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Regulatory Properties of Copolymer I in Th17 Differentiation by Altering STAT3 Phosphorylation

Chunhua Chen,* Xuebin Liu,† Bing Wan,† and Jingwu Z. Zhang2*

Th17 and Th1 play an important role in multiple sclerosis for which copolymer I (COP-I) is a treatment option. We described here that the treatment effect of COP-I correlated with its unique regulatory properties on differentiation and survival of Th17 in experimental autoimmune encephalomyelitis mice, which was mediated through down-regulation of STAT3 phosphorylation. The effect of COP-I on Th17 differentiation required CD14+ monocytes through IL-6 signaling as a key mediator to regulate STAT3 phosphorylation and subsequent RORγt expression in Th17 cells. The observed effect was markedly dampened when monocytes were genetically deficient for IL-6. Similar regulatory properties of COP-I were demonstrated in human Th17 differentiation. The study revealed the differential regulatory roles and the novel mechanism of action of COP-I chiefly responsible for its treatment efficacy in experimental autoimmune encephalomyelitis and multiple sclerosis. The Journal of Immunology, 2009, 183: 246–253.

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2 Abbreviations used in this paper: EAE, experimental autoimmune encephalomyelitis; COP-I, copolymer I; MS, multiple sclerosis; MOG, myelin oligodendrocyte glycoprotein; GKO, IFN-γ knockout; MBP, myelin basic protein; ROR, retinoic acid-related orphan receptor.

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55). The sequence of the peptide was Met-Glu-Val-Gly-Trp-Tyr-Arg-Ser-Pro-Phe-Ser-Arg-Val-Val-His-Leu-Tyr-Arg-Asn-Gly-Lys, and it displayed a purity of 95% (BioAsia Biotechnology). Immunization was performed by mixing MOG peptide in CFA containing 5 mg/ml heat-killed H37Ra, a strain of *Mycobacterium tuberculosis* (Difco Laboratories). Two hundred nanograms of pertussis toxin (List Biological Laboratories) in PBS were administered i.v. on the day of immunization and 48 h later. For the treatment protocol, COP-I was coemulsified with 300 µg of the MOG peptide in CFA and traditionally administered s.c. at 500 µg/mouse once on EAE induction. Mice were weighed and examined daily for disease symptoms. They were scored for disease severity using the standard scale: 0, no clinical signs; 1, limp tail; 2, paraparesis (weakness, incomplete paralysis of

![Image](http://www.jimmunol.org/)

**FIGURE 1.** Administration of COP-I ameliorates EAE in mice. A, C57BL/6 mice were immunized with MOG peptide to induce EAE. For COP-I-treated group, mice were coimmunized with MOG peptide and 500 µg of COP-I emulsified in CFA simultaneously. Each group consisted of 10 mice and was monitored and scored daily as described in Materials and Methods. Data are representative of three independent experiments. B and C, Histopathology of spinal cord tissue from control EAE mice (top) or COP-I-treated mice (bottom) by H&E staining (B) and Luxol Fast Blue staining (C). D, Serum levels of IL-17 and IFN-γ in COP-I-treated and control mice. Data are presented as mean ± SEM and representative of three independent experiments. Asterisks represent statistical differences between groups; *, p < 0.05; **, p < 0.01.

**FIGURE 2.** Encephalitogenic T cell response and cytokine profile and cellular immunophenotype in EAE mice treated with COP-I. A, Splenocytes isolated from COP-I-treated and control mice 16 days postimmunization were stimulated with the indicated concentrations of MOG peptide and examined for ex vivo proliferation. Data are presented as mean cpm ± SEM of triplicates. B, Supernatants were collected from the above-mentioned cultures after 48 h, and concentrations of the indicated cytokines were measured using ELISA. The values represent mean concentration (picograms per milliliter ± SEM) of triplicate samples. Asterisks represent statistical differences between groups; *, p < 0.05; **, p < 0.01. C, Intracellular staining of IL-17, IFN-γ, and IL-6 in the CNS and spleen. Mononuclear cells were isolated from CNS and spleen of COP-I-treated or control EAE mice and subjected to intracellular staining of IL-17, IFN-γ, or IL-6 as described in Materials and Methods. The plots of IL-17 and IFN-γ were gated on CD4+ T cells, and the plot of IL-6 was gated on CD14+ cells. Data are representative of three independent experiments with similar results.
one or two hind limbs); 3, paraplegia (complete paralysis of two hind limbs); 4, paraplegia with forelimb weakness or paralysis; 5, moribund or death. The animal protocol was approved by the institutional review board of Institute of Health Sciences.

**Histopathology**

Spinal cords for histological analysis were removed from mice and immediately fixed in 4% paraformaldehyde. Paraffin-embedded 5- to 10-μm sections of spinal cord were stained with H&E or Luxol Fast Blue and then examined by light microscopy.

**MOG-specific T cell response ex vivo**

For ex vivo proliferation response, splenocytes isolated from COP-I-treated and control EAE mice were cultured in triplicate in culture medium (RPMI 1640 with 10% FCS, 5 × 10⁻⁵ M β-ME, 2 mM l-glutamine, 1% nonessential amino acids, 1 mM sodium pyruvate, and 100 μg/ml penicillin/streptomycin) at a density of 2 × 10⁵ per well in 96-well plates in the presence or absence of the MOG peptide (5, 10, and 20 μg/ml). All cultures were maintained at 37°C in 5% CO₂ for 72 h. Cells were pulsed with 1 μCi of [³H]thymidine during the last 16–18 h of culture before harvest. [³H]Thymidine incorporation was measured as cpm as detected by a MicroBeta counter (PerkinElmer).

**Measurement of cytokine production**

Splenocytes (1 × 10⁶ per well) isolated from COP-I-treated and control EAE mice were stimulated with the MOG peptide (20 μg/ml) in culture medium. Supernatants were collected after 48 or 72 h and diluted for measurement of IFN-γ, IL-17, IL-6, TGF-β, IL-4, and IL-10 using ELISA kits (R&D Systems) according to the manufacturer’s instructions. For serum cytokine measurement, blood was collected from the heart, and serum samples were collected through centrifugation. Serum IL-17 and IFN-γ levels were determined using ELISA kits (BD Biosciences). A standard curve was performed for each plate and used to calculate the absolute concentrations of the indicated cytokines.

**Isolation of mononuclear cells from mouse CNS tissue**

Mononuclear cells of CNS tissue were first prepared from brain and spinal cord using gradient centrifugation as described previously (25). In brief, mice were perfused with 30 ml of PBS via the heart to eliminate peripheral blood. The dissociated brain and spinal cord tissue were centrifuged in a Percoll gradient (GE Healthcare). Mononuclear cells at the interface between the two gradients (37% and 70% Percoll) were collected and washed by centrifugation with medium.

**Flow cytometric analysis**

For intracellular cytokine staining, isolated CNS-infiltrating cells were stimulated with 50 ng/ml PMA and 500 ng/ml ionomycin (Sigma-Aldrich) in the presence of GolgiPlug (BD Biosciences) for 5 h. To analyze MOG-specific T cells, mouse splenocytes were stimulated with 20 μg/ml MOG peptide for 48 h, followed by restimulation with 50 ng/ml PMA and 500 ng/ml ionomycin in the presence of GolgiPlug for 5 h. For intracellular staining of IL-6, cells were stimulated with 100 ng/ml LPS overnight, and staining of IL-6, cells were stimulated with 50 ng/ml PMA and 500 ng/ml ionomycin (Sigma-Aldrich) for 24 h. CD4⁺ T cells were stained and subjected to immunoblot analysis for the expression of the indicated JAK/STAT signaling molecules and their phosphorylation levels. β-Actin was loaded as a control throughout the experiments. T-bet expression was analyzed in separate experiments using a different β-actin loading control. The intensity of each band was analyzed using Quantity One software, and the ratio of the COP-I-treated group to the control group for each item is presented as fold. Data are representative of at least two independent experiments. B, mRNA levels of RORγ and RORα in abovementioned cells were analyzed by real-time PCR. Data are representative of three independent experiments. Asterisks represent statistical differences between groups; *, p < 0.05; **, p < 0.01.

**Quantitative real-time PCR**

Total RNA was isolated from cell pellets using RNeasy Mini Kit (Qiagen), and first-strand cDNA was subsequently synthesized using SuperScript RT Kit (Qiagen) according to the manufacturer’s instructions. mRNA expression was determined by real-time PCR using SYBR Green Master Mix under standard thermocycler conditions (Applied Biosystems). Data were collected and quantitatively analyzed on an ABI Prism 7900 Sequence Detection System (Applied Biosystems). Mouse β-actin gene or human GAPDH gene, respectively was used as an endogenous control for sample normalization. Results were presented as fold relative to the expression of mouse β-actin or human GAPDH. Sequences of PCR primer pairs were as follows: mouse β-actin, forward 5’-TGTCACACCCCTCCAGAGTGT-3’ and reverse 5’-AGCTTGA-AAACTGAGAATGAG-3’; mouse RORγ, forward 5’-GCTTGCAGAATGAGGC-3’ and reverse 5’-GAAGATGTGATGCGG-3’; human RORα, forward 5’-GCTGCAACTGCTGATTACA-3’ and reverse 5’-CTTCGGCAATTTTACACGAT-3’; human RORγ, forward 5’-CGGGCCTCAATGCTGACA-3’ and reverse 5’-GCCACGATTGGGCTTCA-3’;

**FIGURE 3. Regulation of JAK/STAT signaling pathway by COP-I treatment. A, MOG-reactive splenocytes isolated from COP-I-treated or control EAE mice were restimulated with MOG peptide at the concentration of 20 μg/ml for 24 h; CD4⁺ T cells were isolated and subjected to immunoblot analysis for the expression of the indicated JAK/STAT signaling molecules and their phosphorylation levels. β-Actin was loaded as a control throughout the experiments. T-bet expression was analyzed in separate experiments using a different β-actin loading control. The intensity of each band was analyzed using Quantity One software, and the ratio of the COP-I-treated group to the control group for each item is presented as fold. Data are representative of at least two independent experiments. B, mRNA levels of RORγ and RORα in abovementioned cells were analyzed by real-time PCR. Data are representative of three independent experiments. Asterisks represent statistical differences between groups; *, p < 0.05; **, p < 0.01.**
cytes isolated from COP-I-treated or stimulated with MOG peptide (20 μg/ml) from groups; independent experiments. Asterisks represent statistical differences between groups; *p < 0.05; **p < 0.01.

**FIGURE 4.** COP-I-inhibited mouse Th17 and Th1 differentiation in vitro. Naive CD4⁺ T cells were cultured with irradiated CD4⁺ monocytes in the presence or absence of indicated concentrations of COP-I for 4 days under Th17-polarizing conditions (A) or Th1-polarizing conditions (B). Intracellular staining of IL-17 and IFN-γ were analyzed in CD4⁺ T cells; and mRNA levels of RORγt and RORα in Th17 differentiation system were determined by real-time PCR (C). Data are representative of three independent experiments. Asterisks represent statistical differences between groups; *, p < 0.05; **, p < 0.01.

**FIGURE 5.** IL-6 produced by CD14⁺ monocytes is involved in the MOG-reactive Th17 response. A, CD4⁺ MOG-reactive T cells were restimulated with MOG peptide (20 μg/ml) in the presence of CD14⁺ monocytes isolated from COP-I-treated or control EAE mice for 72 h. [³H]Thymidine incorporation and IL-17 and IL-6 levels in culture supernatants were determined. The phosphorylation of STAT3 in CD4⁺ cell populations was analyzed by flow cytometry. B–D, Splenocytes isolated from wild-type (WT) or IL-6 knockout (IL-6 KO) mice were incubated with control peptides or COP-I (40 μg/ml) for 6 h. CD4⁺ monocytes were isolated and cocultured with CD4⁺ MOG-reactive T cells in the presence of MOG peptide (20 μg/ml). [³H]Thymidine incorporation (B) and IL-17 and IL-6 levels in culture supernatants (C) were determined. The phosphorylation of STAT3 in CD4⁺ T cells was analyzed by flow cytometry (D). Data are representative of two independent experiments. Asterisks represent statistical differences between groups; *, p < 0.05; **, p < 0.01.
The treatment effect of COP-I correlated with the reduced levels of Th17 and Th1 in EAE

We first investigated whether the treatment effect of COP-I correlated with selective reduction in pathogenic Th17 and Th1 differentiation among CD4⁺ T cells capable of differentiating into various pro- and anti-inflammatory functional lineages in EAE. As illustrated in Fig. 1A, treatment with COP-I showed significant efficacy in both delaying the onset and reducing severity of EAE as compared with that of control mice, which was consistent with the histological analysis of the affected spinal cord tissue (Fig. 1, B and C). The serum levels of IL-17 and IFN-γ were significantly reduced in EAE mice treated with COP-I (Fig. 1D). When challenged with the eliciting MOG peptide, T cells derived ex vivo from treated EAE mice displayed a markedly dampened proliferative response accompanied by significantly reduced levels of IL-17, IL-6, and IFN-γ in CD4⁺ T cells (Fig. 2, A and B). The observed effect of COP-I was highly selective for the three indicated cytokines as the other cytokines, including IL-4 and IL-10, were not affected. As shown in Fig. 2C, flow cytometric analysis of intracellular IL-17 and IFN-γ in CD4⁺ T cells revealed a marked inhibitory effect of COP-I on Th17 and Th1 cells in both spinal cord and spleen in treated EAE mice, whereas IL-6-producing CD14⁺ monocytes were equally affected. The results indicate that COP-I treatment correlated with selective inhibition of Th17 and Th1 and reduced production of IL-6 in monocytes.

The effect of COP-I on Th17 and Th1 differentiation was mediated through the JAK/STAT signaling pathway

Because differentiation and survival of Th17 and Th1 are related to the JAK/STAT signaling pathway, we further investigated our hypothesis that COP-I could interact with respective JAK/STAT signaling molecules through IL-6 signaling and selectively alter their functions, leading to decreased downstream RORγt expression. To this end, cell lysates of purified CD4⁺ MOG-reactive T cells were obtained from COP-I-treated or control EAE mice and subjected to immunoblot analyses using Ab pairs for nonphosphorylated and phosphorylated forms of the indicated JAK and STAT signaling molecules. As illustrated in Fig. 3A, COP-I treatment specifically inhibited phosphorylation of JAK2, TyK2, STAT3, and STAT4 as well as the expression levels of T-bet. The findings were consistent with the roles of STAT3 and STAT4 in Th17 and Th1 differentiation (26, 27). It was evident that the expression of transcription factors RORγt and RORα critically required for Th17 differentiation was markedly inhibited in CD4⁺ T cells derived from EAE mice treated with COP-I as compared with that of control EAE mice (Fig. 3B). Furthermore, COP-I was shown to inhibit in vitro differentiation of Th17, which correlated with the reduction in

FIGURE 6. COP-I inhibited IL-6 production in CD14⁺ monocytes and impaired human Th17 differentiation in vitro. Purified human CD4⁺ T cells were cultured with CD14⁺ monocytes in the presence or absence (control) of COP-I (40 μg/ml) for 5 days under Th17-polarizing conditions. A, Intracellular staining of IL-17 and IFN-γ were measured by flow cytometry. The plots of IL-17 and IFN-γ were gated on CD4⁺ T cells. B, The phosphorylation of STAT3 in CD4⁺ T cells was determined by flow cytometry. Shaded histogram, isotype control; open histogram, level of p-STAT3. C and D, CD4⁺ T cells and CD14⁺ cells in the differentiation system were isolated, respectively; mRNA levels of RORγt and RORα in CD4⁺ T cells (C) and mRNA level of IL-6 in CD14⁺ cells (D) were determined by real-time PCR respectively. Data are representative of three independent experiments. Asterisks represent statistical differences between groups; *, p < 0.05; **, p < 0.01.
IL-17 and IL-6. In contrast, the observed effect of CD14 by marked reduction of STAT3 phosphorylation and levels of MOG-reactive T cells was significantly decreased as accompanied as opposed to their untreated counterparts, the proliferation of CD14

The inhibitory effect of COP-I treatment on IL-6 production in CD14+ monocytes prompted us to speculate that the observed regulatory properties of COP-I on Th17 differentiation were mediated at the JAK-STAT level.

Role of IL-6 and CD14+ monocytes in the effect of COP-I on Th17 differentiation

The inhibitory effect of COP-I treatment on IL-6 production in CD14+ monocytes prompted us to speculate that the observed regulatory properties of COP-I on Th17 differentiation were mediated through reduced IL-6 production in monocytes, resulting in altered STAT3 phosphorylation mediated by IL-6 signaling. To this end, CD14+ monocytes isolated ex vivo from treated EAE mice or treated in vitro with COP-I were cocultured with purified CD4+ MOG-reactive T cells derived from EAE mice to quantitatively measure Th17 differentiation and the related events. As shown in Fig. 5A, when exposed to CD14+ monocytes treated with COP-I as opposed to their untreated counterparts, the proliferation of MOG-reactive T cells was significantly decreased as accompanied by marked reduction of STAT3 phosphorylation and levels of IL-17 and IL-6. In contrast, the observed effect of CD14+ monocytes was completely abolished when wild-type CD14+ monocytes were substituted by those derived from IL-6 gene knockout mice (Fig. 5, B–D). Taken together, the results confirmed that the effect of COP-I on Th17 differentiation was mediated through reduced IL-6 production in treated monocytes and subsequently altered IL-6 signaling in CD4+ T cells.

Furthermore, the observed regulatory effect of COP-I on Th17 differentiation was confirmed in human in vitro experimental system involving CD4+ T cells and CD14+ monocytes. As illustrated in Fig. 6, A and B, both the percentage of Th17 and STAT3 phosphorylation were significantly decreased in CD4+ T cells when treated with COP-I at the indicated concentrations commonly used in vitro analysis. mRNA levels of RORγt and RORα in CD4+ T cells and IL-6 in CD14+ monocytes were decreased consistently (Fig. 6, C and D).

Differential roles of γ-IFN and IL-17 in the regulatory properties of COP-I in EAE

As both Th1 and Th17 cells were amenable to COP-I treatment in EAE as described here, we asked which pathogenic T cell subset(s), i.e., Th1 and/or Th17, was chiefly responsible for the efficacy of COP-I seen in the treatment of EAE. To this end, GKO EAE mice immunologically dominated by Th17-mediated inflammation were treated with COP-I using the same treatment protocol and the experimental conditions as those for wild-type EAE mice. As shown in Fig. 7, the efficacy pattern seen in GKO EAE mice was similar to that of their wild-type counterparts, indicating that the treatment effect of COP-I was predominantly mediated through Th17 and independent of IFN-γ.

Discussion

In this study, we provide compelling evidence indicating that in addition to its effect on IFN-γ-producing Th1 cells, COP-I has a unique regulatory property on Th17 differentiation that is critically involved in autoimmune pathologies such as MS and its animal model, EAE. Although COP-I has been shown previously to modulate other immune cell subsets, such as CD4+CD25+Foxp3+ regulatory T cells (28) and type-II monocytes (24), we demonstrate here that COP-I regulates Th17 differentiation and survival in both EAE and a human in vitro system. The finding is consistent with the newly described pathological role of Th17 in autoimmune disease and has important implications in our understanding of the mechanism of action of COP-I in the treatment of MS. Our study provides a novel aspect of the regulatory mechanism induced by COP-I with respect to its observed effect of COP-I on Th17 and Th1. First, the study addresses the mechanism underlying the regulatory effect of COP-I in EAE. In this regard, we show that COP-I treatment inhibits STAT3 and STAT4 phosphorylation in CD4+ encephalitogenic T cells, altering Th17 and Th1 differentiation and the clinical course of EAE. The inhibition of STAT3 and STAT4 phosphorylation is consequent to altered upstream JAK signaling
events. The JAK/STAT signaling pathway is one of the major signaling networks that regulate T cell differentiation into various functional lineages and their activities (29). In particular, STAT3, activated by IL-6 and IL-23, is an essential regulator to control lineage commitment of Th17 cells (30, 31). IL-6 binds to the gp130 receptor, resulting in the activation of JAK1; this leads to activation of STAT3 by tyrosine phosphorylation. STAT3 has been shown to act as an upstream regulator for RORγt, a transcription regulator of Th17 differentiation (32). On the other hand, STAT4 has been shown to play a role in IL-23-dependent expansion of Th17 cells (35). Here, the regulatory properties of COP-I on Th17 and Th1 differentiation are consistent with its predicted effect on the JAK/STAT signaling pathway. Secondly, as demonstrated previously (23, 24, 36, 37), COP-I affects the T cell reactivity through regulating multiple subsets of T cells and monocytes and involving multiple regulatory properties. In particular, it is capable of regulating both Th1 and Th17 cells that are jointly involved in the systemic as well as CNS inflammation in EAE as described here. In this context, our results indicate that the efficacy of COP-I treatment in EAE is mainly attributable to its regulatory property on Th17 differentiation and independent of Th1/IFN-γ as evident in the experiments involving GKO EAE mice. This is important in our understanding of the mechanism of action of COP-I in the treatment of EAE and MS.

Furthermore, the study confirms the essential roles of IL-6 and monocytes in the regulatory property of COP-I on Th17 differentiation. The results described here revealed that COP-I primarily interacts with monocytes and inhibits the production of IL-6, critically required for Th17 differentiation through STAT3 activity in T cells. This is evident by the markedly diminished effect of COP-I on Th17 differentiation when IL-6 deficient monocytes were used. There is mounting evidence suggesting that CD4+ T cells are highly dynamic in their ability and plasticity to differentiate into various functional lineages, many of which we have not identified specific markers for (38). This dynamic properties of CD4+ T cells are uniquely influenced by the cytokine milieu in which they are situated. That is, CD4+ T cells are able to sense the changes in cytokine milieu in health and autoimmune disease and differentiate into distinct functional subsets. The findings described here further demonstrate that COP-I acts on both monocytes and T cells to regulate the cytokine milieu that in turn alters differentiation and dynamic switch of T cells of pathogenic and regulatory functions.

Taken together, the study provides another novel example that the effect of COP-I in cytokine regulation and subsequent T cell lineage differentiation has direct consequences in altering the clinical course of autoimmune disease. Although COP-I has been shown to have a broad spectrum of regulatory or stimulatory properties on T cells and monocytes, there are key check points that are specifically altered by COP-I and attributable directly to the treatment efficacy whereas other properties of COP-I may be less relevant to the treatment effect of COP-I. In this regard, our understanding of the role of COP-I in the treatment of MS will help to identify relevant biomarkers that can be used to evaluate the treatment and the key pathological component(s) involved in MS for future drug design.

Disclosures
The authors have no financial conflict of interest.

REGULATION OF IL-17 BY COP-I THROUGH IL-6/STAT3

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