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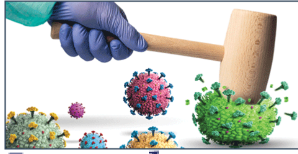

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Retinoic Acid Increases Foxp3$^+$ Regulatory T Cells and Inhibits Development of Th17 Cells by Enhancing TGF-β-Driven Smad3 Signaling and Inhibiting IL-6 and IL-23 Receptor Expression[1]

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The de novo generation of Foxp3$^+$ regulatory T (Treg) cells in the peripheral immune compartment and the differentiation of Th17 cells both require TGF-β, and IL-6 and IL-21 are switch factors that drive the development of Th17 cells at the expense of Treg cell generation. The major vitamin A metabolite all-*trans* retinoic acid (RA) not only enforces the generation of Treg cells but also inhibits the differentiation of Th17 cells. Herein we show that RA enhances TGF-β signaling by increasing the expression and phosphorylation of Smad3, and this results in increased Foxp3 expression even in the presence of IL-6 or IL-21. RA also inhibits the expression of IL-6Rα, IRF-4, and IL-23R and thus inhibits Th17 development. In vitro, RA significantly promotes Treg cell conversion, but in vivo during the development of experimental autoimmune encephalomyelitis it does not increase the frequency of Treg cells in the face of an ongoing inflammation. However, RA suppresses the disease very efficiently by inhibiting proinflammatory T cell responses, especially pathogenic Th17 responses. These data not only identify the signaling mechanisms by which RA can affect both Treg cell and Th17 differentiation, but they also highlight that in vivo during an autoimmune reaction, RA suppresses autoimmunity mainly by inhibiting the generation of effector Th17 cells. ***The Journal of Immunology,*** **2008, 181: 2277–2284.**

Proinflammatory Th1 cells have long been linked to the induction of organ-specific autoimmune diseases (1). However, the fact that loss of IFN-γ or lack of IFN-γ signaling results in increased susceptibility rather than resistance to autoimmunity has led to the hypothesis that a T cell subset other than Th1 cells might be crucial in the induction of autoimmunity (2). This observation provided the impetus for the identification of a new subset of effector T cells called Th17 cells, which produce the hallmark cytokine IL-17A (also called IL-17). Th17 cells are highly proinflammatory cells, and emerging data suggest that they orchestrate tissue inflammation and organ-specific autoimmune diseases (3). On the other hand, CD4$^+$CD25$^+$ regulatory T (Treg)[5] cells are a subset of CD4$^+$ T cells that express Foxp3 and are important in inhibiting inflammation and mediating self-tolerance (4). Therefore, Treg and Th17 cells antagonize functions of each other. Whereas Th17 cells are crucial for inducing tissue inflammation and autoimmunity, Treg cells prevent tissue inflammation, autoimmunity, and mediate self-tolerance (5).

Treg cells are normally generated in the thymus upon contact with self-Ag (natural Treg, nTreg) and are seeded to the peripheral immune compartment, where they regulate immune responses throughout the lifespan of the individual (4). Treg cells can also be induced in the peripheral immune compartment by conversion of naive CD4$^+$ T cells into Foxp3$^+$ T cells by TGF-β (induced Treg, iTreg) (4). TGF-β also supports the maintenance of Foxp3 expression and suppressive function of nTreg cells (6). TGF-β signals by binding its cell-surface serine/threonine kinase receptors, which in turn phosphorylate Smad2 and Smad3. Phosphorylated Smad2 and Smad3, together with Smad4, enter the nucleus to regulate the expression of target genes, either directly or in association with other transcription factors (7). A number of factors have been identified to be able to enhance or inhibit the TGF-β-mediated conversion of Treg cells. Cytokines (such as IL-6 and IL-21) and various metabolites, such as retinoic acid, have been shown to regulate the conversion of TGF-β-mediated Foxp3$^+$ Treg cells (8,

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[5] Abbreviations used in this paper: Treg, regulatory T; EAE, experimental autoimmune encephalomyelitis; IRF4, IFN regulatory factor 4; iTreg, induced regulatory T; LN, lymph node; MOG, myelin oligodendrocyte glycoprotein; nTreg, natural regulatory T; RA, retinoic acid; RAR, retinoic acid receptor; ROR, retinoic acid-related orphan receptor; RXR, retinoic X receptor; 7-AAD, 7-aminoactinomycin D; Th0, neutral T cell differentiation conditions.

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9). IL-6, an acute-phase protein induced early during inflammation, has been shown to suppress TGF-β-mediated Foxp3 induction but in turn induce the generation of Th17 cells from differentiating naive T cells (3). IL-6 binds its cell-surface receptors, IL-6Rα and a shared common receptor gp130, to recruit and phosphorylate STAT3 (9). IL-6 or IL-21, in combination with TGF-β, induces the expression of retinoic acid receptor-related orphan receptor (ROR)γt in T cells, a transcription factor important for the development of Th17 cells (9). RORγt also up-regulates the expression of IL-23R, and engagement of IL-23R by IL-23 further induces RORγt expression and reinforces and stabilizes the development and function of Th17 cells (3, 9, 10).

In addition to RORγt, IFN regulatory factor 4 (IRF4), a transcription factor induced by TCR/CD3 signaling, has recently been reported to be also required for Th17 differentiation (11). Lack of IRF4 in CD4$^+$ T cells led to decreased RORγt expression and IL-17 production with an associated defect in Foxp3 down-regulation in Th17 cultures, due to a reduced IL-6 response. However, IL-6-induced phosphorylation of STAT3 is normal in IRF4$^{-/-}$ cells, suggesting that the downstream events of STAT3 phosphorylation in the IL-6 signaling pathway are impaired in the absence of IRF4.

Retinoic acid (RA) is an active metabolite of vitamin A that regulates various cellular functions such as proliferation, differentiation, and cell death in a variety of cell types through two specific families of nuclear receptors, the retinoic acid receptors (RAR, including α, β, and γ subtypes) and the retinoic X receptors (RXR, also including α, β, and γ subtypes), which function as ligand-inducible transcription factors (12–15). All-*trans* RA binds preferentially to RAR, whereas 9-*cis* RA binds to both RAR and RXR (12, 13). In the immune system, RA plays important roles in regulating the functions of various types of immune cells (15). For example, RA prevents activation-induced cell death of T cells and inhibits Th1 but enhances Th2 responses (16, 17). RA in vivo suppresses inflammatory responses and tissue damage and ameliorates a variety of autoimmune diseases in animal models, including experimental autoimmune encephalomyelitis (EAE), rheumatoid arthritis, type I diabetes, inflammatory bowel disease, and lupus (18–22). However, the beneficial effect of RA in these diseases was so far thought to be due to its inhibition of Th1 responses. A number of recent reports have demonstrated that RA produced by CD103$^+$ dendritic cells in the gut preferentially induces iTreg cells (23–26) but inhibits Th17 differentiation (26), and this may be one of the mechanisms by which RA suppresses autoimmune reactions in vivo. However, the biochemical and molecular mechanism by which RA promotes iTreg cell conversion and suppresses Th17 cell generation has not been addressed.

Herein we report that RA indeed affects both Treg cell conversion and Th17 differentiation by influencing the components of TGF-β, IL-6/IL-21, and TCR/CD3 signaling pathways, which are involved in the development of Th17 and Treg cells. Interestingly, although RA in vitro dramatically enhances the development of Treg cells, RA in vivo does not increase the frequency of Treg cells significantly in an autoimmune disease setting but mainly inhibits the generation of effector T cells. We conclude that the beneficial effect of RA in autoimmunity is mainly due to its inhibition of pathogenic Th17 responses.

Materials and Methods

Mice and reagents

C57BL/6 mice were purchased from The Jackson Laboratory. *Foxp3gfp*.KI mice were generated and maintained as described previously (27). Mice were maintained and all animal experiments were done according to the animal protocol guidelines of Harvard Medical School. Myelin oligodendrocyte glycoprotein (MOG)$_{35-55}$ peptide was synthesized by Quality Controlled Biochemicals and was >80% pure, as determined by HPLC. All-*trans* RA and DMSO were purchased from Sigma-Aldrich. All-*trans* RA (100 mM) was solved in DMSO (vehicle) and stored at −80°C as aliquots before use. All of the fluorescence-conjugated Abs were obtained from eBioscience and BD Biosciences. All of the Ab pairs for ELISA were purchased from BD Biosciences. All cytokines were purchased from eBioscience and R&D Systems.

Naive CD4$^+$ T cell purification and stimulation

Naive CD4$^+$ T cells (CD4$^+$CD62L^{high}CD25$^-$) were purified by FACS sorting following a MACS bead isolation of CD4$^+$ cells. Naive CD4$^+$ cells were activated with plate-bound anti-CD3 (2 μg/ml) and soluble anti-CD28 (2 μg/ml) in 96-well plates. All-*trans* RA (10 nM) was included in the cultures. Cultures were also supplemented with IL-6 (20 ng/ml), IL-21 (200 ng/ml), IL-6 (20 ng/ml), or IL-21 (200 ng/ml) plus TGF-β (2 ng/ml) for Th17 cell differentiation, and TGF-β (2 ng/ml) for Treg cell conversion.

Flow cytometry

For intracellular cytokine staining, cells were stimulated in culture medium containing PMA (30 ng/ml, Sigma-Aldrich), ionomycin (500 ng/ml, Sigma-Aldrich), and monensin (GolgiStop, 1 μl/ml, BD Biosciences) in a cell incubator with 10% CO_2 at 37°C for 4 h. After staining surface markers, cells were fixed and permeabilized using Cytofix/Cytoperm and Perm/Wash buffer (BD Biosciences) according to the manufacturer's instructions. Then, cells were stained with fluorescence-conjugated cytokine Abs at 25°C for 30 min before analysis. In most of the staining experiments, if possible, 7-aminoactinomycin D (BD Biosciences) was also included to gate out the dead cells. MOG tetramer staining has been described previously (27, 28). All data were collected on a FACSCalibur (BD Biosciences) and analyzed with FlowJo software (TreeStar).

RNA isolation, real-time PCR, and Western blot

Total RNA isolation, TaqMan quantitative PCR, and Western blot were performed as described previously (28, 29). All primers and probes for real-time PCR were purchased from Applied Biosystems.

Immunization and EAE evaluation

Female B6 mice (8–12 wk old) were immunized s.c. in the flanks with an emulsion containing MOG$_{35-55}$ (100 μg/mouse) and *M. tuberculosis* H37Ra extract (3 mg/ml, Difco Laboratories) in CFA (100 μl/mouse). Pertussis toxin (100 ng/mouse, List Biological Laboratories) was administered i.p. on days 0 and 2. RA was i.p. injected every other day (450 μg/mouse/time point). Mice were monitored and assigned grades for clinical signs of EAE using the following scoring system: 0, healthy; 1, limp tail; 2, impaired righting reflex or waddled gait; 3, hind limb paralysis; 4, total limb paralysis; 5, moribund or death.

Proliferation assay and ELISA

Draining lymph node (LN) cells were isolated from treated mice and plated in round-bottom 96-well plates (BD Biosciences) in culture medium with various concentrations of Ag. After 48 h, plates were pulsed for 16 h with 1 μCi [^{3}H]thymidine per well. Proliferation was measured as cpm by using a Wallac liquid scintillation counter (PerkinElmer). Cytokine production in the supernatant of cell cultures was measured by ELISA as described previously (28, 29).

Statistics

The clinical score and inidence of EAE were analyzed by Fisher's exact test, and other comparisons were analyzed by Student's *t* test. $p < 0.05$ was considered significant.

Results

Differential effects of RA on the development of Th17 and Foxp3$^+$ Treg cells

TGF-β plays an important role in Foxp3$^+$ Treg cell conversion and, together with IL-6 or IL-21, in Th17 development (30, 31). Several recent studies have shown that RA synergizes with TGF-β to induce iTreg cells (23–26) and reciprocally inhibit development of Th17 cells induced by TGF-β plus IL-6 (26). To understand the mechanism by which RA enhances iTreg cell conversion and inhibits Th17 differentiation, we first studied whether RA would affect the TGF-β-induced development of Foxp3$^+$ Treg and Th17

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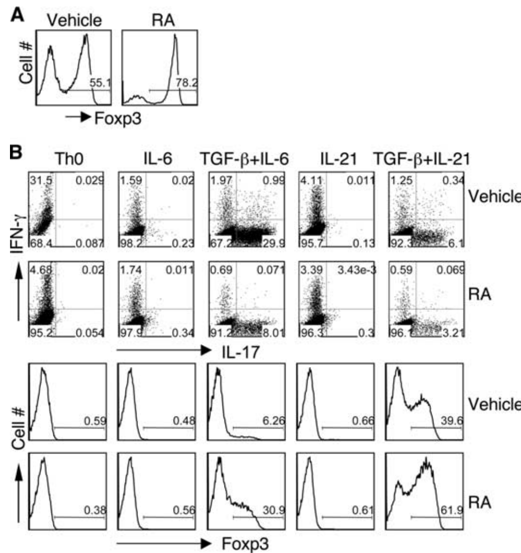

FIGURE 1. Differential effect of RA in the development of Foxp3$^+$ Treg and Th17 cells. Naive CD4$^+$ T cells were activated with anti-CD3 and anti-CD28 for 4 days under indicated conditions in the absence or presence of RA or vehicle (DMSO). Aliquots of cells cultured with TGF-β (*A*) or different combinations of cytokines (*B*) were fixed, permeabilized, and stained intracellularly with PE-Foxp3 mAb and analyzed by flow cytometry. Cells were also reactivated with PMA and ionomycin for 4 h in the presence of GolgiStop, and then stained with allophycocyanin-IFN-γ and PE-IL-17 mAb and analyzed by flow cytometry. Numbers in lower right quadrants indicate the percentage of IL-17-producing cells (*B*). Data are representative of three to five experiments.

cells. Naive CD4$^+$ T cells were activated with anti-CD3 and anti-CD28 plus TGF-β. Under these conditions, 55% of T cells became Treg cells, as measured by Foxp3 expression (Fig. 1*A*). Addition of RA further increased the frequency of Foxp3$^+$ Treg cells (78%). In the absence of TGF-β, RA did not induce any Foxp3 expression (Fig. 1*B*). Similarly, whereas TGF-β plus IL-6 or IL-21 induced IL-17, RA alone or in combination with IL-6 or IL-21 had no effect on IL-17 production. However, RA inhibited both TGF-β plus IL-6- and TGF-β plus IL-21-induced IL-17 production (Fig. 1*B*). In both cases, the inhibition of IL-17 by RA was associated with an increased expression of Foxp3, suggesting that RA inhibited the capacity of both IL-6 and IL-21 to suppress the induction of Foxp3 by TGF-β. As reported previously (18), RA also strongly inhibited IFN-γ production in the neutral T cell differentiation conditions (Th0). These data indicate that although RA alone does not affect either IL-17 or Foxp3 expression, RA can enhance TGF-β-induced Foxp3$^+$ Treg cell conversion and reciprocally inhibit IL-6- and IL-21-induced Th17 differentiation in a TGF-β-dependent manner.

RA enhances the expression and phosphorylation of Smad3 but not Smad2 in the presence of TGF-β

TGF-β signals through Smad2 and Smad3 to regulate various target genes, including *Foxp3* (7). Therefore, we examined whether RA affected *Smad2* and *Smad3* transcription during Treg cell conversion. As shown in Fig. 2*A*, RA did not affect the TGF-β-induced expression of *Smad2* mRNA. In contrast, RA clearly increased the TGF-β-induced expression of *Smad3* mRNA.

We then determined the levels of total and phosphorylated Smad (p-Smad) proteins by Western blot. Neither expression nor phosphorylation of Smad2 was affected by RA in any condition, whereas the TGF-β-induced expression of Smad3 and its phosphorylation (p-Smad3) were markedly increased in the presence of RA (Fig. 2*B*). RA alone apparently increased the expression of

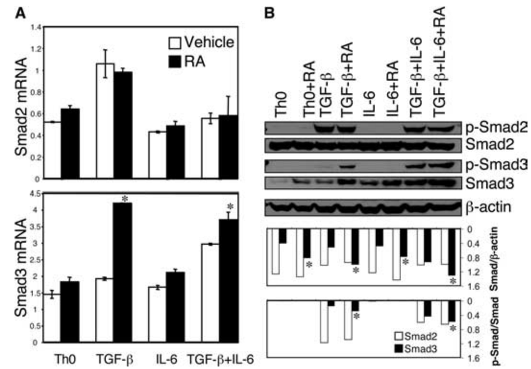

FIGURE 2. RA enhanced TGF-β-induced expression and phosphorylation of Smad3 but not Smad2. Naive CD4$^+$ T cells were activated with anti-CD3 and anti-CD28 for 24 h under indicated conditions with or without RA. *A*, Total RNA was isolated and expression of Smad2 and Smad3 mRNA was determined by TaqMan quantitative PCR. *B*, Cell lysates from indicated conditions were separated by SDS-PAGE gel, and total Smad and p-Smad were detected by immunoblotting. For quantification, the ratio of total Smad/β-actin and the ratio of p-Smad/total Smad were determined. Data are representative of three experiments. *, $p < 0.05$ when RA treatment was compared with vehicle treatment.

Smad3 but had no effect on its phosphorylation (Fig. 2*B*). These data suggest that RA in the presence of TGF-β specifically enhances the expression and phosphorylation of Smad3, which then up-regulates the expression of target genes, including *Foxp3*.

RA inhibits TGF-β-induced expression of IL-6Rα

Besides inducing Smad3 and Foxp3, RA also inhibited the generation of Th17 cells by TGF-β plus IL-6. We wanted to determine whether the increase in TGF-β-induced Foxp3 expression was sufficient to explain Th17 inhibition. Thus, we analyzed IL-6 signaling in the presence of RA. First, we determined whether RA affected the expression of IL-6 receptors.

IL-6 receptors consist of the IL-6Rα and gp130 subunits. Interestingly, TGF-β but not IL-6 or IL-21 up-regulated the expression of both *IL-6Rα* and *gp130* mRNA in CD4$^+$ T cells (Fig. 3*A*). TGF-β-induced gp130 up-regulation was not affected by RA. However, RA strongly inhibited the up-regulation of *IL-6Rα* mRNA by TGF-β (Fig. 3*A*). Consistent with the quantitative PCR

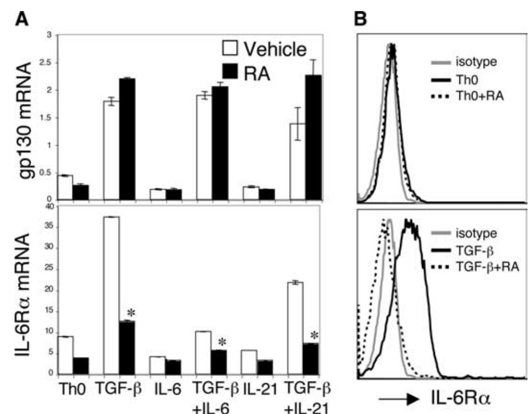

FIGURE 3. RA blocked TGF-β-induced up-regulation of IL-6Rα expression. Naive CD4$^+$ T cells were activated under indicated conditions with or without RA. After 48 h, cells were collected for the determination of gp130 and IL-6Rα mRNA by TaqMan quantitative PCR (*A*) and surface expression of IL-6Rα protein by flow cytometry (*B*). Data are representative of three experiments. *, $p < 0.05$ when RA treatment compared with vehicle treatment.

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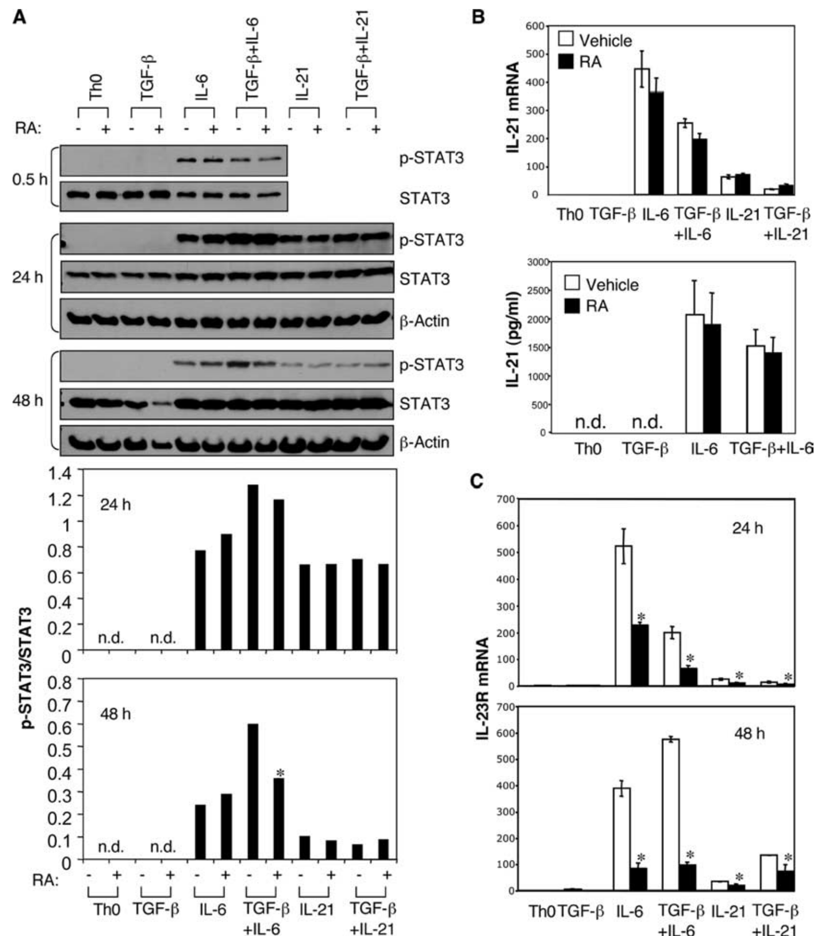

FIGURE 4. Effect of RA on STAT3 phosphorylation, IL-21 production, and IL-23R expression in CD4$^+$ cells cultured with different combinations of TGF-β, IL-6, and IL-21. Naive CD4$^+$ T cells were activated under indicated conditions with or without RA. Cells were collected for the determination of STAT3 phosphorylation by immunoblotting at various time points, and the ratio of p-STAT3/total STAT3 was analyzed (*A*). IL-21 mRNA expression was analyzed at 24 h by TaqMan quantitative PCR and IL-21 production in 96-h culture supernatants was analyzed by ELISA (*B*). Expression of IL-23R mRNA at 24 h and 48 h was analyzed by TaqMan quantitative PCR (*C*). Data are representative of three to five experiments. *, $p < 0.05$ when RA treatment was compared with vehicle treatment.

data, surface expression of IL-6Rα on CD4$^+$ T cells was up-regulated by TGF-β, which was blocked by RA (Fig. 3*B*). Similar results were observed in the condition where RA was included together with TGF-β plus IL-21 (data not shown). Collectively, these data suggest that the RA-mediated inhibition of Th17 differentiation not only relates to an increased Foxp3 expression, but also to blocking of the TGF-β-induced up-regulation of the IL-6Rα and thus prevention of IL-6 signaling.

Effect of RA on STAT3 phosphorylation, IL-21 production, and IL-23R expression during Th17 differentiation

Naive CD4$^+$ T cells activated by IL-6 secrete IL-21 through STAT3. In concert with TGF-β, IL-21 induces RORγt and IL-17 expression. IL-21, which amplifies its own production by an autocrine feedback loop, also acts upstream of RORγt mainly through STAT3 to induce the expression of IL-23R. Engagement of IL-23R by IL-23 induces further RORγt expression and stabilizes the Th17 phenotype (3, 9, 10). Given that RA inhibited TGF-β-induced up-regulation of IL-6Rα, we then examined whether RA could directly influence downstream components of the IL-6 signaling pathway.

We first examined STAT3 phosphorylation (p-STAT3) induced by IL-6 in the presence of RA. After 30 min, neither TGF-β nor RA affected IL-6-induced STAT3 phosphorylation (Fig. 4*A*). Because the effect of RA may not be achieved as early as 30 min, we therefore determined STAT3 phosphorylation after longer treatments. After 24-h culture, a sustained level of p-STAT3 was observed by the treatment with IL-6 alone. This level was further increased in the presence TGF-β (Fig. 4*A*), which is in agreement with the observation that TGF-β could up-regulate the expression of IL-6 receptors. After 24 h, RA still did not affect IL-6- or IL-21-induced STAT3 phosphorylation irrespective of whether TGF-β was present. Notably, unlike in the condition with IL-6, addition of TGF-β did not increase IL-21-induced STAT3 phosphorylation, indicating that TGF-β may not affect the expression of the IL-21 receptor. However, after 48-h culture, RA decreased the IL-6 plus TGF-β-driven enhancement of p-STAT3 to the level obtained with IL-6 alone (Fig. 4*A*). These data demonstrate that while TGF-β enhances IL-6-induced p-STAT3 by upregulating the expression of IL-6 receptor subunits, this enhancement is inhibited by RA. The data also illustrate that although RA decreases the level of p-STAT3 induced by IL-6 plus TGF-β, it does not inhibit STAT3 phosphorylation induced by IL-6 alone, IL-21 alone, or IL-21 plus TGF-β (Fig. 4*A*). Taken together, these data suggest that RA is unlikely to directly affect IL-6- or IL-21-mediated STAT3 phosphorylation. Instead, RA can indirectly decrease the level of IL-6-induced p-STAT3 in the presence of TGF-β by inhibiting the TGF-β-induced up-regulation of IL-6Rα.

We next examined the IL-6- and IL-21-induced IL-21 expression in the presence of RA. As shown in Fig. 4*B*, both IL-6 and IL-21 enhanced expression of *IL-21* mRNA. However, IL-6 is a stronger inducer of IL-21 than IL-21 itself. Addition of RA did not affect the expression of *IL-21* mRNA or IL-21 protein (Fig. 4*B*).

Both IL-6 and IL-21 induce IL-23R expression and both IL-6-induced and IL-21-induced expression of IL-23R is independent on TGF-β (32). However, irrespective of the stimulation condition, the expression of IL-23R is dependent on the transcription factor RORγt. As the expression of RORγt has been shown to be decreased in the presence of RA (26, 33), we predicted that the expression of IL-23R would also be decreased by RA treatment.

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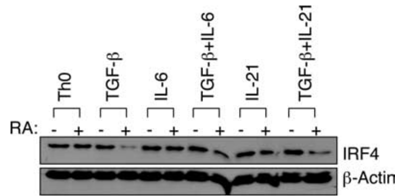

FIGURE 5. RA decreased expression of IRF4 protein in the presence of TGF-β, IL-6, or IL-21. Naive CD4$^+$ T cells were activated under indicated conditions with or without RA. Cells were collected after 48 h, lysed, and detected for IRF4 protein expression by immunoblotting. Data are representative of three experiments.

Indeed, the expression of IL-23R was strongly suppressed by RA irrespective of whether T cells were activated in the presence of IL-6 alone or IL-6 plus TGF-β. RA similarly inhibited the IL-23R expression when incubated with IL-21 alone or TGF-β plus IL-21, although the IL-23R up-regulation with IL-21 was much lower than that with lL-6 (Fig. 4*C*).

RA decreased IRF4 expression in both Th17 and Treg cell cultures

Given that IRF4, a transcription factor induced by TCR/CD3 signaling, is also required for Th17 differentiation (11), we studied the effect of RA on IRF4 expression during the development of Th17 and Treg cells. When RA was added to the stimulation cocktail, a significant decrease of IRF4 expression could only be observed in conditions containing TGF-β. RA did not affect IRF4 expression under Th0 condition or conditions containing IL-6 or IL-21 alone (Fig. 5). These data suggest that RA down-regulates IRF4 expression through cooperating with TGF-β signaling, which is TGF-β dependent.

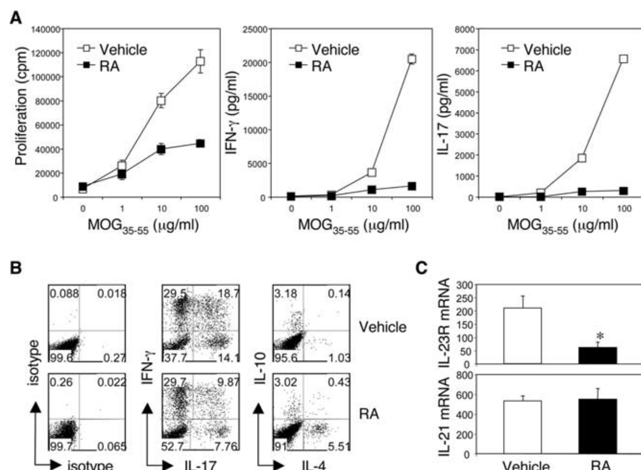

FIGURE 6. Treatment with RA decreased pathogenic Th17 and Th1 responses and IL-23R expression in CD4$^+$ T cells. Foxp3gfp.KI mice were immunized with MOG$_{35–55}$/CFA, and RA was i.p. injected every other day from day 0 to day 8. On day 10, (*A*) draining lymph node cells were isolated and restimulated with MOG$_{35–55}$. Proliferation and cytokine production in 48-h culture supernatants were measured by (3) [^{3}H]thymidine incorporation and ELISA assays, respectively; (*B*) isolated spleen cells were cultured with 5 μg/ml of MOG$_{35–55}$ for 5 days. Cytokine production from CD4$^+$ cells was determined by intracellular cytokine staining following 4-h activation with PMA and ionomycin; (*C*) expression of *IL-23R* and *IL-21* mRNA was measured in CD4$^+$ T cells purified from spleen cells by TaqMan quantitative PCR. $*$, $p < 0.05$ when RA treatment was compared with vehicle treatment. Data are the average or representative of 4–6 mice in each group.

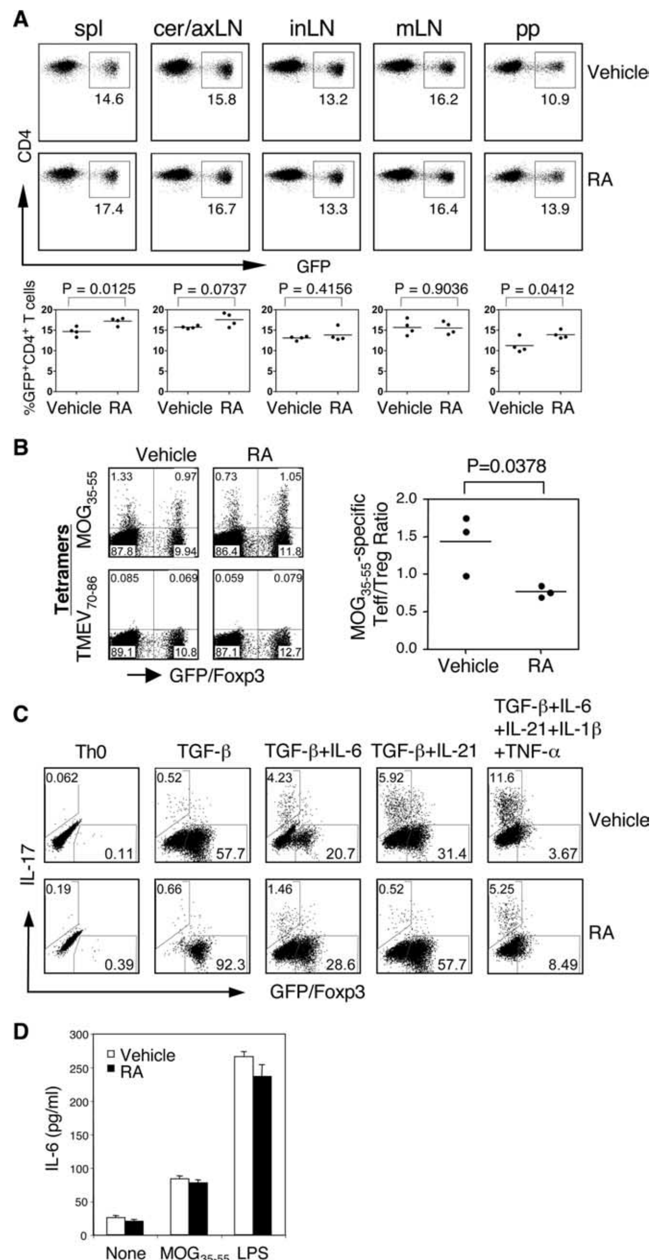

FIGURE 7. RA in vivo did not measurably increase the population of Foxp3$^+$ Treg cells. *Foxp3gfp*.KI mice were immunized with MOG$_{35–55}$/CFA, and RA was i.p. injected every other day from day 0 to day 8. On day 10, (*A*) the frequency of GFP$^+$ Treg cells was determined in different lymphoid compartments by flow cytometry. Spl indicates spleen; cer/ax, cervical/axillary; in, inguinal; m, mesenteric; pp, Peyers patch; (*B*) spleen cells were restimulated with 2 μg/ml of MOG$_{35–55}$ for 5 days, and then stained with PE-conjugated IA^b tetramer, allophycocyanin-anti-CD4, and 7-aminoactinomycin D (7-AAD). The MOG$_{35–55}$/IA^b tetramer-positive cells were determined in the live CD4$^+$ cell population (CD4$^+$7-AAD$^-$). Theiler's murine encephalomyelitis virus (TMEV)$_{70–86}$/IA^s tetramers were used as a negative control. *C*, Inflammatory cytokines IL-6, TNF-α, and IL-1β inhibited RA-enhanced Foxp3 expression but could not rescue RA-inhibited IL-17 production. Naive CD4$^+$ T cells (CD4$^+$CD62L^{high}Foxp3/GFP$^-$) purified from *Foxp3gfp*.KI mice were activated with anti-CD3 and anti-CD28 under indicated conditions in the absence or presence of RA for 5 days. Cells were reactivated with PMA and ionomycin for 4 h in the presence of GolgiStop. Then, cells were stained with allophycocyanin-anti-CD4, PE-anti-IL-17, and 7-AAD and analyzed by flow cytometry for IL-17 production and Foxp3/GFP expression in gated CD4$^+$7-AAD$^-$ cells. *D*, Isolated spleen cells from immunized mice on day 10 were cultured with 5 μg/ml of MOG$_{35–55}$ peptide or 500 ng/ml LPS. IL-6 production was determined in 48-h cultures by ELISA. None, no treatment.

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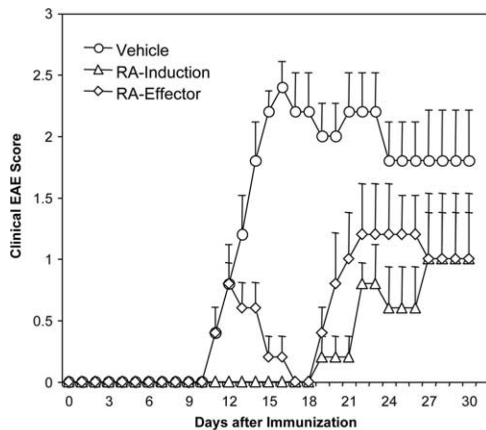

FIGURE 8. Treatment with RA inhibited EAE both during the induction and after disease onset. C57BL/6 mice were immunized with MOG_{35-55}/CFA, and RA was i.p. injected every other day starting from day 0 (RA-Induction) or after all mice in the group showed signs of clinical EAE (RA-Effector). Mice were evaluated daily for the signs of EAE. EAE was significantly ($p < 0.05$) reduced in mice in the "RA-Induction" group after day 10 and in the "RA-Effector" group after day12 compared with the vehicle-treated mice.

In vivo treatment with RA decreased Ag-specific Th17 and Th1 responses but had only minor effects on the Foxp3$^+$ Treg cell population

RA enhanced TGF-β-induced Foxp3$^+$ Treg cell conversion but reciprocally inhibited TGF-β plus IL-6/IL-21-induced Th17 cell differentiation in vitro. Therefore, we next examined in vivo effects of RA on T cell responses and the development of EAE, which serves as an animal model for multiple sclerosis (34). EAE is mediated by myelin-reactive Th17 and Th1 cells, and Treg and Th2 cells have been shown to inhibit EAE (5, 34). From our in vitro data we predicted that RA would increase the Treg cell population but decrease encephalitogenic Th17 responses and thus inhibit development of EAE.

We first determined the effect of RA on the development of Ag-specific effector T cell responses. *Foxp3gfp*.KI mice were immunized with MOG_{35-55}/CFA and i.p. treated with RA in corn oil every other day starting from day 0. On day 10, draining LN cells from these MOG_{35-55}-immunized *Foxp3gfp*.KI mice treated with RA were tested for T cell proliferation and cytokine production. Compared with vehicle-treated animals, cells from RA-treated mice showed decreased proliferation upon Ag restimulation in vitro (Fig. 6*A*). In line with the decreased proliferation, RA dramatically inhibited the production of IL-17 as well as IFN-γ upon Ag-specific restimulation, suggesting that RA strongly inhibited the priming/expansion of pathogenic Th17 and Th1 responses in vivo. However, the decrease of IFN-γ and IL-17 in the culture supernatants may reflect a decrease in the production of these effector cytokines or a decrease in the frequency of IL-17- and IFN-γ-producing T cells. By intracellular cytokine staining we could clearly see a decreased frequency of IL-17- and IL-17/IFN-γ-producing CD4$^+$ T cells in RA-treated mice (Fig. 6*B*). Furthermore, we found that CD4$^+$ T cells from RA-treated mice showed a strong reduction of *IL-23R* mRNA expression but no change in *IL-21* mRNA expression (Fig. 6*C*), thus corroborating the in vitro data. These data suggest that the reduced Th17 response was also partly due to the decreased expression of IL-23R after RA treatment in vivo.

We then analyzed the in vivo effect of RA on Treg cells in these immunized *Foxp3gfp*.KI mice, which allowed us to track Foxp3$^+$ Treg cells based on GFP expression (27). We determined the frequency of Foxp3/GFP$^+$ cells among CD4$^+$ T cells in different lymphoid compartments on day 10 after immunization. Surprisingly, RA treatment only slightly increased the frequency of Foxp3$^+$ Treg cells among CD4$^+$ T cells in the spleen and Peyers patches but not in cervical and axillary, inguinal, or mesenteric LNs from MOG_{35-55}-immunized mice (Fig. 7*A*). Furthermore, the MOG_{35-55}/IA^b tetramer staining demonstrated that RA treatment did not increase the frequency of tetramer-reactive MOG_{35-55}-specific Foxp3$^+$ Treg cells. However, RA treatment inhibited dramatically the frequency of Ag-specific effector T cells and therefore reduced the ratio of MOG_{35-55}-specific effector T vs Treg cells (Fig. 7*B*).

We have shown that IL-6 inhibits the generation of iTreg cells, and in MOG/CFA-immunized mice or under strongly inflammatory conditions (in the presence of IL-6, TNF-α, and IL-1β), Ag-specific Treg cells are not generated de novo but are derived from nTreg cells (27). Therefore, we first determined the effect of IL-6, TNF-α, and IL-1β on the generation of Foxp3$^+$ iTreg cells induced by TGF-β. As an inflammatory cytokine, IL-6 showed a stronger inhibitory capacity in TGF-β-induced Foxp3 expression than did IL-21, although both cytokines could induce the production of IL-17, which was dramatically inhibited by RA treatment (Fig. 7*C*). However, addition of all of the inflammatory cytokines (IL-6, TNF-α, and IL-1β) into Th17 differentiation cultures strongly inhibited the enhancement of Foxp3 by RA, but could not abrogate the inhibitory effects of RA on IL-17 production (Fig. 7*C*). Also, spleen cells from RA-treated mice produced similar amounts of IL-6 upon treatment with Ag or LPS (Fig. 7*D*), suggesting that RA treatment did not decrease IL-6 production.

RA treatment inhibits EAE both during the induction and the effector phase of the disease

Although RA treatment in vivo under inflammatory conditions (i.e., immunization with CFA) has only minor effects on the Foxp3$^+$ Treg cell population, pathogenic Th17 and Th1 responses nevertheless were strongly inhibited by RA. Therefore, based on the shift in the ratio of MOG_{35-55}-reactive effector and regulatory T cells, RA would inhibit development of EAE. Indeed, when mice were immunized with MOG_{35-55}/CFA to induce EAE, treatment with RA from day 0 not only dramatically delayed the disease onset but also strongly decreased the disease severity, compared with the control group (Fig. 8 and Table I). Furthermore, when administrated therapeutically after the onset of clinical signs of EAE, RA significantly ameliorated the disease course (Fig. 8 and Table I). These results confirm that RA treatment in vivo not only

Table I. *EAE in RA- and vehicle-treated mice*

Treatment	Incidence	Mean Day of Onset (Mean ± SEM)	Mean Maximum Score (Mean ± SEM)
Vehicle	10 of 10 (100%)	12.20 ± 1.23	2.40 ± 0.52
RA-Induction	8 of 10 (80%)	21.25 ± 1.39^{a}*	1.75 ± 0.89^{a}*
RA-Effector	10 of 10 (100%)	11.60 ± 0.52*	1.00 ± 0.94*

^{a} Represents the mice that showed clinical signs of EAE (8 of 10 mice).
*, $p < 0.05$ when RA-treated mice were compared to vehicle-treated mice.

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inhibits proinflammatory Th17 and Th1 responses, but also has functional consequences in that it dramatically suppresses the progression and severity of autoimmune inflammation.

Discussion

RA, the physiological vitamin A metabolite, has recently been shown to enhance TGF-β-induced Foxp3 expression and Treg cell conversion (23–26) and to decrease Th17 differentiation and IL-17 production (26). In this study, we confirmed these results and determined the mechanism by which RA increased Foxp3 expression but reciprocally decreased IL-17 production. Furthermore, we analyzed whether RA exerted similar effects in vivo during an autoimmune reaction. We found that RA affected components in the TGF-β, IL-6/IL-21, and TCR/CD3 signaling pathways that are involved in the development of Treg and Th17 cells. Moreover, treatment with RA prevented autoimmune disease mainly by decreasing pathogenic Th17 and Th1 responses; however, under this in vivo setting RA had no significant effect on Treg cells, possibly due to ongoing inflammation.

RA strongly increased TGF-β-induced Foxp3 expression in vitro and thus enhanced Treg cell conversion. Under these conditions, RA enhanced TGF-β signaling by increasing the expression and phosphorylation of Smad3, which is one of the key elements responsible for the induction of *Foxp3*. RA exerts its regulatory effects by binding to its receptor RAR, which is a transcription factor by itself. RA alone in the absence of TGF-β could increase the expression of Smad3; however, the increased Smad3 phosphorylation and Foxp3 expression by RA are completely dependent on TGF-β. On the other hand, it has been reported that the expression of RAR can be increased by TGF-β signaling (35). Therefore, RA and TGF-β could cooperatively augment their mutual signaling to further enhance Foxp3 expression. Moreover, RA may also enhance Smad3-driven transactivation by promoting a direct RAR-Smad3 interaction since a direct RAR/Smad3 interaction has been demonstrated previously (36). RA has also been reported to increase histone acetylation of the *Foxp3* promoter regions (37). Since histone acetylation is associated with increased gene activation and expression (38), this may contribute to the RA-mediated increase in TGF-β-induced *Foxp3* transcription.

In addition to the positive effect on TGF-β-induced Foxp3 expression and Treg cell conversion, RA also inhibited IL-6/IL-21-mediated Th17 differentiation. Inhibition of Th17 differentiation by RA appears to be achieved by multiple mechanisms: 1) enhanced TGF-β-induced Foxp3 expression by RA counteracts Th17 differentiation; 2) inhibition of the IL-6 receptor up-regulation by RA decreases Th17 differentiation; 3) RA down-regulates IRF4 expression and thus impairs IL-6-induced inhibition of Foxp3 expression; and 4) RA inhibits IL-23R expression and thus impairs the stabilization and further maturation of the Th17 phenotype. The inhibition of IL-6 receptor expression not only decreases IL-6-initiated Th17 differentiation directly but also counteracts the inhibitory effect of IL-6 on Foxp3 induction and results in increased Foxp3 levels that further inhibit the induction of IL-17. RA seems to have only minor effects on proximal IL-6 or IL-21 signaling events. RA did not considerably affect either STAT3 phosphorylation or IL-21 expression induced by IL-6 or IL-21. However, RA reduced the expression of IL-23R induced by either IL-6 or IL-21 alone or in combination with TGF-β. This might be due to the fact that the RA/RAR complex binds to the promoter of the *IL-23R* gene and transcriptionally represses IL-23R expression. Alternatively, RA binding to RAR might exert a direct inhibitory effect on IL-6/IL-21 signaling downstream of STAT3 since the expression of IL-23R is a downstream event of RORγt function, and inhibition of RORγt expression by RA has recently been reported (26, 33). Both RAR and RORγt belong to a large superfamily of ligand-inducible nuclear receptors that includes RXR and ROR, steroid, vitamin D, and thyroid hormone receptors, estrogen receptor, peroxisome proliferator-activated receptors, and a number of orphan receptors whose ligands are presently unknown (15). Direct interactions among the family members (such as RAR/RXR heterodimers and RXR/vitamin D3 receptor heterodimers) and between the transcription factors from other families (such as vitamin D3 receptor/c-Jun and RAR/STAT3 interactions) have been reported (15, 39). Therefore, it is possible that RA interferes with the transactivation function of STAT3 and/or RORγt by inducing direct RAR/STAT3 and/or RAR/RORγt interactions and thus inhibiting the IL-6/IL-21-induced expression of IL-23R, in addition to RA caused decrease of RORγt expression. Conversely, it will be interesting to determine whether RAR also binds to Foxp3, similar to NFAT (40), and enhances Foxp3 function.

Although down-regulation of IL-23R by RA may not play a role for Th17 differentiation in our in vitro APC-free systems, in which there is no source of IL-23 cytokine, IL-23/IL-23R signaling is required for stabilizing the development and pathogenic function of Th17 cells in vivo (10). Indeed, RA-treated MOG_{35-55}-immunized mice have a decrease in the generation of pathogenic Th17 responses, which is associated with a decreased expression of IL-23R. In addition to its direct inhibition of pathogenic Th17 and Th1 responses, RA also exerts this effect indirectly by decreasing the ability of APCs to generate Th1 and Th17 cells (data not shown), although RA does not affect the production of IL-6 in these cells (Fig. 7*D*). It has previously been demonstrated that RA impairs the secretion of IL-12 from APCs and therefore decreases Th1 differentiation (41, 42). Whether RA-treated dendritic cells have an impaired IL-23 production remains to be determined. Taken together, we propose that in vivo, the inhibitory effects of RA on the generation of Th17 cells are due to direct inhibitory effects on Th17 cells and modulation of APC function.

The decreased ratio of Ag-specific effector vs regulatory T cells upon treatment with RA attenuates autoimmunity. Herein we provide evidence that this change in the effector T-to-regulatory T cell ratio is mainly due to a decrease of effector T cells in RA-treated mice. Unlike the in vitro observation that RA strongly enhances Treg cell conversion, RA treatment in vivo only slightly increases the population of Treg cells. Interestingly, Mucida et al. also observed a remarkable reduction of Th17 cells but not measurable enhancement of the differentiation of $Foxp3^+$ Treg cells in mice infected with *Listeria monocytogenes* and treated with RA, and they suggested that TGF-β might be a limiting factor in the lack of Treg cell differentiation induced by exogenous RA in vivo (26). Herein we show that this minor expansion of Treg cells by RA in vivo appears to be due to the strong induction of IL-6 as well as TNF-α and IL-1 during the inflammatory process induced by MOG_{35-55}/CFA immunization, as addition of all of these inflammatory cytokines into Th17 differentiation cultures in vitro strongly inhibited the enhancement of Foxp3 by RA, but could not abrogate the inhibitory effects of RA on Th17 cells (Fig. 7*C*). This is also consistent with our previous data showing that IL-6 inhibits the generation of iTreg cells, and in MOG/CFA-immunized mice or under strongly inflammatory conditions, Ag-specific Treg cells are not generated de novo but are derived from nTreg cells (27).

The significant reduction of EAE by RA is associated with a decrease in Th17 and Th1 responses. However, the observation that IFN-γ signaling may not be absolutely required for the induction of EAE (2, 3) suggests that the decreased Th17 response is most likely responsible for the reduction of EAE by RA. Also, the decreased IL-23R expression by RA impairs IL-23 signaling, which further dampens the encephalitogenicity of Th17 cells, since

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IL-23 signaling has been shown to be crucial for full expression of the pathogenic phenotype in Th17 cells (10). RA treatment after disease onset still attenuates the severity of EAE, suggesting that RA also inhibits established disease by regulating not just the expansion but most likely the effector functions of pathogenic Th17 and Th1 cells. Accordingly, we observed that addition of RA into in vitro cultures of LN or spleen cells from mice with EAE also strongly inhibited $CD4^+$ T cell proliferation and their production of IL-17 and IFN-γ upon antigenic restimulation (data not shown).

Although under inflammatory conditions in vivo, RA has minor effects on increasing the frequency of Treg cells, it is likely that the blockade of TGF-β-induced up-regulation of IL-6Rα and the decrease of IRF4 expression by RA would reduce IL-6-mediated Treg cell inhibition (43) and thus enhance their suppressive function, which may also play a role in the strong inhibition of pathogenic Th1/Th17 responses in RA-treated mice during EAE.

In summary, we demonstrated that RA both in vitro and in vivo inhibits the development of Th17 cells by interfering with signaling events in the TGF-β and IL-6/IL-21/IL-23 pathways that are involved in the development and function of these cells. Although RA strongly promotes Treg cell conversion in vitro, RA treatment in vivo does not considerably increase the population of Treg cells in the face of ongoing inflammation. However, in the absence of strong inflammatory conditions, RA may be able to increase Treg cells in vivo as well. Therefore, RA regulates the balance between pathogenic Th17 and protective Treg cells by enhancing TGF-β signaling and by suppressing IL-6/IL-21/IL-23-driven signaling.

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Disclosures

The authors have no financial conflicts of interest.

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