Enhanced M2 macrophage polarization in high n-3 polyunsaturated fatty acid transgenic mice fed a high-fat diet

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Scope: Diet-induced obesity and consequent insulin resistance are caused, in part, by macrophage polarization and accumulation in peripheral tissues. Here, we examined the effects of endogenously synthesized n-3 PUFAs on macrophage chemotaxis and polarization.

Methods and results: Fat-1 mice and wild-type (WT) littersmates were fed a 60% calorie high-fat diet (HFD) for 10 weeks. Bone marrow macrophages (BMMs) from fat-1 and WT mice were used in in vitro chemotaxis assays and macrophage polarization studies. WT mice fed a HFD exhibited glucose intolerance, insulin resistance, and lipid accumulation and macrophage infiltration in liver and adipose tissue. However, these metabolic and inflammatory phenotypes were not observed in HFD-fed fat-1 mice. In flow cytometric analysis, M1 macrophage infiltration into adipose tissue was markedly attenuated in fat-1 mice. Consistently, results from in vitro experiments indicated that n-3 PUFAs prevented adipocyte conditioned medium-mediated macrophage chemotaxis, stimulated M2 polarization, and suppressed M1 polarization. The inhibition of macrophage migration by n-3 PUFAs was associated with suppression of multiple kinases, such as IkB kinase, AKT, and focal adhesion kinase.

Conclusion: Our results indicate that n-3 PUFAs play a crucial role in macrophage polarization and chemotaxis, and thus regulate the development of HFD-induced tissue inflammation and metabolic derangements.

Keywords: Chemotaxis / Inflammation / Macrophage / n-3 PUFAs / Polarization

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1 Introduction

Adipose tissue inflammation is an essential event in the development of insulin resistance in high-fat diet (HFD)-induced obesity. A number of studies have shown that blood-derived F4/80+CD11b+CD11c+ macrophages, which are referred to as classically activated M1 macrophages, infiltrate the adipose tissue in obese animals and humans [1]. These M1 macrophages are attracted by chemokines, such as C-C chemokine ligand 2 (CCL2)/MCP-1 and leukotriene B4, and...
Contribute to chronic low grade inflammation through the release of proinflammatory cytokines, such as tumor necrosis factor-α (TNF-α), IL-1β, and IL-6 [2, 3]. Another group of macrophages (F4/80+CD11b+CD11c−), which were originally described as alternatively activated M2 macrophages, are involved in wound healing and immunoregulation [4]. The importance of adipose tissue macrophages (ATMs) in inflammatory responses and subsequent metabolic derangements has been well documented in genetic studies. Genetic deletion of CCL2 or its receptor, C-C chemokine receptor 2 (CCR2), exhibits a reduction in adipose tissue macrophages and an improvement of glucose homeostasis on a HFD [2, 5]. In contrast, transgenic overexpression of CCL2 in adipocytes increases macrophage infiltration and decreases insulin sensitivity [2]. Therefore, strategies interfering with macrophage infiltration could be clinically beneficial.

To manage macrophage chemotaxis, pharmacological interventions are used, although these interventions are often associated with adverse side effects [6]. Alternatively, calorie restriction has been strongly recommended. However, patients usually fail to achieve such changes because they are unable or unwilling to modify their dietary habits. In this regard, dietary interventions targeting reduction of adipose tissue inflammation, independent of weight loss, have received a great deal of attention. Dietary fish oils, which are rich in n-3 polyunsaturated fatty acids (PUFAs), are considered to be anti-inflammatory and thus may counteract some of the damaging effects of obesity [7]. The incorporation of n-3 PUFAs into the membranes of immune cells, at the expense of arachidonic acid, is thought to be important with regard to anti-inflammatory effects of n-3 PUFAs [8]. This gives rise to less inflammatory eicosanoids, and this shift ultimately affects cytokine production. Dietary or endogenously synthesized n-3 PUFAs suppresses T-cell activation [9], monocyte chemotaxis [10], macrophage M1 polarization [11], and dendritic cell maturation [12], resulting in a decrease of adipose tissue inflammation and a lower incidence of type 2 diabetes. These immunomodulating effects of n-3 PUFAs seem to be dependent on the suppression of NF-κB and JNK pathways, through the binding to the G protein-coupled receptor 120 [3, 13]. Our group has recently provided in vivo and ex vivo evidence that n-3 PUFAs also protect pancreatic β cells against diabetogenic insult via suppression of ER stress [14]. However, it is difficult to ascertain the contribution of n-3 PUFAs per se, without the potential confounding effects of other dietary components, such as nutrient composition, total caloric intake, duration of feeding, and contamination of trace elements. To address this issue, we used fat-1 mice, which contain the fat-1 gene from Caenorhabditis elegans and are able to convert n-6 to n-3 PUFAs in vivo [15]. As a result of the endogenously synthesized n-3 PUFAs in blood and tissues, these mice exhibit the beneficial effects of n-3 PUFAs in various inflammatory disease models, such as pancreatitis [16], asthma [9], and rheumatoid arthritis [17]. Although the mice are not fed an n-3 PUFAs rich diet, they have abundant n-3 PUFAs and reduced levels of n-6 PUFAs in their organs. The resulting profile of n-6/n-3 PUFAs has been shown to be comparable to those obtained by dietary supplementation [16]. The mice therefore provide a controlled approach for evaluating the effects of a balanced n-6/n-3 PUFAs ratio, one that does not introduce the confounding factors that result from enforcing different test diets. To date, the fat-1 transgenic mouse model has been widely used, and has demonstrated that balancing the n-6/n-3 PUFAs ratio can protect against a wide variety of diseases, including chronic inflammatory diseases and cancer [3].

Given the above background, we considered whether metabolic derangements observed in HFD-induced obese mice would be inhibited in fat-1 mice. Special attention was paid to the effects exerted by n-3 PUFAs on the changes in macrophage subsets and inflammatory biomarker (cytokine/adipokine) expression in local tissues. Bone marrow macrophages (BMMs) from wild type (WT) or fat-1 mice were used in in vitro chemotaxis assays and macrophage polarization studies.

## 2 Materials and methods

### 2.1 Animals

Dr. J.X. Kang at Harvard Medical School (Boston, MA, USA) kindly provided the fat-1 transgenic mice. Mice were housed in a laminar flow cabinet and maintained on AIN-76A diets containing 5% corn oil provided ad libitum. Male C57BL/6 mice, each weighing 25–30 g, were purchased from Orient Bio (Seoul, Korea). All animal experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals, published by the US National Institutes of Health (NIH Publication No. 85-23, revised 2011). The current study protocol was also approved by the Institutional Animal Care and Use Committee of Chonbuk National University (Permit No. CBNU 2015–056).

### 2.2 Genotyping and phenotyping of fat-1 mice

Heterozygous fat-1 mice and littersmates were bred on the same C57BL/6 genetic background and all mice genotyped. Ear punches and tail tips were incubated with STE buffer (100 mM Tris, 5 mM EDTA, 0.2% SDS, and 200 mM NaCl, pH 7.4) and 0.25 mg/mL Proteinase K for 6 h at 55°C and submitted to a 2-step PCR with Taq polymerase (Clontech, Mountain View, CA, USA) and specific forward (5′-CTGCACCCACGCTTCCCAACCC-3′) and reverse (5′-CACACGGAGATTTGAGATT-3′) primers. Amplification of a 264 bp band confirmed the fat-1 genotype. The amount of n-3 PUFAs and n-6 PUFAs was measured by electrospray ionizing MS.
2.3 Electrospray ionization MS

Liver and adipose tissues were extracted with hexane using a blade type homogenizer (Auto Mill, Tokyo, Japan). Plasma was extracted with methanol. The extracts were diluted with methanol (1:9, v/v). Standards for linoleic acid (LA, C18:2), α-linolenic acid (ALA, C18:3), arachidonic acid (AA, C20:4), eicosapentaenoic acid (EPA, C20:5), and docosahexaenoic acid (DHA, C22:6) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Solutions of LA, ALA, AA, EPA, and DHA were prepared in analytical grade methanol (Merck, Darmstadt, Germany). An optimized multiple reaction monitoring method was developed using ultra-performance liquid chromatography (UPLC) coupled with MS/MS (Acquity system, Waters, Milford, USA). A UPLC system was coupled to a Xevo TQ-S triple quadrupole mass spectrometer (Waters). Chromatographic separations were carried out using reversed phase C18 column (2.1 × 50 mm, 1.7 μm; Waters) maintained at 30°C. LA, ALA, AA, EPA, and DHA were separated using a gradient elution with a flow rate of 0.5 mL/min. Mobile phase solvent A was 5 mM ammonium formate (Sigma-Aldrich) in water and solvent B was 5 mM ammonium formate in acetonitrile. The samples were eluted according to the following linear gradient from 70 to 100% B buffer for 10 min. Ions were generated in negative ionization mode using electrospray ionization interface. Peaks were identified by comparison with PUFA standards, and the integrated peak area of each resolved peak was used to calculate the percentage of each PUFA.

2.4 HFD feeding

Fat-1 mice and age-matched littermates older than 6 weeks were fed either a standard laboratory chow diet or a 60% HFD (Supporting Information Table 1, Research Diet, New Brunswick, NJ, USA) ad libitum for 10 weeks. Percentage body fat was determined using a Bruker Minispec mq 7.5 NMR analyzer (Bruker Optics, Ettlingen, Germany). Mice were placed in a clear, plastic cylinder (50-mm diameter) and kept immobile by insertion of a tight-fitting plunger. Fat and lean mass were recorded within 2 min. The accuracy and precision of the instruments were determined by cross-calibration, measuring the same groups of mice with different adiposities.

2.5 Glucose and insulin tolerance tests

At the ninth week after HFD feeding, the intraperitoneal glucose tolerance test and the insulin tolerance test were performed over a 3-day interval. Glucose tolerance test was performed after 16 h fast, before and after mice were intraperitoneal injected glucose at dose 1 g/kg body weight, blood glucose concentrations were measured from the tail vein at 0 (baseline), 15, 30, 60, 90, and 120 min. For ITT, after 6 h fast, glucose levels were likewise measured from tail vein after an intraperitoneal injection at 0.75 units/kg body weight of human insulin (Sigma-Aldrich). Mice were sacrificed on the tenth week.

2.6 Biochemical analysis

Blood samples were collected after overnight fasting. Plasma glucose level was assayed using the glucose oxidase method (Sigma-Aldrich). Insulin was measured using specific ELISA kits (Millipore Billerica, MA, USA). For liver TG quantification, liver tissues were homogenized and extracted in a mixture of chloroform, methanol, and DW (2:1:1 ratio).

2.7 Stromal vascular cells isolation and FACS analysis

Epididymal fat pads were excised from 10-week-old HFD-fed wild type and fat-1 mice, rinsed three times in a Krebs-Ringer (KR) buffer (119 mM NaCl, 4.74 mM KCl, 1.19 mM KH2PO4, 1.19 mM MgSO4·7H2O, 20 mM NaHCO3, 10 mM HEPES, and 2.54 mM CaCl2·2H2O, pH 7.4), and then homogenized in a KR buffer containing type II collagenase (Sigma-Aldrich), 3.5% BSA, and 6 mM glucose. Tissues were shaken for 1 h at 37°C and filtered through a 100 μm filter (BD Biosciences, San Jose, CA, USA) and then centrifuged at 1000 rpm for 5 min. SVF pellets were incubated with an ACK lysis buffer (Invitrogen, Carlsbad, CA, USA) for 3 min prior to centrifugation at 1000 rpm for 5 min and then resuspended in a KR buffer. Stromal vascular cells (SVCs) were incubated in a FACS buffer containing 2% fetal bovine serum (FBS, GIBCO, Pascagoula, MS, USA) with Fc Block (BD Biosciences) for 30 min at 4°C prior to staining with fluorescently labeled primary antibodies [F4/80 (2 μg/mL, BM8), CD11b (0.8 μg/mL, M1/70), and CD11c (1.6 μg/mL, N418)] or isotype control antibodies [rat IgG2a (2 μg/mL, eBR2a), rat IgG2b (1 μg/mL, eB149/10H5), and hamster IgG (1 μg/mL, eBio299Arm)] for 30 min at 4°C. Primary and isotype control antibodies were obtained from eBioscience (San Diego, CA, USA). The cells were gently washed three times and resuspended in FACS buffer. SVCs were analyzed using FACS Calibrator and Flowjo (BD Biosciences). Unstained, single stained, and fluorescence minus one controls were used for setting compensation and gates.

2.8 Histology

Adipose and liver tissues were removed and immediately placed in fixative (10% formalin solution in 0.1 M PBS). Histological sections (4 μm for liver and 6 μm for adipose tissue) were cut from formalin-fixed paraffin-embedded tissue blocks. Specimens were stained with hematoxylin-eosin (H&E) to identify morphological changes. For
immunohistochemical analysis, tissue sections were treated with a microwave antigen retrieval procedure using a 10 mM sodium citrate buffer. After blocking endogenous peroxidase activity, the sections were incubated with Protein Block Serum-Free (DAKO, Carpinteria, CA, USA) to block non-specific staining. Sections were then incubated with an anti-F4/80 antibody (1:100, BM8, and rat IgG2a, Abcam, Cambridge, UK) or SirT1 (1:100, H-300, and rabbit IgG, Santa Cruz Biotechnology, Dallas, TX, USA). Peroxidase activity was detected with a 3-amin-9-ethyl carbazole (Millipore). To measure the adipocyte area, sections were observed under an Axiovert 40 CFL microscope (Carl Zeiss, Oberkochen, Germany) and measured using iSolution DT 36 software (Carl Zeiss). Liver inflammation was graded on liver biopsies using a modified histologic activity index [18]. Briefly, liver inflammation was defined as high grade if mice had more than four foci in a 200× field (inflammation score 3) or two to four foci in a 200× field (inflammation score 2). Inflammation was defined as low grade if mice had fewer than 2 foci in a 200× field (inflammation score 1) or no foci (inflammation score 0). For each animal, 3–5 areas in four different sections were analyzed. The data were first averaged per section and then per animal.

2.9 Cell culture

3T3-L1 fibroblasts were obtained from American Type Culture Collection (Manassas, VA, USA). To prepare the adipocyte conditioned medium (CM), 3T3-L1 fibroblasts were grown in a DMEM (Lonza, Walkersville, MD, USA) containing 10% FBS. Confluent cells were treated with differentiation medium [MDI: DMEM, 10% FBS, 1 µM dexamethasone (Sigma-Aldrich), 10 µg/mL insulin (Roche Diagnostics, Indianapolis, IN, USA), and 0.5 mM isobutylmethylxanthine (Sigma-Aldrich)] for 2 days. The medium was then replaced with DMEM containing 10 µg/mL insulin for 2 days and maintained for 2 days without insulin. All adipocyte CM were collected at 6 days after the initial exposure to the differenti-ation cocktail.

For BMM cultures, bone marrow was isolated from femurs and tibias of WT and fat-1 mice and cultured in α-MEM (GIBCO) supplemented with 10% FBS. Floating cells were used as BMMs. BMMs were differentiated into macrophages by cultivation in α-MEM media supplemented with 30% L929-generated CSF-1 for 6 days. For M1 and M2 differentiation, BMMs were exchanged into α-MEM, which did not contain CSF-1, and were then stimulated with LPS (10 ng/mL, Sigma-Aldrich) + IFN-γ (50 U/mL, Invitrogen) or IL-4 (10 ng/mL, Invitrogen) + macrophage colony-stimulating factor (M-CSF, 10 ng/mL, GIBCO), respectively.

2.10 In vitro migration assay

For migration assays, BMMs (1 × 10^5) were seeded in the 12-well upper chamber of a cell culture insert with an 8-µm pore membrane (BD Biosciences) in FBS-free media. Adipocyte CM was added to the lower chamber, and cells were incubated for 3 h. Polyethylene terephthalate membranes separating the upper and lower chambers were fixed with 4% paraformaldehyde in PBS for 20 min and stained with crystal violet for 20 min.

2.11 Western blot

BMMs were homogenized in Mammalian Protein Extraction Reagent (Thermo, Waltham, MA, USA). Homogenates containing 20 µg of total protein were separated by 7.5 or 10% SDS-PAGE and transferred to PVDF membranes (Millipore). After blocking with 5% skim milk, blots were probed with primary antibodies against IKKβ (1:2500, 10A9B6, mouse IgG, Santa Cruz Biotechnology), Ac-p65 (1:2500, Q04206, rabbit IgG, Assay Biotechnology, Sunnyvale, CA, USA), p65 (1:2500, 93H1, rabbit IgG, Abcam), JNK (1:2500, 56G8, rabbit IgG, Cell Signaling, Beverly, MA, USA), p-JNK (1:2500, 98F2, rabbit IgG, Cell Signaling), ERK (1:2500, 137F5, rabbit IgG, Cell Signaling), p-ERK (1:2500, 98F2, rabbit IgG, Cell Signaling), p-FAK (1:2500, rabbit IgG, Cell Signaling), AKT (1:2500, 2H10, mouse IgG, Cell Signaling), p-AKT (1:2500, rabbit IgG, Cell Signaling), p-IKKα/β (1:2500, 16A6, rabbit IgG, Cell Signaling), p65 (1:2500, C22B4, rabbit IgG, Cell Signaling), and p-Src (1:2500, 7G9, mouse IgG, Cell Signaling). Horseradish peroxidase-conjugated goat anti-rabbit IgG (1:2500, Enzo Life Sciences, Farmingdale, NY, USA) and goat anti-mouse IgG (1:2500, Enzo Life Sciences) secondary antibodies were used for visualization. Signals were detected with a Las-4000 imager (GE Healthcare Life Science, Pittsburgh, PA, USA).

2.12 RNA isolation and real-time RT-PCR

Total RNA was extracted from tissues or BMMs using TRIzol reagent (Invitrogen). RNA was precipitated with isopropanol and dissolved in diethylpyrocarbonate-treated distilled water. First-strand cDNA was generated with oligo dT-adaptor primers by reverse transcriptase (TaKaRa, Tokyo, Japan). Specific primers were designed using qPrimerDepot (http://mouseprimerdepot.nci.nih.gov, Supporting Information Table 2). Real-time RT-PCR reactions comprised a final volume of 10 µL, containing 10 ng of reverse-transcribed total RNA, 200 nM of forward and reverse primers, and a PCR master mixture. RT-PCR was performed in 384-well plates using an ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA). Reverse transcription and PCR were performed using a One-Step RT-PCR kit (Invitrogen). PCR fragments were separated by 2% agarose gel electrophoresis followed by staining with ethidium bromide.
Table 1. Composition and ratio of n-6 and n-3 PUFAs in wild type and fat-1 mice

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<tbody>
<tr>
<td>WT plasma (µg/mL)</td>
<td>28.24 ± 16.3</td>
<td>0.21 ± 0.1</td>
<td>1.98 ± 1.0</td>
<td>0.35 ± 0.2</td>
<td>2.70 ± 1.6</td>
<td>9.3</td>
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<tr>
<td>Fat-1 plasma (µg/mL)</td>
<td>14.37 ± 7.2a</td>
<td>0.75 ± 0.4b</td>
<td>0.64 ± 0.32b</td>
<td>0.71 ± 0.4b</td>
<td>3.10 ± 1.8</td>
<td>3.3</td>
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<tr>
<td>WT liver (µg/mg)</td>
<td>61.90 ± 1.67</td>
<td>4.45 ± 2.13</td>
<td>13.61 ± 0.23</td>
<td>3.19 ± 0.27</td>
<td>6.93 ± 0.17</td>
<td>5.2</td>
</tr>
<tr>
<td>Fat-1 liver (µg/mg)</td>
<td>52.93 ± 2.73ab</td>
<td>6.82 ± 0.69b</td>
<td>10.95 ± 0.36b</td>
<td>5.36 ± 0.31b</td>
<td>7.42 ± 0.13b</td>
<td>3.2</td>
</tr>
<tr>
<td>WT eWAT (µg/mg)</td>
<td>34.23 ± 2.12</td>
<td>1.23 ± 0.11</td>
<td>2.79 ± 0.28</td>
<td>0.44 ± 0.05</td>
<td>0.76 ± 0.08</td>
<td>15.0</td>
</tr>
<tr>
<td>Fat-1 eWAT (µg/mg)</td>
<td>32.39 ± 4.41</td>
<td>1.92 ± 0.12b</td>
<td>1.40 ± 0.15b</td>
<td>0.84 ± 0.13a</td>
<td>1.01 ± 0.10a</td>
<td>8.8</td>
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Values are expressed mean ± SEM (n = 3–6). Ratio of n-6/n-3 was calculated from n-6 PUFAs (LA and AA) versus n-3 PUFAs (ALA, EPA, and DHA).

a) p < 0.05
b) p < 0.01 versus WT.

LA, linoleic acid; ALA, α-linolenic acid; AA, arachidonic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; PUFAs, polyunsaturated fatty acids; eWAT, epididymal adipose tissue.

2.13 Statistical analysis

Data are expressed as mean ± SEM. Statistical comparisons were performed using one way and two way analysis of variance. Duncan’s method was used for pairwise comparisons between each factor. Stata 14 (Stata Corp, College Station, TX, USA) was used to conduct all analysis and a value of p < 0.05 was accepted as an indication of statistical significance.

3 Results

3.1 Liquid chromatographic analysis shows increased n-3 PUFAs content in fat-1 mice

We first quantified the major n-3 and n-6 PUFAs in plasma, liver, and adipose tissues of fat-1 mice and their WT littermates. Results showed that the content of n-3 PUFAs (ALA, EPA, and DHA) was significantly different between the genotypes, with higher levels in the plasma, liver, and adipose tissues of fat-1 mice (Table 1). Conversely, levels of n-6 PUFAs (AA and LA) were lower in fat-1 mice compared with WT mice. Fat-1 mice had plasma, liver, and adipose tissue n-6/n-3 ratios of 3.3, 3.2, and 8.8, respectively, compared to ratios of 9.3, 5.2, and 15.0, respectively, in WT mice.

3.2 HFD-induced ectopic fat accumulation and glucose intolerance are attenuated in fat-1 mice

Six-week-old fat-1 mice and WT littermates were fed either normal chow diet (NCD) or 60% HFD for 10 weeks. On a NCD, there were no differences in body weight gain and food intake between WT and fat-1 mice (Figs. 1A and B). However, on a HFD, fat-1 mice showed decreased body weight and fat percentage relative to WT mice (Figs. 1A and C).

For mice on a HFD, WT mice developed glucose intolerance, as indicated by a higher fasting glucose levels and a higher area under curve on glucose tolerance test (Figs. 1D and E). Conversely, in fat-1 mice, both fasting glucose levels and glucose tolerance test curves were similar to those observed in NCD-fed mice. In addition, insulin sensitivity was markedly increased in fat-1 mice after HFD feeding, as demonstrated by lower fasting insulin levels and increased glucose excursion during the insulin tolerance test (Figs. 1F and G).

3.3 Macrophage infiltration into liver and adipose tissue is diminished in fat-1 mice

Increased macrophage infiltration in peripheral tissues, such as liver and adipose tissue, is a hallmark of obesity-induced tissue inflammation and insulin resistance [1]. We therefore examined macrophage infiltration into the liver and adipose tissue and the mRNA expression of inflammatory genes. Liver weight and TG content were similar between genotypes in mice on a NCD, but significantly decreased in fat-1 mice on a HFD (Figs. 2A and B). Microscopy of liver histology by H&E staining revealed a lower grade of inflammation in fat-1 mice relative to WT mice under the HFD condition (Figs. 2C and D). To assess macrophage infiltration into liver tissue, we counted F4/80-positive cells as a pan-marker for macrophages. Accumulation of cells immunopositive for F4/80 was significantly lower in fat-1 mice fed a HFD compared with WT mice (Fig. 2E). Real-time RT-PCR analysis also confirmed the decreased accumulation of macrophages and inflammation in fat-1 mice compared with WT mice under HFD conditions, with decreased expression of macrophage genes (CCL2 and CCR2), cytokine genes (IL-1β, IL-6, and TNF-α) and an adhesion molecule (ICAM-1) (Fig. 2F). Less liver tissue damage in fat-1 mice, as evidenced by decreased serum ALT and AST levels, correlated well with the degree of inflammation (Fig. 2G).

HFD feeding induced a large increase in epididymal white adipose tissue (eWAT) mass in WT mice, but had less of an effect in fat-1 mice, resulting in lower wet weights of eWAT (Fig. 3A). Consistent with this finding, adipocyte size under HFD conditions was also smaller in fat-1 mice.
Adipose tissue macrophages (ATMs) often surround and ingest dying or dead adipocytes to form crown-like structures (CLSs). To assess macrophage infiltration into eWAT, we counted CLS numbers in the tissue. As shown in Figs. 3B and C, fat-1 mice had fewer CLSs, suggesting that the degree of adipose inflammation was reduced in fat-1 mice compared with WT mice. Consistent with this finding, mRNA levels of a variety of proinflammatory genes, including macrophages (CD11c, F4/80, CCL2, and CCR2), cytokines (IL-1β, IL-6, and TNF-α) and adhesion molecule (ICAM-1), were downregulated, while the mRNA level of a representative anti-inflammatory, adipokine adiponectin, was upregulated in fat-1 HFD mice (Fig. 3E). 

To characterize the ATMs, we prepared stromal vascular cells from adipose tissues of WT and fat-1 mice and analyzed them using FACS. FACS analysis revealed that HFD feeding resulted in lower percentages of F4/80+CD11b+CD11c+ macrophages in adipose tissue of fat-1 mice relative to WT mice (Figs. 4A and B).

### 3.4 In vitro chemotaxis and M1 polarization are attenuated in BMMs from fat-1 mice

To investigate the mechanism by which hepatic and adipose inflammation are suppressed in fat-1 mice, we compared in vitro cell migration in response to adipocyte CM. BMMs isolated from WT or fat-1 mice were layered onto a transwell insert for migration in the presence of CM in the lower well. Results indicate that fat-1 BMMs experienced suppressed migration compared with those from WT mice (Fig. 5A). In addition, the mRNA levels of M1 marker genes, such as CCL2, NOS2, IL-6, TNF-α, IL-1β, CCL3, Ccr2, and Clec7a, and adhesion molecule (ICAM-1), were markedly decreased in M1 macrophages of fat-1 mice compared with those of WT mice, whereas M2 marker genes (Arg1, Il-10, Clec7a, MGL1, CD206, and Ym-1) were greater in M2 macrophages from fat-1 mice (Fig. 5B).

To prime macrophages for LPS responses, we treated BMMs with LPS. With this stimulation, BMMs from fat-1 mice produced less cytokines compared to those of WT BMMs (Fig. 5C). Consistently, nuclear factor κB (NF-κB) signaling pathway was downregulated in fat-1 BMMs (Fig. 5D).

### 3.5 Adipocyte CM-mediated NF-κB, FAK, and AKT pathways are suppressed in BMMs from fat-1 mice

Previous studies reported that macrophage migration was associated with activation of multiple signaling pathways, including NF-κB, focal adhesion kinase (FAK), AKT, and mitogen-activated protein kinases (MAPKs) [19–23]. To elucidate the molecular mechanisms of endogenously synthesized n-3 PUFAs on the inhibitory action on macrophage
Figure 2. Effects of fat-1 expression on hepatic inflammation. (A, B) At the end of the study, liver mass and liver TG were determined. (C) Liver tissues were subjected to H&E staining or immunostained with an antibody against F4/80 or Sirt1. Arrows indicate inflammatory cell clusters. Bar = 250 μm. (D, E) Inflammation scores were determined and F4/80+ macrophages were counted. (F) Expression of macrophage infiltration-related genes was determined by real-time RT-PCR. (G) Plasma levels of ALT and AST were measured by specific ELISA. Values are mean ± SEM (n = 3–5). *p < 0.05, WT NCD versus WT HFD; #p < 0.05, fat-1 NCD versus fat-1 HFD; $p < 0.05, WT HFD versus fat-1 HFD.

Figure 3. Effects of fat-1 expression on adipose inflammation. (A) At the end of the study, weights of epididymal white adipose tissue (eWAT) were compared. (B–D) eWAT was stained with H&E or immunostained with an antibody against F4/80, and the mean surface area of adipocytes and number of crown-like structures (CLSs) was determined. Bar = 250 μm. (E) Expression of macrophage infiltration-related genes was determined by real-time RT-PCR. Values are mean ± SEM (n = 3–5). *p < 0.05, WT NCD versus WT HFD; #p < 0.05, fat-1 NCD versus fat-1 HFD; $p < 0.05, WT HFD versus fat-1 HFD.
migration, we examined those signaling pathways. NF-κB activity (as reflected by p65 phosphorylation) was suppressed in BMMs from fat-1 mice relative to those of WT mice (Fig. 6A). To investigate the upstream molecules involved in NF-κB suppression, we examined the phosphorylation of IκB kinase (IKK). Results found that adipocyte CM-induced phosphorylation of IKKα/β was suppressed in BMMs from fat-1 mice (Fig. 6A), indicating that endogenously synthesized n-3 PUFAs inhibit IKK/NF-κB axis in BMMs. In addition, an increase of Sirt1 expression and a decrease of p65 acetylation were observed in BMMs from fat-1 mice. These results are corroborated by those of immunohistochemical findings in liver tissues (Fig. 2C). FAK phosphorylation at Tyr397 markedly increased 30 min after CM stimulation in WT BMMs, which was attenuated in fat-1 BMMs (Fig. 6B). However, the pSrc level did not vary between genotypes, suggesting that the decrease in the FAK phosphorylation in fat-1 BMMs is not dependent on Src activity. Ten minutes after CM stimulation, the phosphorylation of AKT increased in WT BMMs, but not in fat-1 BMMs (Fig. 6B). We examined whether the lack of change in phosphorylation of AKT in fat-1 BMMs was mediated by the induction of phosphatase and tensin homolog (PTEN). Regardless of the presence of fat-1 genes in BMMs, the expression levels of PTEN were similar, indicating that the change in the p-AKT is mediated through upstream modulators other than PTEN. Finally, ERK and p38 MAPK were not significantly different in BMMs of both genotypes, while JNK was suppressed in BMMs from fat-1 mice.

4 Discussion

The glucose intolerance observed in obesity and type 2 diabetes is related to the inflammation in insulin target tissues, such as liver, skeletal muscle, and adipose tissue [1]. This study found that fat-1 mice fed a HFD had improved glucose tolerance, less lipid accumulation, and lower inflammation in both liver and adipose tissue, consistent with previous reports [24–26]. The protective effect of endogenously synthesized n-3 PUFAs was associated with reduced proinflammatory macrophage infiltration, as manifested by decreases in F4/80-positive macrophages in IHC and F4/80-CD11b-CD11c+ cells in flow cytometry. The mechanism whereby fat-1 expression suppresses macrophage infiltration into local tissues lies in the reduction of macrophage chemotaxis and a shift of macrophage polarization from M1 to M2. These anti-inflammatory effects of fat-1 expression are associated with the suppression of NF-κB, AKT, and FAK pathways.

A number of animal and human studies have demonstrated that regular supplementation of n-3 PUFAs exerts metabolically beneficial effects by affecting weight gain, lipid accumulation in liver and adipose tissue, adipokine secretion, and more [27]. Experiments using fat-1 mice also display similar results. White et al. [26] reported that hemizygous fat-1 (+/-) mice are protected against 8-week HFD-induced insulin resistance without changes in weight gain, body fat mass, or hepatic steatosis. Li et al. [24] reported that, when fed a HFD for 12 weeks, homozygous fat-1 (+/-) mice strongly resisted weight gain, insulin resistance, and hepatic steatosis. Similar to the latter report, this study also found that homozygous fat-1 mice fed a HFD for 10 weeks exhibited a resistance to HFD-induced adiposity, hepatic steatosis, and insulin resistance compared with WT mice. We reasoned that doubling the expression of the fat-1 transgene (hemizygous versus homozygous) would increase the endogenous conversion of n-6 PUFAs to n-3 PUFAs and allow for greater correction of the metabolic phenotypes in HFD-fed mice. Despite the differences in metabolic phenotypes in hemizygous and homozygous fat-1 mice, no difference in the degree of macrophage...
Figure 5. Regulation of cell migration and macrophage polarization by fat-1 expression. (A) BMMs (1 × 10^5) were transferred to the transwell insert and incubated for another 3 h for migration in the presence or absence of adipocyte CM in the lower well. Representative microphotographs of migration assay after staining cells with crystal violet. Bar = 50 μm. Cells that migrated to the lower bottom of the inserts were counted and the quantification results are expressed as arbitrary units (AU). Values are mean ± SEM (n = 3). Statistical comparisons were performed using a one-way analysis of variance. * p < 0.05, DMEM versus WT CM; # p < 0.05, DMEM versus fat-1 CM; $ p < 0.05, WT CM versus fat-1 CM. (B) BMMs were treated with either 10 ng/mL LPS and 50 U/mL IFN-γ (for M1 polarization) or 10 ng/mL IL-4 and 10 ng/mL M-CSF (for M2 polarization) for 6 h. mRNA levels of M1 and M2 markers were analyzed by PCR. (C) BMMs from WT and fat-1 mice were treated with PBS (vehicle, VEH) or 10 ng/mL LPS for 24 h and then culture supernatant was harvested. Cytokine levels in the culture supernatant were analyzed by ELISA. Values are mean ± SEM (n = 4). * p < 0.05, WT VEH versus WT LPS; # p < 0.05, fat-1 VEH versus fat-1 LPS; $ p < 0.05, WT LPS versus fat-1 LPS. (D) BMMs were treated with 10 ng/mL LPS for the indicated time periods and NF-κB signaling pathway was analyzed by Western blotting.

infiltration was observed. This finding is in agreement with several fish oil supplementation studies [28, 29], suggesting that the metabolic effects of n-3 PUFAs are, at least in part, a result of inhibition of macrophage infiltration and concomitant cytokine production.

Macrophages are heterogeneous immune cells, ranging from proinflammatory M1 macrophages to anti-inflammatory M2 macrophages [4]. It is widely accepted that an enhanced M1/M2 macrophage ratio within the adipose tissue of HFD-fed mice aggravates tissue inflammation and impairs systemic insulin sensitivity [30, 31]. The molecular mechanism by which M1 macrophages contribute to insulin resistance is one where the macrophage secretes proinflammatory cytokines, such as TNF-α and IL-6. Both TNF-α and IL-6 are known to interfere with insulin signaling pathways and suppress the release of adiponectin, an adipokine with insulin sensitizing abilities [32]. In histological and flow cytometric analyses, we observed that n-3 PUFAs had an effect of reducing infiltration of CD11b^+CD11c^+ M1 macrophages in adipose tissue. In support of these results, PCR analysis showed a decrease in M1 marker gene expression and an increase in M2 marker gene expression in BMMs from fat-1 mice, suggesting a switch of macrophage polarization toward an M2 phenotype. The absolute reduction in M1 macrophage numbers led to decreases in TNF-α, IL-6, and MCP-1 and an increase in adiponectin in adipose tissue of HFD-fed fat-1 mice. Notably, BMMs from fat-1 mice exhibited a reduced cell migration toward adipose tissue and adipocyte cytokines. Collectively, endogenously synthesized n-3 PUFAs ameliorated adipose tissue inflammation and systemic insulin resistance induced by a HFD by the following mechanisms: (1) switching adipose tissue-infiltrating macrophages from a proinflammatory M1 phenotype toward an anti-inflammatory M2 phenotype, (2) reducing adipose chemokine-mediated cell migration, and (3) decreasing proinflammatory cytokine secretion while increasing adiponectin secretion.

Systemic insulin resistance in obesity is closely associated with hepatic steatosis [1]. As observed in adipose tissue, WT mice fed a HFD had microscopic evidence of hepatic steatosis and inflammation (nonalcoholic steatohepatitis, NASH).
Accordingly, levels of liver TG and tissue injury marker enzymes were significantly higher in HFD-fed WT mice compared with NCD-fed WT mice. In contrast, we did not observe those metabolic and inflammatory phenotypes in HFD-fed fat-1 mice. These results suggest that n-3 PUFAs also have a protective effect against the development of NASH.

To date, the fat-1 transgenic mouse model has been widely used, and has demonstrated that balancing the n-6/n-3 PUFAs ratio can protect against a wide variety of diseases, including inflammatory and metabolic diseases [33]. Bhattacharya et al. [34] fed mice with 10% fish oil for 6 months and observed an anti-inflammatory effect of n-3 PUFAs. The fish oil supplemented mice obtained a serum n-6/n-3 PUFAs ratio of 0.41, which is lower than we observed in fat-1 mice. Despite metabolic benefits in animal experiments, human studies have not demonstrated such clear effects of fish oils. In metaanalyses of controlled supplementation trials, dietary EPA + DHA and fish/seafood consumption does not produce major changes in biomarkers of glucose-insulin homeostasis in subjects with DM, while plant-derived ALA is associated with trends toward lower risk [35]. Because plant sources of n-3 PUFAs are potentially more widely available on a global basis, further clinical investigation is needed.

Molecular mechanisms underlying the macrophage migration inhibitory action of n-3 PUFAs have been suggested, including altered synthesis of eicosanoids-derived lipid mediators (maresin, resolin, and protectin) or their receptors [36–39]. Here, we suggest that, at the molecular level, n-3 PUFAs inhibit macrophage chemotaxis by the suppression of multiple signaling pathways, including those of NF-κB, FAK, and AKT. Because NF-κB is a molecular target for macrophage-mediated inflammation, we investigated changes in the NF-κB signaling pathway, and results indicated that fat-1 gene expression in BMMs resulted in lower levels of p-p65 and Ac-p65 following CM stimulation. We further observed increases in Sirt1 levels in both BMMs and liver tissues of fat-1 mice. These results are consistent with our previous findings, as well as the findings of others [40, 41]. Collectively, fat-1 gene expression regulates macrophage-mediated inflammation, in part by inducing Sirt1 expression, which results in the suppression of the NF-κB pathway.

In our current work, we have used endogenously produced n-3 PUFAs to explore their effects on adipocyte-mediated macrophage chemotaxis and M1/M2 polarization in the context of obesity. Thus, we present direct evidence that n-3 PUFAs suppress proinflammatory M1 polarization while they enhance anti-inflammatory M2 polarization in ATM and cause anti-inflammatory and insulin sensitizing effects.

This work was supported by the Bio & Medical Technology Development Program (No. NRF-2012M3A9B2027975), the Basic Science Research Program (No. 2013012280 and 2014R1A1A3053867), and the Medical Research Center Program (No. 2008–0062279).

B.H.P. and E.J.B. conceived the idea, designed the experiments, and wrote the manuscript. M.Y.S., J.W., and Y.L. conducted the experiments. J.L. and K.S.K. analyzed the data. B.H.P. had primary responsibility for final content. All authors read and approved the final manuscript.

The authors declare no conflict of interest.

5 References


