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SOCS3 Transactivation by PPARγ Prevents IL-17–Driven Cancer Growth

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Abstract

Activation of the transcription factor PPARγ by the n-3 fatty acid docosahexaenoic acid (DHA) is implicated in controlling proinflammatory cytokine secretion, but the intracellular signaling pathways engaged by PPARγ are incompletely characterized. Here, we identify the adapter-encoding gene SOCS3 as a critical transcriptional target of PPARγ. SOCS3 promoter binding and gene transactivation by PPARγ was associated with a repression in differentiation of proinflammatory T-helper (T₃/₁) cells cells. Accordingly, T₃/₁ cells induced in vitro displayed increased SOCS3 expression and diminished capacity to produce interleukin (IL)-17 following activation of PPARγ by DHA. Furthermore, naïve CD4 T cells derived from mice fed a DHA-enriched diet displayed less capability to differentiate into T₃/₁ cells. In two different mouse models of cancer, DHA prevented tumor outgrowth and angiogenesis in an IL-17–dependent manner. Altogether, our results uncover a novel molecular pathway by which PPARγ–induced SOCS3 expression prevents IL-17–mediated cancer growth. Cancer Res; 73(12): 3578–90. ©2013 AACR.

Introduction

Inflammation plays contrasting roles during cancer progression. Acute inflammation can be beneficial for cancer regression as shown by the requirement of HMGB1 and interleukin (IL)-1β for the induction of CD8 T-cell polarization and anticancer immune responses (1, 2). In contrast, proinflammatory mediators can support growth of established cancers by supporting an inflammatory milieu within the tumor microenvironment that favors tumor cell survival and neoangiogenesis (3). IL-17–producing CD4⁺ T cells [T-helper (T₃/₁) cells] have recently emerged as a key T-cell subset that produces proinflammatory mediators such as IL-17A, IL-17F, IL-21, and TNF-α and promotes autoimmunity (4). T₃/₁ cells can be induced from naïve T cells using stimulation with TGF-β and IL-6 and are characterized by the expression of their lineage-specific transcription factor RORγt (5). Although Rorc is indispensable for T₃/₁ cell induction, STAT3 is also an essential transcription factor for T₃/₁ cell differentiation as it directly regulates il17a/f and Rorc gene transcription (6, 7). IL-17 secretion from T₃/₁ cells has been proposed to contribute to tumor progression through the promotion of tumor neoangiogenesis (8).

The beneficial anti-inflammatory properties of dietary intake of (n-3) polyunsaturated fatty acids (PUFA) were initially illustrated by epidemiologic studies reporting a lower incidence of cardiovascular diseases in Eskimos compared with Western populations (9, 10). Administration of the n-3 PUFA docosahexaenoic acid (DHA) was later shown to dampen proinflammatory mediator secretion in patients with cancer (11). Activation of the transcription factor PPARγ has been proposed to account for the ability of DHA to prevent proinflammatory cytokine release (12).

Although DHA anticancer efficacy has been reported in preclinical mouse models of established tumors (13), whether the anticancer activity of DHA relies on its ability to modulate the production of proinflammatory cytokines remains elusive. Here, we report that activation of the PPARγ transcription factor by DHA in developing T₃/₁ cells induces Socs3 expression and interferes with the STAT3 signaling pathway, thereby inhibiting T₃/₁ cell differentiation. In addition, DHA prevents in vivo tumor growth of B16F10 melanoma and 4T1 mammary adenocarcinoma tumors in an IL-17–dependent manner. Altogether, not only our study shows that DHA administration prevents tumor angiogenesis and growth in an IL-17–

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dependent manner, but it also uncovers a previously unrecognized molecular pathway by which PPARγ-induced SOCS3 expression in T<sub>H</sub>17 cells prevents IL-17 secretion.

Materials and Methods

Mouse strains

All animals were bred and maintained according to both the Federation of Laboratory Animal Science Associations (FELASA) and the Animal Experimental Ethics Committee Guidelines (University of Burgundy, Dijon, France). Animals were used between 6 and 22 weeks of age. Female C57BL/6, BALB/c and nude mice were purchased from Centre d’élevage Janvier and from Charles River Laboratories. RORγt-eGFP reporter mice were obtained from Dr. Eberl (Institut Pasteur, Paris, France) and have been previously described (14). IL-17α<sup>−/−</sup> mice were obtained from Dr. Y. Iwakura (University of Tokyo, Tokyo, Japan; ref. 15). γδTCR-deficient mice were kindly provided by Dr. Bernhard Ryffel (University and CNRS, Orléans, France). Rag2<sup>−/−</sup> mice were provided by the CDTA (Cryopréservation, Distribution, Typage et Archivage animal) and distributed by EMMA (European Mouse Mutant Archive, a service funded by the EC FP7 Capacities Specific Programme). PPARβ- and PPARγ-deficient mice were provided by Dr. Béatrice Desvergne (Centre for Integrative Genomics, University of Lausanne, Lausanne, Switzerland) and have been described previously (16, 17).

Diets

To investigate the effect of dietary DHA in mice, we constituted 2 diet groups. One was fed a control diet containing sunflower oil, and the other group of mice was fed a DHA-enriched diet containing Omegavie DHA90 TG (Polaris Nutrition) for at least 3 weeks before starting experiments. Diets were prepared as described by Triboulot and colleagues (Supplementary Fig. S1; ref. 18).

To investigate the effect of dietary DHA in 2 human healthy volunteers (2 of the authors), 860 mg/day of DHA (NutraSea) was provided as a diet supplement for 3 weeks. NAIVE CD4<sup>+</sup> T cell differentiation into T<sub>H</sub>17 cells was then assessed.

Fatty acid analysis

Total lipids were extracted from the diets according to the method of Bligh and Dyer (19), then transmethylated by BF3/methanol after saponification and fatty acids were analyzed by gas–liquid chromatography using C<sub>15:0</sub> as internal standard with a Becker gas chromatograph (Becker Instruments) equipped with a 50 m capillary glass column packed with carbowax 20 m (Spiral-RD) as described previously (18).

Direct quantification of nonesterified fatty acid in plasma was conducted by a one-step method of derivatization and extraction using (trimethylsilyl)diazomethane (Sigma) in the presence of C<sub>15:0</sub> as internal standard (20) with subsequent analysis by conventional gas–liquid chromatography as described earlier.

Tumor growth experiments

B16F10 melanoma and 4T1 mammary adenocarcinoma cancer cells were cultured at 37 °C under 5% CO<sub>2</sub> in RPMI-1640 with GlutaMax-1 (Lonza) supplemented with 10% (v/v) fetal calf serum (Lonza), 1% penicillin, streptomycin, amphotericin B (Gibco), 4 mmol/L HEPES (Gibco), and 1 mmol/L sodium pyruvate (Gibco). 4T1 cells were kindly provided by Dr. Trad (Université de Bourgogne, Dijon, France) and B16F10 cells were obtained from American Type Culture Collection. All cells were routinely tested for Mycoplasma contamination using Mycoalert Mycoplasma Detection Kit (Lonza) and found negative. To induce tumor formation, 3 × 10<sup>5</sup> B16F10 or 10<sup>5</sup> 4T1 cancer cells were injected subcutaneously into nude or immunocompetent mice. In vivo IL-6 and IL-17 neutralization were respectively achieved by injecting 200 μg intraperitoneally (i.p.) of an anti-IL-6 (clone MP5-20F3; BioXCell) or an anti-IL-17 antibody (clone 17F3; BioXCell) on day 0, 1, 2, 3, 4, and 6 following tumor implantation. In vivo IL-1 blockade was carried out by injecting 0.5 μg IL-1 receptor antagonist (IL-1Ra; Kinerei from Biovitrum) 3 times a week. All experiments were carried out in accordance with guidelines prescribed by the Ethics Committee at the University of Burgundy.

CD31 immunostaining

Tumors were cryosliced from mouse biopsies and permeabilized with 0.1% Triton X-100. Sections were probed with a rat monoclonal against CD31 (BD Biosciences—Pharmingen) followed by a secondary antibody coupled to horseradish peroxidase (HRP) and counterstained with Harris’s hematoxylin.

In vitro T-cell differentiation

NAIVE CD4<sup>+</sup> T cells (CD4<sup>+</sup>CD62L<sup>−/−</sup>CD44<sup>hi</sup>) were obtained from spleens and lymph nodes of C57BL/6 wild-type (WT) or PPARβ- or PPARγ-deficient mice. CD4<sup>+</sup> T cells were purified from spleen and lymph nodes with anti-CD4 microbeads (Miltenyi Biotec), then were further sorted as naive CD4<sup>+</sup>CD62L<sup>−/−</sup>CD44<sup>lo</sup> T cells. Isolated naïve T cells were routinely 98% pure. Isolated naïve CD4<sup>+</sup> T cells were stimulated with plate-bound antibodies against CD3 (145-2C11, 2 μg/mL; BioXCell) and CD28 (PV-1, 2 μg/mL; BioXCell) and polarized into effector CD4<sup>+</sup> T lymphocyte subsets with cytokines in presence or not of DHA (Sigma-Aldrich). Mouse IL-4 (20 ng/mL), IL-6 (25 ng/mL), IL-12 (10 ng/mL), and TGF-β (2 ng/mL) were all purchased from MiltenyiBiotec. Anti-IL-4 (clone 11B11) and anti-IFN-γ (clone XMG1.2; 10 μg/mL) antibodies were obtained from Bio-XCell. In some experiments, the PPARγ agonist troglitazone and the antagonist GW9662 have been added at final concentrations of 0.75 and 0.1 μmol/L, respectively (Cayman Chemical). The PPARα and PPARγ antagonists (GW6471 and GSK0660; Sigma-Aldrich) have been supplemented to reach a final concentration of 0.24 and 0.16 μmol/L, respectively. Cells were classically harvested on day 3 (unless otherwise specified) for detection of cytokines by ELISA and quantitative real-time PCR (qRT-PCR) analysis.

Experiments on human CD4<sup>+</sup> T cells were carried out using peripheral blood from healthy volunteers (provided by the "Etablissement Français du Sang" Besançon, France). Written informed consent was obtained from all healthy blood donors. NAIVE CD4<sup>+</sup> T cells were isolated with the human CD4<sup>+</sup> T Cell
Isolation Kit II (MiltenyiBiotec), followed by stimulation with the Expansion/Activation Kit (MiltenyiBiotec), and differentiated for 3 days into T\(_0\) cells without cytokines or T\(_g\)17 cells in presence of TGF-\(\beta\) (2 ng/mL), IL-6 (10 ng/mL), and IL-23 (10 ng/mL).

**siRNA transfection**
For transfection experiments, naïve CD4\(^+\) T cells were transfected in vitro with Silencer Select Predesigned siRNA specific for murine SOCS3 (ID: s72013; Ambion, Life Technologies) or murine PPAR\(\gamma\) (ID: 160219; Ambion) or Silencer Negative Control N\(^1\) (Ambion) with transfection reagent TransIT-TKO (Mirus Bio LLC) according to the manufacturer’s instructions. Twenty-four hours after transfection, CD4\(^+\) T cells were restimulated with anti-CD3 and anti-CD28, differentiated in T\(_0\) or T\(_g\)17 conditions as described earlier and cultured for an additional 24 or 48 hours before analysis.

**Measurement of cytokines**
After 72-hour polarization, cell culture supernatants were assayed by ELISA for mouse IL-4 (BD Biosciences), IL-17a (Biologend), IFN-\(\gamma\) (BD Biosciences), or human IL-17 (Biologend) according to the manufacturer’s protocol.

For intracellular cytokine staining, cells were cultured for 5 days and then stimulated for 4 hours at 37°C in culture medium containing phorbol 12-myristate 13-acetate (PMA; 50 ng/mL; Sigma-Aldrich), ionomycin (1 \(\mu\)g/mL; Sigma-Aldrich), and monensin (GolgiStop; 1 \(\mu\)L/mL; BD Biosciences). After staining for surface markers and 7-amino-actinomycin D (7-AAD) to exclude dead cells, cells were fixed and permeabilized according to the manufacturer’s instructions (Cytofix/Cytoperm Kit; BD Biosciences), then stained for intracellular products. Monoclonal antibodies (mAbs) used for flow cytometry analyses were as follows: fluorescein isothiocyanate (FITC)–conjugated anti-CD4 (eBiosciences), Alexa Fluor 647–conjugated anti-IL-17 (eBio 17B7; eBiosciences), and phycocyanin (PE)–conjugated anti-IL-4 (554435; BD Biosciences) or PE-conjugated anti-IFN-\(\gamma\) (554412; BD Biosciences). All events were acquired by a BD LSRII cytometer equipped with BD FACSDiva software (BD Biosciences) and data were analyzed using FlowJo software (Tree Star).

**Immunoblot analysis**
Purified naïve T cells were differentiated for 24 to 48 hours into T\(_0\) or T\(_g\)17 cells with or without DHA (20 \(\mu\)mol/L), then collected and pelleted by centrifugation (5 minutes, 1,500 \(\times\) g). Cells were lysed in boiling buffer [1% SDS, 1 mmol/L sodium orthovanadate, and 10 mmol/mL Tris (pH 7.4)] containing protease inhibitor cocktail for 20 minutes at 4°C. Cell lysates were subjected to sonication (10 seconds at 10%) and protein concentration was assessed using the Bio-Rad DC Protein Assay Kit. Proteins were then denatured, loaded, and separated on SDS-PAGE and transferred on nitrocellulose membranes (Schleicher & Schuell). After blocking with 5% bovine serum albumin (BSA) in TBS–0.1% Tween 20 (TBST), membranes were incubated overnight with primary antibody diluted in TBST containing 1% BSA, washed and incubated for 1 hour with secondary antibody diluted in TBST–1% BSA. After additional washes, membranes were incubated with luminol reagent (Santa Cruz Biotechnology) and exposed to X-ray films. The following mouse mAbs were used: anti-STAT3 (Cell Signaling Technology), anti-SOCS3 (Novus Biologicals), and anti-\(\beta\)-actin (Sigma-Aldrich). The following rabbit polyclonal antibody was used: anti-phospho-STAT3-Tyr705 (Cell Signaling Technology). Secondary antibodies HRP-conjugated polyclonal goat anti-mouse (Jackson ImmunoResearch) and polyclonal rabbit anti-goat immunoglobulins (Dako) were also used.

**Chromatin immunoprecipitation assay**
Cells were differentiated for 24 hours into T\(_g\)17 cells with or without DHA. Chromatin immunoprecipitation (ChiP) was conducted according to the manufacturer’s instructions (ChiP-IT Express Enzymatic; Active Motif). Briefly, cells were fixed in a solution containing 37% formaldehyde for 10 minutes and quenched with 0.125 mol/L glycine. Chromatin was isolated and sheared to an average length of 300 to 500 bp by Enzymatic Cocktail. Twenty-five micrograms of DNA were immunoprecipitated with a PPAR\(\gamma\)-specific antibody (H-1000; Santa Cruz Biotechnology). After chromatin elution, cross-links were reversed by a Reverse Cross-linking Buffer and qRT-PCR was conducted. Data were normalized to Actin-\(b\) \(C\) values and expressed in fold enrichment according to immunoglobulin (Ig) values. Primers designed to assess ChiP assay are as follows: Actb 5'-actctttgcaacccacattc-3' and 5'-agctgctgttctccaaatc-5'; Sox3 1679 5'-gtgacatctcttcaggtt-3' and 5'-geacccctcctctcttcttt-3'; and Sox3 576 5'-ccccagcttcttgcag-3' and 5'-tgagaaagggagcgagagaa-3'.

**Quantitative real-time PCR**
Total RNA from T cells was extracted with TrizReagent (Ambion), reverse transcribed using M-MLV Reverse Transcriptase (Invitrogen), and was analyzed by qRT-PCR with the SYBR Green method according to the manufacturer’s instructions using the 7500 Fast Real-Time PCR System (Applied Biosystems). Expression was normalized to the expression of mouse Actb. Primers designed to assess gene expression are as follows: Actb 5'-agggagaaaaatcgcgcc-3' and 5'-ttctttgcagctcctgt-3'; Tbx21 5'-actcttgattgggggtaa-3' and 5'-tcaacagccacagacag-3'; Gata-3 5'-aggattcctgctcttccttt-3' and 5'-gctctggactcctataa-3'; Foxp3 5'-ctggctgaagagaaga-3' and 5'-tgaggagaggtagtaa-3'; Rorc mus musculus 5'-ggtgataacccttgatgtaa-3' and 5'-ctggcaaaagaccaccac-3'; Rora 5'-ccctactgtctctctcaca-3' and 5'-tgcacacatctctcctg-3'; Abr 5'-ctcttttgaaactcgc-3' and 5'-ggccagacttctttttag-3'; il7f 5'-ttcttgagctcctggtc-3' and 5'-tcatcctgcctcctctcctc-3'; il21 5'-aaccaggaacatgc-3' and 5'-ttgaatttgtaaacagacaa-3'; il22 5'-tttgctggtctcctccttc-3' and 5'-gcctggactcctcatac-3'; cd4 5'-ctgctcaacagagatc-3' and 5'-gtctgctgtatctctctcctc-3'; Tfgb 5'-caacaggtctcctcctaa-3' and 5'-ggagctgctgactac-3'; Rorc homo sapiens 5'-aaggacaggaaggaagga-3' and 5'-gaacactcctggtc-3'; Sox3 mus musculus 5'-aactctgcgagtc-3' and 5'-aaggacaggaagga-3'; Sox3 homo sapiens 5'-tggagacaggaagga-3' and 5'-aaggacaggaagga-3'; gpr120 5'-gatttctcctatgcggttgg-3' and 5'-tcgctggactcctataa-3'.
DHA-enriched diet reduces the capacity of naïve CD4⁺ T cells to differentiate into T<sub>H</sub>17 cells. Cell-sorted naïve CD4⁺CD62L<sup>hi</sup>CD44<sup>lo</sup> T cells were isolated from mice under a control or a DHA-enriched diet and differentiated into T<sub>H</sub>0, T<sub>H</sub>1, T<sub>H</sub>2, Treg, or T<sub>H</sub>17 cells in the presence of anti-CD3 and anti-CD28 for 72 hours. A and B, qRT-PCR analysis of Tbx21, Gata3, Foxp3 (A) and Rorc (B) mRNA expression. Expression is presented relative to Actb expression. C, ELISA analyses of IL-17 (left), IFN-γ (middle), and IL-4 (right) in supernatants of CD4⁺ T cells differentiated for 3 days. D, flow cytometry analysis of intracellular staining for IFN-γ, IL-4, and IL-17, respectively, in naïve CD4⁺ T cells polarized in T<sub>H</sub>1, T<sub>H</sub>2, and T<sub>H</sub>17 conditions assessed on day 5 of culture. Numbers beside outlined areas indicate percentage cells in gate. Representative data from 1 of 3 independent experiments are shown. NS, not significant.
Socs3 Induction by PPARγ Limits IL-17 Secretion

**Results**

In vivo administration of DHA impairs mouse T<sub>H17</sub> cell differentiation from naïve CD4<sup>+</sup> T cells

Despite reports suggesting that DHA could dampen the production of proinflammatory cytokines from innate immune cells (21, 22), whether DHA affects naïve CD4<sup>+</sup> T-cell differentiation in a cell-intrinsic manner has not been explored. To test this, we first examined the ability of naïve CD4<sup>+</sup>CD62L<sup>+</sup> T cells...
Figure 4. PPARγ transactivates Socs3 upon activation by DHA. A, expression of mouse GPR120 (top) and IL-17 release from control or GPR120 siRNA-transfected T17 cells (bottom) were detected after 48 hours of polarization with or without DHA by Western blotting and ELISA, respectively. B, relative
isolated from mice fed a control or DHA-enriched diet for 3 weeks to differentiate into effector T-cell subsets following activation in absence of antigen-presenting cells. DHA-enriched diet led to accumulation of DHA within splenocytes as previously described (Supplementary Fig. S2; ref. 18). Upon examining T-cell polarization, DHA failed to significantly alter the expression of the transcription factors Tbx21, Gata3, and Foxp3 that respectively specify the Th1, Th2, and regulatory T cell (Treg) lineages (Fig. 1A). However, naïve CD4+ T cells isolated from mice under a DHA-enriched diet featured a poor capacity to differentiate into Th17 cells under Th17-polarizing conditions, as illustrated by their weak expression of the Th17-cell transcription factor Rorc (Fig. 1B). By assessing IL-17a secretion from Th17 cells generated from mice under a DHA-enriched diet by ELISA, we found reduced IL-17a secretion in comparison with controls, whereas IFN-γ or IL-4 production from Th1 and Th2 cells were not affected (Fig. 1C). Intracellular staining data confirmed dampened IL-17a secretion from Th17 cells from mice receiving DHA in vivo, whereas the respective production of IFN-γ or IL-4 from Th1 and Th2 cells remained unaffected (Fig. 1D). Overall, these results suggest that DHA selectively affects Th17 cell differentiation in vivo.

**DHA inhibits mouse and human Th17 cell differentiation in a cell-intrinsic manner**

To test whether DHA directly affects Th17 cell development, we differentiated naïve CD4+ T cells without antigen-presenting cells under Th17 cell-polarizing conditions in the presence of DHA. We observed a marked reduction of Rorc expression in Th17 cells upon DHA treatment, confirming that DHA impairs Th17 cell differentiation (Fig. 2A). This inhibition is specific for DHA as other PUFAs such as eicosapentaenoic or arachidonic acids did not impair Th17 cell differentiation (Fig. 2A). To study further the impact of DHA on Th17 differentiation, we generated Th17 cells from Rorγt-eGFP reporter mice with increasing doses of DHA. DHA reduced the proportion of Rorγt-expressing cells in a dose-dependent manner, corroborating the DHA capacity to inhibit Th17 cell differentiation (Fig. 2B). In addition, we also explored the ability of DHA to affect other Th17-related genes and found that DHA impaired the expression of Rora, Ahr, Il17f, il21, and il22, suggesting that DHA globally affects the Th17 cell differentiation program (Fig. 2C). IL-23 is essential to maintain the proinflammatory Th17 cell program both in vitro and in vivo (23). Because DHA blunted the primary differentiation of naïve CD4 T cells into effector Th17 cells, we explored the ability of DHA to influence IL-23 capacity to restimulate engaged Th17 cells. We found that DHA was also able to reduce IL-17a secretion from engaged Th17 cells restimulated with IL-23 (Fig. 2D). To test whether DHA skewed Th17 cell differentiation toward another CD4 effector T-cell subset, we monitored the expression of Tbx21, Gata3, and Foxp3 genes in Th17 cells treated with DHA. We found that DHA failed to induce any of the transcription factors defining the Th1, Th2, or Treg lineages, indicating that DHA restricts but does not skew Th17 cell differentiation in vitro (Fig. 2E). We tested whether DHA also affects human Th17 cell differentiation. For this, we first differentiated naïve human CD4+ T cells isolated from the blood of healthy volunteers into Th17 cells in the presence of DHA. In line with our findings with mouse T cells, we found that DHA also prevented human Th17 cell differentiation (Fig. 2F). Similarly, upon assessing Th17 cell induction from naïve human CD4 T cells obtained from healthy individuals before or after 3 weeks of DHA intake as a diet supplement, we found that oral DHA intake decreased Th17 cell differentiation (Fig. 2G). Altogether, these findings indicate that DHA restrains mouse and human Th17 cell differentiation.

**DHA interferes with Th17 cell differentiation by inducing Socs3**

The transcription factor STAT3 was shown to transactivate the Il17a/f and the Rorc promoters (24, 25). Because DHA dampened IL-17a/f secretion and Rorc expression in developing Th17 cells, we hypothesized that DHA negatively regulates STAT3 activation during Th17 cell differentiation. To test this, we induced Th17 cells from naïve CD4+ T cells generated without DHA (Fig. 3A). In contrast, CD4 T cells incubated under Th17-polarizing conditions with DHA featured a weak level of p-STAT3, confirming that DHA reduces STAT3 signaling in Th17 cells (Fig. 3A). Because Socs3 is a major regulator of STAT3 phosphorylation in Th17 cells, we hypothesized that the DHA-mediated downregulation of p-STAT3 may be due to increased Socs3 expression. We found that Socs3 mRNA and protein expression was induced by DHA treatment in mouse and human Th17 cells, suggesting that the DHA-mediated downregulation of p-STAT3 may be due to increased Socs3 expression. The Socs3 transcription factor was shown to transactivate the Il17a/f and the Rorc promoters (24, 25). Because DHA dampened IL-17a/f secretion and Rorc expression in developing Th17 cells, we hypothesized that DHA negatively regulates STAT3 activation during Th17 cell differentiation. To test this, we induced Th17 cells from naïve CD4+ T cells generated without DHA (Fig. 3A). In contrast, CD4 T cells incubated under Th17-polarizing conditions with DHA featured a weak level of p-STAT3, confirming that DHA reduces STAT3 signaling in Th17 cells (Fig. 3A). Because Socs3 is a major regulator of STAT3 phosphorylation in Th17 cells, we hypothesized that the DHA-mediated downregulation of p-STAT3 may be due to increased Socs3 expression. We found that Socs3 mRNA and protein expression was induced by DHA treatment in mouse and human Th17 cells, suggesting that the DHA-mediated downregulation of p-STAT3 may be due to increased Socs3 expression. The Socs3 transcription factor was shown to transactivate the Il17a/f and the Rorc promoters (24, 25).
to restrain IL-17 and RORγt expression in T<sub>H</sub>17 cells (Fig. 3C and D). Collectively, our findings suggest that DHA restrains T<sub>H</sub>17 cell differentiation by interfering with STAT3 signaling through SOCS3 induction.

**DHA prevents T<sub>H</sub>17 cell differentiation in a PPARγ-dependent manner**

To unravel the mechanism accounting for SOCS3 induction in developing T<sub>H</sub>17 cells upon DHA treatment, we investigated the intracellular signaling pathways engaged by DHA on T<sub>H</sub>17 cells. GPR120 was recently identified as an omega-3 fatty acid receptor (26). To test the involvement of GPR120 in the DHA-driven impairment of T<sub>H</sub>17 cell differentiation, we downregulated GPR120 expression with siRNA in naïve T cells and analyzed the effect of DHA during T<sub>H</sub>17 differentiation. Our results showed no significant differences in T<sub>H</sub>17 polarization between cells transfected with control or GPR120 siRNA, indicating that the decreased T<sub>H</sub>17 cell induction upon DHA treatment does not depend on GPR120 (Fig. 4A). PPARγ is another proposed receptor for omega-3 fatty acid. Synthetic PPARγ ligands have been reported to induce Socs3 expression and inhibit STAT3 activation (27). Given that DHA interfered with T<sub>H</sub>17 cell differentiation through STAT3 signaling pathway blockade, we hypothesized that the effects of DHA on T<sub>H</sub>17 cells relied on the transcription factor PPARγ. Gene expression analysis revealed that the known PPARγ target genes MMP9, Cyp27, and Clu were induced in DHA-treated T<sub>H</sub>17 cells, suggesting...
that PPARγ signaling is activated by DHA treatment on T_{H}17 cells (Fig. 4B). In addition, the DHA effect on T_{H}17 cell differentiation was markedly reduced in the presence of a PPARγ inhibitor GW9662, but not with a PPARδ inhibitor GSK0660 or PPARα inhibitor GW6471, as measured by IL-17 secretion and Rorc expression (Fig. 4C). Moreover, PPARγ agonist troglitazone neutralized IL-17 secretion from T_{H}17 cells to a comparable extent to DHA, suggesting that PPARγ activation is involved in DHA effects on T_{H}17 cells (Fig. 4C). To validate the elective involvement of PPARγ in the intracellular effects of DHA on T_{H}17 cells, we downregulated PPARγ expression with siRNA and tested the ability of DHA to inhibit T_{H}17 cell differentiation in absence of PPARγ. We found that silencing PPARγ completely abolished the ability of DHA to restrain T_{H}17 cell induction (Fig. 4D). Although PPARγ agonists induce Socs3 expression (27, 28), the direct link between PPARγ and SOCS3 remains unclear. To test whether the DHA-driven Socs3 induction is directly due to PPARγ activation, we conducted a bioinformatic analysis of the Socs3 promoter and found 2 putative binding sites for PPARγ at positions −1679 and −576 from the transcription start site. By conducting ChIP, we found that PPARγ binds to the Socs3 promoter only at position −576 in DHA-treated T_{H}17 cells as compared with control T_{H}17 cells (Fig. 4E). We further checked the specificity of this interaction by down-regulating PPARγ expression by siRNA and confirmed that
the amount of PPARγ-binding Socs3 promoter was accordingly reduced (Fig. 4E). Finally, we tested the effects of DHA on naïve T cells from WT-, PPARγ-, or PPARβ-deficient mice. In line with our results obtained with the PPAR inhibitors, we found that while DHA could suppress T\(_{17}\) cell differentiation from WT- and PPARβ-deficient T cells, PPARγ-deficient T cells were totally unaffected by DHA treatment (Fig. 4F). Altogether, these findings show that the ability of DHA to hamper T\(_{17}\) cell differentiation is strictly dependent on PPARγ-dependent Socs3 expression.

**DHA prevents tumor growth in vivo in an IL-17-dependent manner**

Although DHA was proposed to promote cancer cell death (29), we found that DHA did not exert direct cytotoxic effects on mouse melanoma B16F10 and in 4T1 mammary adenocarcinoma tumor cells in vitro at concentrations that could be reached in vivo (60 μmol/L; Fig. 5A). However, we noted that the DHA-enriched diet delays subcutaneous tumor growth in vivo in both cancer models (Fig. 3B). In contrast, DHA did not show any antitumor efficacy in nude mice in both models suggesting that DHA mediates its anticancer function through T cells (Fig. 5C and Supplementary Fig. S3). Given that DHA impeded proinflammatory cytokine release, which has been proposed to contribute to B16F10 and 4T1 tumor growth (8, 30–32), we tested whether DHA affected tumor growth in vivo in these 2 tumor models by affecting IL-1, IL-6, or IL-17 release. DHA still exhibited in vivo anticancer activity against B16F10 tumors in absence of IL-1 and IL-6 (Supplementary Fig. S4). However, DHA failed to exhibit any effect on tumor progression in IL-17a\(^{-/-}\) mice, suggesting that the anticancer effects of DHA are dependent on IL-17 (Fig. 5D). These results were confirmed by neutralizing IL-17 in vivo in the 4T1 tumor model (Fig. 5D). As a control, IL-17 mAb did not exert any direct cytotoxic effect on tumor cells, ruling out any direct effect of IL-17 on tumor cell growth (Supplementary Fig. S5).

Because γ\(\delta\) T cells and T\(_{17}\) cells are major IL-17 sources in vivo, we investigated whether DHA anticancer efficacy relied on γ\(\delta\) T cells and IL-17–producing CD4 T cells. We found that the DHA-enriched diet could prevent tumor growth in γ\(\delta\)TCR-deficient mice, ruling out any significant contribution of these cells in DHA anticancer activity (Fig. 6A). However, upon testing DHA antitumor effect in T-cell-deficient Rag2\(^{-/-}\) mice reconstituted with either IL-17\(^{+/+}\) or IL-17\(^{-/-}\) αβ CD4 T cells, we failed to note any therapeutic benefit of the DHA diet in mice that had received IL-17–deficient CD4 T cells (Fig. 6B). We also measured T\(_{17}\) cell responses from WT mice under the control or DHA-enriched diet. In line with our previous findings, draining lymph node CD4 T cells from B16F10 tumor-bearing mice under the DHA-enriched diet expressed lower amounts of IL-17 and Rorc than controls (Fig. 6C). Together these data suggest that DHA may affect tumor growth via this capacity to regulate IL-17 secretion from T\(_{17}\) cells.

IL-17A is a proangiogenic factor that favors neovascularization and tumor growth in vivo via its capacity to induce VEGF production (8, 33). In line with the documented angiogenic properties of IL-17A, we noted that B16F10 tumors from mice under DHA-enriched diet featured decreased tumor vasculature reflected by reduced vegfr and vegf expression and reduced number of CD31-positive blood vessels (Fig. 6D). Finally, to determine the role of VEGF in the antitumor effect of DHA, we treated tumor-bearing mice fed or not a DHA-enriched diet with the VEGF receptor (VEGFR)-blocking mAb, DC101. We observed that while DHA and anti-VEGF therapy exhibited similar antitumor effects, these treatments failed to synergize (Fig. 6E). Collectively, these data suggest that the activity of DHA relies on the prevention of IL-17–dependent tumor neoangiogenesis.

**Discussion**

Although activation of the transcription factor PPARγ has been associated with reduced inflammation, the intracellular series of events consecutive to PPARγ activation remain incompletely understood and the actual involvement of PPARγ in the anti-inflammatory effects of PPARγ ligands remains debated (34, 35). In this study, we propose what we believe to be a novel molecular pathway by which PPARγ contributes to limit the secretion of the proinflammatory cytokine IL-17. DHA activates PPARγ, which binds to the Socs3 promoter and drives Socs3 expression in nascent T\(_{17}\) cells, leading to reduced STAT3 phosphorylation, Rorc expression and IL-17 secretion. Finally, DHA prevents tumor growth in vivo through the down-regulation of IL-17 production.

Although our data indicate that DHA inhibits T\(_{17}\) cell induction through downregulating the STAT3 signaling pathway, the strong inhibitory effect of DHA on T\(_{17}\) cell differentiation suggests that DHA could act at several levels on the T\(_{17}\) cell differentiation program. Indeed, activation of PPARγ has been shown to prevent the clearance of corepressors from the Broyt promoter (36), resulting in repressed T\(_{17}\) cell generation. We cannot rule out that this signaling pathway plays an additional role in DHA effect on differentiating T\(_{17}\) cells.

The links between inflammation and cancer have been thoroughly documented as illustrated by data showing an increased risk of patients suffering from inflammatory bowel disease to develop cancer compared with healthy individuals (37). Chronic inflammation can indeed contribute to cell transformation and cancer formation. Dietary supplementation of DHA has been shown to alleviate the severity of intestinal inflammation (38, 39) in experimental models of colitis and in inflammatory bowel disease in humans (40, 41). These findings possibly explain why dietary (n-3) PUFAs intake was associated with decreased risk of cancer (42, 43). However, recent data indicate that DHA also improves outcome of chemotherapy in patients with metastatic breast cancer, suggesting that it not only affects cancer incidence, but can be therapeutically used to prevent tumor growth (44). Our study brings up evidence that the anticancer effects of DHA could rely on its ability to dampen IL-17 levels in vivo.

The role of T\(_{17}\) cells during cancer growth remains intensely debated. Adoptive transfer experiments have shown the anticancer potential of both mouse and human T\(_{17}\) cells (45, 46). It was further shown recently that the remarkable ability of T\(_{17}\) cells to eliminate tumors in these settings relied on their ability to self-renew and transdifferentiate into IFN-γ–producing cells in vivo (46). However, in some human
cancers IL-17 was shown to be associated with poor prognosis (47–49). Mouse and human studies revealed an association between IL-17 levels in the tumor bed, VEGF, and blood vessels number (33, 50). In addition, IL-17 has the capacity to directly trigger VEGF release from VEGFR-expressing cells such as cancer cells and stromal cells (33). These data support the contention that IL-17 is an important factor, which drives VEGF-dependent neoangiogenesis during cancer growth. Anti-VEGF therapies are being implemented for cancer treatment in humans but are expensive. In this regard, our study supports the antiangiogenic property of a DHA-enriched diet via its capacity to regulate IL-17 production and VEGF-related angiogenesis. If such results could be confirmed in patients with cancer, DHA should be considered as a potential antiangiogenic candidate for future clinical trials.

Altogether, our study unraveled that DHA-mediated PPARγ activation limits T17 cell differentiation by inducing SOCS3 expression. Because our results suggest that the activity of DHA is relevant in humans, DHA administration might prove beneficial in diseases where IL-17 cytokine secretion is undesirable.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

References


