

SOCS3 Transactivation by PPAR γ Prevents IL-17–Driven Cancer Growth

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Abstract

Activation of the transcription factor PPAR γ by the n-3 fatty acid docosahexaenoic acid (DHA) is implicated in controlling proinflammatory cytokine secretion, but the intracellular signaling pathways engaged by PPAR γ are incompletely characterized. Here, we identify the adapter-encoding gene *SOCS3* as a critical transcriptional target of PPAR γ . *SOCS3* promoter binding and gene transactivation by PPAR γ was associated with a repression in differentiation of proinflammatory T-helper (T_H)17 cells. Accordingly, T_H17 cells induced *in vitro* displayed increased *SOCS3* expression and diminished capacity to produce interleukin (IL)-17 following activation of PPAR γ by DHA. Furthermore, naïve CD4 T cells derived from mice fed a DHA-enriched diet displayed less capability to differentiate into T_H17 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 PPAR γ -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)-1 β 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 (T_H)17 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). T_H17 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 ROR γ t (5). Although *Rorc* is indispensable for T_H17 cell induction, STAT3 is also an essential transcription factor for T_H17 cell differentiation as it directly regulates *il17a/f* and *Rorc* gene transcription (6, 7). IL-17 secretion from T_H17 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 PPAR γ 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 PPAR γ transcription factor by DHA in developing T_H17 cells induces *Socs3* expression and interferes with the STAT3 signaling pathway, thereby inhibiting T_H17 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 PPAR γ -induced SOCS3 expression in T_H17 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. ROR γ t-eGFP reporter mice were obtained from Dr. Eberl (Institut Pasteur, Paris, France) and have been previously described (14). *IL-17a*^{-/-} mice were obtained from Dr. Y. Iwakura (University of Tokyo, Tokyo, Japan; ref. 15). $\gamma\delta$ TCR-deficient mice were kindly provided by Dr. Bernhard Ryffel (University and CNRS, Orléans, France). Rag2^{-/-} 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). PPAR β - and PPAR γ -deficient mice were provided by Pr. 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 Nutritional Lipids) 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 (Nutrixel) was provided as a diet supplement for 3 weeks. Naïve CD4⁺ T-cell differentiation into T_H17 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_{15:0} 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_{15:0} 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% CO₂ 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 × 10⁵ B16F10 or 10⁵ 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 i.p. 30 μg of IL-1 receptor antagonist (IL-1Ra; Kineret 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

Naïve CD4⁺ T cells (CD4⁺CD62L^{hi}CD44^{lo}) were obtained from spleens and lymph nodes of C57BL/6 wild-type (WT) or PPAR β - or PPAR γ -deficient mice. CD4⁺ T cells were purified from spleen and lymph nodes with anti-CD4 microbeads (Miltenyi Biotec), then were further sorted as naïve CD4⁺CD62L^{hi}CD44^{lo} T cells. Isolated naïve T cells were routinely 98% pure. Isolated naïve CD4⁺ 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⁺ 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 PPAR γ agonist troglitazone and the antagonist GW9662 have been added at final concentrations of 0.75 and 0.1 μmol/L, respectively (Cayman Chemical). The PPAR α and PPAR β 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⁺ 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. Naïve CD4⁺ T cells were isolated with the human CD4⁺ T Cell

Isolation Kit II (MiltenyiBiotec), followed by stimulation with the Expansion/Activation Kit (MiltenyiBiotec), and differentiated for 3 days into T_H0 cells without cytokines or T_H17 cells in presence of TGF- β (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 γ* (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_H0 or T_H17 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 (Biolegend), IFN- γ (BD Biosciences), or human IL-17 (Biolegend) 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 μ g/mL; Sigma-Aldrich), and monensin (GolgiStop; 1 μ 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 (mAb) used for flow cytometry analyses were as follows: fluorescein isothiocyanate (FITC)-conjugated anti-CD4 (GK1.5; BD Biosciences), Alexa Fluor 647-conjugated anti-IL-17 (eBio 17B7; eBiosciences), and phycoerythrin (PE)-conjugated anti-IL-4 (554435; BD Biosciences) or PE-conjugated anti-IFN- γ (554412; BD Biosciences).

All events were acquired by a BD LSR-II 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_H0 or T_H17 cells with or without DHA (20 μ 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/L 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- β -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_H17 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 γ -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_t values and expressed in fold enrichment according to immunoglobulin (Ig) values. Primers designed to assess ChIP assay are as follows: *Actb* 5'-actctttgcagccacattcc-3' and 3'-agcgtctggttccaataact-5'; *Socs3* 1679 5'-gtcagattccttctcaggtt-3' and 5'-gcacccttcccttttttt-3'; and *Socs3* 576 5'-cccagtccttgcctgatt-3' and 5'-tgagagagggggaccaggagaaa-3'.

Quantitative real-time PCR

Total RNA from T cells was extracted with TriReagent (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'-atggaggggaatacagccc-3' and 5'-ttctttgagctcctcgtt-3'; *Tbx21* 5'-atcctgtaatggctgtggg-3' and 5'-tcaaccagcaccagacagag-3'; *Gata-3* 5'-aggatgtcctcctcctcctt-3' and 5'-gcctgaggactctaccataa-3'; *Foxp3* 5'-ctcgtctgaaggcaggtca-3' and 5'-tggcagagaggtattgaggg-3'; *Rorc mus musculus* 5'-gggtataaaccctagtgga-3' and 5'-ctgcaaagaagaccacacc-3'; *Rora* 5'-cccctactgttctctacca-3' and 5'-tgccacatcactctctctg-3'; *Ahr* 5'-ctccttctgcaaatcctgc-3' and 5'-ggccaagagctctttgatg-3'; *il17f* 5'-ttgatgcagcctgagtgct-3' and aattccagaaccgctccagt-3'; *il21* 5'-aaaacaggcaaaagctgcat-3' and 5'-tgacattgtgaacagctgaaa-3'; *il22* 5'-tcgccttgatctcactc-3' and gctcagctcctgacatca-3'; *cd4* 5'-cctgtgcaagaagcagagtg-3' and 3'-gttctgctgattcccctcc-5'; *Tgfb1* 5'-caaccagtccttctctaaa-3' and 3'-ggagagccctggatacaac-5'; *Rorc homo sapiens* 5'-aagcaggagcaatggaagt-3' and 3'-gcaatctcatctcggaaaa-5'; *Socs3 mus musculus* 5'-aactgtctggtggtgacat-3' and 5'-aaggccggagatttgcct-3'; *Socs3 homo sapiens* 5'-tggatggagcgggaggt-3' and 5'-acgggacatcttccactcaggtcct-3'; *gpr120* 5'-gatttctcctatgcggttg-3' and 5'-

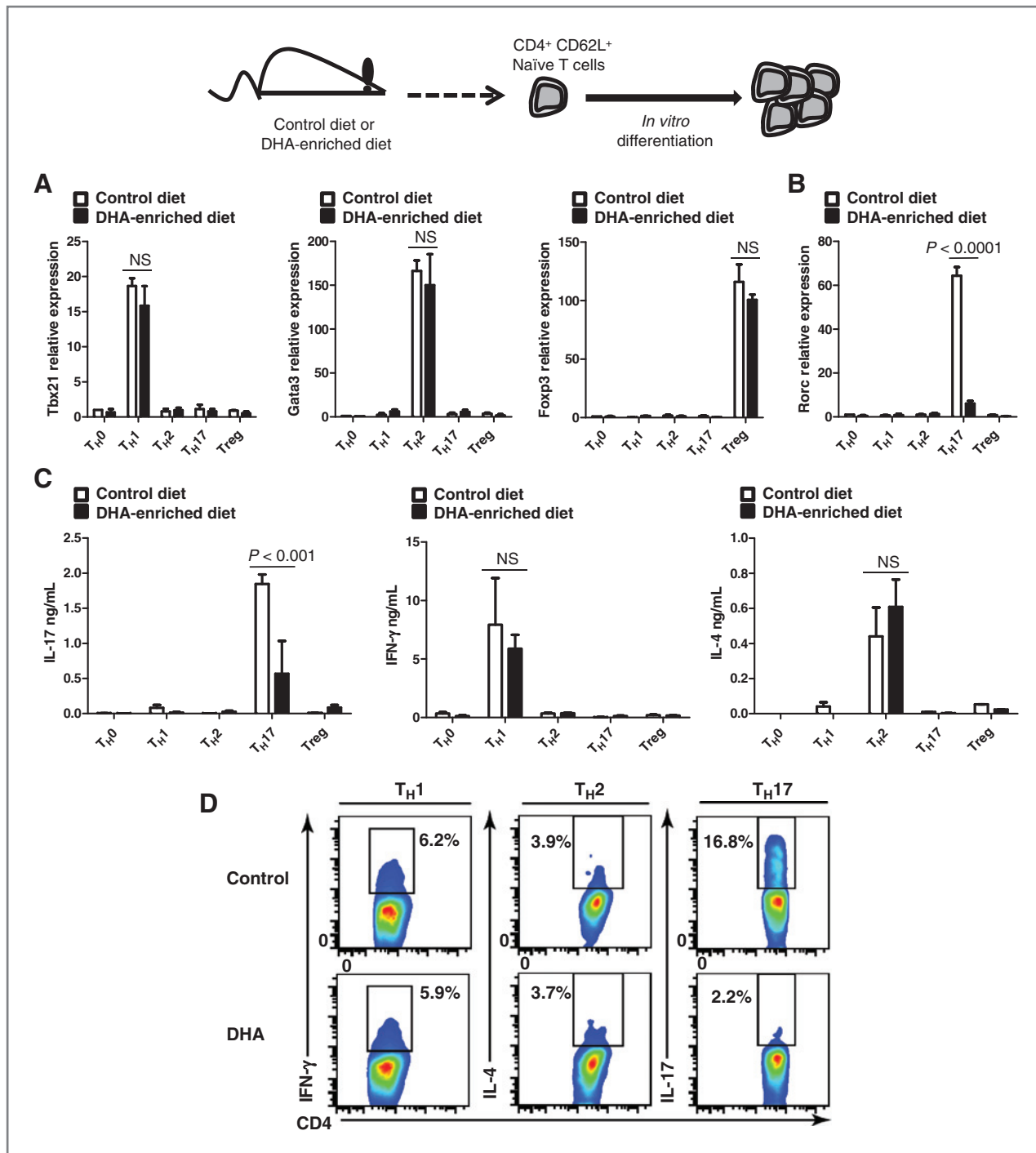
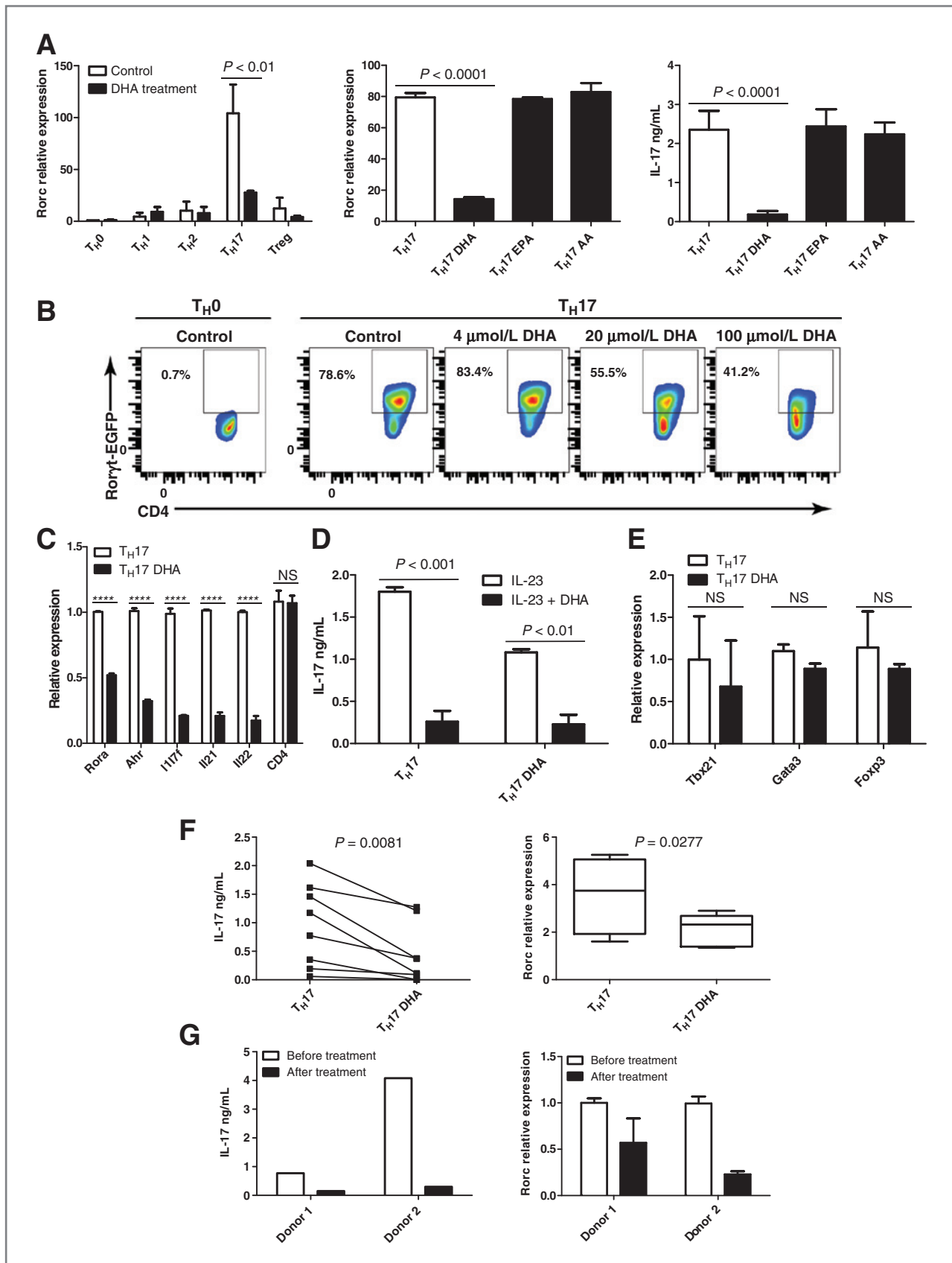


Figure 1. DHA-enriched diet reduces the capacity of naive CD4⁺ T cells to differentiate into T_H17 cells. Cell-sorted naive CD4⁺CD62L^{hi} CD44^{lo} T cells were isolated from mice under a control or a DHA-enriched diet and differentiated into T_H0, T_H1, T_H2, Treg, or T_H17 cells in the presence of 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 naive CD4⁺ T cells polarized in T_H1, T_H2, and T_H17 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.

ccctctgcatcttgttc-3'; *mmp9* ctgtcggtgtggttcagt-3' and agac-gacatagacggcatcc-3'; *cyp275*-gggcactagccagattcaca-3' and 5'-ctatgtgctgcacttccc-3'; *clu* 5'-ccattgtcccagatcagca-3' and 5'-

aggaggagcgcactggag-3'; *vegfr* 5'-aagagagtctggcctgett-3' and 5'-ctgctcggtgtctgctt-3'; and *vegfr* 5'-aatgctttctccgctctgaa-3' and 5'-gcttctacagcacagca-3'.



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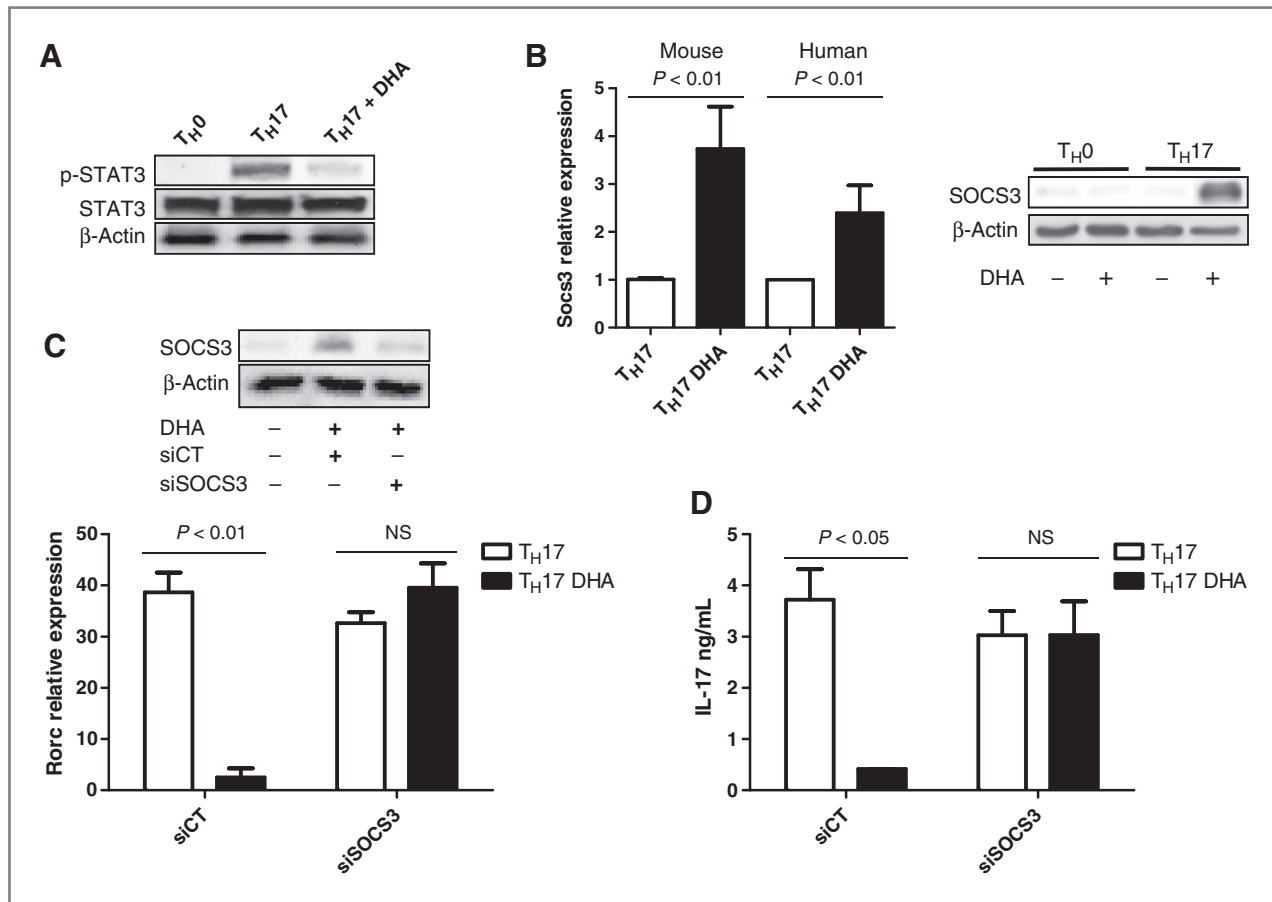


Figure 3. DHA treatment impairs STAT3 phosphorylation and upregulates SOCS3 in TH17 cells. **A**, expression of mouse p-STAT3 in TH0- and TH17-differentiated cells for 24 hours treated or not with DHA was determined by Western blotting. **B**, SOCS3 mRNA expression level was assessed in mouse and human control TH17 cells or DHA-treated counterparts after 3 hours of differentiation by qRT-PCR analysis (left) and activation of mouse protein SOCS3 was examined by immunoblotting (right) in CD4⁺ T cells polarized under TH0 or TH17 conditions 24 hours long in presence or not of DHA. **C**, CD4⁺ T cells were transfected with a control siRNA (siCT) or a siRNA specific for SOCS3 (siSOCS3) for 24 hours before being differentiated for further 24 hours into control or DHA TH17-cell driving conditions, then SOCS3 expression was determined by Western blotting (top) and *Rorc* mRNA induction (bottom) monitored by qRT-PCR. **D**, TH17 cells were generated and transfected as in **C** and IL-17 release was assayed by ELISA. Representative data from 1 of 3 experiments are shown. NS, not significant.

Statistical analyses

Statistical analysis was conducted using Prism software (Graph Pad software). For the analysis of experimental data, comparison of continuous data was achieved by the Mann-Whitney *U* test and comparison of categorical data by Fisher exact test, as appropriate. All *P* values are two-tailed. *P* values less than 0.05 were considered significant. Data are represented as mean \pm SD.

Results

In vivo administration of DHA impairs mouse TH17 cell differentiation from naïve CD4 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 T-cell differentiation in a cell-intrinsic manner has not been explored. To test this, we first examined the ability of naïve CD4⁺ CD62L⁺ T cells

Figure 2. DHA impairs TH17 differentiation *in vitro*. **A**, RNA isolated from naïve CD4⁺ CD62L^{hi} cells differentiated into TH0, TH1, TH2, Treg, or TH17 cells with anti-CD3 and anti-CD28 antibodies in the presence or absence of DHA (20 μ mol/L) was subjected to qRT-PCR relative to the expression of mRNA-encoding mouse *Actb* to examine the expression of *Rorc* at 72 hours following activation (left). qRT-PCR analysis of *Rorc* expression and ELISA of IL-17 in supernatants of TH17 cells polarized with or without DHA, EPA (eicosapentaenoic acid, n-3 PUFA), or AA (arachidonic acid, n-6 pufa) on day 3 of culture (right). **B**, naïve CD4⁺ T cells from ROR γ t-eGFP reporter mice were differentiated into TH17 cells for 72 hours in the presence of indicated escalating doses of DHA and the proportion of ROR γ t-expressing cells was assessed by flow cytometry. Numbers beside outlined areas indicate percentage cells in each gate. **C** and **E**, TH17 cells were generated as in **A** and the expression of the *Rora*, *Ahr*, *il17f*, *il21*, *il22*, and *CD4* genes (**C**) and *Tbx21*, *Gata3*, and *Foxp3* genes (**E**) was assessed by qRT-PCR. **D**, TH17 cells were generated with or without DHA as in **A**, first round, and reactivated for further 48 hours in presence of IL-23 with an additional DHA treatment or not (second round), then the levels of IL-17 released from these cells after the second round of stimulation were determined by ELISA. **F**, human TH17 cells were generated from naïve peripheral blood CD4⁺ T cells isolated from healthy volunteers polarized for 72 hours *in vitro* and treated or not with DHA, then IL-17 secreted in supernatants and *Rorc* mRNA level were assessed, respectively, by ELISA (left) and qRT-PCR (right). **G**, same analyses as in **F** but carried out from differentiated TH17 cells generated before or after a 3-week long DHA supplementation diet. Representative data from 1 of 3 experiments are shown. ****, *P* < 0.0001; NS, not significant.

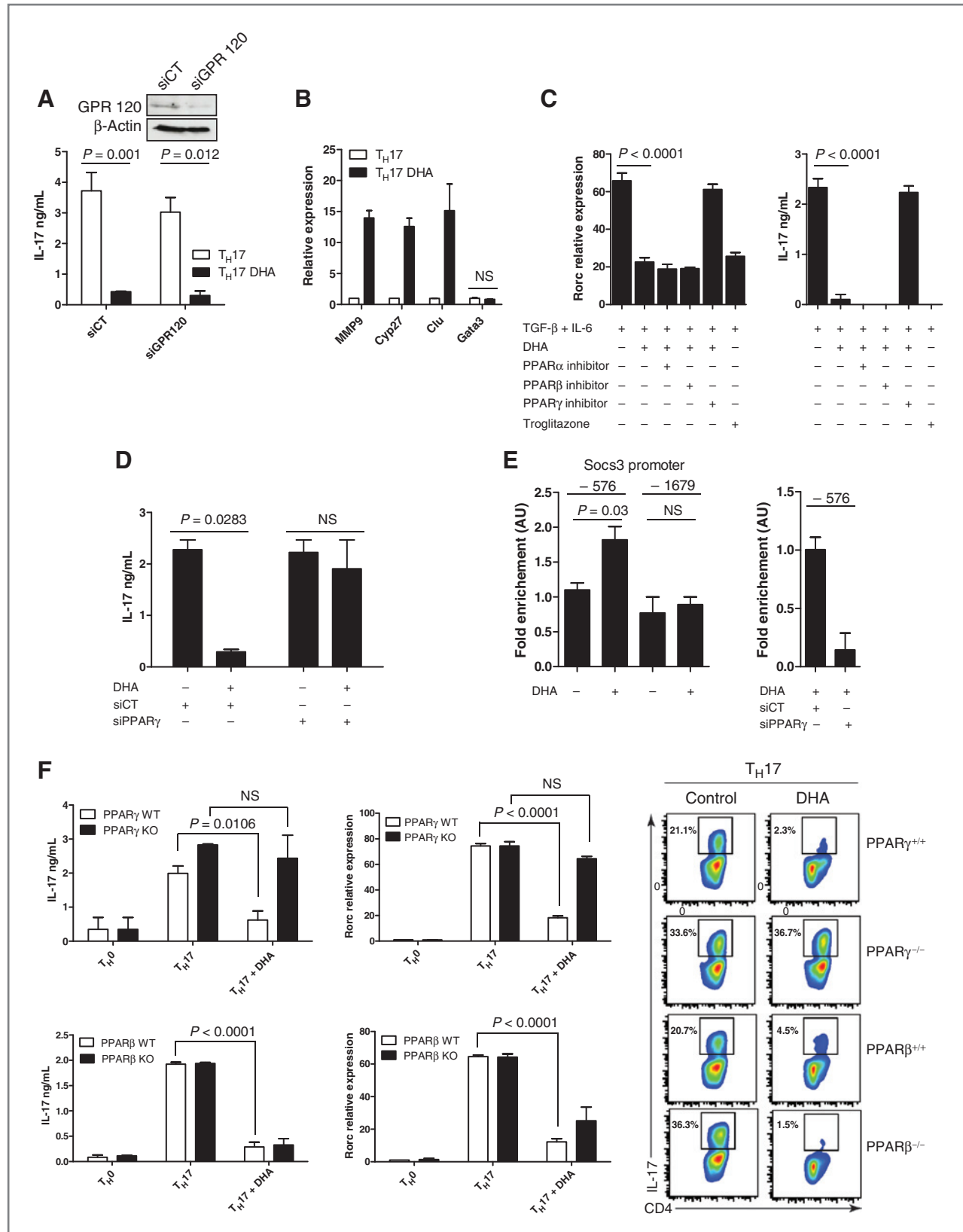


Figure 4. PPARγ transactivates Socs3 upon activation by DHA. A, expression of mouse GPR120 (top) and IL-17 release from control or GPR120 siRNA-transfected T_H17 cells (bottom) were detected after 48 hours of polarization with or without DHA by Western blotting and ELISA, respectively. B, relative

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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 T_H1, T_H2, 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 T_H17 cells under T_H17-polarizing conditions, as illustrated by their weak expression of the T_H17-cell transcription factor *Rorc* (Fig. 1B). By assessing IL-17a secretion from T_H17 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 T_H1 and T_H2 cells were not affected (Fig. 1C). Intracellular staining data confirmed dampened IL-17a secretion from T_H17 cells from mice receiving DHA *in vivo*, whereas the respective production of IFN- γ or IL-4 from T_H1 and T_H2 cells remained unaffected (Fig. 1D). Overall, these results suggest that DHA selectively affects T_H17 cell differentiation *in vivo*.

DHA inhibits mouse and human T_H17 cell differentiation in a cell-intrinsic manner

To test whether DHA directly affects T_H17 cell development, we differentiated naïve CD4⁺ T cells without antigen-presenting cells under T_H17 cell-polarizing conditions in the presence of DHA. We observed a marked reduction of *Rorc* expression in T_H17 cells upon DHA treatment, confirming that DHA impairs T_H17 cell differentiation (Fig. 2A). This inhibition is specific for DHA as other PUFAs such as eicosapentaenoic or arachidonic acids did not impair T_H17 cell differentiation (Fig. 2A). To study further the impact of DHA on T_H17 differentiation, we generated T_H17 cells from Ror γ t-eGFP reporter mice with increasing doses of DHA. DHA reduced the proportion of Ror γ t-expressing cells in a dose-dependent manner, corroborating the DHA capacity to inhibit T_H17 cell differentiation (Fig. 2B). In addition, we also explored the ability of DHA to affect other T_H17-related genes and found that DHA impaired the expression of *Rora*, *Ahr*, *il7f*, *il21*, and *il22*, suggesting that DHA globally affects the T_H17 cell differentiation program (Fig. 2C). IL-23 is essential to maintain the proinflammatory T_H17 cell program both *in vitro* and *in vivo* (23). Because DHA blunted the primary differentiation of naïve CD4 T cells into effector T_H17 cells, we explored the ability of DHA to influence IL-23 capacity to restimulate engaged T_H17

cells. We found that DHA was also able to reduce IL-17a secretion from engaged T_H17 cells restimulated with IL-23 (Fig. 2D). To test whether DHA skewed T_H17 cell differentiation toward another CD4 effector T-cell subset, we monitored the expression of *Tbx21*, *Gata3*, and *Foxp3* genes in T_H17 cells treated with DHA. We found that DHA failed to induce any of the transcription factors defining the T_H1, T_H2, or Treg lineages, indicating that DHA restricts but does not skew T_H17 cell differentiation *in vitro* (Fig. 2E). We tested whether DHA also affects human T_H17 cell differentiation. For this, we first differentiated naïve human CD4⁺ T cells isolated from the blood of healthy volunteers into T_H17 cells in the presence of DHA. In line with our findings with mouse T cells, we found that DHA also prevented human T_H17 cell differentiation (Fig. 2F). Similarly, upon assessing T_H17 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 T_H17 cell differentiation (Fig. 2G). Altogether, these findings indicate that DHA restrains mouse and human T_H17 cell differentiation.

DHA interferes with T_H17 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 T_H17 cells, we hypothesized that DHA negatively regulates STAT3 activation during T_H17 cell differentiation. To test this, we induced T_H17 cells from naïve CD4⁺ T cells *in vitro* in presence or absence of DHA. As expected, we noted a robust STAT3 (p-STAT3) phosphorylation in T_H17 cells generated without DHA (Fig. 3A). In contrast, CD4 T cells incubated under T_H17-polarizing conditions with DHA featured a weak level of p-STAT3, confirming that DHA reduces STAT3 signaling in T_H17 cells (Fig. 3A). Because SOCS3 is a major regulator of STAT3 phosphorylation in T_H17 cells (24), 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 T_H17 cells, suggesting that the DHA-mediated downregulation of T_H17 cell differentiation could be related to SOCS3 induction (Fig. 3B). To investigate the involvement of SOCS3 in the capacity of DHA to control T_H17 cell differentiation, we downregulated SOCS3 expression in T cells differentiated under T_H17-cell skewing conditions with DHA using siRNA. We found that SOCS3 silencing blunted the capacity of DHA

expression of PPAR γ target genes (*MMP9*, *Cyp27*, and *Clu*) and *Gata3* as control within T_H17 cells exposed or not to DHA *in vitro*. C, mouse T_H17 cells were differentiated as described in Materials and Methods with control or DHA in presence or absence of specific PPARs ligands (PPARs inhibitors and troglitazone). *Rorc* mRNA level was monitored in these cells (left) and the amount of IL-17 secreted was quantified by ELISA (right) at 3 days following activation. D, ELISA of IL-17 in supernatants of T_H17 cells treated or not with DHA for 48 hours after transfection with control (siCT) or PPAR γ siRNA (siPPAR γ). E, ChIP analysis of the interaction between PPAR γ and Socs3 promoter *in vitro* differentiated T_H17 cells transfected either with siCT or siPPAR γ after DHA treatment or not. Analysis on the 2 putative binding sites for PPAR γ on Socs3 promoter (–1679 and –576) is shown. F, naïve CD4⁺ T cells from PPAR γ - (top) and PPAR β (bottom)-deficient mice or WT counterparts were polarized into T_H0, T_H17, and DHA-treated T_H17 lymphocytes. At day 3 of culture, IL-17 secretion and *Rorc* expression levels were monitored by ELISA and qRT-PCR, respectively. At day 5 and after restimulation, flow cytometry of the intracellular production of IL-17 by these cells in presence or not of DHA was assessed (right). Representative data from 1 of 3 experiments are shown. NS, not significant; KO, knockout.

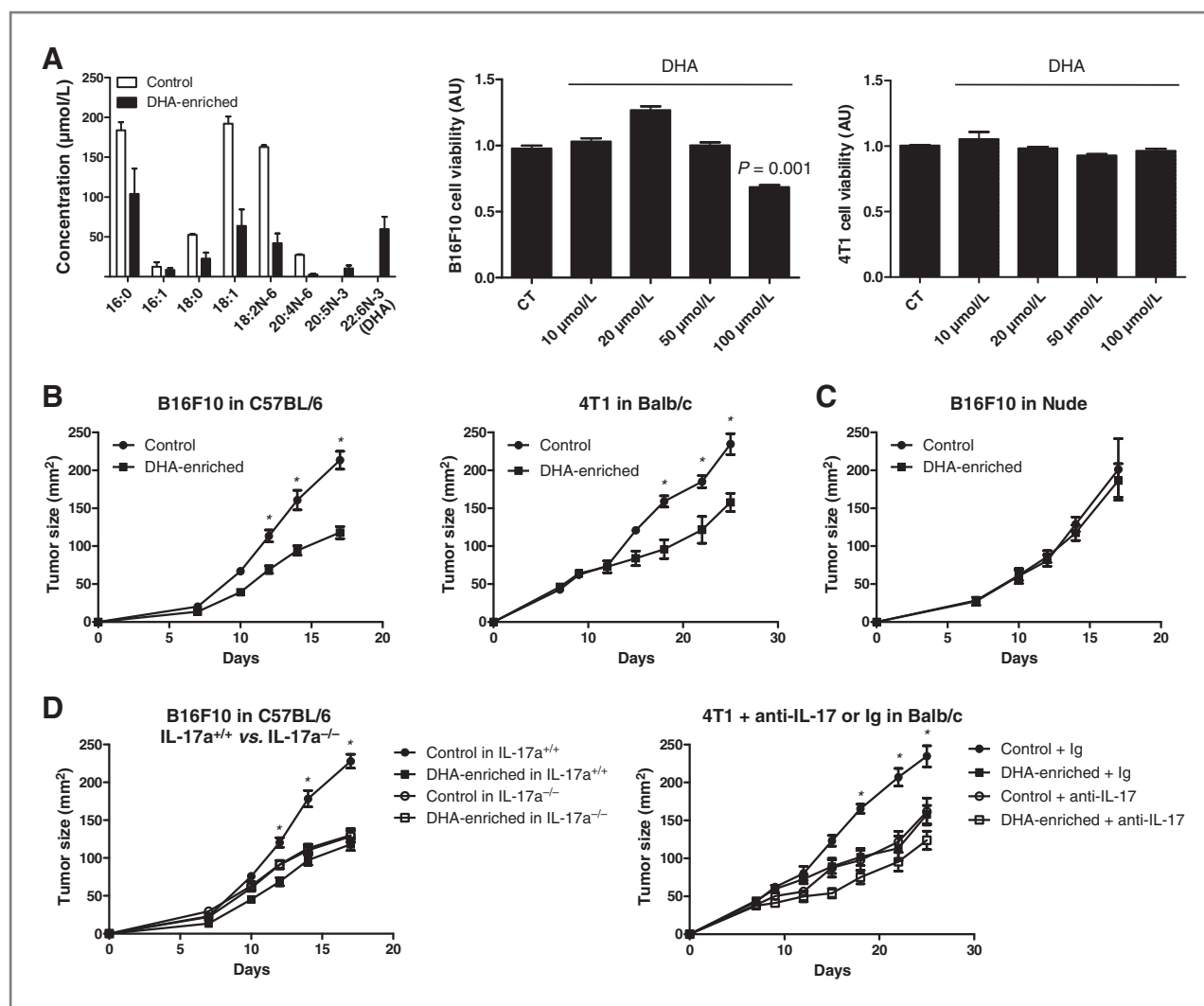


Figure 5. DHA prevents tumor outgrowth *in vivo* in an IL-17-dependent manner. A, plasma major fatty acids concentration of mice under control or DHA-enriched diet was assessed by gas chromatographic analysis (left). B16F10 melanoma and 4T1 mammary adenocarcinoma tumor cells (respectively, middle and right) were incubated with increasing doses of DHA for 72 hours and the cell viability was assessed by MTT assay. B, C57BL/6 (left) and BALB/c mice (right) under control or DHA-enriched diet were respectively inoculated with B16F10 and 4T1 tumor cells and tumor growth was monitored over 3 weeks. C, same as in B, but B16F10 were injected subcutaneously in Nude mice. D, monitoring of tumor growth of respectively B16F10 melanoma cells in C57BL/6 and *IL17a*^{-/-} mice (left) and of 4T1 in BALB/c mice injected with an anti-IL-17 antibody (200 µg/day injected on day 0, 1, 2, 3, 4, and 6) or control rat immunoglobulin G (IgG). All along these experiments, animals were either given control or DHA-enriched diet *, *P* < 0.05.

to restrain IL-17 and RORγt expression in T_H17 cells (Fig. 3C and D). Collectively, our findings suggest that DHA restrains T_H17 cell differentiation by interfering with STAT3 signaling through SOCS3 induction.

DHA prevents T_H17 cell differentiation in a PPARγ-dependent manner

To unravel the mechanism accounting for SOCS3 induction in developing T_H17 cells upon DHA treatment, we investigated the intracellular signaling pathways engaged by DHA on T_H17 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_H17 cell differentiation, we downregulated GPR120 expression with siRNA

in naïve T cells and analyzed the effect of DHA during T_H17 differentiation. Our results showed no significant differences in T_H17 polarization between cells transfected with control or GPR120 siRNA, indicating that the decreased T_H17 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_H17 cell differentiation through STAT3 signaling pathway blockade, we hypothesized that the effects of DHA on T_H17 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_H17 cells, suggesting

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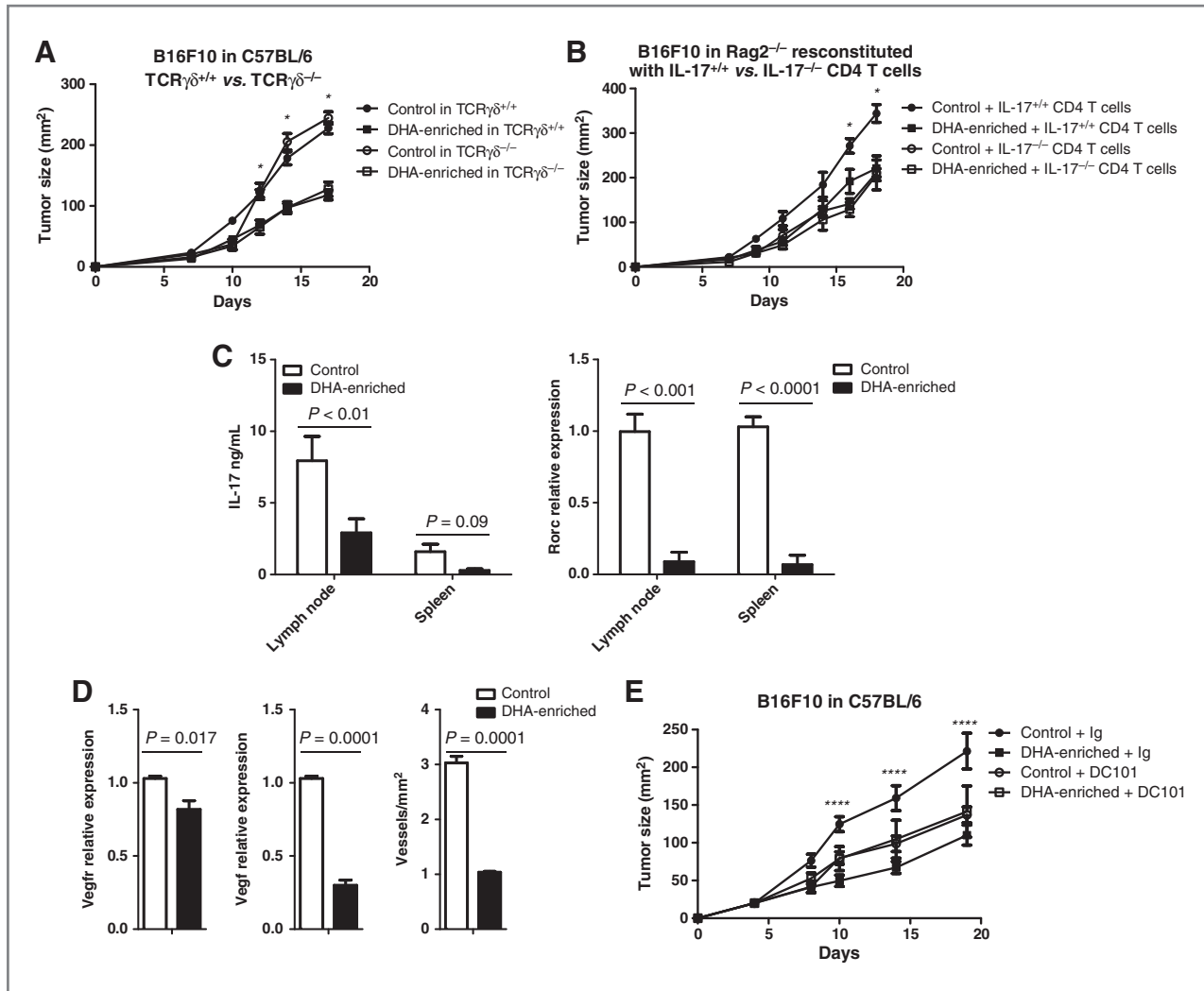


Figure 6. DHA limits CD4 T cell-derived IL-17 secretion and tumor angiogenesis. **A**, C57BL/6 $\gamma\delta$ TCR-deficient mice under control or DHA-enriched diet were respectively inoculated with B16F10 tumor cells and tumor growth was monitored over 3 weeks. **B**, Rag2^{-/-} mice received intravenous $\alpha\beta$ CD4 T cells isolated from either IL-17^{+/+} or IL-17^{-/-} mice and were subsequently fed a control or DHA-enriched diet. Five weeks later, mice were injected with B16F10 melanoma cancer cells and tumor growth was monitored. Pooled data from 2 experiments are shown. **C**, CD4⁺ T cells isolated from draining lymph nodes and spleen of B16F10 tumor-bearing immunocompetent mice were activated 3 days with anti-CD3 and anti-CD28 antibodies. Then, IL-17 release was controlled by ELISA (left) and Rorc mRNA expression level was determined by qRT-PCR analysis (right). **D**, qRT-PCR analysis of *Vegfr* and *Vegf* mRNA level in B16F10 tumor extracts from immunocompetent tumor-bearing mice treated as in Fig. 5B and number of CD31-positive blood vessels in these tumors. **E**, same as in Fig. 5B but C57BL/6 mice were treated or not with an anti-VEGFR2 (clone DC101) antibody (200 μ g/day injected on day 1 and 5). Representative data from 1 of 3 experiments are shown. *, $P < 0.05$; ****, $P < 0.0001$.

that PPAR γ signaling is activated by DHA treatment on T_H17 cells (Fig. 4B). In addition, the DHA effect on T_H17 cell differentiation was markedly reduced in the presence of a PPAR γ inhibitor GW9662, but not with a PPAR β inhibitor GSK0660 or PPAR α inhibitor GW6471, as measured by IL-17 secretion and *Rorc* expression (Fig. 4C). Moreover, PPAR γ agonist troglitazone neutralized IL-17 secretion from T_H17 cells to a comparable extent to DHA, suggesting that PPAR γ activation is involved in DHA effects on T_H17 cells (Fig. 4C). To validate the elective involvement of PPAR γ in the intracellular effects of DHA on T_H17 cells, we downregulated PPAR γ expression with siRNA and tested the ability of DHA to inhibit T_H17 cell differentiation in absence of PPAR γ . We

found that silencing PPAR γ completely abolished the ability of DHA to restrain T_H17 cell induction (Fig. 4D). Although PPAR γ agonists induce *Socs3* expression (27, 28), the direct link between PPAR γ and SOCS3 remains unclear. To test whether the DHA-driven *Socs3* induction is directly due to PPAR γ activation, we conducted a bioinformatic analysis of the *Socs3* promoter and found 2 putative binding sites for PPAR γ at positions -1679 and -576 from the transcription start site. By conducting ChIP, we found that PPAR γ binds to the *Socs3* promoter only at position -576 in DHA-treated T_H17 cells as compared with control T_H17 cells (Fig. 4E). We further checked the specificity of this interaction by downregulating PPAR γ expression by siRNA and confirmed that

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the amount of PPAR γ -binding *Socs3* promoter was accordingly reduced (Fig. 4E). Finally, we tested the effects of DHA on naïve T cells from WT-, PPAR γ -, or PPAR β -deficient mice. In line with our results obtained with the PPAR inhibitors, we found that while DHA could suppress T_H17 cell differentiation from WT- and PPAR β -deficient T cells, PPAR γ -deficient T cells were totally unaffected by DHA treatment (Fig. 4F). Altogether, these findings show that the ability of DHA to hamper T_H17 cell differentiation is strictly dependent on PPAR γ -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 μ mol/L; Fig. 5A). However, we noted that the DHA-enriched diet delays subcutaneous tumor growth *in vivo* in both cancer models (Fig. 5B). 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 impedes 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 $\gamma\delta$ T cells and T_H17 cells are major IL-17 sources *in vivo*, we investigated whether DHA anticancer efficacy relied on $\gamma\delta$ T cells and IL-17-producing CD4 T cells. We found that the DHA-enriched diet could prevent tumor growth in $\gamma\delta$ 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^{-/-} $\alpha\beta$ 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_H17 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_H17 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 PPAR γ has been associated with reduced inflammation, the intracellular series of events consecutive to PPAR γ activation remain incompletely understood and the actual involvement of PPAR γ in the anti-inflammatory effects of PPAR γ ligands remains debated (34, 35). In this study, we propose what we believe to be a novel molecular pathway by which PPAR γ contributes to limit the secretion of the proinflammatory cytokine IL-17. DHA activates PPAR γ , which binds to the *Socs3* promoter and drives *Socs3* expression in nascent T_H17 cells, leading to reduced STAT3 phosphorylation, *Rorc* expression and IL-17 secretion. Finally, DHA prevents tumor growth *in vivo* through the downregulation of IL-17 production.

Although our data indicate that DHA inhibits T_H17 cell induction through downregulating the STAT3 signaling pathway, the strong inhibitory effect of DHA on T_H17 cell differentiation suggests that DHA could act at several levels on the T_H17 cell differentiation program. Indeed, activation of PPAR γ has been shown to prevent the clearance of corepressors from the *Roryt* promoter (36), resulting in repressed T_H17 cell generation. We cannot rule out that this signaling pathway plays an additional role in DHA effect on differentiating T_H17 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_H17 cells during cancer growth remains intensely debated. Adoptive transfer experiments have shown the anticancer potential of both mouse and human T_H17 cells (45, 46). It was further shown recently that the remarkable ability of T_H17 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 PPAR γ activation limits T_H17 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.

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