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Therapeutic Effect of Ergotope Peptides on Collagen-Induced Arthritis by Downregulation of Inflammatory and Th1/Th17 Responses and Induction of Regulatory T Cells

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Rheumatoid arthritis (RA) is a systemic autoimmune disease that results in a chronic and inflammatory disorder. Dynamic balance of helper T cells (Th) 1 and 17 and regulatory T cells (Treg) is broken in RA. Since there is no cure for RA at present, it is necessary to find a truly effective and convenient treatment. Several studies have intended to induce ergotopic regulation to treat autoimmune diseases. This study was undertaken to find potential ergotope peptides and investigate their effects in treating the animal model of RA and their underlying regulatory mechanisms. First, we selected functional ergotope peptides from 25 overlapping peptides derived from the interleukin 2 receptor (IL-2R) α chain, and then used these peptides to treat collagen-induced arthritis (CIA). We showed ergotope peptides as immunomodulatory factors with great benefits at the clinical and pathologic levels. This effect was associated with inhibition of type II collagen (CII)-specific proliferation and autoantibody production as well as induction of antiergotypic immune response, downregulation of both Th1 and Th17 cells and their related components, and emergence of Treg cells that had suppressive action on autoreactive T cells. We also proved that cytotoxic T lymphocyte–associated antigen-4 (CTLA-4) and IL-10 are two important mediators that are critical to Treg suppressive function. Inhibition of Th1 and Th17 in established CIA could be attributed to ergotope-induced Treg cells. Our findings reveal that ergotope peptides induce regulatory immune responses and restore immune tolerance, suggesting that treatment with ergotope peptides may be a novel approach to therapy for RA patients and has good application prospects, with cheap, effective, convenient, wide-spectrum features.
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INTRODUCTION

Rheumatoid arthritis (RA) is a chronic inflammatory disease of polyarticular arthritis affecting approximately 1% of adults worldwide (1,2). It typically leads to deformity and destruction of the joints, as well as systemic disorders throughout the body.

Although the etiology and pathogenesis of RA remain unknown, immunological hyperreactivity caused by many T cells, mostly cluster of differentiation (CD) 4 and plasma cells, is generally considered to be an important contributor to its development. Both clinical and experimental evidence strongly suggest that helper T cell (Th) 1 responses and the principal effector cytokine interferon (IFN)-γ mediate the synovial inflammatory cascade, synovial hyperplasia and joint destruction in arthritis (3–6). In 2005, Th17 was defined as a new subset of Th cells (7,8). Th17 cells are characterized by production of interleukin (IL)-17, which is elevated in RA patients and in the CIA mouse model, involving synovitis and articular cartilage damage (9–11). In contrast, regulatory T cells (Treg), known as suppressor T cells, are a subpopulation of T cells that modulate the immune system and abrogate autoimmune disease by expressing coinhibitory molecules, such as cytotoxic T lymphocyte–associated antigen-4 (CTLA-4), or secreting antiinflammatory cytokines, such as IL-10 (12,13). In a steady state, Th1, Th17 and Treg may remain in dynamic

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balance. When this delicate balance is broken, it can lead to autoimmune disease (14). Decreased counts and functional defects of Treg in RA have been reported to be associated with a high tumor necrosis factor (TNF)-α level, which decreases forkhead box p3 (FOXP3) expression or defects in CTLA-4 expression (15–17). Thus it is meaningful for clinical treatment and assessment to study Th subsets involved in RA as well as the balance of effector T cells and regulatory T cells in RA patients and CIA, a frequently used animal model of human RA.

Regarding the current therapy, nonsteroidal antiinflammatory drugs (NSAIDs) and disease-modifying antirheumatic drugs (DMARDs) are mainly used to reduce acute inflammation, thereby decreasing pain and improving function. As biotherapy is developed, several approaches to immunotherapy are applied to prevent and/or treat RA. Due to their crucial roles in the pathogenesis of RA, biological agents targeting inflammatory cytokines or autoreactive T cells, including TNF inhibitors, IL-1 inhibitors, IL-6 inhibitors, T cell costimulatory blockers and T cell vaccines, have led to effective therapy for RA (18,19). A phase I study of T cell vaccination (attenuated autoimmune T cells) in 13 RA patients was first reported in 1993 (20). Later, our study found that a potentially beneficial immunomodulatory response toward activated T cells in general, specifically against peptides derived from the IL-2Rα-chain (so-called antiergotypic T cells), was obtained by vaccinating RA patients with expanded, activated and irradiated autologous synovial T cells (21).

Immunization with attenuated activated autoreactive T cells induces a response in syngeneic animals that can induce protection or recovery from autoimmune disease (22,23). This process has been termed T cell vaccination (TCV) (24). TCV activates both antiidiotypic and antiergotypic T cells. These regulators provide a useful view of the physiology of T cell regulation of the immune response (25). Ergotope is an activation marker whose level of expression is upregulated during the course of T cell activation, and ultimately it is presented to and induces antiergotypic regulators. Since expression of CD25 and hot shock protein (HSP) 60 are induced upon T cell activation, it was suggested that CD25 (interleukin-2 receptor α chain, IL-2Rα) and HSP60 might serve as the ergotopes (26–28). HSP60, acting as ergotope, was found to control both the effector T cells and the regulatory HSP60-specific T cells that control them (28).

The aim of this study was to screen the functional peptide(s) of the IL-2Rα chain, investigate the activities of the functional peptide(s) in an experimental model of RA and reveal the potential mechanisms. Administration of ergotope peptides showed great benefits at the clinical and pathologic levels. This effect was associated with inhibition of CII-specific proliferation and induction of antiergotypic immune response, downregulation of both Th1 and Th17 cells and their related components of the disease, and emergence of Treg cells that had suppressive actions on CII-specific T cells. This study has important implications for understanding and treating patients with RA with functional ergotope peptides that have good application prospects with cheap, effective, convenient, wide-spectrum therapy.

MATERIALS AND METHODS

Ethics Statement

The animal protocol used in this study was approved by the Institutional Review Board of Shanghai Jiao Tong University School of Medicine. All mice received humane care in compliance with the 2011 Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (29).

Animals and Arthritis Induction

Male DBA/1 mice 6 to 8 wks old were purchased from Shanghai SLAC Laboratory Animal Co. Ltd., and were housed in the animal care facility of Shanghai Jiao Tong University School of Medicine under pathogen-free conditions according to Institutional Animal Care and Use Committee guidelines. To induce CIA, mice were injected subcutaneously in the base of the tail with 0.1 ml emulsion containing 150 µg chick type II collagen (CII; Chondrex) and 200 µg Mycobacterium tuberculosis (strain H37RA, Difco). On d 21, mice were given a subcutaneous booster injection of 50 µg CII in complete Freund's adjuvant (CFA).

Preparation of T Cell Vaccine and TCV Treatment

Spleen mononuclear cells (MNCs) were prepared from CIA mice by grinding through a nylon mesh. The resulting MNCs were incubated at 5×10^5 cells/well with irradiated antigen-presenting cells (APCs) in complete medium containing 50 IU/well recombinant mouse IL-2 and 20 µg/mL CII in a 96-well plate. The culture medium was changed every 3 d and irradiated APCs were added every week. After 21 d of culture, cell lines were harvested. T cell vaccine (1×10^7 irradiated T cells for each mouse) was administered subcutaneously 2 wks before establishment of CIA. Mice were euthanized at the peak of CIA (about 35 d after first immunization).

Ergotope Peptide Synthesis and Treatment

Twenty-five overlapping peptides and three control peptides containing 20 amino acids were synthesized according to the amino acid sequence of murine CD25 (IL-2Rα) and CD132 (IL-2Rγ).The sequences are as follows: peptide 1 (p1, aa 1–20) meprllmlgf lsltivpscr; peptide 2 (p2, aa 11–30) lsltivpscr aelclydppe; peptide 3 (p3, aa 21–40) aelclydppe vpnatfkals; peptide 4 (p4, aa 31–50) vpnatfkals ykngtilnce; peptide 5 (p5, aa 41–60) ykngtilnce ckrgfrrlke; peptide 6 (p6, aa 51–70) ckrgfrrlke lvymrclgns; peptide 7 (p7, aa 61–80) lvymrclgns wssncqctsn; peptide 8 (p8, aa 71–90) wssncqctsn shdksrkqvt; peptide 9 (p9, aa 81–100) shdksrkqvt aqlehqkeqq; peptide 10 (p10, aa 91–110) aqlehqkeqq tttdmqkptq; peptide 11 (p11, aa 101–120) tttdmqkptq smhqenltgh; peptide 12 (p12, aa 111–130)

smhqenltgh crepppwkhe; peptide 13 (p13, aa 121–140) crepppwkhe dskriyhfve; peptide 14 (p14, aa 131–150) dskriyhfve gqsvhyecip; peptide 15 (p15, aa 141–160) gqsvhyecip gykalqrgpa; peptide 16 (p16, aa 151–170) gykalqrgpa isickmkcgk; peptide 17 (p17, aa 161–180) isickmkcgk tgwtqpqltc; peptide 18 (p18, aa 171–190) tgwtqpqltc vderehhrfl; peptide 19 (p19, aa 181–200) vderehhrfl aseesqgsrn; peptide 20 (p20, aa 191–210) aseesqgsrn sspesetscp; peptide 21 (p21, aa 201–220) sspesetscp itttdfpqpt; peptide 22 (p22, aa 211–230) itttdfpqpt ettamtetfv; peptide 23 (p23, aa 231–250) ltmeykvava sclfllisil; peptide 24 (p24, aa 241–260) sclfllisil llsgltwqhr; peptide 25 (p25, aa 251–268) llsgltwqhr wrksrrti; control peptide 1 (IL-2Rγ 71–90) nctwnsssep qatnltlhyr; control peptide 2 (IL-2Rγ 201–220) livnheprfs lpsvdelkry; control peptide 3 (IL-2Rγ 341–360) algegpggsp cslhspywpp.

There are two modes of peptide administration: pulse administration, which was giving 1000 μg peptide in 500 μl volume intraperitoneally 2 wks before first immunization for prevention or 1 wk post first immunization for treatment, and every-other-day administration, which was giving 200 μg peptide in 200 μl volume intraperitoneally five times, starting from the same time points, for prevention or treatment. Mice in the control group were injected in the same way with the same volume of sterile phosphate-buffered saline (PBS).

Assessment of Arthritis

Mice were evaluated by two independent blinded examiners every day according to the following clinical score assessment system: grade 0 = no swelling; 1 = mild, with definite redness and swelling of the ankle or wrist or apparent redness and swelling limited to individual digits, regardless of the number of affected digits; 2 = moderate redness and swelling of ankle or wrist; 3 = severe redness and swelling of the entire paw, including digits; and 4 = maximally inflamed limb with involvement of multiple joints. Each limb was scored, yielding a maximum possible score per animal of 16.

Histologic Analysis

For histologic analysis, mice were euthanized by cervical dislocation at about 45 d after first immunization. The paws from four to six animals were randomly collected by two independent experimenters, fixed in 4% buffered formaldehyde, decalcified in ethylene diaminetetraacetic acid, embedded in paraffin and cut into 4 μm sections, which were then stained with hematoxylin and eosin. Histopathological changes were evaluated by optical microscope. Specifically, we assessed cell infiltration, cartilage destruction and bone erosion.

Mononuclear Cell Preparation

Splenic MNC suspensions from mice were prepared by grinding through a nylon mesh. Erythrocytes were osmotically lysed. MNCs were suspended in RPMI 1640 (Gibco, Life Technologies) supplemented with 200 U/mL penicillin, 200 U/mL streptomycin, 55 μM β2-mercaptoethanol and 10% heat-inactivated fetal bovine serum.

Enzyme-Linked Immunospot (ELISPOT) Assay

CD4 + T cells were isolated from the mouse spleens with CD4 isolation kits (Miltenyi Biotec). MACS-isolated splenic CD4 + T cells (2×10^5) were added to purified antimouse IL-10–coated ELISPOT assay plates (eBioscience) and coincubated with control or ergotope peptides at a final concentration of 10μg/mL, in the presence of APCs. All ELISPOT assay plates were incubated for 48 h and developed with biotin-labeled antimouse IL-10 antibody, peroxidase-conjugated streptavidin and aminoethylcarbazole dye solution (Sigma-Aldrich). The spots were counted microscopically, and the numbers of IL-10–secreting CD4 + T cells were calculated from the number of spots in triplicate wells.

Proliferation Assay of CII-Specific T Cells

Mice were euthanized at the peak of CIA. Splenic MNCs were prepared and incubated (5×10^5cells/well) in the absence and presence of CII antigen (0, 5, 10, 20 μg/mL) for 48 h. [^{3}H] thymidine (1 μCi/well) was added during the last 24 h of culture. The counts per minute (cpm) value was then detected by liquid scintillation counter.

In Vitro Suppression Assay

Spleens from the mice of each group were obtained at d 35 post first immunization. CD4 + CD25 + T cells (Treg) and CD4 + CD25 – T cells were purified by using a mouse regulatory T cell isolation kit (Miltenyi Biotec) according to the manufacturer's protocol. The average purity of CD4 + T cells and CD4 + CD25 + T cells was >95–98% as determined by flow cytometry (not shown). CD4 + T and Treg cells were cocultured at ratios of 1:0, 1.25:1, 2.5:1, 5:1, 10:1 and 20:1, stimulated with anti-CD3 (1 μg/mL) and anti-CD28 antibody (1 μg/mL), and cultured for 72 h. [^{3}H] thymidine (1 μCi/well) was added during the last 24 h of culture. The cpm value was then detected by liquid scintillation counter. Inhibition rate was determined by using the following formula:

$$\text{inhibition rate (\%)} = (R–r)/R*100,$$

where R = cell proliferation in Teff:Treg at a ratio of 1:0 (cpm) and r = cell proliferation in the indicating coculture ratio (1.25:1, 2.5:1, 5:1, 10:1, 20:1 [cpm]).

In function-blocking experiments, CD4 + T and Treg cells were cocultured at a ratio of 1.25:1. Anti-IL-10, anti-CTLA-4 and anti-IL-10 + anti-CTLA-4 blocking antibodies were added to the cultures at the beginning, and cells were cultured for 3 d. At the last 24 h of culture, [^{3}H] thymidine (1 μCi/well) was added. The cells were harvested and [^{3}H] thymidine incorporation was counted with a liquid scintillation counter.

Flow Cytometric Analysis

Splenocytes from each group were isolated on d 14 post immunization. For intracellular IL-17 and IFN-γ staining, splenocytes were stimulated for 5 h with 50 ng/mL phorbol myristate

acetate (Sigma-Aldrich), 500 ng/mL ionomycin (Sigma-Aldrich) and GolgiPlug (BD Pharmingen) at the recommended concentrations. Cells were stained with fluorescein isothiocyanate–conjugated anti-CD4, fixed and permeabilized with Cytofix/Cytoperm solution (BD Pharmingen), and then labeled with allophycocyanin (APC)-conjugated anti–IFN-γ (eBioscience) and phycoerethrin (PE)-conjugated anti–IL-17 (eBioscience). To analyze Treg, APC-conjugated anti-CD4 (eBioscience) and PE-conjugated anti-CD25 (eBioscience) were used for surface staining. Cells were fixed and made permeable for 60 min at 4°C using a Fixation and Permeabilization Buffer Set (eBioscience) and were stained with FITC-conjugated anti-FOXP3 (eBioscience) for 60 min at 4°C in permeabilization buffer. Cells were stained with APC-conjugated anti–CTLA-4 to cell surface molecule. For intracellular staining of CTLA-4, surface stained cells were fixed and permeabilized with a Cytofix/Cytoperm buffer set (BD Pharmingen) and then stained with APC-conjugated anti–CTLA-4. Cells were analyzed on a FACSCalibur flow cytometer.

Cytokine Measurement

The levels of cytokines were determined by enzyme-linked immunosorbent assay (ELISA) using IFN-γ, IL-17, transforming growth factor (TGF)-β and IL-10 kits (eBioscience). Serum samples were collected from each group of mice at the peak of disease and also adoptive transfer mice. Mice were euthanized on d 14 post first immunization. Then 200 μl aliquots of splenic MNC suspensions (5×10^6/mL) were added into 96-well round-bottom microtiter plates and stimulated with CII (20 μg/mL). After 48 h of incubation at 37°C in 5% CO_2 and humidified atmosphere, the supernatants were harvested. The cytokines in the supernatants as well as sera were detected according the ELISA kit instructions.

Immunoassay of Serum Anti-CII Antibody Level

The levels of anti-CII and total IgG, IgG1 and IgG2a were measured by ELISA as described (30). Chicken CII (1 μg/mL) was coated onto microtiter plates overnight at 4°C. After blocking with 1% bovine serum albumin in PBS, serially diluted serum samples were added and incubated for 1 h at room temperature. After washing, goat antimouse IgG, IgG1 and IgG2a coupled with horseradish peroxidase (eBioscience) were added and incubated for 1 h at 37°C. Following five washes, tetramethylbenzidine was added and stopped by 2N H_2SO_4. Absorbance was then measured at 450 nm on a microplate reader (BioRad, Hercules). A standard serum composed of a mixture of sera from arthritic mice was added to each plate in serial dilutions and a standard curve was constructed. The standard serum was defined as 1 unit, and the antibody titers of serum samples were determined by the standard curve.

RNA Isolation

Total RNA was isolated from splenic MNCs (1×10^6) with CII (20μg/mL) stimulation for 1 d using an RNeasy Mini Kit (Qiagen). Genomic DNA was removed from total RNA prior to cDNA synthesis using an RNase-free DNase Set (Qiagen). First-strand cDNA synthesis was performed for each RNA sample using a Sensiscript RT Kit (Qiagen). Random hexamers were used to prime cDNA synthesis.

Real-Time Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Analysis of Gene Expression

Primer Express software (ABI) was used to design primers from published cDNA sequences. Basic local alignment search tool searches were conducted on the primer nucleotide sequences to ensure gene specificity. The primer sequences were as follows: *β-actin*, forward 5′-TTCAACACCCCAGCCATGT-3′ and reverse 5′-GTGGTACGACCAGAG GCATACA-3′; *IFN-γ*, forward 5′-GA TGCATTCATGAGTATTGCCAAGT-3′ and reverse 5′-GTGGACCACTCGGATG AGCTC-3′; *T-bet*, forward 5′-GGTGTC TGGGAAGCTGAGAG-3′ and reverse 5′-TCTGGGGTCACATTGTTGGAA-3′; *IL-17*, forward 5′-CTCCAGAAGGCC CTCAGACTAC-3′ and reverse 5′- GGGT CTTCATTGCGGTGG-3′; *signal transducers and activators of transcription (STAT)3*, forward 5′-TGCCCATGGCTACCTGTT-3′ and reverse 5′-GAACCTCCTGGGCTTA GTCC-3′; *RAR-related orphan receptor γt (RORγt)*, forward 5′-GGAGCTCTGCC AGAATGAGC-3′ and reverse 5′-CAAGG CTCGAAACAGCTCCAC-3′; *FOXP3*, forward 5′-GGCCCTTCTCCAGGACAGA-3′ and reverse 5′-GCTGATCATGGCTGGG TTGT-3′. Relative quantification of gene expression was performed using the ABI Prism 7900 sequence detection system. SYBR Green master mix (ABI) was used for real-time RT-PCR to detect the abundance of PCR products among samples. Thermocycler conditions comprised an initial holding at 50°C for 2 min, then 95°C for 10 min. This was followed by a two-step PCR program consisting of 95°C for 15 s and 60°C for 60 s for 35 cycles. Data were collected and quantitatively analyzed on an ABI Prism 7900 HT sequence detection system (ABI). The β-actin gene was used as an endogenous control to normalize the differences in the amount of total RNA in each sample. All quantities were expressed as -fold values relative to a calibrator.

Adoptive Transfer of Arthritis

DBA/1 mice were immunized with CII and CFA in the presence or absence of peptide treatment. After 14 d, total CD4 + T cells, CD4 + CD25– T cells and CD4 + CD25 + T cells derived from CIA or peptide-treated CIA mice were resuspended in PBS and transferred (2×10^7 cells combined from the mice in the same group) into naive DBA/1 through intravenous injection, and then the mice were immunized with CII on the same day. Arthritis development was observed in recipient mice, as described above.

Statistical Analysis

All values are expressed as the mean ± SD. Student *t* test was used to analyze the differences between the groups. One-way ANOVA was initially

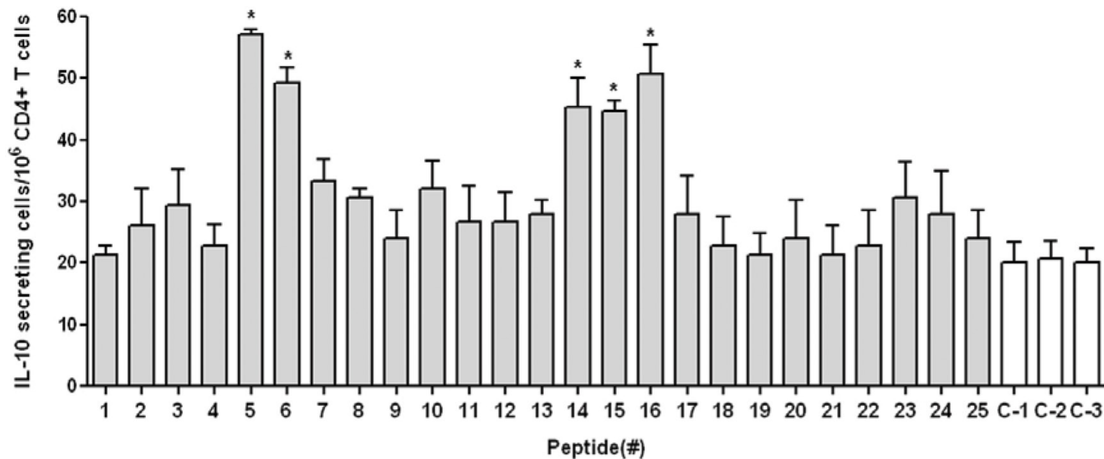

Figure 1. Screening for ergotope peptides. Twenty-five overlapping peptides and three control peptides (abbreviated C-1, C-2 and C-3), each containing 20 amino acids, were designed according to the amino acid sequence of murine CD25 (interleukin-2 receptor α chain, IL-2Rα) and CD132 (interleukin-2 receptor γ chain, IL-2Rγ). CD4 + T cells were isolated from the draining lymph nodes of TCV-treated mice at d 45 and then stimulated with different peptides at a dose of 10 μg/mL in the presence of APC for 48 h. IL-10 + cells were detected by ELISPOT assay. Triplicate wells were used. *: $P < 0.05$ versus controls. Data are representative of three separate experiments with similar results.

performed to determine whether an overall statistically significant change existed before using the two-tailed paired or unpaired Student *t* test. A value of $P < 0.05$ was considered statistically significant.

RESULTS

Ergotope Peptides with Immune Regulation were Screened

In previous studies, we found that TCV vaccine could induce antiergotypic regulation in patients with RA and in mice with CIA (21). To further investigate the functional sites of murine IL-2Rα, we synthesized the overlapping peptide derivatives based on the structural features of IL-2Rα to generate peptide libraries. ELISPOT analysis was used to detect the IL-10–secreting cells in the different peptide-stimulated CD4 + T cells that were isolated from the draining lymph nodes of TCV-treated mice. IL-10 spot-forming cells were significantly increased when cells were stimulated with peptide 5 (IL-2R41–60), peptide 6 (IL-2R51–70), peptide 14 (IL-2R131–150), peptide 15 (IL-2R141–160) and peptide 16 (IL-2R151–170) (Figure 1). These peptides were considered to be potential functional sites of IL-2Rα that could serve as immunoreactive ergotope peptides and were used in the later experiments.

Ergotope Peptide Treatment Ameliorated the Development of CIA

CIA is a mouse model induced by immunization with an emulsion of CFA and type II collagen that shares features of the human autoimmune disease rheumatoid arthritis (31). In this study, we used the CIA model to examine the effects of ergotope peptides. Two different modes of administration, pulse and every-other-day administration, were utilized in the peptide-treated groups. P5–6 and P14–16 peptide treatment of CIA mice resulted in a progressive decrease in the severity of CIA as compared with untreated mice (CIA) and control peptide treated mice (Figures 2A and B). There was no significant difference between CIA mice and control peptide-treated mice, but P5–6 and P14–16 peptides had a preventive effect (Figure 2A) as well as a therapeutic effect (Figure 2B). Furthermore, every-other-day administration was more effective than pulse administration. Thus, we applied therapeutic treatment with every-other-day administration to investigate the potential mechanisms. Histopathologic analysis of joints showed that peptide treatment significantly abrogated the chronic inflammation of synovial tissue, pannus formation, cartilage destruction and bone erosion that are characteristic of CIA (Figure 2C). Individuals with rheumatoid arthritis have autoreactive antibodies in their blood targeting their own body tissues. High levels of circulating antibodies have a strong correlation with development of RA and CIA. Ergotope peptide treatment resulted in reduced serum levels of CII-specific IgG, particularly IgG2a antibodies (Figure 2D).

To find the possible mechanisms, we determined the proliferation of splenic MNCs isolated from the CIA group, the control peptide (C-P) treated group, the P5–6 treated group and the P14–16 treated group in response to CII *in vitro*. CIA mice under ergotope peptide treatment significantly inhibited expansions of CII-specific mononuclear splenocytes (Figure 3A). CD4 + T cells from each group were sorted by MACS and stimulated with CII-specific T cells (TCV), control peptide,

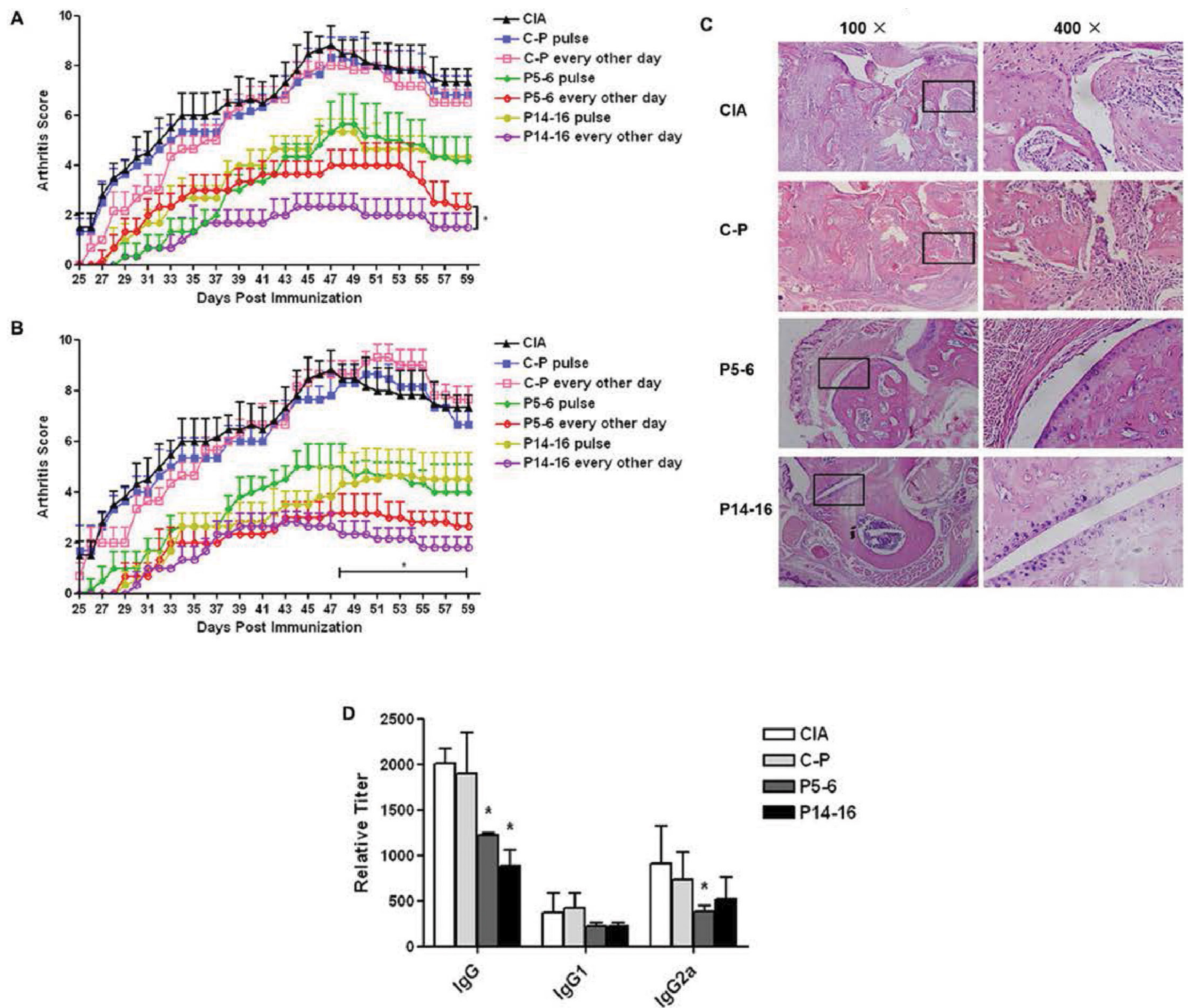

Figure 2. Preventive and therapeutic effects of ergotope peptides on CIA. Mice were randomly divided into seven groups, eight mice in each: CIA, control peptide treated (C-P), peptides 5–6 (P5–6) and peptides 14–16 (P14–16). Two different modes of administration were applied in all treated groups. The pulse administration regimen was giving 1000 μg peptide in 500 μl volume through intraperitoneal injection at 14 d before first immunization for prevention (A), or at 7 d post first immunization for treatment (B), while every-other-day administration was giving 200 μg peptide in 200 μl volume intraperitoneally five times starting 14 d before first immunization for prevention, or 7 d after first immunization for treatment (B). Mice in the CIA group were injected in the same way as control, with the same volume of sterile PBS every other day. (A and B) Clinical scores were assessed daily. Data are represented as mean ± SD (mice/group). *: $P < 0.05$, P14–16 every other day versus P5–6 every other day. (C) Histopathological changes of joints were examined in the CIA group, C-P treated group, and P5–6 and P14–16 every-other-day groups on d 45. Representative sections stained with hematoxylin and eosin in each group (B) are shown (left panel, × 100; right panel, × 400). (D) The levels of IgG, IgG1 and IgG2a in sera were measured by ELISA at the peak of disease in the groups described in (C). Data are represented as mean ± SD. *: $P < 0.05$, versus C-P group. Data are representative of three separate experiments with similar results.

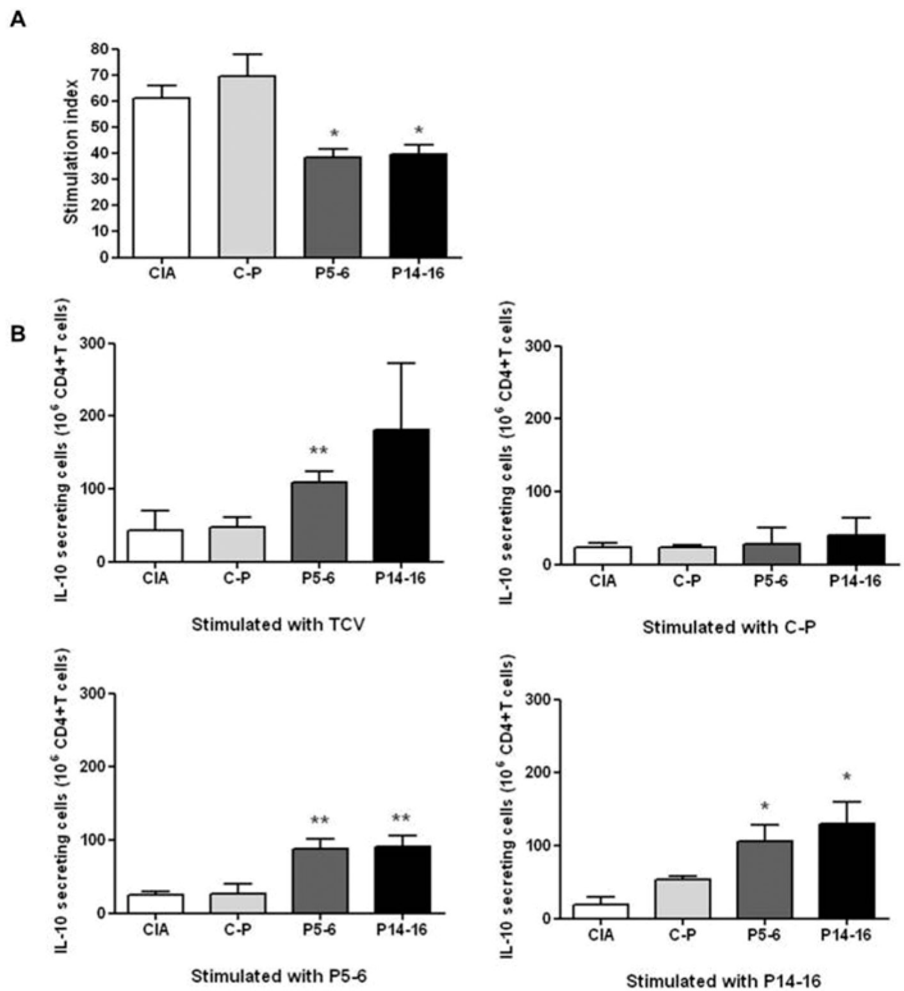

Figure 3. Inhibition of CII-specific proliferative response and induction of antiergotypic immune response by ergotope peptide treatment. (A) Mice from the CIA, C-P treated, P5–6 treated and P14–16 treated every-other-day groups were euthanized at the peak of disease. Splenic mononuclear cells were isolated and cultured at the density of 5×10^5/well with stimulation of CII antigen (20 μg/mL) *in vitro*. H3–TdR (1 μCi/well) was added at 48 h and cell proliferation was assessed at 72 h. The stimulation index is displayed normalized to the unstimulated controls. (B) CD4 + T cells were isolated from the spleens of mice in four groups treated with CII-specific T cells (TCV), C-P, P5–6 and P14–16 at a final concentration of 10 μg/mL in the presence of APC. ELISPOT assay was used to enumerate IL-10–producing cells. Data are represented as mean ± SD. *: $P < 0.05$ versus C-P group.

P5–6 and P14–16. Compared with CD4 + T cells from the CIA and C-P groups, CD4 + T cells from the ergotope peptide–treated group could recognize TCV and generated more IL-10–secreting cells (Figure 3B). Additionally, those CD4 + T cells also recognized peptides 5–6 and peptides 14–16, which triggered more IL-10 spot formations, suggesting that ergotope peptide treatment induced antiergotypic immune response *in vivo*.

Ergotope Peptides Restored the Balance of Th Subsets

It was reported that effector T cell subsets that include Th1 and Th17 cells were involved in the pathogenesis of RA, while Treg with immune suppression functions were decreased in RA (32). The intricate balance between Th17, Th1 and Treg cell function was central in understanding the immunological mechanisms of autoimmune diseases (33). We therefore investigated the influence of peptide treatment on Th subsets. Flow cytometry analysis showed that the percentages of Th1 and Th17 cells were significantly decreased in ergotope peptide–treated mice on d 14, when P14–16–treated mice showed even much less Th1 (0.87 ± 0.12) (Figure 4A, top) and Th17 (3.17 ± 0.19) (Figure 4A, bottom) compared with the P5–6–treated group (Th1: 2.05 ± 0.07; Th17: 6.23 ± 1.18). In the advanced stage of CIA, not Th1 but Th17 decreased in CIA mice with ergotope treatment (data not shown). Similarly, P14–16 treatment was more effective at impacting Th17 (data not shown). In addition, splenic MNCs from CIA mice produced slightly higher levels of IFN-γ and IL-17, which are inflammatory cytokines secreted by Th1 and Th17 cells, respectively (Figure 4B), and lower levels of TGF-β and IL-10 (Figure 4D), antiinflammatory cytokines secreted mainly by Treg. P14–16 treatment significantly enhanced IL-10 production from splenocytes stimulated with CII. After treatment with ergotope peptide, CD4 + CD25 + FOXP3 + T cells were induced (Figure 4C) as well as their releasing cytokines, especially IL-10 (Figure 4D). Downregulation of systemic inflammatory cytokines (Figure 4B) and induction of antiinflammatory mediators (Figure 4D) were observed in sera, accompanied by Th subset changes. Moreover, gene expression of the Th1-related cytokine *IFN-γ* and its transcriptional factor *T-bet* were decreased, as well as that of the Th17 cytokine *IL-17* and its transcriptional factors, *STAT3* and *RORγt* (Figure 4E). In contrast, *FOXP3* mRNA was upregulated after ergotope peptide treatment (Figure 4E).

The Immunosuppression Function of Treg Cells Resumed

Our results indicate that the number of Treg cells was increased in mice treated with ergotope peptides. It is important to

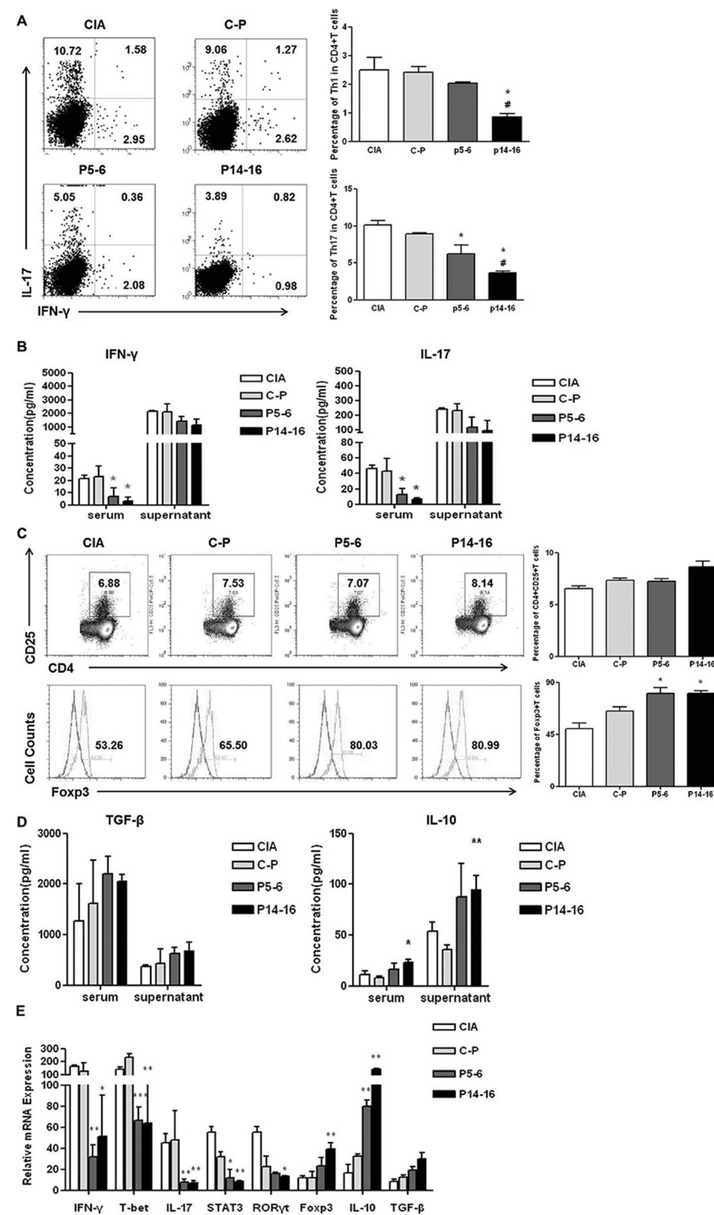

Figure 4. Downregulation of Th1 and Th17 and induction of regulatory T cells in CIA by ergotope peptide treatment. (A) Splenic mononuclear cells were isolated on d 14 post first immunization from untreated (CIA) or C-P, P5–6 or P14–16 treated mice with CIA and stimulated *in vitro* with CII (20 μg/mL) for 48 h and analyzed for CD4 and intracellular cytokine expression by flow cytometry. Dot plots (left) show representative double staining for IL-17A and IFN-γ in gated CD4 + T cells. Percentages of CD4 + IFN-γ + (Th1) and CD4 + IL-17A + (Th17) are shown on the right. Values are the mean ± SD of eight mice per group and pooled from three independent experiments. *: $P < 0.05$ versus C-P group; #: $P < 0.05$ versus P5–6 group. (B) Cytokine production was assessed by ELISA in sera and in supernatants by splenic mononuclear cells with CII stimulation (20 μg/mL) for 48 h. Values are the mean ± SD of eight mice per group. (C) Regulatory T cells were analyzed in the splenocytes from indicated groups by flow cytometry. Percentages of CD4 + CD25 + T cells are shown in the representative dot plots (upper panel). Histograms show the expression of FOXP3 + cells (dark gray lines) in gated CD4 + CD25 + cells from splenocytes, while light gray lines indicate antibody isotype controls. Data are representative of eight mice per group. Statistical analysis is displayed on the right. Values are the mean ± SD. *: $P < 0.05$ versus C-P group. (D) Treg-secreted cytokines TGF-β and IL-10 were measured by ELISA in sera and in supernatants by splenic mononuclear cells with CII stimulation (20 μg/mL) for 48 h. Values are the mean ± SD of eight mice per group. (E) The expressions of Th cell related mRNA, *IFN-γ*, *T-bet*, *IL-17*, *STAT3*, *RORγt*, *FOXP3*, *IL-10* and *TGF-β* in splenic mononuclear cells were detected by real-time qPCR at CII (20 μg/mL) stimulation for 24 h. *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$ versus C-P group.

better understand the functions of Treg. CTLA-4 is a member of the immunoglobulin superfamily, which is expressed on the surface of T cells and transmits an inhibitory signal to T cells through interaction with CD80/CD86, which are members of the B7 family (34,35). The costimulation blocking agent CTLA-4Ig was applied successfully for the treatment of RA, having not only a systemic effect, but potentially also a local one (36). In peptide-treated CIA mice, we found that extracellular (Figure 5A) and intracellular (Figure 5B) expressions of CTLA-4 in CD4 + CD25 + T cells were augmented, especially in the P14-16–treated group. The immunosuppressive function of CD4 + CD25 + regulatory T cells (Treg) from the group treated with ergotope peptides was significantly increased when we cocultured them with CD4 + CD25– effect T cells (Teff). Proliferation of Teff was conspicuously inhibited by Treg cells in the mice treated with ergotope peptides. As the proportion of Treg in the cultures increased, the extent of Teff proliferation decreased (Figure 5C). Moreover, Treg cells from the CIA and C-P treated groups had less ability to inhibit proliferation of Teff, suggesting an impaired function of Treg cells in CIA mice. Intriguingly, treatment with ergotope peptides restored the impaired suppressive function of Treg to some extent.

Treg cells are thought to suppress target cells by three mechanisms: cell-cell contact, local secretion of inhibitory cytokines and local competition for growth factors (37,38), in which CTLA-4 and IL-10 play important roles. We wanted to find out whether ergotope treatment restored Treg function through CTLA-4 and IL-10 pathways. As shown in Figure 5D, we blocked these two pathways using CTLA-4 and IL-10 function-blocking antibodies in the coculture system of Teff and Treg at a ratio of 5:1 and measured the proliferation response by H^3–TdR. Blocked with IL-10 and CTLA-4 antibodies, Treg suppression was reduced in each group (Figure 5D). Compared to the CIA and C-P treated groups, the ergotope peptide–treated group showed more obvious suppressive dysfunction under a blockade of IL-10 and CTLA-4, suggesting that ergotope peptides may have restored Treg function by upregulating the expression of CTLA-4 and the secretion of IL-10.

Ergotope Peptide–Treated Treg Cells Have Suppressive Function *In Vivo*

To further confirm the therapeutic effect of ergotope peptides and the mechanism by which they upregulate the quantity and quality of Treg cells, we determined the *in vivo* functions of ergotope peptide–treated T cells using adoptive transfer models. On d 14 post adoptive transfer, clinical scores were assessed. Mice received CD4 + CD25 + T cells from ergotope peptide–treated CIA mice, which dramatically reduced the arthritis (Figure 6A). Moreover, the serum levels of IgG and IgG2a were decreased in the mice that were transferred with CD4 + CD25 + T cells from ergotope peptide–treated donors (Figure 6B). Meanwhile, their CII-specific T cell proliferation was apparently inhibited (Figure 6C). These results demonstrate that the efficacy of ergotope peptides ameliorates CIA by inhibition of CII antigen-specific T cell activation and proliferation. It was also observed that the inflammatory cytokines IFN-γ and IL-17 were decreased and the antiinflammatory cytokines TGF-β and IL-10 were increased in both sera and the supernatants of CII-stimulated splenocytes of the recipient mice transferred with CD4 + CD25 + T cells from ergotope peptide–treated CIA mice (Figure 6D). The mRNA expressions of Th1, Th17 and Treg related cytokines and transcription factors displayed similar trends (Figure 6E).

DISCUSSION

Rheumatoid arthritis, a form of inflammatory arthritis, is an autoimmune disease without effective and specific treatment. In addition to its complicated risk factors, the pathogenesis of RA is not entirely clear, making it difficult to develop new drugs with therapeutic value and fewer side effects. Besides commonly used drugs like DMARDs and NSAIDs, which alleviate current symptoms and prevent future destruction of the joints, immunotherapies targeting inflammatory components have recently been discovered and developed. Therapeutic approaches include not only specific cytokine inhibitors, but also a T cell vaccine.

TCV has been reported to be effective in several T cell–mediated autoimmune diseases, including experimental autoimmune encephalomyelitis and experimental arthritis (39). Immunization with inactivated autoreactive T cells (TCV) can be used as a powerful means of activating antiidiotypic and antiergotypic T cells to regulate the autoreactive T cells potentially involved in autoimmune conditions (40–42). Our previous study found that T cell vaccination led to induction of CD4 + Tregs and CD8 + cytotoxic T cells specific for T cell vaccine by production of antiinflammatory IL-10 and cytotoxic activity (21). These CD4 + T cells may represent the so-called antiergotypic T cells seen in experimental animals vaccinated with activated T cells. Cohen and colleagues and other investigators have demonstrated that IL-2 receptor α-chain (CD25) acts as one of these potential ergotopes (26,43). Compared with those from health controls, peripheral CD4 + T cells from RA patients had lower CD25 peptide response, which was restored after treatment with TCV. Our findings demonstrate that T cell vaccination induces regulatory immune responses that are associated with improved clinical and laboratory variables in RA patients (21). Therefore, it is meaningful to find the ergotope peptide(s) with immunological activity.

Since TCV can induce antiergotypic response in CIA mice *in vivo*, we screened 25 overlapping peptides of CD25 to stimulate CD4 + T cells derived from TCV-treated mice and measured IL-10–secreting cells by ELISPOT. A significant increase in IL-10–producing cells was detected in cells treated with peptides 5, 6, 14, 15 and 16 compared with three control peptides that were

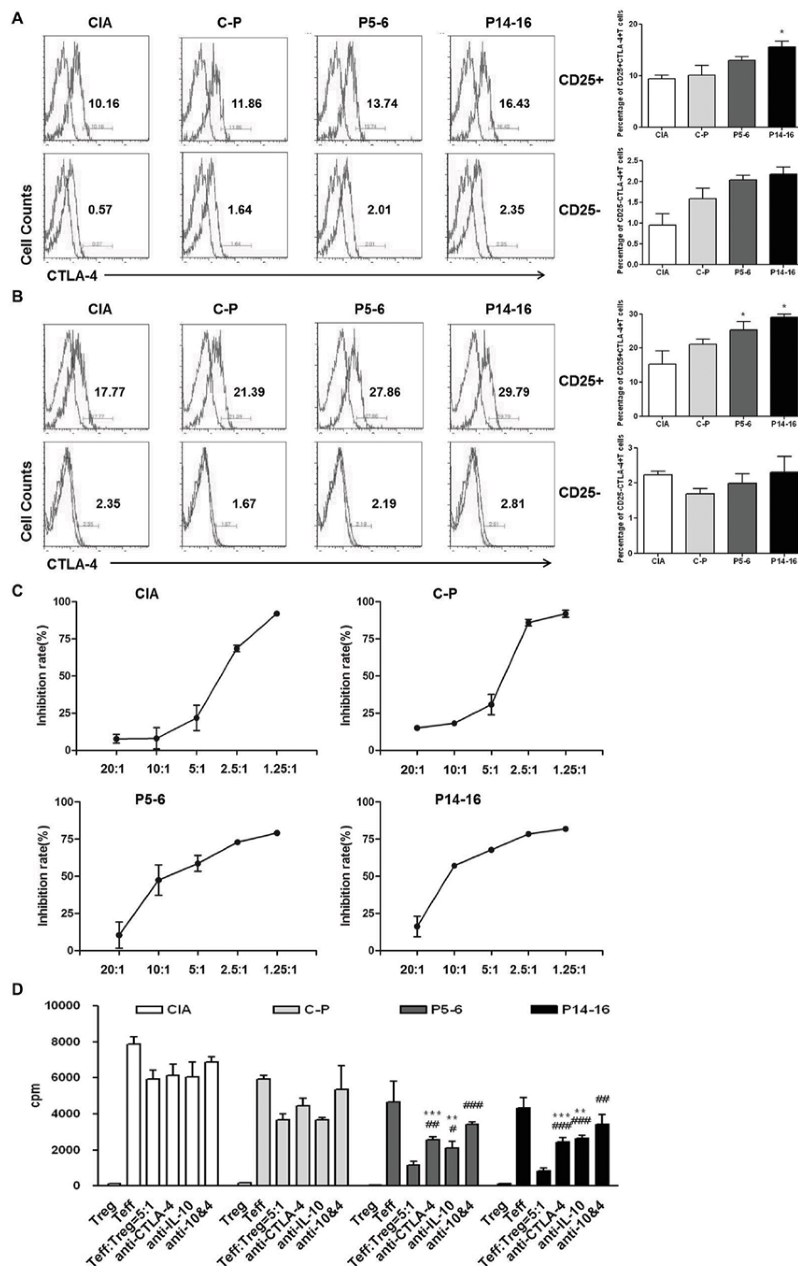

Figure 5. Treg function affected by ergotope peptide treatment. Flow cytometric analysis of CTLA-4 extracellular (A) and intracellular (B) expressions in CD4 + CD25 + (upper panel) and CD4 + CD25– (lower panel) splenocytes from untreated, C-P, P5–6 and P14–16 treated groups are shown. Statistical analysis is shown on the right. Values are the mean ± SD. *: $P < 0.05$ versus C-P group. (C) Inhibition rate was evaluated by H^3–TdR incorporation assay using the formula described in the Materials and Methods section. CD4 + CD25– T cells were isolated from mice with CIA and then cocultured with increasing numbers of CD4 + CD25 + regulatory T cells from untreated, C-P, P5–6 and P14–16 treated CIA mice on d 35 post first immunization for 3 d. Ratio indicates CD4 + CD25– T cells:regulatory T cells. Values are the mean ± SD of five mice per group from three independent experiments (each performed in triplicate). (D) Splenocytes were isolated from untreated, C-P, P5–6 and P14–16 treated mice with CIA on d 35 post first immunization; CD4 + CD25– effect T cells (Teff) and CD4 + CD25 + regulatory T cells (Treg) were sorted by MACS. H^3–TdR incorporation assay was used to test the cell proliferation of Treg, Teff, Teff coculture with Treg at a ratio of 5:1. Anti–IL-10, anti–CTLA-4 and anti–IL-10 + anti-CTLA-4 blocking antibodies were added to the culture and cells were stimulated with anti-CD3 and anti-CD28 for 72 h. H^3–TdR (1 μCi/well) was added at the last 16 h. Data are the mean ± SD of five mice per group from three independent experiments (each performed in triplicate). **: $P < 0.01$, ***: $P < 0.001$ versus C-P group; #: $P < 0.05$, ##: $P < 0.01$, ###: $P < 0.001$ versus Teff:Treg = 5:1 of internal group.

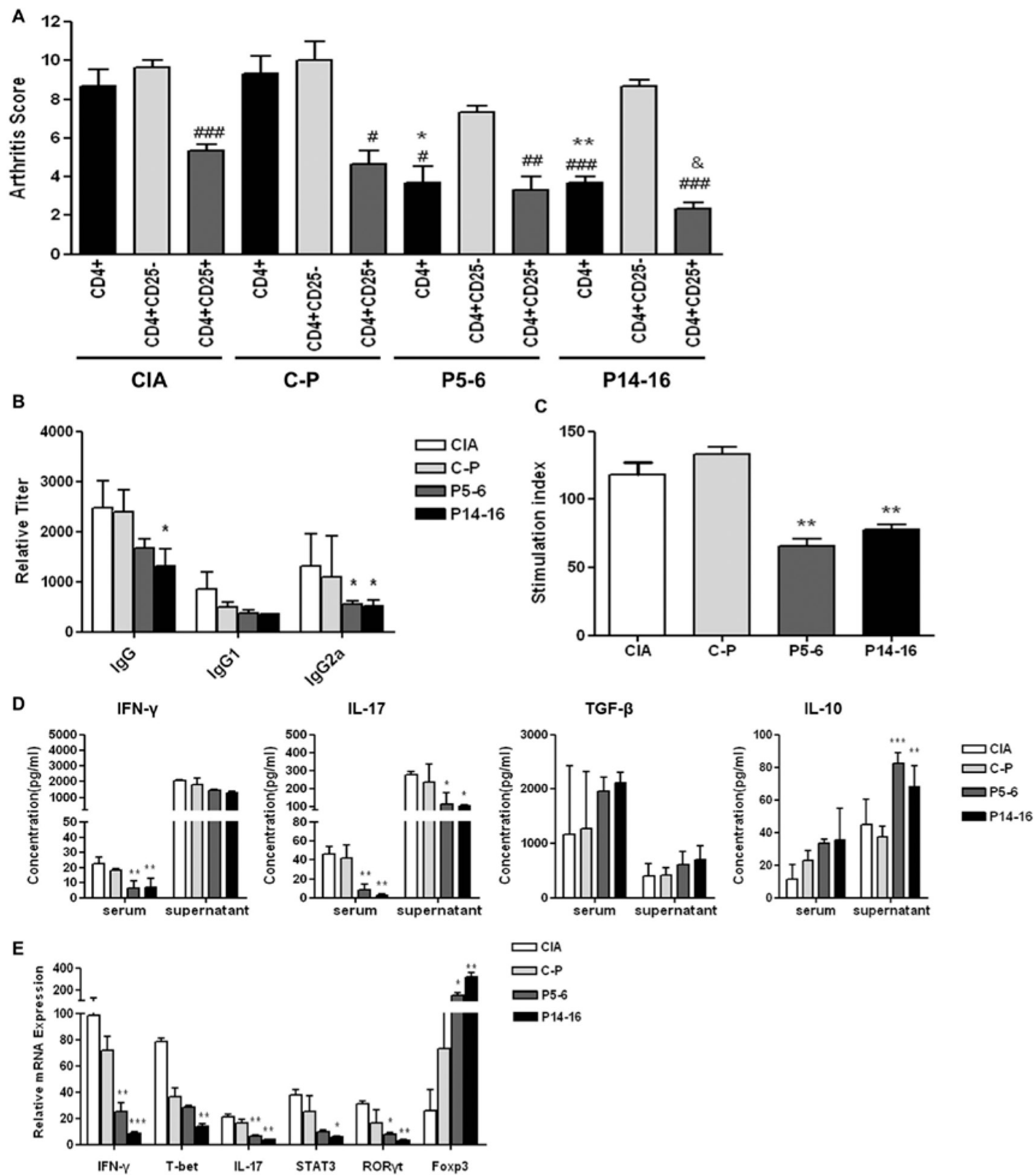

Figure 6. Prevention of CIA by *in vivo* ergotype peptide–induced Treg. (A) Total CD4 + T cells, CD4 + CD25-T cells or CD4 + CD25 + T cells derived from CIA or peptide-treated CIA mice were transferred into naive DBA/1 through intravenous injection. On the same day, naïve mice were induced with CII. Severity of arthritis was assessed by scoring clinical features 2 wks after the adoptive transfer. Values are the mean ± SD of 10 mice per group. *: $P < 0.05$, **: $P < 0.01$ versus C-P group; #: $P < 0.05$, ##: $P < 0.0$, ###: $P < 0.001$ versus internal CD4 + CD25–; &: $P < 0.05$ versus C-P group. (B) The levels of CII-specific IgG, IgG1 and IgG2a in sera collected on d 14 post adoptive transfer were determined by ELISA. Data are represented as mean ± SD. *: $P < 0.05$ versus C-P group. (C) Mononuclear cells from the spleens of transferred mice were isolated and stimulated with CII at 20 μg/mL. H^3–TdR (1μCi/well) was added at 48 h and cell proliferation was assessed at 72 h. The stimulation index normalized to unstimulated controls is displayed. Data are represented as mean ± SD. **: $P < 0.01$ versus C-P group. (D) The expressions of Th1, Th17 and Treg related cytokines IFN-γ, IL-17, TGF-β and IL-10 were measured by ELISA in sera and in supernatants by splenic mononuclear cells with CII stimulation (20 μg/mL) for 48 h. Values are the mean ± SD of 10 mice per group. *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$ versus C-P group. (E) mRNA levels of *IFN-γ*, *T-bet*, *IL-17*, *STAT3*, *RORγt* and *FOXP3* in splenic mononuclear cells stimulated with CII (20μg/mL) for 24 h were measured by real-time qPCR. Values are represented as mean ± SD. *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$ versus C-P group. Data are representative of three separate experiments with similar results.

randomly intercepted according to CD132 (IL-2Rγ). These IL-10 + CD4 + T cells may be ergotope-induced Treg cells. Selected functional ergotope peptides had both preventive and therapeutic effects in mice with CIA *in vivo*, reducing the frequency of arthritis, ameliorating symptoms and preventing joint damage. In addition, ergotope peptide treatment reduced autoantibody production in mice with CIA, in which CII-specific T cell proliferation was inhibited and IL-10–producing CD4 + T cells were induced, suggesting that ergotope peptides induced an antiergotypic immune response in CIA.

Since the mechanisms of these phenomena are incompletely understood, we investigated immunoregulation in ergotope peptide administration during CIA. It is widely known that effective immunological homeostasis relies on a continual balance among a number of factors, including helper T cell activation and regulatory T cell suppression. When homeostasis is disrupted, the host becomes susceptible to autoimmunity, in which autoreactive T cells contribute to the pathogenesis. Our further *in vivo* experiments demonstrated that ergotope peptide treatment had an impact on Th1 and Th17 cells. Downregulation of Th1 and Th17 cells was observed in ergotope peptide–treated mice, especially in the P14–16 group. The transcriptional factor of Th1 cells, T-bet and Th17, as well as STAT3 and RORγt was decreased, as well as the levels of cytokines IFN-γ and IL-17. The inhibition of Th1 and Th17 in established CIA could be attributed to ergotope-induced Treg cells.

Regulatory T cells play a pivotal role in the maintenance of tolerance as well as the control of immune activation by expressing the negative regulatory molecule CTLA-4 or secreting cytokines such as IL-10. The lineage-specific transcription factor FOXP3 is essential in Treg cell biology (13,44). Defects in CD4 + CD25 + Treg have been reported in RA (15,45). After TCV treatment, RA patients showed augmentation of CD4 + CD25 + Treg cells, which expressed a high level of transcription factor FOXP3 and secreted inhibitory cytokine IL-10 (21). Furthermore, these CD4 + IL-10–producing Treg cells induced by TCV were found to react specifically with peptides derived from IL-2 receptor α-chain, indicating that ergotope peptide treatment can induce Treg directly. The efficiency of inhibition in established arthritis by ergotope peptides and ergotope peptide–induced Treg cells could be explained by the fact that in addition to expanding the CD4 + CD25 + FOXP3 + Treg population, ergotope peptides also induced an efficient Treg population, in terms of both cytokine production and suppressive activity. The ergotope peptide–induced Treg cells secreted high levels of IL-10, but not TGF-β, and they were very strong suppressors of CD4 + CD25– effector T cell proliferation. Ergotope peptide treatment increased the surface and intracellular expression of CTLA-4, a costimulatory molecule that negatively regulates T cell activation, in CD4 + CD25 + Treg rather than in CD4 + CD25– T cells (46). CTLA-4 is constitutively expressed by CD4 + CD25 + FOXP3 + Treg and blockade of CTLA-4 interferes with Treg function (47). CTLA-4–deficient Treg failed to inhibit cytokine production associated with homeostatic expansion and was unable to prevent colitis (47,48). Our results suggest that the upregulation of CTLA-4 may contribute to the suppressive function of Treg cells.

To further study the potential pathway through which restored Treg cells act after ergotope peptide treatment, anti–CTLA-4 and anti–IL-10 antibodies were used separately or added together in the Treg/Teff system. The suppressive function abrogated when the blocking antibodies were added. The application of combined antibodies impaired mostly Treg suppressive ability, which was obvious in Treg from ergotope peptide–treated mice with CIA. These results verified that CTLA-4 and IL-10 are two important mediators that are critical to Treg suppressive function.

Moreover, we also checked the *in vivo* function of ergotope peptide–induced Treg cells by adoptive transfer. Treg cells from ergotope peptide–treated mice prevented CIA progression not only in clinical scores, but also in regulation of inflammatory cell activation and cytokine production. Downregulation of proinflammatory cytokines IFN-γ and IL-17 in sera and in the supernatants of CII-stimulated splenic MNCs, and increased antiinflammatory cytokines IL-10 and TGF-β, which ameliorate the disease, were also found in the adoptive transfer model with ergotope peptide–induced Treg, showing that the latter gained inhibitory function both *in vitro* and *in vivo*.

CONCLUSION

In summary, this study identified ergotope peptides as an immunomodulatory factor with the capacity to reduce the inflammatory response *in vivo* at both gene and protein levels and to restore immune tolerance. This study provides clues to the development and progression of RA. Although our findings provide evidence that ergotope peptides may be a novel preventive and therapeutic approach to the treatment of RA and may be prior to TCV, whose *in vitro* amplification is relatively expensive and time-consuming and requires manpower and material resources, further studies are warranted to elucidate potential mechanisms as well as dosage and administration, and to define safe and effective clinical strategies. Moreover, the polymorphism of human major histocompatibility (MHC) genes is so great that in a mixed population there are not two individuals with exactly the same set of MHC genes and molecules, with the exception of identical twins; hence it is necessary to screen functional peptides under the background of RA-associated MHC. To explore the unrevealed problems, our next step will be to focus on the *in vitro* effects of ergotope peptides on RA patients.

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DISCLOSURE

The authors declare that they have no competing interests as defined by *Molecular Medicine*, or other interests that might be perceived to influence the results and discussion reported in this paper.

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