Fish Oil Rich Diet Promotes Hematopoiesis and Alters Hematopoietic Niche

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The self-renewal and differentiation of hematopoietic stem cells (HSCs) in bone marrow are essential to replenish all blood cell types, but how this process is influenced by diet remains largely unclear. Here we show that diet rich in fish oils promotes self-renewal of hematopoietic stem cells and extramedullary hematopoiesis. Chronic intake of fish-oil rich diet increases the abundance of HSCs, alters hematopoietic microenvironment and intriguingly, induces the expression of matrix metalloproteinase 12 (MMP12) in the bone marrow. Pointing to a direct effect of fish oil on MMP12 expression, omega-3 polyunsaturated fatty acids (n-3 PUFAs) induce the expression of MMP12 in a dose-dependent manner in bone marrow cells. Importantly, downregulation of MMP12 activity using an MMP12-specific inhibitor attenuates diet-induced myelopoiesis in both bone marrow and spleen. Thus, fish-oil rich diet promotes hematopoiesis in the bone marrow and spleen, in part, via the activity of MMP12. Taken together, these data provide new insights into diet-mediated regulation of hematopoiesis.

The hematopoietic system provides the body with a constant supply of all blood lineages. In order to maintain hematopoietic homeostasis, hematopoietic stem cells (HSCs) have the clonal capacity to provide life-long regeneration of all blood lineages. Bone marrow provides hematopoietic microenvironment or niche to support and regulate the self-renewal, migration and differentiation of HSCs (1). Many cellular components including osteoclasts, osteoblasts, mesenchymal progenitor cells and adipocytes as well as components of the extracellular matrix (ECM) play regulatory roles in the homeostasis of HSCs (2–5). The interplay between matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs) also serves an important role in hematopoiesis through remodeling the ECM in the hematopoietic niche of the bone marrow (6, 7). Injuries to these components brought by inflammation and/or metabolic conditions influence the size and fate of HSCs. Indeed, systemic inflammation alters the regulation of HSCs and increases more granulocytes and monocytes in peripheral tissues (8, 9). Moreover, a number of intracellular regulatory molecules including FoxOs, mTORC1, Fbw7, Egr1, Pbx1, pRb, c-Cbl, Myc, and Bmi1 mediate the processes of dormancy, cycling, self-renewal, differentiation, and survival of HSCs (10–15). The LKB1/AMPK signaling pathway, a metabolic sensing pathway, plays a key role in the HSCs self-renewal and differentiation (16, 17). These studies have provided strong link between HSCs homeostasis and metabolism. However, how intermediary metabolites influence stem cell behavior remains largely unclear, especially in regards to the effect of dietary fatty acids.

Of particular interest are two biologically active fatty acids (FA), omega-3 (n-3) and omega-6 (n-6) polyunsaturated fatty acids (PUFAs). Both n-3 and n-6 FAs are long-chain PUFAs of 18–22 carbons in length with first double-bond positioned at the third or sixth carbon atom, respectively, from the methyl end of the FAs. N-3 PUFAs are present in vegetable oil, but the richest source is fish oil. Unlike n-6 PUFAs which promote inflammation and insulin resistance, n-3 PUFAs are believed to have anti-in-

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flammatory properties (18–20). Indeed, n-3 PUFAs are being used in the prevention and treatment of coronary artery disease (CAD), diabetes, hypertension, arthritis, cancer and other inflammatory and autoimmune disorders in humans (21, 22). However, its effect on stem cell proliferation and differentiation remains largely unknown.

We speculate that diet rich in n-3 PUFAs is likely to play a critical role in the homeostasis of HSCs by influencing the niche and/or energy metabolism of stem cells and thereby their proliferative potential. Indeed, here we show that diet rich in fish oils promotes hematopoiesis in the bone marrow and spleen in mice. The increased abundance of HSCs and hematopoietic progenitors in the spleen is associated with morphological changes in hematopoietic microenvironment of the bone and the upregulation of MMP12, also known as macrophage elastase, in bone marrow. The effect of dietary PUFAs on hematopoietic microenvironment is likely mediated in part by MMP12 as downregulation of MMP12 activity attenuates dietary PUFAs-induced hematopoiesis.

Materials and Methods

Mice and diets

6-week-old male CD45.2$^+$ or CD45.1$^+$ C57BL/6 wildtype mice (from The Jackson laboratory) were fed with low-fat diet (LFD, 2.9 kcal/g, 13% kcal from fat) (Teklad 2914), high-fat diet (HFD, 5.24 kcal/g, 60% kcal from fat) (Bioserv F3282), or high fish oil diet (5.24 kcal/g, 60% kcal from fat) (Bioserv F5424) for 4 weeks. Fatty acid compositions of each diet provided by the manufacturers are shown in Table 1. Of note, LFD vs. high fat diets may not only vary in fat content, but also in the source of protein (grain vs. casein) as well as sucrose and cornstarch. In some experiments, mice on high fish oil diet were orally gavaged on alternate days with an MMP12 inhibitor MMP408 (Calbiochem, Darmstadt, Germany) at 10 mg/kg body weight as previously described (23) for a total 4 weeks. All animal protocols have been approved by the Cornell Institutional Animal Care and Use Committee, and by the Scientific Investigation Board of Jiangsu University.

Antibodies and flow cytometry

Single cell suspension from spleen and bone marrow was prepared at 4°C as previously described (24). After incubation with rat serum to block Fc receptors for 15 minutes, cells were incubated with the following antibodies for 20 minutes: CD11b-PE, Sca-1-FITC, c-Kit-Percp, CD-45.1-PE, CD45.2-Biotin, Biotin-Alexa 488, Lin-PE (containing B220, CD4, CD8, Gr-1, Mac-1, Ter-119) and Ter-119-FITC from BD Biosciences (San Diego, CA). Following three washes with PBS, cells were analyzed using the FACSCalibur flow cytometer (BD Biosciences). Data were analyzed using the FlowJo or CellQuest software.

Table 1. Fatty acid compositions of each diet (mg/g) in this study. N-3 fatty acids are italicized. Shaded area indicates the percent of calories derived from three macronutrients.

Diet	Telkad 2914	Bioserv F3282	Bioserv F5424
	(LFD)	(HFD)	(Fish oil)
Capric (C10:0)	-	0.4*	-
Lauric (C12:0)	-	0.7	-
Myristic (C14:0)	-	4.7	24.3
Palmitic (C16:0)	5	85.3	51.9
Palmitoleic (C16:1n7)	-	9.7	32.1
Stearic (C18:0)	1	48.4	12.5
Oleic (C18:1n9)	7	147.7	58.9
Linoleic (C18:2n6)	20	36.6	34.9
Linolenic (C18:3n3)	1	3.6	5.2
Eicosapentaenoic Acid (C20:5n3)		-	*40.2*
Docosapentaenoic Acid (C22:5n3)		-	*15*
Docosahexanoic Acid (C22:6n3)		-	*26.2*
Calorie of fat	13%	59%	59%
Calorie of protein	20%	15%	15%
Calorie of sugar	67%	26%	26%

*, g/kg diet

Colony forming unit (CFU) assay in vitro

Colony forming unit assay was performed using Methylcellulose Complete Media (R&D Systems) per manufacture's protocol. Briefly, murine splenocytes or bone marrow cells were counted and resuspended in IMDM containing 2% fetal calf serum (FCS) followed by the addition of mouse methylcellulose complete medium. After vigorous vortex, 1.1 ml cell-medium mixture with a total of 2×10^5 splenocytes or 1×10^4 bone marrow cells were carefully plated into 35 mm culture plate using a syringe. The plates were then incubated at 37°C with 5% CO_2 for 8 days. CFU granulocyte and macrophage (CFU-GM), burst-forming unit erythroids (BFU-E), and CFU granulocyte erythrocyte macrophage megakaryocyte (CFU-GEMM) blood lineage

precursor cells were scored based on their colony morphologies under a light microscope. CFU-GEMM colonies included multiple progenitors, and had a dense core but without distinct border. In CFU-GM colonies, oval-shaped monocytic lineage cells and/or bright round granulocytic lineage cells formed grainy or gray dense core and individual cells were easy to see. BFU-E colonies were made up of tiny and irregular erythroid lineages cells and the clusters were relatively scattered without an obviously dense core.

Spleen colony forming cell (CFU-S) assay in vivo

CFU-S assay was performed as previously described (25) with minor modifications. Briefly, 5×10^4 splenocytes in PBS were injected i.v. into sublethally irradiated C57BL/6 male mice (1000 cGy). Twelve days after transplantation, spleens were harvested and fixed in Bouin's solution. The number of macroscopic colonies per spleen was counted.

Competitive repopulation assay

Bone marrow cells from C57BL/6 mice either on LFD ($CD45.2^+$) or high fish-oil diet ($CD45.1^+$) were mixed at 1:1 ratio and injected i.v. into lethally irradiated $CD45.2^+$ C57BL/6 recipients at a dose of 2×10^6. Recipients were maintained on LFD with drinking water supplemented with neomycin sulfate (σ-Adlrich). Three months later, peripheral blood was collected from the retro-orbital venous sinus of the recipients and analyzed for the relative abundance of $CD45.1^+$ or $CD45.2^+$ cells.

Bone marrow cell purification

Bone marrow cells were collected, adjusted to 1×10^6/ml with RPMI-1640 supplemented with 10% FCS, seeded into plastic flasks and incubated at 37°C. Six hours later, attached cells were used as bone marrow stromal cells, while nonadherent cells were washed with PBS and fractionated into CD11b-positive and -negative populations using anti-CD11b-biotin (eBioscience, San Diego, CA) and streptavidin-labeled magnetic particles (STEMCELL Co.). Cells were harvested for further q-PCR analysis.

Docosahexaenoic acid (DHA) treatment

Bone marrow cells were collected and adjusted cell concentration to 1×10^6/ml with RPMI-1640 supplemented with 10% FCS. Cells were seeded into 6-well plates with or without DHA for 24 hours at 37°C followed by Q-PCR analysis.

Western blot

Western blot was performed as we previously described (26) with the following antibodies for: MMP12 (rabbit, 1:200, Santa Cruz), ERK (mouse, 1:1000, eBioscience clone 5AD13MA), phospho-ERK(mouse, 1:1000, eBioscience clone MILAN8R), and β-actin-HRP (mouse, 1:1,000, Santa Cruz). Secondary antibody was HRP-conjugated goat antirabbit IgG (1:10,000, Santa Cruz). Protein levels were quantitated using the Quantity software (Saizhi Inc, Beijing, China).

Quantitative-PCR (Q-PCR)

Splenocytes or bone marrow cells from both femurs and tibias were harvested, snap frozen in liquid nitrogen and stored in −80°C until further analysis. Total RNA was extracted with Trizol and reverse-trancribed into cDNA using ReverAid First Strand cDNA Synthesis Kit (Thermo Scientific). Q-PCR analysis was performed using SYBR Green Premix ExTaq™ (Takara), containing SYBR PrimixExTaq™ (5μl), cDNA (0.5μl), primers (0.4μl) and ddH_2O (4.1μl). The relative mRNA levels of target genes were quantitatived using the $2^{-\Delta\Delta Ct}$ method and normalized to housekeeping gene *Gapdh*. Primer sequences used in this study are: murine *Mmp-9*: forward 5′-AAATGGTGCCCCATGTCACTTT –3′, reverse 5′- GCTCCGTGTAGAGTCTCTCACTAGG –3′; murine *MMP12*: forward 5′- TTACAGGATCTATAATTACACTCCGGAC –3′, reverse 5′-GCAAAAAGTATCATAATGTCAGCCT –3′; murine *Gapdh*: forward 5′- GGCATTGCTCTCAATGACAA-3′, reverse 5′-TGTGAGGGAGATGCTCAGTG –3′.

Immunofluorescent staining and immunohistochemistry

H&E staining was performed by the Cornell Histology Core Facility as previously described (27). For CD11b staining, cryosections of spleen tissue were stained with anti-CD11b-PE. Quantitation of $CD11b^+$ cells was counted from three different microscopic fields. For immunohistochemistry of MMP12, femurs were fixed in 4% paraformaldehyde, and decalcified in 10% EDTA solution at 4°C for three weeks prior to be processed in paraffin. After antigen retrieval, sections were blocked with 3% peroxide-methanol (CWBIO Inc. Beijing, China) to ablate endogenous peroxidase, and then incubated with anti-MMP12 primary antibody (1:200) followed by antirabbit IgG HRP at 37°C for 30 minutes. Positive signals were visualized using 3,3-diaminobenzidin (CWBIO Inc., Beijing, China) and counterstained with hematoxylin. After dehydration in xylene, slides were sealed with coverslips and all images were taken using Zeiss light microscope.

Statistical analysis

Data were shown as mean ± SEM for all experiments. Student *t* test was used when comparing means of two groups, while one-way ANOVA were used for statistical analysis of multiple groups. In all cases, $P < .05$ was considered as statistical significance.

Results

Fish oil rich diet promotes myelopoiesis in the spleen

To explore the effect of high fish oil diet on hematopoiesis, we separated wildtype littermates into three dietary groups, LFD, high fish oil diet and HFD. While other diets do not contain any –3 PUFAs, high fish oil diet contains high levels of n-3 PUFAs, including eicosapentaenoic acid (C20:5n3), docosapentaenoic acid (C22:5n3) and DHA (C22:6n3). By contrast, HFD contains higher content of saturated stearic (C18:0) and monounsaturated oleic acid (C18:1n9) relative to high fish oil diet. The content of n-6 PUFAs, mainly in the form of linoleic acid

(C18:2n6), was about the same between HFD and high fish oil diet (Table 1).

Following 4 weeks of feeding, the HFD cohort gained a significant amount of body weight, while the fish oil cohort exhibited a moderate increase of body weight (Figure 1A). Spleens of the fish oil cohort were enlarged and weighed significantly more than the other two cohorts (Figure 1A). Although total number of circulating white blood cells was comparable among the cohorts (Figure 1B), $FSC^{low}SSC^{hi}$ granulocytes in the spleen were significantly increased by 4-fold in the fish oil cohort when compared to other two cohorts (Figure 1C). Pointing to the impact of n-3 PUFAs (rather than the impact of high fat), HFD had no obvious effect on the granulocyte population in the spleen compared to that of the LFD cohort (Figure 1C). While both high fish oil diet and HFD increased the abundance of $FSC^{hi}SSC^{low}$ monocytes in the spleen, and the effect of high fish oil diet was much more pronounced (Figure 1C). This was further confirmed by flow cytometric analysis of $CD11b^+$ myeloid cells in the spleen (Figure 1D). Indeed, while HFD increases the abundance of myeloid cells in the spleen as we previously shown (26), fish oil diet exerted more profound impact on this population (Figure 1D-F). Moreover, histological assessment of the spleen revealed the alteration of splenic compartmentalization in the fish oil cohort (Figure 1G) with a large number of $CD11b^+$ monocytes present in the marginal zone (Figure 1H-I). Thus, 4-week intake of high fish oil diet promotes myelopoiesis in the spleen.

High fish oil diet induces extramedullary hematopoiesis in the spleen. To understand how high fish oil intake induced

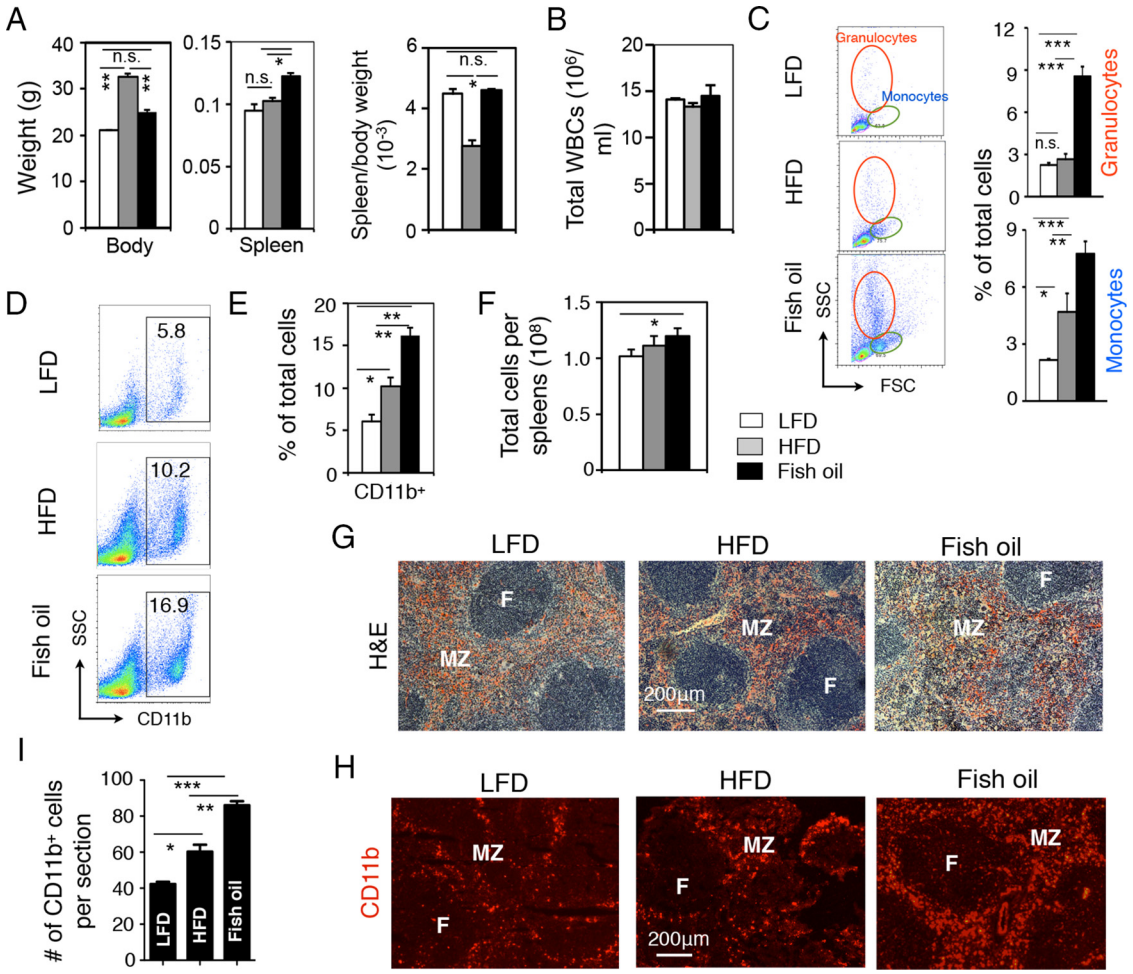

Figure 1. High fish oil diet promotes myelopoiesis in the spleen. A, Body weight (left), spleen weight (middle) and the ratio of spleen weight to body weight of littermates on different diets for 4 weeks. B, Total leukocytes in the circulation. N = 5 mice each group. C, Representative flow cytometric analysis (FSC/SSC) of splenocytes. Distinct myeloid cell populations, granulocytes (red cycle) and monocytes (green), are indicated and quantitated on the right. N = 5 mice per cohort. D–E, Representative flow cytometric analysis (D) and quantitation (E) of $CD11b^+$ cells in the spleens of mice on different diets for 4 weeks. Numbers in dot plots indicated the percent of $CD11b^+$ cells in the spleen from one experiment. N = 6 mice per cohort. F, Total numbers of splenocytes in mice fed with different diets for 4 weeks. G, Representative H&E images showing the loss of splenic compartmentalization in the high fish-oil cohort. MZ, marginal zone. F, Follicular. H–I, Representative CD11b staining (H) and quantitation (I) of $CD11b^+$ cells in the spleen. Data shown as mean ± SEM. *, $P < .05$; **, $P < .01$; ***, $P < .001$; n.s., not significant by ANOVA.

splenic myelopoiesis, we next examined the status of extramedullary hematopoiesis, ie, hematopoiesis occurring in organs other than bone marrow (28). We measured the levels of hematopoietic progenitors (Lin^- $c\text{-}Kit^+Sca\text{-}1^-$) and HSCs ($Lin^-$ $c\text{-}Kit^+$ $Sca\text{-}1^+$) in the spleen. As shown in Figure 2A, high fish oil diet increased the percent of Lin^- $c\text{-}Kit^+Sca\text{-}1^-$ hematopoietic progenitors and Lin^- $c\text{-}Kit^+$ $Sca\text{-}1^+$ HSCs by 4- and 10-fold, respectively, when compared to the HFD cohort. As a control, fish oil diet did not affect the percent of nonprogenitor Lin^- $c\text{-}Kit^-$ $Sca\text{-}1^+$ populations in the spleen.

We next measured the impact of high fish oil diet on proliferative capacity of splenic HSCs and progenitors using the colony forming unit assays (CFU) both in vitro and in vivo. Figure 2B shows representative images of GEMM, GM and BFU-E colonies after in vitro culture of splenocytes in methylcellulose complete culture medium. Numbers of GEMM, GM and BFU-E colonies were all significantly increased in mice fed with the high fish oil diet while HFD had a negligible to moderate effect (Figure 2B). Similarly, percent of $Ter\text{-}119^+$ erythroid precursors was significantly upregulated by nearly 6-fold in the spleens of the high fish oil cohort (Figure 2C). These changes were associated with elevated expression of stem cell factor (SCF) and granulocyte- macrophage colony stimulating factor (GM-CSF), two factors involved in the hematopoietic cell proliferation (29), in the spleens of the fish oil cohort (Figure 2D). To further demonstrate cell-autonomous effect of the diet on hematopoiesis, we performed the spleen colony forming unit (CFU-S) assay in vivo where splenocytes from different cohorts were transferred into sublethally irradiated mice on LFD. Indeed, donor splenocytes from the high fish oil cohort formed significantly more colonies in the spleens of recipients when compared to other dietary cohorts (Figure 2E). Thus, high fish oil diet enhances extramedullary hematopoiesis and increases

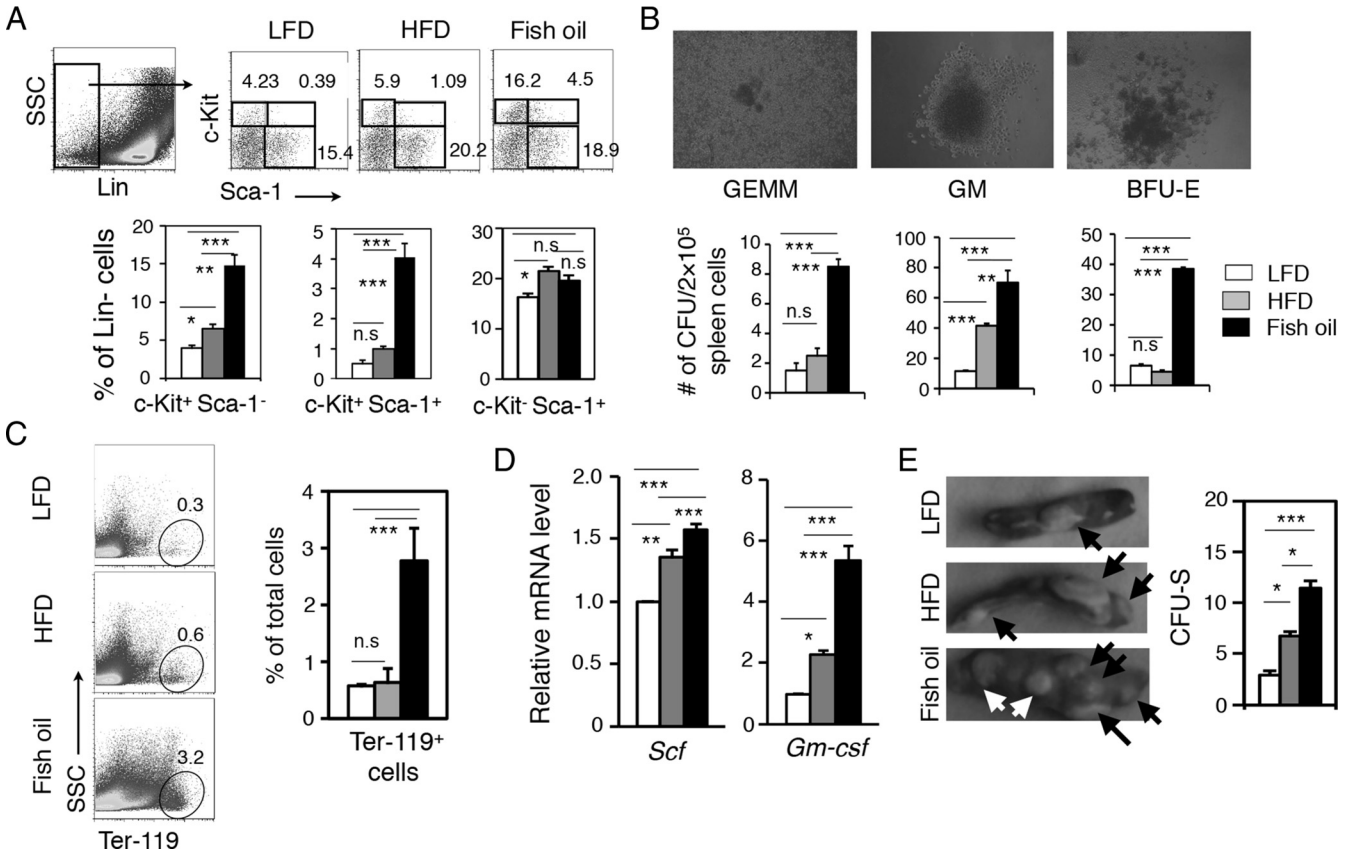

Figure 2. Fish oil rich diet increases extramedullary hematopoiesis in the spleen. A, Representative flow cytometric analysis (upper) and quantitation of hematopoietic progenitors (Lin^- $c\text{-}Kit^+$ $Sca\text{-}1^-$), HSCs (Lin^- $c\text{-}Kit^+$ $Sca\text{-}1^+$) and Lin^- $c\text{-}Kit^-$ $Sca\text{-}1^+$ cells in the spleen. Numbers in dot plot indicate the percent of each population in lineage negative (Lin^-) bone marrow cells from one mouse (N = 5 mice each group). B, Representative images of GEMM, GM and BFU-E in the colony forming unit assays of splenocytes from mice on different diets. Quantitation of colonies shown below (N = 5 mice each group). C, Representative flow cytometric analysis (left) and quantitation of $Ter\text{-}119^+$cells in the spleen. N = 5 mice per group. D, Q-PCR analysis of *Scf* and *Gm-csf* in the spleen. E, CFU-S assay showing colonies (arrows) in the spleens of irradiated recipients 12 days following the transfer of 5×10^4 splenocytes from donor mice on different diets. Quantitation of colonies shown on the right (N = 4 mice per group). Data shown as mean ± SEM. *, $P < .05$; **, $P < .01$; ***, $P < .001$; n.s., not significant by ANOVA.

progenitor and primitive stem cell populations in the spleen.

High fish oil diet increases HSCs self-renewal in the bone marrow. To address mechanistically how high fish oil diet induced extramedullary hematopoiesis in the spleen, we next examined the status of hematopoiesis in the bone marrow. While total numbers of bone marrow cells were comparable among the cohorts after 4 weeks of feeding (Figure 3A), Lin$^-$ c-Kit$^+$ Sca-1$^+$ HSCs, but not Lin$^-$ c-Kit$^+$ Sca-1$^-$ progenitors, were significantly increased by 50%–60% in the fish oil cohort compared to the other two cohorts (Figure 3B). In line with the observation that fish oil diet had no effect on the number of progenitors in the bone marrow, CFU assays in vitro revealed no significant differences in the GEMM, GM and BFU colony formation among the three cohorts (Figure 3C). We next compared repopulating capability of HSCs using an in vivo competitive repopulation assay where a mixed bone marrow cells from the fish oil (CD45.1$^+$) and LFD (CD45.2$^+$) cohorts at the ratio of 1:1 were transferred into lethally irradiated recipients. Three months later, the ratio of CD45.1$^+$ to CD45.2$^+$ lymphocytes changed to near 1.5:1 in peripheral blood, pointing to a higher proliferative capacity and self-renew of HSCs of the fish oil cohort (Figure 3D-E). Collectively, these data suggest that high fish oil diets promote self-renewal of HSCs in the bone marrow.

Fish-oil rich diet alters hematopoietic niche and induces MMP12 expression in the bone marrow. We next addressed how fish oil diet affected hematopoietic stem cell (HSC) self-renewal in the bone marrow. As the homeostasis of HSCs largely depends on the niche, we assessed the change of hematopoietic microenvironment in the bone marrow. Strikingly, fish-oil rich diet reduced the amount of medulla ossiumrubra or the red marrow in distal tibia of the bone (Figure 4A), pointing to a possible altered hematopoietic microenvironment. Given the important role of MMP-9 and 12 in hematopoiesis and tissue remodeling (30, 31), we next determined whether MMPs were responsive to fish oil diet or n-3 PUFAs. Unlike *Mmp9*, *Mmp12* mRNA level in the bone marrow was highly responsive to fish-oil rich diet, but not HFD (Figure 4B). This upregulation was further confirmed at the protein level using Western blot (Figure 4C) and immunohistochemical staining of MMP12 in the bone marrow (Figure 4D).

We next analyzed the effect of fish oil diet on MMP12 expression in different cellular components made up of the hematopoietic niche including stromal cells and macrophages. Fish oil-rich diet dramatically enhanced *Mmp12* expression in stromal cells, while having a moderate effect on CD11b$^+$ macrophages or CD11b$^-$ bone marrow cells (Figure 4E). Moreover, as extracellular signal-regulated kinase (ERK)-mediated signaling pathway is known to regulate the expression of *Mmp12* (32), we next measured ERK phosphorylation in bone marrow cells. Indeed, fish oil rich diet promoted ERK phosphorylation of bone marrow cells when compared to that of LFD cohort (Figure 4F).

To directly assess the effect of n-3 PUFAs on *Mmp12*

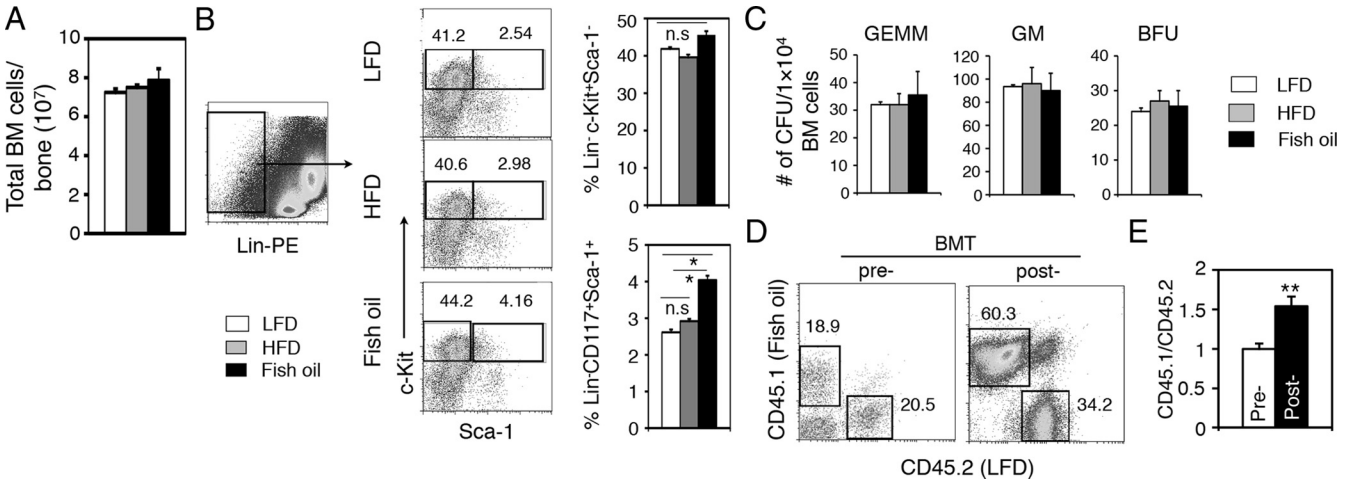

Figure 3. Fish oil rich diet increases the frequency of HSCs in bone marrow. A, Total bone marrow cell numbers per femur bone of mice on different diets for 4 weeks. N = 5 mice per cohort. B, Representative flow cytometric images (left) and quantitation of the percent of hematopoietic progenitors (Lin$^-$ c-Kit$^+$ Sca-1$^-$), HSCs (Lin$^-$ c-Kit$^+$ Sca-1$^+$) and Lin$^-$ c-Kit$^-$ Sca-1$^+$ cells in the bone marrow. N = 5 mice per cohort. C, Quantitation of GEMM, GM and BFU-E in the bone marrow using colony forming unit (CFU) assay. N = 5 mice per cohort. D, Competitive bone marrow proliferation assay. Representative flow cytometric data showing the percent of CD45.1$^+$ (on fish oil diet) and CD45.2$^+$ (LFD) cells before and after BMT. The numbers in graphs show the percent of gated cells in total blood leukocytes. E, Quantitation of the ratio of CD45.1$^+$ to CD45.2$^+$ cells from D (n = 6 mice per group). Data shown as mean ± SEM. *, $P < .05$; **, $P < .01$; n.s., not significant by ANOVA (B and C) and Student's *t* test (E).

expression in bone marrow cells, we treat with bone marrow cells with DHA (n-3 PUFA). DHA increased the expression of *Mmp12* in a dose-depend manner, but had no obvious effect on *Mmp9* (Figure 4G). Thus, MMP12 expression in the bone marrow is responsive to n-3 PUFAs.

MMP12 activity contributes to fish oil diet-induced myelopoiesis. Lastly, we asked whether there was a causal relationship between MMP12 expression and diet-induced myelopoiesis. To this end, mice on fish oil rich diet were orally gavaged with MMP12-specific inhibitor MMP408 (23) on alternate days for a total of 4 weeks (Figure 5A). MMP408 treatment reduced $CD11b^+$ myeloid cells in the spleen by nearly 50% (Figure 5B-C). In both spleen and bone marrow, percent of Lin^- $c\text{-}Kit^+$ $Sca\text{-}1^+$ HSCs was decreased while Lin^- $c\text{-}Kit^+$ $Sca\text{-}1^-$ progenitors were not affected (Figure 5D-G). These data were further confirmed by using the CFU-S assay where MMP408 reduced the number of colonies formed by the donor splenocytes from mice on high fish oil diet (Figure 5H). Thus, chronic intake of fish oil rich diet promotes HSCs self-renewal and proliferation, at least in part, via MMP12.

Discussion

Both HFD and fish-oil rich diets are known to alter myeloid cell differentiation (26, 33–35), but underlying molecular mechanisms were not well understood. Here, our

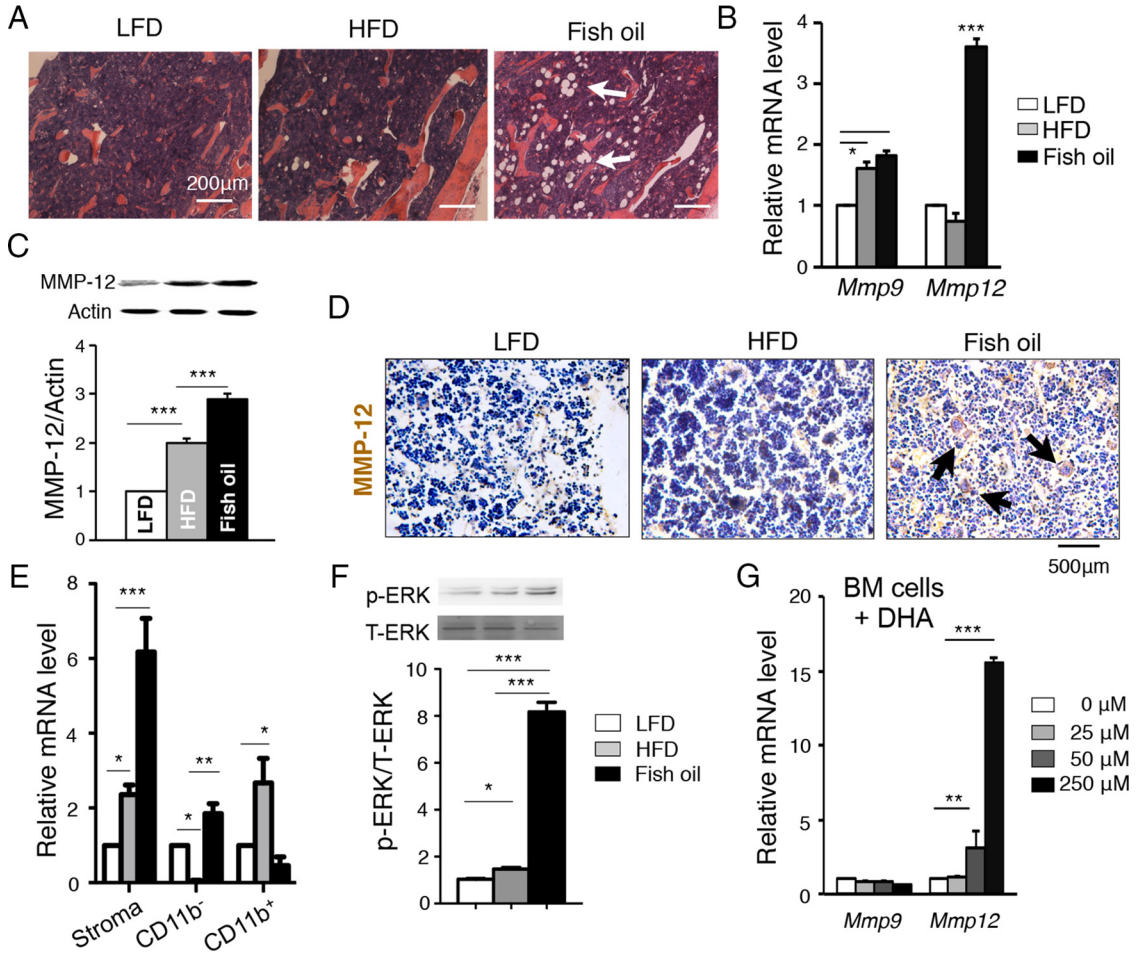

Figure 4. Fish oil rich diet alters hematopoietic niche and induces the expression of MMP12 in bone marrow. A, Representative H&E images showing the structure of medulla ossiumrubra (ie, red marrow) in distal tibia of mice on different diets for 4 weeks (n = 2 mice each group). Arrows point to disrupted red marrow. B, Q-PCR analysis for *Mmp9* and *Mmp12* mRNA levels in bone marrow (N = 5 mice per cohort). C, Western blot analysis (upper) and quantitation of MMP12 protein level in bone marrow (n = 3 per group). D, Representative images showing immunohistochemical staining of MMP12 (brown) in bone marrow of mice on different diets (n = 2 mice each group). E, Q-PCR analysis for mRNA levels of *Mmp12* in different components (stromal cells, $CD11b^+$ cells and $CD11b^-$ nonstromal cells) of bone marrow (n = 6 mice each group). F, Western blot analysis for p- and total (T-) ERK in bone marrow cells (upper) and quantitation (n = 3 per group). G, Q-PCR analysis of mRNA levels of *Mmp9* and *Mmp12* in bone marrow cells treated with DHA at the indicated concentrations in vitro. For Q-PCR analysis, data are normalized to the level of the house-keeping gene *Gapdh* and shown as fold change to that of WT LFD mice or nontreated bone marrow (three repeats). Data shown as mean ± SEM. *, $P < .05$; **, $P < .01$; ***, $P < .001$ by ANOVA.

data show that fish-oil rich diet, not HFD, promotes extramedullary hematopoiesis in the spleen with elevated HSCs and progenitors. In the bone marrow, fish-oil rich diet alters hematopoietic microenvironment and increases the abundance of HSCs. MMP12 is likely involved in fish-oil diet-induced hematopoiesis as inhibition of MMP12 partially attenuates myelopoiesis in both the spleen and bone marrow. This is consistent with early studies showing an important role of MMP12 in hematopoiesis (30, 36, 37). Thus, these data suggest that dietary fish oil intake may alter hematopoietic niche in the bone marrow and thereby affecting self-renewal of HSCs.

The size of HSCs pool largely depends on their intrinsic properties and hematopoietic microenvironment of the bone (1, 38). Many cellular elements, including stromal cells, adipocytes, osteoblasts and multiple components of the extracellular matrix constitute the hematopoietic niche to regulate self-renewal and differentiation of HSCs (39, 40). Moreover, as the renewal of HSCs, progenitors and even stromal cells is highly active, the metabolism of these cells may be influenced by the changes in dietary composition. Indeed, previous studies have shown that different PUFAs and their metabolites have distinct osteoclastogenic effect (41, 42), suggesting their potential roles in hematopoiesis. A recent study showed that adipocytes are present in the bone marrow of HFD mice, which may modify hematopoietic microenvironment and alter myelopoiesis and lymphopoiesis (43). Our data show that fish-oil rich diet alters hematopoietic microenvironment and induces the expression of MMP12 in stromal cells and hence hematopoiesis. However, whether other cell types, such as adipocytes, play a role in fish oil diet-induced bone marrow remodeling remain to be determined.

Both MMP9 and MMP12 belong to the family of zinc-dependent proteases and degrade the components of extracellular matrix in hematopoietic microenvironment, thereby playing a critical role in hematopoiesis under both physiological and pathological conditions. MMP9 is involved in the recruitment of HSCs from the bone marrow (44–46) and in the absence of MMP9, HSCs mobilization is impaired (44). MMP12 secreted by macrophages and osteoclasts is involved in inflammation and tissue remodeling through the degradation of ECM (47). MMP12-deficient mice are defective in macrophage recruitment and tissue inflammation under many pathological settings in-

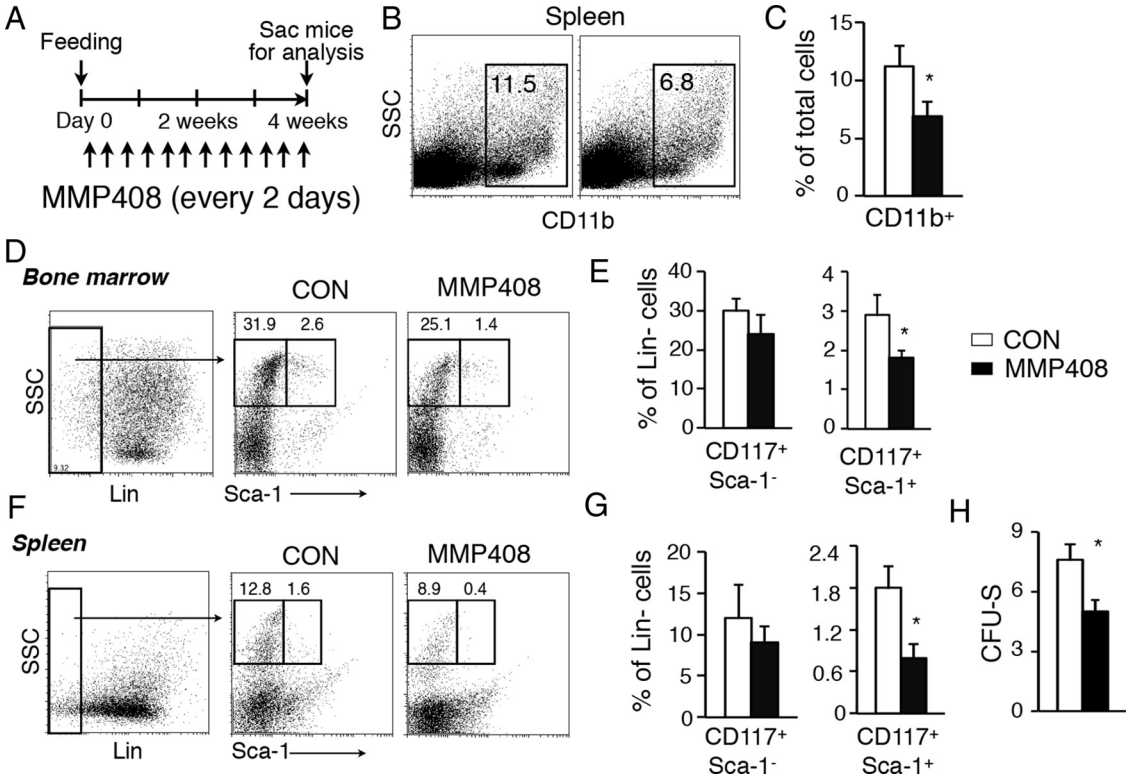

Figure 5. Inhibition of MMP12 attenuates fish oil diet-induced myelopoiesis. 8-week-old B6 male mice on fish oil rich diet were gavaged on alternate days with or without the MMP12 inhibitor MMP408 for 4 weeks. A, A schematic outline of the experiment. B, Representative flow cytometric analysis of $CD11b^+$ myeloid cells in spleen with quantitation shown in C. D–G, Representative flow cytometric analyses of hematopoietic progenitor (Lin^- c-Kit^+ Sca-1^-) and HSCs (Lin^- c-Kit^+ Sca-1^+) in bone marrow (D) and spleen (F) with quantitation shown in (E) and (G), respectively. H, CFU-S assay was performed as described in Figure 2E, with the exception that donor mice on fish oil diet were treated with or without MMP408 (n = 6–8 mice each group). Data shown as mean ± SEM. *, $P < .05$ by Student's *t* test.

cluding emphysema (48, 49). While it remains unclear how hematopoiesis is altered in MMP12-deficient mice, overexpression of MMP12 promotes myelopoiesis and increases the frequencies and numbers of myeloid progenitors (30, 36, 37). Moreover, our observation that n-3 PUFAs regulate *Mmp12* expression in stromal cells of the bone marrow is interesting. More studies are required to identify key transcription factors and signaling pathways linking n-3 PUFAs to *Mmp12* expression.

Additionally, the effect of n-3 PUFA-rich diet may be mediated through the alteration in membrane lipid composition. Lipid microdomains, highly enriched in glycosphingolipids and cholesterol, are important signaling platforms. It has been reported that n-3PUFAs may alter cell signaling and functions by disrupting lipid raft formation in various cell types (50, 51). In addition, n-3 PUFA-rich diet may affect the activation and differentiation of T, B cells and antigen presenting cells through disrupting membrane domain organization and thereby altering signaling networks (52–54). Thus, dietary n-3 PUFAs may regulate hematopoiesis and hematopoietic niche through the modification of lipids rafts on HSCs and hematopoietic stromal cells.

In the 1970s, n-3 PUFAs-rich fish oil was initially realized as an underlying cause for the low rate of coronary heart disease (CHD) in Eskimos (55). Since then, many health benefits of N-3 PUFAs have been reported, esp. in many inflammatory diseases, including rheumatoid arthritis (RA), diabetes, cardiovascular diseases and allergy (56–58). In 2004, FDA approved that fish oil can be used as a prescription drug in cardiovascular diseases. Consistently, our recent study showed that n-3 PUFAs-rich fish oil diet may induce myeloid-derived suppressor cell differentiation to suppress tissue inflammation (59). As this new study suggests that a chronic intake of fish oil rich diet may affect the hematopoietic system in mice, more studies are required in humans to investigate its long-term impact on hematopoiesis in healthy individuals as well as patients with bone marrow transplantation (BMT).

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