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Chronic intake of high fish oil diet induces myeloid-derived suppressor cells to promote tumor growth

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Conflict of interest

The authors declare no competing financial interests.

Abstract

Omega-3 polyunsaturated fatty acids enriched fish oil exerts beneficial anti-inflammatory effects in animal models with acute and chronic inflammatory diseases. Myeloid-derived suppressor cells (MDSCs), comprised of myeloid progenitors and precursors of myeloid cells, play vital roles in cancer. How fish oil affects the generation of MDSCs and the tumor development remains largely unexplored. Here, we show that dietary intake of high fish oil diet suppresses CD8⁺ T cells activation and proliferation in vivo via elevated levels of MDSCs. Mechanistically, high fish oil diet induces the expression of immunosuppressive cytokine IL-10 and promotes myelopoiesis in the spleen as well as other peripheral tissues. The immature myeloid cells in the spleen exhibit morphological and functional characteristics of MDSCs with the capability to downregulate CD8⁺ T cells activation. Depletion of MDSCs using anti-Gr-1 antibody decreases the growth of subcutaneously transferred B16 melanoma in mice on high fish oil diet. Interestingly, diet-induced production of MDSCs is not solely dependent of the spleen, as splenectomy has no effect on the tumor progress. Our data show that the liver functions as an alternative extramedullary hematopoiesis organ to support MDSCs differentiation and maintain tumor growth. Taken together, our study provides a novel insight into the physiological effects of fish oil and points to MDSCs as a possible mediator linking dietary fish oil intake and immunosuppression in cancer immunosurveillance.

Keywords

Polyunsaturated fatty acids; Fish oil; MDSCs; CD8⁺ T cell; Liver; Cancer

Introduction

The balance of immunosuppressive and pro-inflammatory immune cells dictates the outcome of inflammation [1]. Recently, a new type of immunosuppressive cells, myeloid-derived suppressor cells (MDSCs), has been identified [2]. As a heterogeneous population of myeloid precursors and progenitors, MDSCs are most commonly identified by cell surface markers CD11b⁺ Gr-1⁺ in rodents and CD11b⁺ CD14⁻ in humans [3, 4]. They are induced in cancer, obesity, autoimmune diseases and infection diseases [5, 6]. Many hematopoietic niche-associated factors, including macrophage colony-stimulating factor (M-CSF), stem cell factor (SCF), granulocyte/macrophage colony-stimulating factor (GM-CSF) and matrix metalloproteinases-12 (MMP-12) have been shown to promote the expansion of MDSCs [7–9]. The mechanisms underlying the immunosuppression effect of MDSCs are diverse depending on the experimental systems and may include, but not limited to, inducible nitric oxide synthase (iNOS), arginase, nitric oxide and reactive oxygen species (ROS) [3, 4]. Accumulating evidence suggests that MDSCs may contribute to tumor development and play an important regulatory role in inflammation and immunity [5].

Western societies are characterized by a high intake of saturated and omega-6 (n-6) polyunsaturated fatty acids (PUFA) and a low intake of n-3 PUFA. n-6 PUFA and its metabolites, represented by arachidonic acid (C20:4 n-6) and its intermediates, may promote inflammation [10]. Indeed, chronic intake of high-fat Western diet (HFD) elevates chronic inflammation and instigates inflammation-associated complications such as cardiovascular

diseases and type-2 diabetes [11, 12]. Similar to the situation in cancer and infection, we recently reported that obesity promotes the generation of immature myeloid cells in the bone marrow, which are differentiated to MDSCs [13]. In adipose tissue and liver, MDSCs downregulate CD8⁺ T cell activation, ameliorate chronic inflammation and insulin resistance [13].

Omega-3 polyunsaturated fatty acids, such as docosahexaenoic acid (DHA; C22:6n-3) and eicosapentaenoic acid (EPA; C20:5n-3), share same enzymes in desaturation, elongation and β -oxidation with n-6 PUFAs [14]. But, in contrast to n-6 PUFAs, its metabolites, such as prostaglandin E3, are less inflammatory than eicosanoids from n-6 PUFAs, and then exert anti-inflammatory effects [15]. As humans cannot synthesize n-3 PUFAs by themselves, it needs acquiring from n-3 PUFAs rich seafood-derived oils [16]. Diets rich in fish oil (containing high n-3 PUFAs) reduce the secretion of pro-inflammatory cytokines and decrease the expression of adhesion molecules on inflammatory immune cells [17]. Thus, fish oil has been used to prevent and treat chronic inflammation diseases such as atherosclerosis. Multiple mechanisms have been proposed to explain the immune regulating functions of fish oil, including changes in membrane lipid compositions of immune cells, the production of inflammatory cytokines etc. [18]. At the molecular level, several targets, such as peroxisome proliferator-activated receptor gamma (PPAR γ), Toll-like receptors (TLR) and G-protein coupled receptor 120 (GPR120), have been reported to be involved in n-3 PUFAs mediated anti-inflammatory responses [19–21]. At the cellular level, n-3 PUFAs may promote the differentiation of regulatory T cells and inhibit the proliferation of Th17 cells, which collectively downregulate inflammation in autoimmune diseases [22, 23]. However, the effect of n-3 PUFAs on MDSCs in vivo remains to be well explored.

In this study, we test the hypothesis that fish oil may mediate anti-inflammatory properties in part through its effect on MDSCs differentiation. Indeed, our data demonstrate that chronic intake of high fish oil diet elevates the levels of MDSCs, which in turn suppress antigen-specific CD8⁺ T cell proliferation. Dramatically, chronic intake of high fish oil diet promotes tumor growth in vivo in part via the production of MDSCs.

Materials and methods

Animal and diet feeding models

Wild-type (JAX 000664), CD45.1 (JAX 002014) and OT-I (JAX 003831) mice on the C57BL/6J background were purchased from Jackson Laboratory (JAX) and bred in the specific pathogen-free facility. Six-week-old mice were fed with 13 % low-fat diet (LFD, Teklad 2914), 59 % high-fat diet (HFD, Bioserv F3282) or 59 % high fish oil diet (FO, Bioserv F5424) for 4 weeks. Fish oil diet contains 40 mg EPA and 26 mg DHA per gram diet (vs. 0 in HFD) (Table 1). All animal experimental protocols have been approved by the Cornell Institutional Animal Care and Use Committee and by the Scientific Investigation Board of Jiangsu University, China.

Antibodies and flow cytometry

CD4-PE, CD8-PE, CD69-FITC, B220-FITC, NK1.1-PE, CD3-FITC, Gr-1-PE, CD11b-FITC, CD45-PerCP and iso-type controls were purchased from BioLegend (San Diego, CA). Single-cell suspension from the liver, spleen, blood and mesenteric lymph nodes were prepared as described [24]. All cells were incubated with fluorescence-conjugated mAbs in the presence of 2.4G2 or rat sera, and fluorescence-conjugated isotype mAbs were used as background fluorescence. Flow cytometric analyses were performed on a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA), and data were analyzed using the FlowJo or Cell-Quest software.

CD8⁺ T suppression assays

Single-cell suspensions were prepared from OT-I mice (with OVA_{257–264} TCR-specific CD8⁺ T cells), and then CD8⁺ T cells were purified using the anti-CD8-biotin and streptavidin-magnetic beads (Stem cell Co.). For in vivo experiments, 1×10^7 OT-I CD8⁺ T cells were labeled with 5 μ M CFSE [24] and transferred i.p. into mice fed on different diets. Twenty-four hours later, mice were injected i.v. with 1×10^6 OVA_{257–264}-loaded bone marrow-derived dendritic cells (mDC) or 5×10^6 OVA_{257–264}-loaded splenocytes. Four days later, spleens and mesenteric lymph nodes were collected and analyzed for T cell activation using flow cytometry. The OVA_{257–264}-loaded mDC or splenocytes were prepared by incubating these cells with 5 mM OVA_{257–264} at 37° C for 6 h followed by three washes with cold PBS.

For in vitro experiments, CD11b⁺ Gr-1⁺ MDSC cells were purified from spleens of mice on different diets using anti-Gr-1-biotin and streptavidin-magnetic beads. OT-I CD8⁺ T cells, OVA_{257–264}-loaded mDC cells were prepared as described previously [24]. Purified OT-I CD8⁺ T cells were adjusted to 2×10^6 cells/ml, and 100 μ l per well were added into 96-well cell culture plate with mDC cells and MDSC cells at the ratio of 10:1:10 (CD8⁺ T cells : mDCs : MDSCs). Five days later, cells were harvested and labeled with anti-CD8-PE and 7-AAD for flow cytometry analysis. The CD8⁺ cell numbers were counted for 1 min as the number of 7-AAD[−] CD8⁺ cells. In some experiments, OT-I CD8⁺ T cells were labeled with 5 μ M CFSE prior to the co-culture. CFSE intensity was analyzed by flow cytometry. To test whether cell–cell interaction was required, MDSCs and CD8⁺ T cells were cocultured in 24-well transwells plates (Corning) with MDSCs at the bottom and CD8⁺ T cells at the top separated by a 3 μ m filter.

Tumor animal models

The B16 subcutaneous tumor model was performed as previously described with modifications [25]. Briefly, B16 melanoma cells were cultured in RPMI-1640 (PAA, GE Healthcare) with 10 % fetal bovine serum (PAA, GE Healthcare) at 37 °C and 5 % CO₂. For the subcutaneous tumor model, B16 melanoma cells in exponential growth phase were harvested, washed with ice-cold PBS, resuspended in ice-cold PBS at the concentration of 2×10^6 cells/ml. Recipients were injected s.c. with 100 μ l cell solution on the right back flank. Tumor size was measured with calipers every three days. Tumor volume (V_t) was calculated based on the formula $V_t = 1/2 \times a \times b \times b$, where a and b means the shortest and longest diameter of the tumor, respectively. Mice were killed on day 21 post-injection. In some

experiments, recipient mice on high fish oil diet were injected with anti-Gr-1 or isotype IgG2b antibody (eBioscience) at 6 µg/g body weight every 3 days.

Splenectomy

The removal of spleen was operated as described [26]. Briefly, mice were anaesthetized with pentobarbital sodium and cleaned with 70 % ethyl alcohol (vol/vol) at the left hypochondrium. After making an incision, the spleen was carefully removed after associated vessels were cauterized. For control mice, the abdomen was incised without removing the spleen.

Wright's staining

CD11b⁺ Gr-1⁺ MDSC cells were purified from spleen using magnetic beads and adjusted to 2×10^5 cells/ml. 200 µl of cell suspension was transferred to slide. After air dry, smear of MDSC cells was covered with Wright's staining solution for 2 min, and then followed by same volume of Wright's staining buffer for more 4 min. Then, slides were washed with slowly running water and photographed under a light microscope (Zeiss, Germany).

Quantitative reverse transcribed polymerase chain reaction (Q-PCR)

Spleen tissues and purified CD11b⁺ Gr-1⁺ MDSC cells were harvested and snap frozen in liquid nitrogen. Total RNA samples were prepared and analyzed as described [13]. Comparative quantitation of transcripts was normalized to the ribosomal *l32* gene or GAPDH levels. Primer sequences used were listed in Table 2.

ELISA analysis

After being anesthetized with sodium pentobarbital and blood sample of mice was collected to prepare serum. The level of IL-6 in serum was quantified using mouse IL-6 ELISA Ready-SET-GO (eBioscience).

Statistical analysis

Data were shown as mean ± SEM for all experiments. Statistical analysis was performed using one-way ANOVA analysis. In all cases, $p < 0.05$ was considered as statistical significance.

Results

Chronic intake of high fish oil diet suppresses CD8⁺T cell activation and proliferation in vivo

To investigate the dietary effect on the immune system, C57BL/6 WT mice were fed with different diets, LFD, HFD or high fish oil diet for 4 weeks followed by characterization of lymphocytes in spleen. In comparison of fatty acid composition between isocaloric HFD and high fish oil diet, the major difference lie in the amount of n-3 PUFA (Table 1). Interestingly, mice on fish oil diet exhibited increased total splenocyte number but lower percent of CD8⁺, CD4⁺ T cells and CD3⁺ NK1.1⁺ NK cells when compared to those in mice on other diets (Fig. 1a, b). Expression of the early activation marker CD69 on CD8⁺T cells

was downregulated in mice on fish oil diet (Fig. 1c, d). Pointing to a CD8⁺ T-specific effect, CD69⁺ CD4⁺ T cells were not affected by fish oil diet (Fig. 1c, d). No difference in the percent of total or activated CD8⁺ T cells was observed in mice fed a HFD vs. LFD (Fig. 1b–d), pointing to the effect of fish oil and n-3 PUFA in inducing these changes in CD8⁺ T cells.

To further corroborate these observations, we directly assessed the effect of fish oil diet on CD8⁺ T cell proliferation in vivo. CFSE-labeled OVA_{257–264}-specific CD8⁺ T cells from OT-I mice and OVA_{257–264}-loaded splenocytes were consecutively transferred into three cohorts that have been on different diets for 4 weeks. The proliferation of CFSE⁺ CD8⁺ T cells in both the spleen and mesenteric lymph nodes was dramatically reduced in the fish oil group (Fig. 1e). Intermediate phenotype was observed in those of HFD mice (Fig. 1e). These data point to a profound effect of dietary lipids, especially n-3 PUFA, on CD8⁺ T cell proliferation. Taken together, high fish oil diet reduces the activation and proliferation of CD8⁺ T cells in mice.

Chronic intake of high fish oil diet promotes the expansion of CD11b⁺ Gr-1⁺ myeloid cells

We next investigated how high fish oil diet suppresses CD8⁺ T cell activation. Q-PCR analysis revealed that expression of inflammatory cytokine IL-6 as well as *Chi3l3* in the spleen was increased by chronic intake of high fish oil diet (Fig. 2a, b). Both of these genes have been linked to myeloid suppressive cells [27, 28]. Indeed, circulating levels of IL-6 was elevated in high fish oil cohorts compared to the LFD cohort (Fig. 2c). These observations promoted us to examine whether myeloid cells in the spleen were increased following fish oil intake. Indeed, both the percent and total numbers of CD11b⁺ Gr-1⁺ cells were significantly elevated in the spleen of mice on high fish oil diet (Fig. 2d–f). The fish oil diet-induced increase in myeloid cells was not limited to the spleen as similar increase was found in the circulation and, to a lesser extent, the liver (Fig. 2g). Thus, we conclude that high fish oil intake promotes myelopoiesis in vivo.

Dietary fish oil intake enhances the production of myeloid-derived suppressor cells in the spleen

The differential effects of fish oil diet on CD8⁺ T cells and Gr-1⁺ CD11b⁺ myeloid cells prompted the question whether dietary fish oil induces MDSCs in the spleen. Morphologically, purified splenic Gr-1⁺ CD11b⁺ cells resemble many characteristics of myeloid cells (Fig. 3a). Moreover, purified Gr-1⁺ CD11b⁺ cells from fish oil-fed mice expressed higher levels of IL-6, TNF- α and arginase-1 compared to those from LFD-fed mice (Fig. 3b). Functionally, purified Gr-1⁺ cells from fish oil-fed mice inhibited the activation of OVA_{257–264}-specific CD8⁺ T cells by peptide-loaded mDCs compared to purified Gr-1⁺ cells from LFD-fed mice (Fig. 3c). This was further confirmed by CFSE proliferation assays where coculture with Gr-1⁺ cells from fish oil-fed mice downregulated the activation of CD8⁺ T cells (Fig. 3d). Such suppressive effect depended on the cell–cell contact between Gr-1⁺ cells and CD8⁺ T cells as demonstrated in the transwell study (Fig. 3d). Thus, we conclude that dietary fish oil intake induces splenic MDSCs with immunosuppressive functions.

Chronic intake of fish oil diet promotes tumor growth via MDSCs

As MDSCs are tightly associated with tumor development and growth [29–31], we next explored the effect of fish oil on tumor growth in vivo. After fed on different diets for 4 weeks, mice were subcutaneously transplanted with B16 melanoma cells (Fig. 4a). While both HFD and high fish oil diet promoted B16 melanoma growth, more pronounced effect was seen in mice fed a fish oil diet (Fig. 4b). To address whether the effect of high fish oil diet on tumor growth is mediated by the induction of MDSCs, we depleted MDSCs with anti-Gr-1-specific antibody (Fig. 4c, d). Indeed, tumor growth was significantly attenuated compared to the control cohort where mice were injected with an isotype IgG control (Fig. 4e).

The effect of diet-induced MDSCs on tumor growth is independent of splenic microenvironment

Several studies have shown that the differentiation of MDSCs is largely dependent of splenic microenvironment [26, 32]. To address whether the effect of high fish oil diet on MDSCs and tumor growth depends on the splenic microenvironment, we performed splenectomy following 4-week feeding of high fish oil diet. After a two-week recovery, mice were subcutaneously transplanted with B16 melanoma cells and tumor size was measured every 3 days (Fig. 5a). Much to our surprise, there was no difference in tumor growth between the cohorts with and without spleen (Fig. 5b, c). Indeed, more CD11b⁺ Gr-1⁺ MDSCs were detected in the liver of the splenectomized fish oil diet-fed cohort (Fig. 5d, e). Thus, we conclude that in the absence of spleen, the liver provides special microenvironment to support MDSCs differentiation.

Discussion

Since the discovery of health benefits of n-3 PUFA rich fish oil in cardiovascular disease, anti-inflammation effect of fish oil has been extensively studied. Fish oil is prescribed as a drug for the prevention and treatment of cardiovascular diseases. Nonetheless, the anti-inflammatory properties of fish oil may disturb immunosurveillance; however, the underlying molecular mechanism remains largely unknown. Here we show that dietary fish oil enhances the production of MDSCs, which in turn suppress CD8⁺ T cells and promote tumor growth. While the differentiation of MDSCs predominantly occurs in the spleen, it also occurs in the liver microenvironment in the absence of spleen. Thus, our data reveal a novel mechanism by which high fish oil diet downregulates inflammatory response.

Polyunsaturated fatty acids are critical for the integrity and function of cell membranes and regulate various cellular processes including inflammation, cell signaling and gene expression [33]. Lipid mediators derived from n-3 to n-6 PUFAs play opposite roles in inflammation as such that the ratio of n-3 to n-6 PUFAs in the diet is tightly associated with the outcome of immune response [34]. Previous studies have shown that increased consumption of n-3 PUFAs reduces the production of n-6 PUFAs-derived metabolites, such as prostaglandin E2 [35–37]. Moreover, DHA inhibits IL-12 and IL-23 production and prevents the upregulation of MHC II and costimulatory molecules [38]. High consumption of n-3 PUFAs promotes a shift in macrophage polarization and protects the liver and muscle

from pathological damage [39, 40]. Moreover, studies have shown that 2-week feeding of fish oil diet significantly decreases the numbers of CD8⁺ T cells in the lungs of influenza virus-infected mice, resulting in a high morbidity and mortality [41]. Similarly, fish oil consumption has been reported to decrease the expression of CD69 on CD8⁺ T cells, and thereby increases the risk of colon cancer [42]. However, the molecular mechanism underlying the suppression remains unclear, although several have been proposed, including direct suppression of T cell activation [43, 44] and the induction of Foxp3⁺ regulatory T cells during infection [42]. Our data demonstrates that fish oil effect on T cell activation may be due to the expansion of MDSCs, which are capable of suppressing CD8⁺ T cells via a direct cell–cell contact. However, whether MDSCs immunosuppressive effect requires soluble mediators and the nature of these soluble mediators remain unknown.

The spleen usually functions as a reservoir for monocytic or myeloid progenitors, and some studies have shown that the removal of spleen will restore immune functions of lymphocytes and promote tumor regression [26, 32]. Interestingly, our data show that dietary fish oil induces MDSCs production in peripheral tissues and promotes tumor growth. The effect of fish oil on tumor growth is indeed in part mediated by MDSCs as depletion of MDSCs slows down tumor growth. In contrast to previous reports [26, 32], the effect of MDSCs on tumor growth is independent of the spleen. Several studies had reported that MDSCs undergo extramedullary proliferation in response to tumor-derived growth factors [45, 46] and can be generated directly from bone marrow-derived hematopoietic stem cells *ex vivo* [47]. It is generally appreciated that the liver works as an extramedullary hematopoiesis organ similar to the spleen [48–50]. Thus, in the absence of the spleen, the liver can provide a microenvironment to support MDSCs differentiation from hematopoietic stem cells and progenitors. Indeed, the liver can function as the site for MDSCs accumulation and immunosuppression in tumor models [51]. Therefore, more MDSCs are produced in the liver to suppress immune functions and promote tumor growth in the splenectomy model fed a fish oil diet.

CD11b⁺ Gr-1⁺ MDSCs represent a heterogeneous population of immature myeloid cells and are identified in several pathological inflammatory conditions, such as tumor, autoimmune diseases and obesity [5]. Mediators released from inflammatory microenvironment in these diseases such as prostaglandin E2 derived from arachidonic acid (n-6 PUFAs) promote differentiation and production of MDSCs. Our previous study shows that MDSCs are increased in chronic HFD-fed mice and contribute to immune homeostasis in obesity [13]. An increasing of CD11b⁺ Gr-1⁺ MDSCs was found in mice fed with HFD for over 8 weeks, but not at 4 weeks [13]. This finding was further confirmed by this study where a modest induction of MDSCs was observed following 4-week HFD feeding (Fig. 2c). These data support the model where MDSCs are mainly induced by chronic inflammation in obese mice to negatively regulate inflammatory injury in a feedback loop.

Our data further show that 4-week feeding of fish oil diet strongly induces the expansion of MDSCs in peripheral tissues, but the underlying molecular mechanism remains unclear. Previous studies have suggested that biological functions of PUFAs are largely dependent on their metabolites such as prostaglandin E3. More future studies are needed to elucidate the molecular pathways underlying the effect of fish oil. Although it remains speculative as

to how fish oil induces MDSCs expansion, our results demonstrate the role of MDSCs in linking fish oil intake to immunosuppression and tumor growth. Given that n-3 PUFA-enriched fish oil has been widely used as a prescription drug and the intake of fish oil in humans is likely much less than that in our study, our data suggest that the exact pharmacological effects of long-term intake of fish oil may deserve careful investigation.

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Abbreviations

CFSE	5,6-carboxyfluorescein diacetate, succinimidyl ester
<i>Chi3l3</i>	Chitinase 3 like 3
COX-2	Cyclooxygenase-2
DHA	Docosahexaenoic acid
EPA	Eicosapentaenoic acid
FO	High fish oil diet
GM-CSF	Granulocyte macrophage colony-stimulating factor
GPR120	G-protein coupled receptor 120
HFD	High-fat diet
iNOS	Inducible nitric oxide synthase
LFD	Low-fat diet
LN	Lymph node
M-CSF	Macrophage colony-stimulating factor
mDC	Bone marrow-derived dendritic cells
MDSCs	Myeloid-derived suppressor cells
MMP-12	Matrix metalloproteinases-12
OVA	Ovalbumin
PPARγ	Peroxisome proliferator-activated receptor gamma
PUFAs	Polyunsaturated fatty acids
Q-PCR	Quantitative polymerase chain reaction
ROS	Reactive oxygen species
SCF	Stem-cell factor
SPL	Splenectomy

TLR Toll-like receptors

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Fig 1.

Fish oil feeding inhibits CD8⁺ T cells activation and proliferation in spleen. **a, b** Total splenic cell numbers (**a**) and the percent of CD8⁺ T cells, CD4⁺ T cells, B220⁺ B cells and CD3-NK1.1⁺ cells in total splenocytes (**b**) in spleens of age-matched mice after 4 weeks on different diets. $n = 5$ mice each group. **c, d** Representative *dot plots* (**c**) and quantitation (**d**) of CD69⁺CD8⁺ or CD69⁺CD4⁺ T cells in spleen using flow cytometric analysis of the different diet mice. The *numbers in dot plots* showed the percentage of CD8⁺CD69⁺ or CD4⁺CD69⁺ cells in total *cell number* in spleen. $n = 5$ mice per cohort. **e** OVA₂₅₇₋₂₆₄-specific CD8⁺ T cell proliferation was analyzed in vivo after different diet feeding for 4-week. After 1×10^7 5 μ M CFSE-labeled OT-I CD8⁺ T cells being transferred into mice for 20 h, 5×10^6 OVA₂₅₇₋₂₆₄-loaded splenocytes were transferred. Spleens and mesenteric lymph nodes (LN) were collected and prepared single-cell suspensions for analyzing divided CFSE⁺CD8⁺ cells 4 days later. The *numbers in dot plots* referred to the percent of divided CFSE⁺CD8⁺ cells in all CFSE positive cells. $n = 3$ mice per cohort. Data represent the mean $\pm$ SEM, and are representative of 3 independent experiments. * $p < 0.05$

Fig 2.

Dietary fish oil increases the frequency of CD11b⁺ Gr-1⁺ myeloid cells in peripheral tissues. **a–c** Q-PCR analysis for mRNA levels of cytokine profile (**a**) and *Chi3l3* (**b**) in spleens, and ELISA detection for the levels of IL-6 in sera (**c**) of mice after 4-week feeding. mRNA analysis data are normalized to the level of the corresponding *GAPDH* gene or ribosomal gene *l32*, and shown as fold change. Five mice per cohort. Data are representative of three experiments. **d–f** Representative flow data (**d**), quantitation of the percentage (**e**), and total cell number (**f**) of CD11b⁺ Gr-1⁺ cells in spleens of mice on different diets for 4 weeks. Numbers in dot plots indicate the percent of CD11b⁺ Gr-1⁺ cells in spleen. $n = 6$ mice per cohort. **g** Representative flow data of CD11b⁺ Gr-1⁺ cells in CD45⁺ cells in liver NPCs and blood of mice on different diets for 4 weeks. Numbers in dot plots indicate the percent of CD11b⁺ Gr-1⁺ cells in CD45⁺ cells. Data are representative of five experiments. Value represent the mean $\pm$ SEM. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

Fig 3.

Fish oil induces CD11b⁺ Gr-1⁺ cells with the characteristics of myeloid-derived suppressor cells. **a** Representative images showed Wright's staining of purified CD11b⁺ Gr-1⁺ cells from spleens of mice after 4-week fish oil diet feeding (original magnification $\times 100$). **b** Q-PCR analysis for mRNA levels of cytokine profile in purified CD11b⁺ Gr-1⁺ cells from spleens of mice after 4-week LFD and fish oil diet feeding. Data are normalized to the level of the house keep gene *GAPDH* and shown as folds change. Data are representative of 3 experiments. **c, d** MDSCs from fish oil feeding mice showed suppression functions on antigen-specific CD8⁺ T cells proliferation in vitro. **c** Total CD8⁺ T cell numbers after cocultured with purified mDC and/or MDSCs derived from mice either on LFD (LFD-MDSC) or fish oil diet (FO-MDSC) diets. Cells were purified with magnetic beads and added in the coculture as indicated. **d** CFSE proliferation assay to measure the proliferation of CD8⁺ T cells. Proliferation of CFSE-labeled OVA₂₅₇₋₂₆₄-specific CD8⁺ T cells were measured on day 4 following the coculture with OVA₂₅₇₋₂₆₄ loaded mDC and/or MDSCs from fish oil diet-fed mice (FO-MDSC). Bottom right, transwell were used to separate MDSCs from T cells and mDCs to show the necessity of cell-cell contact. Data shown as mean $\pm$ SEM of triplicate wells and representative of at least three independent experiments. * $p < 0.05$; ** $p < 0.01$

Fig 4.

Fish oil-induced MDSCs promote tumor growth. **a, b** Schematic diagram (**a**) and tumor growth graph (**b**) of B16 melanoma models in different dietary fat feeding mice. Mice were respectively treated with LFD, HFD and fish oil diet for 4 weeks, and then subcutaneously injected with 2×10^5 B16 melanoma cells on the right back flank. Tumor size was measured with calipers every 3 days. Six mice per cohort. Data are representative of four experiments. **c–e** The loss-of-function experiments of CD11b⁺Gr-1⁺ MDSCs in B16 tumor models (**c**), representative *dot plots* to show the depletion of Gr-1⁺ cells in blood after anti-Gr-1 treatment in fish oil diet-fed mice (**d**), tumor sizes were measured every 3 days (**e**). Data were shown as mean $\pm$ SEM. * $p < 0.05$; ** $p < 0.01$

Fig 5.

Splenic microenvironment is dispensable for MDSCs differentiation and tumor growth. **a–c** Schematic diagram for splenectomy (**a**), tumor growth curves of B16 melanoma models in LFD (**b**) and FO diet (**c**) with/without spleens. Spleens of 4-week diet feeding mice were removed. After 2 weeks recovery, mice were subcutaneously transferred with B16 melanoma cells and tumor sizes were analyzed as Fig. 4. **d–e** Representative flow graphs (**d**) and quantitation (**e**) of the percent of CD11b⁺ Gr-1⁺ MDSCs in CD45⁺ liver NPCs in splenectomy and sham control cohorts. $n = 5$ mice per cohort and data from three independent experiments. SPL, splenectomy. Values shown as mean ± SEM. ** $p < 0.01$

Table 1

Fatty acid compositions of each diet (mg/g diet) in this study are shown below

g/kg Diet	Telkad#2914	Bioserv F3282	Bioserv F5424
Capric (C10:0)	–	0.4	–
Lauric (C12:0)	–	0.7	–
Myristic (C14:0)	–	4.7	24.3
Palmitic (C16:0)	5.0	85.3	51.9
Palmitoleic (C16:1n7)	–	9.7	32.1
Stearic (C18:0)	1.0	48.4	12.5
Oleic (C18:1n9)	7.0	147.7	58.9
Linoleic (C18:2n6)	20.0	36.6	34.9
Linolenic (C18:3n3)	1.0	3.6	5.2
<i>Eicosapentaenoic acid (C20:5n3)</i>	–	–	<i>40.2</i>
<i>Docosapentaenoic acid (C22:5n3)</i>	–	–	<i>15.0</i>
<i>Docosahexanoic acid (C22:6n3)</i>	–	–	<i>26.2</i>
Calorie of fat (%)	13	59	59
Calorie of protein (%)	20	15	15
Calorie of sugar (%)	67	26	26

n-3 fatty acids are bold and italic. %, percent calories

Table 2

Primers used in this paper

Gene	Sequences
<i>Chi3l3</i>	5'-GGCTCAAGGACAACAATTTAGG-3' 5'-ACTGTGGAAAAACCGTTGAACT-3'
<i>INF-γ</i>	5'-GCGTCATTGAATCACACCTG-3' 5'-TGAGCTCATTGAATGCTTGG-3'
<i>IL-2</i>	5'-CGGCATGTTCTGGATTTGAC-3' 5'-TCCACCACAGTTGCTGACTC-3'
<i>IL-10</i>	5'-GGAGCAGGTGAAGAGTGATTTT-3' 5'-GGTACAAACGAGGTTTTCCAAG-3'
<i>TGF-β</i>	5'-GAAGGACCTGGGTTGGAAGT-3' 5'-TGGTTGTAGAGGGCAAGGAC-3'
<i>Foxp3</i>	5'-ACTCGCATGTTTCGCCTACTT-3' 5'-AGGGATTGGAGCACTTGTG-3'
<i>IL-1β</i>	5'-TGTGAAATGCCACCTTTTGA-3' 5'-TGAGTGATACTGCCTGCCTG-3'
<i>IL-6</i>	5'-GAGGAGACTTCACAGAGGATAC-3' 5'-GAGGAGACTTCACAGAGGATAC-3'
<i>TNF-α</i>	5'-GAACTGGCAGAAGAGGCACT-3' 5'-GGTCTGGGCCATAGAAGTGA-3'
<i>Arg-1</i>	5'-CTCCAAGCCAAAGTCCTTAGAG-3' 5'-AGGAGCTGTCATTAGGGACATC-3'
<i>GAPDH</i>	5'-GGCATTGCTCTCAATGACAA-3' 5'-TGTGAGGGAGATGCTCAGTG-3'
<i>L32</i>	5'-GAGCAACAAGAAAACCAAGCA-3' 5'-TGCACACAAGCCATCTACTCA-3'