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Dihydroartemisinin Ameliorates Inflammatory Disease by Its Reciprocal Effects on Th and Regulatory T Cell Function via Modulating the Mammalian Target of Rapamycin Pathway

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Dihydroartemisinin (DHA) is an important derivative of the herb medicine Artemisia annua L., used in ancient China. DHA is currently used worldwide to treat malaria by killing malaria-causing parasites. In addition to this prominent effect, DHA is thought to regulate cellular functions, such as angiogenesis, tumor cell growth, and immunity. Nonetheless, how DHA affects T cell function remains poorly understood. We found that DHA potently suppressed Th cell differentiation in vitro. Unexpectedly, however, DHA greatly promoted regulatory T cell (Treg) generation in a manner dependent on the TGF-βR:Smad signal. In addition, DHA treatment effectively reduced onset of experimental autoimmune encephalomyelitis (EAE) and ameliorated ongoing EAE in mice. Administration of DHA significantly decreased Th but increased Tregs in EAE-inflicted mice, without addition, DHA treatment effectively reduced the onset of experimental autoimmune encephalomyelitis and ameliorated on-going EAE in mice. Importantly, enhanced Akt activity neutralized DHA-mediated effects on T cells in an mTOR-dependent fashion. This study therefore reveals a novel immune regulatory function of DHA in reciprocally regulating Th and Treg cell generation through the modulating mTOR pathway. It addresses how DHA regulates immune function and suggests a new type of drug for treating diseases in which mTOR activity is to be tempered. The Journal of Immunology, 2012, 189: 4417–4425.

Artemisinin is a sesquiterpene lactone isolated from the Chinese plant Artemisia annua (commonly known as Qinghaosu or sweet wormwood) and has been used as a Chinese herbal medicine for the last 2000 y to treat more than 20 different diseases, including fever (1). Since the 1970s, artemisinin and its derivatives have been used extensively as an antimalaria drug by effectively killing multidrug-resistant strains of malarial parasites (2) through specific and selective inhibition on the sarcoplasmic and endoplasmic reticulum calcium ATPase of Plasmodium falciparum (3). Dihydroartemisinin (DHA) is the active metabolite of all artemisinin compounds (artemisinin, artemetate, artemether, and so on) and is also an important derivative available as a drug itself. Apart from its prominent antimalarial effect, DHA affects cellular functions, including tumor cell growth (4), angiogenesis (5), and immune regulation.

Aberrant function of the immune system leads to the development of inflammatory and autoimmune diseases. Immune suppressive agents are therefore needed to treat such diseases. Research directed at the identification and characterization of natural ingredients in plants for use in treating inflammatory disease has been limited, although natural plant compounds are potential sources of new classes of therapeutic agents to control inflammation with reduced side effects. One such promising phytochemical is artemisinin and its derivatives. Increasing evidence suggests that artemisinin and its derivatives possess immune suppressive function. Artemisinin was reported to effectively relieve the symptoms of lupus nephritis in mice (6). In addition, artemisinin derivatives were shown to suppress delayed-type hypersensitivity response (7) and collagen-induced arthritis (8). These findings suggest that the derivatives of artemisinin are promising agents for modulating immune response and for treating inflammatory disease. However, the matter of how artemisinin or its derivatives, such as DHA, influence T cell function remains poorly understood. More importantly, the molecular mechanisms underlying the immune regulatory function of artemisinin and its derivatives remain to be revealed. In this report, we investigated these issues.

We found that DHA treatment modestly inhibited the proliferation of activated T cells. DHA suppressed Th cell function. Particularly, DHA treatment virtually abolished Th17 differentiation. Unexpectedly, however, DHA greatly promoted regulatory T cell (Treg) generation in a manner dependent on the TGF-βR:Smad signal. These findings prompted us to test whether DHA can be used to treat autoimmune disease. We found that DHA treatment effectively reduced the onset of experimental autoimmune encephalomyelitis (EAE) and ameliorated ongoing EAE in mice. Administration of DHA significantly decreased Th but increased

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Abbreviations used in this article: caAkt, constitutively active form of AKT; CFA-MOG, CFA-myelin oligodendrocyte glycoprotein; DHA, dihydroartemisinin; EAE, experimental autoimmune encephalomyelitis; MIT, MSCV-IRES-Thy1.1; mTOR, mammalian target of rapamycin; RORγt, retinoic acid-related orphan receptor γ Treg, regulatory T cell.

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Tregs in EAE-infected mice, without apparent global immune suppression. We further investigated the molecular mechanisms underlying DHA-mediated effects on T cells and found that DHA attenuated the mammalian target of rapamycin (mTOR) signal in T cells. Importantly, we found that enhanced Akt activity neutralized DHA-mediated effects on T cells in an mTOR-dependent fashion. This study therefore reveals a novel immune regulatory function of DHA in reciprocally regulating Th17 cell and Treg generation through modulating the mTOR pathway. It addresses how DHA regulates immune function and suggests a new type of drug for treating diseases in which mTOR activity is to be tempered.

Materials and Methods
Mice and DHA
Wild-type and CD4cre;Snad2fl/fl mice are on C57BL/6 background and kept under specific pathogen-free conditions in the animal care facility at the University of North Carolina at Chapel Hill (Chapel Hill, NC). All mouse experiments were approved by the Institutional Animal Care and Use Committee of the University of North Carolina at Chapel Hill. DHA with a purity of ≥98% was purchased from Chengdu Okay Plant & Chemical or from TCI America. In vitro experiments, DHA was dissolved in DMSO (Sigma-Aldrich). For in vivo injection, DHA was dissolved in a mixture of DMSO and polysorbate 80 (Alfa Aesar) at 6/4 (v/v) ratio.

Flow cytometric analysis
Cells were stained per the manufacturers’ protocols with fluorescence-conjugated Abs for CD4, CD8, CD44, IFN-γ, IL-4, CD62L, Foxp3, and IL-17A, purchased from eBioscience. For cytokine staining, cells were stained per the manufacturers’ protocols with fluorescence-conjugated Abs against p-STAT3, STAT3, p-S6, S6, p-S6K, p-STAT5, STAT5 (Cell Signaling Technology), RORγt (eBioscience), and β-actin (Santa Cruz Biotechnology) were used for immunoblotting per the manufacturers’ protocols.

EAE induction, prevention, and treatment
Mice (12–16 wk old) were immunized s.c. with 50 μg MOG35–55 peptide [MEGVYWSPFRRVYHNGK] (Anspec) and 500 μg Mycobacterium tuberculosis (Difco) emulsified in incomplete Freund’s adjuvant (Difco). In addition, the animals received 200 ng pertussis toxin (List Biological Laboratories) i.p. on days 0 and 2. The severity of EAE was monitored and graded on a clinical score of 0–5: 0 = no clinical signs; 1 = limp tail; 2 = paraparesis (weakness, incomplete paralysis of one or two hind limbs); 3 = paraplegia (complete paralysis of two hind limbs); 4 = paraplegia with forelimb weakness or paralysis; 5 = moribund or death. To prevent EAE development, DHA was prepared at 10 mg/ml in the 6:4 mixture of DMSO and polysorbate 80, injected i.p. at a dose of 25 mg/kg on day 0, and continued for 7 d. To treat ongoing EAE, DHA was injected when mice showed clinical signs of EAE and then continued for 9 d.

Retrovirus-mediated ectopic gene expression in T cells
A retroviral construct expressing a constitutively active form of Akt (caAkt) with a linked Thyl.1 marker [MSCV-IRES-Thyl.1 (MIT)] were kindly provided by Dr. Hongbo Chi (St. Jude Children’s Research Hospital, Memphis, TN). A total of 293 packaging cells were transfected with retroviral constructs, using FuGENE HD transfection reagent (Roche). The recombinant retroviruses were collected 48 and 72 h after transfection. MACS-purified CD4+ T cells were activated for 48 h and then transduced with recombinant retroviruses. Cells were cultured for an additional 3 d and harvested for flow cytometric analysis.

Statistical analysis
Data from at least three sets of samples were used for statistical analysis. Statistical significance was calculated by the Student t test. A p value < 0.05 was considered significant.

Results
DHA treatment suppressed T cell proliferation
To study how DHA influences T cell function, we first investigated whether DHA treatment affected T cell activation, proliferation, and survival. Under culture conditions, DHA showed minimal toxicity to T cells at a dose of 0.4 μg/ml, although it was toxic to T cells at high doses (>1.0 μg/ml). Therefore, we used DHA at a dose of 0.4 μg/ml for studies on cultured T cells. Upon anti-CD3 and anti-CD28 stimulation, DHA did not affect T cell activation because T cell activation markers, such as CD25, CD44, and CD69, were upregulated normally in DHA-treated CD4+ T cells (Fig. 1A). Nonetheless, the numbers of live CD4+ T cells recovered from DHA-treated samples were less than those from mock-treated samples (Fig. 1B). To further investigate the reason for reduced T cell numbers, we assessed how DHA treatment impacted T cell proliferation and survival. DHA treatment moderately inhibited the proliferation of activated CD4+ T cells (Fig. 1C) without apparently affecting cell survival (Fig. 1D). Similarly, DHA treatment marginally suppressed CD8+ T cell proliferation, but not their activation or survival (Supplemental Fig. 1). These findings demonstrated that DHA exerts moderate inhibition on T cell proliferation without apparent effect on T cell activation and survival.

DHA inhibited helper CD4+ T cell differentiation
CD4+ T cells are pivotal in controlling immune responses, in large part by differentiating into distinct types of Th cells to direct various kinds of immune response (9). IFN-γ-producing Th1, IL-4-producing Th2 (10-12), and IL-17A–producing Th17 cells (13–15) are the three major types of Th cells studied extensively. To test whether DHA influences Th differentiation, CD4+ T cells were purified and cultured under Th1, Th2, or Th17 cell-skewing cytokines in the presence of DHA. We found that DHA treatment reduced IFN-γ and IL-4 production by Th1 and Th2 cells, respectively (Fig. 2A, Supplemental Fig. 2A). DHA treatment, however, virtually abrogated IL-17A production by differentiating Th17 cells (Fig. 2A).
Further comprehensive analysis revealed that expression of IL-21, another Th17-promoting cytokine (16), was suppressed by DHA. In addition, the expression of IL-23R, which promotes Th17 cells (17), was inhibited by DHA. Moreover, DHA abrogated the expression of transcription factor retinoic acid-related orphan receptor γ (RORγt), a master regulator controlling Th17

FIGURE 1. The effects of DHA on T cell activation and proliferation. (A) Purified CD4+ T cells were activated with anti-CD3 and anti-CD28 under the treatment of DHA (solid line) and mock (dashed line) for the indicated times. The expression of activation markers CD25, CD44, and CD69 was detected by flow cytometry. Results are representative of at least three experiments. (B) The numbers of CD4+ T cells recovered 4 d after activation in the presence (solid bar) or absence (open bar) of DHA. Means ± SD of three experiments are shown. *p < 0.05. (C) CFSE-labeled CD4+ T cells were activated with the treatment of mock (dashed line) or DHA (solid line) for 4 d. Cell proliferation was monitored by assessing CFSE dilution by flow cytometry. Representative results of three experiments are shown. (D) Purified CD4+ T cells were activated with plate-bound anti-CD3 and anti-CD28 with the treatment of mock and DHA for 48 and 96 h. Cells were stained with Annexin-V and 7-aminoactinomycin D. The percentages of each population were detected by flow cytometry and indicated. Results are representative of at least three experiments.

FIGURE 2. DHA inhibited helper T cell differentiation. (A) Purified CD4+ T cells were cultured under Th1, Th2, and Th17 cell polarizing conditions with mock or DHA treatment. IFN-γ, IL-4, and IL-17A production in CD4+ T cells was assessed by intracellular staining and flow cytometry. Results are representative of at least three experiments. (B) CD4+ T cells were cultured under Th17 cell polarizing conditions for the indicated times in the presence (solid bar) or absence (open bar) of DHA. The mRNA expression of IL-17A, IL-21, IL-23R, and Rorc was detected by quantitative RT-PCR. Means ± SD of triplicates done in one experiment, representative of three, are shown. *p < 0.05, **p < 0.01. (C) CD4+ T cells were activated under Th17 cell polarizing conditions in the presence (+) or absence (−) of DHA for the indicated times. The protein amounts of phosphorylated-STAT3 (p-STAT3), STAT3, RORγt, and β-actin were detected by immunoblotting. Results are representative of three experiments. Densitometry analysis of immunoblotting was also shown. *p < 0.05.
differentiation (18) (Fig. 2B, 2C). Nevertheless, DHA-mediated effects appeared to be gene specific, because the expression of certain Th17-related genes, such as IL-22 (19) and AhR (20), was not impaired by DHA treatment (Supplemental Fig. 2B). In addition, DHA treatment had unnoticeable effects on phosphorylation of STAT3, which is activated by IL-6 and critical for Th17 differentiation (21) (Fig. 2C). These findings suggest that DHA potently suppresses Th17 cell differentiation by interfering with specific molecular programs.

**DHA promoted Treg generation induced by TGF-β**

Foxp3-expressing Tregs constitute a subset of CD4+ T cells critical to suppression of immune response (22, 23). The differentiation programs of Tregs and Th17 cells antagonize each other (24, 25):

Whereas TGF-β promotes the generation of Tregs (26, 27), the presence of IL-6 suppresses Treg generation but promotes Th17 cell generation (24, 28). Because DHA inhibited Th17 cell differentiation (Fig. 2), we investigated whether DHA affected Treg generation under the same condition. Unexpectedly, the proportion of Tregs was greatly increased in DHA-treated cells compared with mock-treated cells (Fig. 3A). Such an increase was not merely due to reduced numbers of non-Treg cells (Supplemental Fig. 2C), because the numbers of Foxp3+ Tregs were also substantially increased (Fig. 3B). Further investigation revealed that DHA-promoted Foxp3+ cells were indeed Tregs, because DHA-promoted Foxp3+ cells displayed potent immune suppressive activity in vitro (Fig. 3C). Intrigued by this observation, we hypothesized that TGF-β–induced Treg generation is promoted by

**FIGURE 3.** DHA promoted Treg generation through TGF-βR:Smad signaling. (A) Purified CD4+ T cells were activated under Th17 polarizing conditions for 4 d with DHA or mock treatment. The fractions of Foxp3-expressing Tregs were determined by Foxp3 intracellular staining and flow cytometry. Means ± SD of three experiments are shown. (B) The total numbers of Tregs recovered from experiments described in (A) were compared. Means ± SD of three experiments are shown. *p < 0.05. (C) Purified CD4+ T cells from Foxp3 reporter mice (27) were activated under Th17 polarizing conditions for 4 d with DHA treatment. Foxp3+ Tregs were sorted and mixed with CFSE-labeled Foxp3 naïve CD4 responder T cells (Tresp.) at different ratios. Cell mixtures were activated with soluble anti-CD3 in the presence of irradiated APCs. At 4 d post activation, the proliferative index of responder T cells was monitored by CFSE dilution and flow cytometry. The division index of responder T cells was determined using FlowJo software to quantitate the degree of Treg-mediated suppression on the proliferation of responder T cells. Means ± SD of three experiments are shown. (D) CD4+ T cells were activated in the presence of varying doses of TGF-β (0–2 ng/ml) with the treatments of mock (dashed line) or DHA (solid line) for 4 d. The percentages of Foxp3+ Tregs were determined by intracellular staining and flow cytometry. Means ± SD of triplicates done in one experiment, representative of three, are shown. *p < 0.05. (E) CD4+ T cells were activated under Th17 polarizing conditions with DHA (+) or mock (−) treatment for the indicated times. The protein amounts of phosphorylated-Smad2 (p-Smad2), Smad2, and β-actin protein were detected by immunoblotting. Results are representative of three experiments. Densitometry analysis of immunoblotting was also shown. **p < 0.01. (F) CD4+ T cells were activated in the presence of 1 ng/ml TGF-β with the treatment of DHA (+) or mock (−) for the indicated times. The protein amounts of p-Smad2, Smad2, and β-actin protein were detected by immunoblotting. Results are representative of three experiments. Densitometry analysis of immunoblotting was also shown. **p < 0.01. (G) CD4+ T cells were activated in the presence of 1 ng/ml TGF-β with the treatment of DHA or mock for 4 d. Cells were simultaneously treated with TGF-βR1 (ALK5) inhibitor SB525334 (+) or remained untreated (−). The fractions of Foxp3+ Tregs were detected by intracellular staining and flow cytometry. Means ± SD of three experiments are shown. (H) CD4+ T cells were purified from wild-type (WT) or CD4cre-Smad2fl/fl mice (Smad2−/−) in the presence of 1 ng/ml TGF-β with the treatment of DHA or mock for 4 d. The fractions of Foxp3+ Tregs were detected by intracellular staining and flow cytometry. Results are representative of at least three experiments. Means ± SD of three experiments are shown.
DHA. Indeed, DHA treatment notably enhanced Treg generation induced by TGF-β (Fig. 3D). Therefore, DHA exerted a reciprocal effect on Th17 cells and Tregs by inhibiting Th17 cell differentiation and promoting Treg generation.

The TGF-βR signal is critical for Treg generation (29, 30). We hypothesized that DHA regulates the TGF-βR signal cascade to promote Treg generation. Indeed, TGF-β-activated Smad2 activation (31), measured by its phosphorylation, was prolonged in DHA-treated cells (Fig. 3E, 3F), although early Smad2 activation in DHA-treated cells appeared to be comparable to that in mock-treated cells (Fig. 3E, Supplemental Fig. 3A). In addition, DHA treatment did not result in a global alteration of cell signaling, because the activities of MAPK, such as p38 and ERK, appeared normal in DHA-treated cells (Supplemental Fig. 3B). These findings suggest that the TGF-βR-Smad2 signaling cascade is important for DHA-mediated promotion of Tregs. To address whether TGF-βR-Smad2 signaling is required for DHA-promoted Treg generation, we blocked TGF-βR function in T cells, using a pharmacological inhibitor, SB525334 (32), and abrogated Smad2 function, using T cells from CD4cre-Smad2fl/fl mice (33, 34), in which Smad2 was deleted specifically in T cells. Disruption of the TGF-βR signal led to greatly reduced Treg generation promoted by DHA (Fig. 3G, 3H) without Th differentiation (Supplemental Fig. 3C). These results suggest that DHA promotes Treg generation through mechanisms dependent on the TGF-βR-Smad2 signal.

DHA treatment prevented the onset of EAE

The findings that DHA reciprocally regulated Th cell and Treg function prompted us to investigate how DHA treatment may influence the development of inflammatory disease. Th17 and Th1 cells contribute to immune pathology in EAE induced by CFA-myelin oligodendrocyte glycoprotein (CFA-MOG) injection (35, 36), a murine model for human multiple sclerosis. Tregs, on the contrary, suppress EAE development (37). We therefore hypothesized that DHA treatment would be therapeutic for EAE. To perform studies in vivo, we identified a nontoxic dose of DHA in mice. Although DHA showed noticeable toxicity at high doses (>100 mg/kg), minimal toxicity was observed when mice were injected with 25 mg/kg of DHA. This dose was therefore used for the studies performed in vivo. We first addressed whether DHA treatment was able to prevent or reduce the onset of EAE. When administered daily during the first 7 d of EAE elicitation, DHA reduced the incidence of EAE onset (Fig. 4A). In addition, the clinical scores of DHA-treated mice that developed EAE were much lower than those of mock-treated mice (Fig. 4B), suggesting that administration of DHA effectively prevented or reduced onset of EAE in mice.

In association with the aforementioned observation, the numbers of lymphocytes infiltrating the spinal cord (Fig. 4C, top panel), as well as Th17 and Th1 cells (Fig. 4D), were decreased in DHA-treated mice compared with mock-treated mice. Of note, DHA administration did not cause systemic immune depletion or suppression, because similar numbers of splenocytes were recovered from DHA- and mock-treated mice (Fig. 4C, lower panel) and CD4+ and CD8+ T cells were activated equally well in DHA- and mock-treated mice (Supplemental Fig. 4). Thus, these findings demonstrated that DHA was effective in preventing EAE onset, in part through suppressing Th differentiation without apparently inhibiting immune function globally.

DHA ameliorated ongoing EAE

Encouraged by the above findings, we further investigated whether DHA was able to treat ongoing EAE in mice. To do this, EAE was elicited in mice. When mice developed clinical signs of EAE (limp tail), we treated them with either DHA or mock. More than 80% of mock-treated mice showed clinical scores >2 and remained so (Fig. 5A, 5B). In contrast, although a large percentage of DHA-treated mice showed clinical scores >2 during the early stage of treatment, the majority of DHA-treated mice became less sick or disease free at the later stage (Fig. 5A, 5B), without relapse after discontinuation of DHA treatment (data not shown). These findings suggest that DHA administration is able to ameliorate ongoing EAE and can be used to treat mice with EAE.

We further investigated whether DHA treatment affected the distribution of Th cells and Tregs in EAE-inflicted mice. The percentages of Th17 and Th1 cells were reduced in DHA-treated mice compared with mock-treated mice (Fig. 5C). In contrast, the

FIGURE 4. DHA treatment prevented the onset of EAE. (A) EAE was elicited in mice with CFA-MOG injection. Mice were also treated with DHA (dashed line) or mock (solid line) during the first 7 d of EAE elicitation. The numbers of mice with clinical scores >1 were recorded. The results for 12 mice in one experiment of three are shown. *p < 0.05. (B) As described in (A), mice were injected with CFA-MOG to elicit EAE and were treated with DHA (dashed line) or mock (solid line). Clinical scores were recorded every day thereafter. The mean clinical scores ± SEM of 12 mice in one experiment of three are shown. (C) As described in (A), mice were injected with CFA-MOG to elicit EAE and were treated with DHA (solid bar) or mock (open bar). The numbers of lymphocytes infiltrating the spinal cord (upper panel) and total splenocytes (lower panel) in mice were counted after treatment. The means ± SD of six mice are shown. (D) As described in (A), mice were injected with CFA-MOG to elicit EAE and were treated with DHA or mock. Lymphocytes were isolated from the spinal cords and spleens of mice with a clinical score of 2. The fractions of IL-17A- and IFN-γ-producing CD4+ T cells were determined by intracellular staining and flow cytometry. Results are representative of at least three experiments.
FIGURE 5. DHA treatment ameliorated ongoing EAE. (A) EAE was elicited in mice by CFA-MOG injection. When mice showed clinical signs of EAE, they were treated either with DHA (dashed line) or with mock (solid line) for the following 9 d. The numbers of mice with clinical scores ≥2 were recorded. The results for 12 mice in one experiment of three are shown. (B) As described in (A), when mice showed clinical signs of EAE, they were treated either with DHA (dashed line) or with mock (solid line) for the following 9 d. EAE clinical scores were recorded every day. Mean clinical scores ± SEM of 12 mice in one experiment of three are shown. *p < 0.05. (C) As described in (A), when mice showed clinical signs of EAE, they were treated either with DHA or with mock. Lymphocytes were isolated from the spinal cords of mice with a clinical score of 2. The fractions of IL-17A– and IFN-γ–producing CD4+ T cells were determined by flow cytometry. Results are representative of at least three experiments. (D) At the end of the experiments described in (A), lymphocytes were isolated from the spinal cords (SC), inguinal lymph nodes (ILN), spleens, and peripheral lymph nodes (PLN) of mice. Tregs were identified by Foxp3 and CD25 staining. A representative result is shown in the right panel. The percentages of Foxp3+ Tregs were determined and plotted in the left panel. Means ± SEM of multiple mice are shown. *p < 0.05.

DHA regulates T cell function through modulating the mTOR pathway

The aforementioned findings suggest that DHA is an agent effective in regulating Th cell and Treg function to suppress immune response. We sought to further understand the molecular mechanisms underlying DHA-mediated effects on T cells. The mTOR pathway is important in promoting Th cell function and in suppressing Treg function (38, 39). Inhibition of the mTOR pathway leads to defective Th cell differentiation and enhanced Treg generation, an observation strikingly similar to DHA-mediated effects. We thus hypothesized that DHA affected the mTOR signal in T cells to reciprocally control Th cell and Treg generation. Indeed, by assessing the phosphorylation of p70S6K and S6, an indicator for the activation of the mTOR pathway (40), we found that DHA treatment reduced mTOR signaling in T cells (Fig. 6A). In addition, cytokine IL-2–promoted mTOR activities also decreased upon DHA treatment (Fig. 6B). Thus, DHA treatment attenuated the mTOR signal.

We therefore prompted to investigate whether mTOR signaling is functionally involved in DHA-mediated effects. To do so, we ectopically expressed caAkt in T cells to enhance the mTOR signal (41). The expression of caAkt suppressed DHA-promoted Treg generation (Fig. 6C). In addition, the expression of caAkt overcame DHA-mediated suppression of Th17 differentiation (Fig. 6D). Thus, caAkt expression neutralized DHA-mediated effects on T cells. More importantly, the neutralization effect of caAkt on DHA-treated T cells depended on mTOR activity, because addition of rapamycin, a classic mTOR inhibitor (42), abolished caAkt-mediated effects on DHA-treated cells (Fig. 6E). These findings suggest that the reduction of mTOR signal upon DHA treatment, albeit modest, is indeed functionally important for DHA-mediated effects on T cells.

Discussion

The widely used antimalaria drug artemisinin and its derivatives showed immunoregulatory effects (6–8). However, the underlying mechanism remains poorly defined. In this study, we investigated the effect of DHA, the derivative and the major metabolite of artemisinin, on T cell function. We found that DHA suppressed Th cell differentiation but promoted Treg generation, suggesting that DHA could be useful in treating inflammatory disease. Indeed, DHA treatment not only prevented the onset of EAE but also ameliorated ongoing EAE in mice, associated with decreased Th cells but increased Tregs. Although another derivative of artemisinin, SM933, was found to suppress EAE (8), it did not apparently affect T cell function. Thus, subtle differences in the molecular structures of artemisinin derivatives may result in substantial changes in their immune regulatory functions. Nonetheless, these findings suggest that artemisinin derivatives constitute a new class of immune regulatory agent that suppresses immune function. DHA, in particular, is effective in dampening T cell function by reciprocally controlling Th cell and Treg generation and in treating T cell-mediated autoimmune and inflammatory diseases.

Ag-specific and -nonspecific immune suppressive agents have been used to treat inflammatory diseases. Because natural compounds generally have low side effects, studies are needed to identify and characterize natural plant ingredients that can be used to treat inflammatory disease. This study identifies DHA, a derivative of the natural compound artemisinin, as an agent that can be used for the development of Ag-specific and -nonspecific immunosuppressants with reduced side effects. We have found that, at effective doses, DHA showed slight side effects on T cells in vitro and in vivo. DHA treatment only modestly inhibited T cell proliferation without discernible effects on T cell activation or
survival in vitro. In addition, DHA administration in vivo minimally affected animal physiological status and the T cell population. At appropriate doses, DHA affected specific functions of T cells: It inhibited Th differentiation but promoted Treg generation. Although DHA abolished Th17 differentiation, it did not affect the immediate signal of IL-6 or TGF-β, and it inhibited the IL21/IL23-RORγt signal axis, but not the IL22-Ahr signal axis. The inability of DHA to affect STAT3 activation suggests that DHA affects the signal downstream of STAT3. Therefore, the effect of DHA is independent of STAT3, but through modulating the TGF-β/Smad and mTOR pathways. The inability of DHA to affect STAT3 function, which is critical for initiating all Th17-associated molecular programs, could be one reason why DHA affects certain, but not all, molecular programs of Th17 cells. These findings suggest that DHA could be used as an agent to fine tune the Th17 response. DHA can therefore be used as an Ag-nonspecific drug against specific T cell function and inflammation, with mild side effects. In addition, because a Treg-based strategy is one of the most important means of developing Ag-specific immune suppressive therapies, an agent able to promote the generation and function of Tregs is desirable for Treg-based therapy. We have found that DHA promoted Treg generation in vitro and in vivo without substantially affecting Treg survival or proliferation. It is therefore reasonable to believe that the incorporation of DHA into current regimens will enhance the efficacies of Treg-based immune therapy. Further studies are warranted to explore such possibilities. Our findings suggest that DHA is a promising agent in facilitating the development of both Ag-specific and Ag-nonspecific therapies for inflammatory diseases.

One limitation of using natural product derivatives to treat disease is the lack of understanding of how they exert their effects at molecular levels, which contributes to reluctance in clinical use. It is important to elucidate the molecular pathways affected by DHA. We found that interfering with the mTOR pathway is functionally critical for DHA-mediated effects in T cells because DHA treatment attenuated the mTOR signal and enhanced Akt activity corrected DHA-mediated effects in T cells in a manner dependent on mTOR. We nonetheless believe that mTOR is not the only

**FIGURE 6.** DHA regulated T cell function by modulating the mTOR pathway. (A) CD4⁺ T cells were activated under Th17 cell polarizing conditions for the indicated times with the treatment of DHA (+) or mock (−). The protein amounts of phosphorylated S6 (p-S6), S6, phosphorylated p70 S6 kinase (p-S6K), S6K, and β-actin were determined by immunoblotting. Results are representative of at least three experiments. Densitometry analysis of immunoblotting was also shown. *p < 0.05. (B) Effector CD4⁺ T cells were stimulated in the presence of 5 ng/ml IL-2 for the indicated times with the treatment of DHA (+) or mock (−). The protein amounts of phosphorylated S6 (p-S6), S6, phosphorylated STAT5 (p-STAT5), STAT5, and β-actin were assessed by immunoblotting. Results are representative of at least three experiments. Densitometry analysis of immunoblotting was also shown. *p < 0.05. (C) CD4⁺ T cells were activated and then transduced with control recombinant virus MIT or with recombinant virus expressing caAkt (MIT-caAkt) in the presence of 1 ng/ml TGF-β under the treatment of DHA (dashed line) or mock (solid line). Foxp3 expression in transduced (+) or untransduced (−) cells was assessed by intracellular staining and flow cytometric analysis. The percentages of Foxp3⁺ Tregs were indicated above the brackets. Results are representative of three experiments. (D) CD4⁺ T cells were activated and then transduced with control recombinant virus MIT or with recombinant virus expressing MIT-caAkt under Th17 cell polarizing conditions with DHA (dashed line) or mock (solid line) treatment. IL-17A expression in transduced (+) or untransduced (−) cells was assessed by intracellular staining and flow cytometric analysis. The percentages of IL-17A⁺ cells were indicated above the curves. Results are representative of three experiments. (E) CD4⁺ T cells were activated under Th17 cell polarizing conditions with the treatment of mock, DHA, or DHA plus rapamycin (DHA+Rapa). Cells were transduced with control recombinant virus MIT (solid line) or with recombinant virus expressing MIT-caAkt (dashed line). Foxp3 and IL-17A expression in transduced cells was assessed by intracellular staining and flow cytometric analysis. The percentages of Foxp3⁺ and IL-17A⁺ cells among transduced T cells are shown above the curves. Results are representative of three experiments.
pathway affected by DHA. DHA treatment attenuated but not abrogated the mTOR signal. In addition, our findings suggest that an enhanced Smad pathway could be another important mechanism underlying DHA-mediated effects on T cells, a mechanism that may or may not depend on the mTOR signal. The possibility of additional signaling pathways affected by DHA in an mTOR-dependent or -independent fashion warrants further investigation.

The findings that DHA attenuated the mTOR signal but enhanced the Smad signal indicate a broad application for DHA in treating diseases other than inflammatory diseases (e.g., cancer) in which mTOR and Smad signals need to be adjusted.

Although the findings in this study suggest that DHA may be beneficial in treating inflammatory disease, they also indicate the potential for an undesirable effect in malaria treatment whereby immune suppression could be detrimental to the clearance of parasites. DHA is effective in clearing malaria-causing parasites; however, DHA-resistant strains do emerge and contribute to the recrudescence of malaria. Given the observed immune suppressive effect of DHA, the emergence of drug resistance may be due to weakened immune function in the host. Thus, to develop more effective malaria treatment, the use of DHA in combination with immune-enhancing agents should be considered. In contrast, DHA-promoted Treg function may benefit malaria treatment by reducing malaria-induced immune dysfunction, as it has been shown that enhanced Treg function paradoxically prevented experimental cerebral malaria via the function of CTLA-4 (43). Therefore, better understanding of the mechanism of how DHA affects immune function is critical in treating malaria more efficiently.

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Disclosures

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