4*−/− and WT mice (n=5/group). Data were frequency of CD4+CD44+ cells.

(F) Gene expression of *Il17a, Il17f, Rorc* or *Ctrp4* mRNA in CD4+ T cells from *Ctrp4*−/− or WT mice (n=5/group) were analyzed by quantitative real-time PCR. Values were normalized against *Gapdh*. Data were shown as mean ± SEM and were from one of three independent experiments with similar results.

Statistical significance was determined using two-tailed unpaired Student t-test or Mann-Whitney U test as appropriate after assessing for distribution; *p < 0.05, **p < 0.001.
Figure 2 Ctrp4 deficiency exacerbates EAE progression with increased infiltration of CD4+ T cells in the CNS.

(A) After immunization with MOG peptide, the progression of disease was monitored. The clinical EAE scores and body weight of Ctrp4−/− (n=10) or WT (n=10) mice following disease induction were shown. Statistical significance was determined using Two-way repeated measures ANOVA.

(B) The spinal cord sections were stained with hematoxylin and eosin (H&E) or Luxol Fast Blue (LFB). Histological images were representative of three mice in each group. Scale bar, 200 μm.

(C-D) Single cells were isolated from the central nervous system on day 18 after EAE induction and stained with the indicated cell type-specific markers. The summary bar graph showed the absolute number of CNS-infiltrating immune cells in control and Ctrp4−/− mice (C). Representative flow cytometry plots of macrophage (CD45hiF4/80+) and microglia (CD45lowF4/80+) infiltrated in CNS were shown (D).

(E-F) Representative flow cytometry plots showed the percentage of IFNγ+/CD4+, IL-17A+/CD4+, IFNγ−IL-17A−/CD4+ in CNS of WT and Ctrp4−/− mice (n = 5 mice per group) on day 18 after EAE induction.

Quantified percentage (E) and absolute cell numbers (F) were shown.

(G-H) Recall response of antigen-specific T cells from the dLN of WT and Ctrp4−/− mice on day 9 after EAE induction. CD4+ T cells were expanded with irradiated autologous presenting cells plus 10 μg/mL MOG peptide for 72 hr and subjected to cell proliferation assay based on BrdU assay (G) and ELISA assay (H) to quantitate the productions of IL-17A and IFN-γ.

(I) Flowcytometric analysis of CFSE-labeled CD4+ T cells and quantification of intracellular cytokine staining at day 5 after in vitro coculturing with irradiated autologous presenting cells plus10 μg/mL MOG peptide. The percentage of IL-17+ and IFN-γ+ cells were gated on CSFe−/CD4+ T cells and data were presented as representative flow plots. Data were shown as mean ± SEM and were from one of three independent experiments with similar results. (C-H) Statistical significance was determined using two-tailed unpaired Student t-test or Mann-Whitney U test as appropriate after assessing for distribution; *P < 0.05, **P < 0.001, ***P < 0.0001.
Figure 3. The protective function of CTRP4 is T-cell intrinsic for IL-6 signaling

(A) Mean clinical scores of chimeric mice generated by (i) transfer of WT bone marrow cells into irradiated WT and Ctrp4−/− recipient mice or (ii) transfer of Ctrp4−/− bone marrow cells into irradiated WT and Ctrp4−/− recipient mice (each n=6-8) following MOG35-55 immunization. Statistical significance was determined using Two-way repeat measure ANOVA and Holm–Sidak post hoc test.

(B) Representative H&E staining of spinal cords sections harvested from chimeric WT and Ctrp4−/− mice showed inflammatory cells infiltration and demyelination at day18 postimmunization. Scale bar, 200 μm.

(C) Flow-cytometric analysis of absolute cell numbers of CNS-infiltrating T cells (CD45+CD3+CD4+ T and CD45+CD3+CD8+ T) and B cells (CD45+CD3-CD19+) at 18 day postimmunization.

(D) Flow-cytometric analysis of absolute numbers of different CNS-infiltrating myeloid cells including monocyte (CD45+CD11b+Ly6C+), neutrophil (CD45+CD11b+Ly6G+) and DC (CD45+CD11c+MHCII+) cells at 18 day postimmunization.

(E) Flow-cytometric analysis of the absolute number of Th1 (IFN-γ+), Th17 (IL-17A+) and Treg (CD25+Foxp3+) cells of CD4+ T cells infiltrated to the CNS harvested at 18 day postimmunization.

(F) Mean clinical scores of CTRP4flox/flox and CD4-cre CTRP4flox/flox mice were monitored after MOG immunization. Data were representative of three independent experiments. Statistical significance was determined using Two-way repeat measure ANOVA.

(G) H&E staining (Left) and LFB staining (Right) of spinal cords sections harvested from CTRP4flox/flox and CD4-cre CTRP4flox/flox mice at day 18 after EAE induction. Data were shown as mean ± SEM and were from one of three independent experiments with similar results. one-way ANOVA with Tukey’s post-test(C-E), *p < 0.05; **p < 0.01, ***P < 0.0001.
Figure 4 Naïve Ctrp4−/− CD4+ T cells display an enhanced Th17 phenotype in vitro

(A) Naïve CD4+CD62LhighCD44lowCD25− T cells were sorted from WT and Ctrp4−/− mice, and differentiated with (1) no cytokine, (2) TGF-β1 and IL-6, or (3) IL-1β, IL-6, and IL-23. Numbers adjacent to outlined areas indicated the percentage of CD4+IL-17A+ cells. The production of IL-17A in the supernatants of different conditions were measured by ELISA (right).

(B-C) Quantitative real-time PCR was used to quantify Rorc transcript expression in WT and Ctrp4−/− CD4+ T cells under Th0 or Th17 differentiation conditions (B) and quantify pathogenic Th17-associated genes expression of Il17a, Il17f, Rorc, Ifng or Il23r mRNA in CD4+ T cells from Ctrp4−/− or WT mice (C). The values were normalized against Gapdh.

(D-E) Representative flow plots of CD4+ T cells stimulated for 72 hr in the presence of TCR stimulations (anti-CD3/anti-CD28 antibodies). The proliferation of WT and Ctrp4−/−CD4+T were measured by CFSE dilution assay (D). The apoptosis of WT and Ctrp4−/−CD4+T were assessed by AV and 7-AAD staining (E).

(F-G) Representative flow plots of naïve CD4+ T cell after 72 hr in vitro Th17 cell differentiated condition to detect proliferation by CFSE dilution assay (F) or apoptosis by AV and 7-AAD staining (G). Data are shown as mean ± SEM and are from one of three independent experiments with similar results.

(A-C) Statistical significance was determined using two-tailed unpaired Student t-test or Mann-Whitney U test as appropriate after assessing for distribution, *P < 0.05, **P < 0.001.
Figure 5 CTRP4 negatively regulates IL-6-induced STAT3 activation through IL-6-IL-6R axis

(A) Jurkat cells were transduced with lentivirus encoding pmCherry-CTRP4 (red) and EGFP-IL-6R (green) and then were observed by laser confocal microscopy. Higher magnification images of boxed areas on lower power images were provided to the right of merged images.

(B) Coimmunoprecipitation of IL-6R and CTRP4 from Jurkat cell lysates. Membrane proteins of Jurkat cell lysates were coimmunoprecipitated by anti-IL-6R antibody (upper panel) or anti-CTRP4 antibody (lower panel). Eluted proteins were analyzed with both anti-IL-6R and anti-CTRP4 antibodies.

(C) The sorted naïve CD4\(^+\) T cells were cultured under Th17 polarization conditions and were collected for coimmunoprecipitation assay. The cell membrane extracts were incubated with anti-IL-6R, and then these proteins were immunoblotted with anti-CTRP4 antibody to verify the physiological CTRP4:IL-6R interaction.

(D) HEK293T transiently transfected with plasmid vectors encoding Myc-tagged CTRP4 together with different Flag-tagged IL-6R truncated forms, followed by immunoblotting analysis with anti-Flag or anti-Myc antibodies.

(E) Recombinant CTRP4 protein was labeled with \(^{125}\)I tracer. The equivalent quantities of Jurkat cell membrane extract were incubated with different 2-fold serial dilution of \(^{125}\)I-CTRP4 in binding buffer. Saturation curves of CTRP4 binding to IL-6R were calculated.

(F) Competitive binding assay were performed by addition of unlabeled-CTRP4 recombinant protein to disturb the interaction between \(^{125}\)I-CTRP4 and IL-6R. IL-6 and OSM were chosen as positive and negative control, respectively.
(G) HEK293T cells were transfected with plasmid vectors encoding EGFP-IL-6R (green) and pmCherry-IL-6 (red) together in the presence or absence of rhCTRP4. The interaction of IL-6 to IL-6R was detected by laser confocal microscopy.

(H) Increasing concentrations of recombinant CTRP4 or irrelevant BSA protein were incubated with solid-phase 200 ng/mL IL-6R protein. The interaction between CTRP4 and IL-6R was detected with anti-CTRP4 antibody by ELISA.

(I) Competitive blockade assays were performed by the addition of 200 ng/mL IL-6 to compete with CTRP4 for binding to IL-6. The competitive binding was detected with anti-IL-6R antibody by ELISA.

(J) Jurkat cells were transduced with retrovirus encoding Flag-tagged IL-6 or Flag-tagged IL-6 plus Myc-tagged CTRP4 together. The interaction between IL-6R and IL-6 from membrane proteins of Jurkat cell lysates were coimmunoprecipitated by anti-Flag antibody. Eluted proteins were analyzed with anti-IL-6R and anti-Flag antibodies, respectively.

(K) Serum levels of CTRP4 and IL-6 were determined by ELISA in physiological conditions before model induction or at the peak of EAE.

(L) Representative flow cytometry plots and percentage of cells after in vitro polarization into Th17 cell. In the presence or absence of rhCTRP4, the percentage of siRNA-nucleolin transfected CD4+ T cells differentiated into Th17 cells was assessed. (H-L) Data were shown as mean ± SEM and were from one of three independent experiments with similar results. The samples derived from the same experiment and gels/blots were processed in parallel (B-D, J). Statistical significance was determined using two-tailed unpaired Student t-test or one-way ANOVA with Tukey’s post-test. *p < 0.05; **p < 0.01.
Figure 6 Reconstitution of CTRP4 inhibits IL-6-mediated STAT3 activation

(A) Purified CD4+ T cells from Ctrp4−/− and WT mice were stimulated with IL-6 (100 ng/mL) for indicated time. Lysates were subjected to western blot analysis for indicated antibodies.

(B) Freshly isolated WT and Ctrp4−/− CD4+ T cells were treated with IL-6 (100 ng/mL) for 30 min. The level of phospho-STAT3 was examined by flow cytometry.

(C) Representative flow cytometry analysis of p-STAT3 or p-STAT1 of CD4+ T cells from Ctrp4−/− and WT EAE-induced mice at the peak of disease.

(D-F) WT CD4+ T cells were polarized to Th17. The intracellular IL-17A was analyzed by flow cytometry (D) and gene expression level of Rorc mRNA was analyzed by quantitative real-time PCR (E). Supernatants were collected to determine levels of IL-17A by ELISA (F).

(G) Purified WT naïve CD4+ T cells were polarized under Th17 conditions with indicated doses of rhCTRP4. The quantification of the percentage of CD4+IL17A+ cells were analyzed by flow cytometry.

(H) Purified WT CD4+ cells were activated with IL-6 (10 ng/mL) or rhCTRP4 (100 ng/mL) alone or treated with IL-6 prior to treatment with rhCTRP4 for 1 hr or without treatment as control. Western blot was performed to analyze the activation of STAT3.

(I) Purified WT CD4+ cells were activated with IL-6 or with IL-6 pretreated with various concentration of rhCTRP4 (100 ng/mL, 500 ng/mL and 1000 ng/mL), followed by western blot to analyze the activation of STAT3 and JAK2. The samples derived from the same experiment and gels/blots were processed in parallel. Representative of three independent experiments with 5 mice per experiment.

(J) Purified WT CD4+ cells were activated with IL-6 (10 ng/mL) prior to treatment with rhCTRP4 (100 ng/mL) for the indicated time. Time-dependent changes in the level of p-STAT3 were evaluated by western blot. Data were shown as mean ± SEM and were from one of three independent experiments with similar results. (D-F) Statistical significance was determined using paired Student t test; (G) Statistical significance was determined using one-way ANOVA with Tukey’s post-test. *p < 0.05;
**p < 0.01 , ns, not significance.

Figure 7: Mechanism underlying the inhibition of Th17 cell differentiation by CTRP4

(A-B) Proliferative response of Ba/F3-gp130-IL6R cells cultured for 2 days in the presence of exogenous rhCTRP4 (100 ng/mL), OSM (100 ng/mL), IL-6 (10 ng/mL), IL-3 (10 ng/mL), rhCTRP4 plus IL-6, OSM plus rhCTRP4 or left untreated (A). (B) Proliferative response of Ba/F3-gp130 cells cultured for 2 days with OSM (100 ng/mL), IL-6 (10 ng/mL), rhCTRP4 (100 ng/mL), hyper-IL-6 (10 ng/mL), the combination of IL6 (10 ng/mL) and IL-6R (10 ng/mL) the combination of IL6 and IL-6R plus rhCTRP4, hyper-IL-6 plus rhCTRP4, or IL-3 (10 ng/mL). The proliferation in indicated culture conditions was determined by the colorimetric CCK8 assay. Results were shown as relative light Units (RLU) and normalized to the growth of cells cultured in medium.

(C) Purified WT CD4+ cells were activated with hyper-IL-6 (10 ng/mL) prior to treatment with rhCTRP4 for 1hr or without treatment as control. Western blot was performed to analyze the activation of STAT3.

(D) Naïve CD4+ T cells were differentiated toward Th17 cells with TGFβ plus the combination of IL-6 (10 ng/mL) and IL-6R (10 ng/mL) in the presence or absence of rhCTRP4 (100 ng/mL); or differentiated toward Th17 cells with TGFβ and hyper-IL6 (10 ng/mL) in the presence or absence of rhCTRP4 (100 ng/mL). Data were shown as mean ± SEM and were from one of three independent experiments with similar results. (A-B,D) Statistical significance was determined using one-way ANOVA with Tukey’s post-test. ns, not significance.; **p < 0.01.
**Figure 8** In vivo administration of rhCTRP4 attenuates the clinical severity

**A** After EAE induction of WT mice, 500 ng/mice rhCTRP4 or control (BSA) were administrated intraperitoneally every day starting on disease onset day 9 postimmunization until sacrificed. The clinical scores were monitored daily and depicted (n=10/group).

**B** Representative images of hematoxylin/eosin (H&E) and Luxol Fast Blue (LFB) staining on spinal cord sections of mice at the peak of EAE. Scale bar, 200 μm.

**C** CNS monocytes were harvested on day 18. The absolute cell numbers of indicated CNS-infiltrating cell populations (gated on CD45+) or the absolute number of CD4+IFNγ+, CD4+IL-17A+ and CD4+IL-17A+ IFNγ+ in CNS were analyzed.

**D** Representative immunofluorescent images and quantification of IL-17A+ cells in the spinal cord of indicated mice at day 18 after immunization. Nuclei were counterstain with DAPI. Scale bar, 100 μm.

**E** Representative immunohistochemistry detection and quantification of p-STAT3(Y705) in the spinal cord of indicated mice at day 18 after immunization. Scale bar, 100 μm.

**F** CD4+ T cells were isolated from Ctrp4-/- and WT mice 18 days after EAE induction and the activity phosphorylation of STAT3 and JAK2 was detected by western blot.

**G-H** Lymph node CD4+ T cells isolated from rhCTRP4-treated group or control group were expanded
with irradiated autologous presenting cells plus 10 μg/mL (G) or indicated concentration (H) of MOG35-55 peptide for 72 hr and subjected to cell proliferation assay to determine T cell recall response based on BrdU assay (G) or quantitate the productions of IL-17A and IFN-γ (H).

(I) Ctrp4−/− female mice were immunized with MOG35-55 peptide to induce EAE. 500 ng/mice rhCTRP4 or control (BSA) were administered intraperitoneally every day starting on disease onset day 9 postimmunization until sacrificed to restore the level of CTRP4. Clinical scores were depicted (n=8/group). Data were shown as mean ± SEM and were from one of three independent experiments with similar results. (A, I) Statistical significance was determined using two-way repeated measure ANOVA. Data were analyzed by unpaired Student t test or Mann-Whitney U test as appropriate after assessing for distribution (C-E, G-H); *p < 0.05; **p < 0.01, ***p < 0.001, ns not significance.

**Figure 9** IL-6 signaling blockade abrogates the protective effects of CTRP4 in EAE

(A) WT mice were intraperitoneally injected with anti-IL6R or control IgG on immunization days 1, 3, 7, 11 and 15 (n=5 mice/group). After EAE induction, mice were treated with rhCTRP4 or BSA daily from day 0 to day 27 after EAE induction. Mean clinical scores were shown progression of disease.

(B) CNS monocytes were harvested on day 18 and quantified the absolute cell numbers of indicated CNS-infiltrating cell populations gated on CD45+.

(C) CNS monocytes were harvested on day 18 and quantified the absolute number of CD4+IFNγ+, CD4+IL-17A− and CD4+IL-17A+ IFNγ+ in CNS after stimulating with PMA and inomycin with GolgiPlug for 5 hr.

(D) Ctrp4−/− and WT mice subjected to MOG-induced EAE were treated with selective STAT3 inhibitor S3I-201 (10 mg/kg/day dissolved in 20% DMSO/80% corn oil). Control mice were injected equal volume of vehicle. Each group was monitored and scored daily (n=7/group).

(E) Representative images of H&E staining and LFB staining of spinal cord sections showed inflammatory cell infiltration and demyelination, respectively. Scale bar, 200 μm. Data were shown as mean ± SEM and analyzed by one-way ANOVA with Tukey’s post-test (B-C). Two-way repeat measure ANOVA and Holm–Sidak post hoc test (A, D), *p < 0.05; ns, not significance.
Figure 10 Mice transferred with MOG-reactive T cells that expand in the presence of rhCTRP4 develop mild EAE

(A) Lymphocytes from the draining LNs of B6.SJL mice (CD45.1+) that were previously immunized with MOG<sub>35-55</sub> in CFA were rechallenged with the MOG<sub>35-55</sub> peptide in the presence of BSA or rhCTRP4 under Th17 polarization condition. The intracellular IL-17A was analyzed via flow cytometry (left) and the production of IL-17A in the supernatants was measured by ELISA (Right).

(B) After PMA/ionomycin stimulation for 5 hr, the representative FACS plots and the frequency of CD4<sup>+</sup>IL-17A<sup>+</sup>, CD4<sup>+</sup>IFNγ<sup>+</sup>, CD4<sup>+</sup>IL-17A<sup>+</sup>IFNγ<sup>+</sup> and CD4<sup>+</sup>IL-17A<sup>+</sup>GM-CSF<sup>+</sup> before the time of adoptive transfer were shown.

(C) Ex vivo-expanded MOG-specific CD4<sup>+</sup> T cells under Th17 polarization conditions in the presence of BSA or rhCTRP4 were analyzed for the expression of indicated gene by quantitative PCR. The values were normalized against gapdh.

(D-G) Ex vivo-expanded MOG-specific CD4<sup>+</sup> pretreated with BSA or rhCTRP4 were transferred into irradiated congenic recipients (CD45.2) to induce EAE. (D) The clinical scores of EAE progression were monitored daily. (E) The mononuclear cells isolated from brain and spinal cord at disease peak stage was analyzed by flow cytometry. The absolute number of CD4<sup>+</sup>T cells of donor and recipient mice in CNS were analyzed. The gated CD45.1<sup>+</sup>CD4<sup>+</sup>IL-17<sup>+</sup> T cells were analyzed for the production of IFNγ and GM-CSF. Representative contour plots (F) showed the percentage and absolute number of CD4<sup>+</sup>IL-17<sup>+</sup>IFNγ<sup>+</sup>, CD4<sup>+</sup>IL-17GM-CSF<sup>+</sup> donor cells in the CNS(G). Data were shown as mean ± SEM and were from one of three independent experiments with similar results. The two-way repeated measure ANOVA was used for (D). Statistical significance was determined using unpaired Student t test or Mann-Whitney U test for (A-C, E and G); *p < 0.05; **p < 0.01, ***p < 0.001, ns not significance.