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Mobilization efficiency is critically regulated by fat via marrow PPARδ

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ABSTRACT

The efficiency of mobilization of hematopoietic stem/progenitor cells from bone marrow into the circulation by granulocyte colony-stimulating factor (G-CSF) is extremely varied in humans and mice and a mechanistic explanation for poor mobilizers is lacking. A mechanism of regulating mobilization efficiency by dietary fat was assessed in mice. Compared to a normal diet, a fat-free diet for 2 weeks greatly increased mobilization. The bone marrow mRNA level of peroxisome proliferator-activated receptor δ (PPARδ), a receptor for lipid mediators, was markedly upregulated by G-CSF in mice fed a normal diet and displayed a strong positive correlation with widely varied mobilization efficiency. It was hypothesized that the bone marrow fat ligand for PPARδ might inhibit mobilization. A PPARδ agonist inhibited mobilization in mice fed a normal diet and enhanced mobilization by a fat-free diet. Mice treated with a PPARδ antagonist and chimeric mice with PPARδ$^{+/-}$ bone marrow showed enhanced mobilization. Immunohistochemical staining and flow cytometry revealed that bone marrow PPARδ expression was enhanced by G-CSF mainly in mature/immature neutrophils. Analysis of bone marrow lipid mediators revealed that G-CSF treatment and a fat-free diet resulted in exhaustion of ω3-polyunsaturated fatty acids such as eicosapentaenoic acid. Eicosapentaenoic acid induced the upregulation of genes downstream of PPARδ, such as *Cpt1α* and *Angptl4*, in mature/immature neutrophils *in vitro* and inhibited enhanced mobilization in mice fed with a fat-free diet *in vivo*. Treatment of wild-type mice with anti-Angptl4 antibody enhanced mobilization as well as bone marrow vascular permeability. Collectively, PPARδ signaling in mature/immature bone marrow neutrophils induced by dietary fatty acids negatively regulates mobilization, at least partially, via Angptl4 production.

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Introduction

Granulocyte colony-stimulating factor (G-CSF) is widely used in the clinic as a standard agent to induce the mobilization of hematopoietic stem/progenitor cells (HSPC) from bone marrow (BM) into the circulation. G-CSF-mobilized HSPC are currently a major source of cells for stem cell transplantation which is a curative therapeutic option for intractable hematologic diseases. According to the current understanding of the mechanism of G-CSF-induced mobilization, in addition to the cytokine's pharmacological effect of expanding BM neutrophils, its neurotropic action through the G-CSF receptor in the sympathetic nervous system (SNS) leads to the suppression of macrophages that support HSPC niche cell function,[1-3] reduction of stromal cell synthesis of factors retaining HSPC in the BM, such as CXCL12,[4-6] and suppression of osteolineage cells through β_2-adrenergic receptors (β_2-AR),[7-10] leading to the passive release of HSPC from the microenvironment rather than their expansion or active migration. Besides the mechanism of mobilization itself, two unfavorable clinical events in G-CSF-induced mobilization have long remained unexplained and unsolved since the clinical application of G-CSF for mobilization. First, donors/patients treated with G-CSF often complain of low-grade fever and bone pain, which can be relieved by the administration of non-steroidal anti-inflammatory drugs. Second, mobilization efficiency is widely variable, and 10% to 20% of healthy donors are poor mobilizers, such that the number of HSPC that can be harvested is insufficient for transplantation. As an explanation of the former problem, we have reported that low-grade fever (and likely bone pain) associated with the administration of G-CSF is due to prostaglandin E_2 (PGE_2) production from mature BM neutrophils stimulated by the SNS through β_3-AR.[11] However, our understanding of the latter problem remains unacceptably inadequate. Poor mobilization is a particularly serious problem for healthy donors for allogeneic transplantation in the National Marrow Donor Program because they receive a certain dose of G-CSF without expected volunteer contribution to the patients. The wide range of mobilization efficiency, which occurs even in genetically identical mice, is currently unpredictable and uncontrollable. Mobilization efficiency may be partially determined by a balance between mobilization-promoting signals, such as SNS-mediated osteolineage suppression, and counteraction to mobilization, such as PGE_2 from neutrophils to support osteoblast activity.[11] Thus, it is clinically essential to elucidate the pathways that counteract mobilization during G-CSF treatment.

Analysis of lipid mediators in the BM lags behind that of other lipid-rich organs such as the liver and brain.[12,13] BM fat has been suggested to modulate hematopoiesis.[14,15] Evidence of lipid mediators of hematopoietic cells as inflammatory/resolving cells is accumulating.[16,17] However, a precise evaluation of total BM fat contents had not been done before our previous report on PGE_2.[11] In addition to fat cells, the BM contains an enormous number of inflammatory cells, such as neutrophils, macrophages, and their precursors, which are constantly stimulated by many marrow factors on their way to maturation and peripheralization throughout the body. Red blood cells and their precursor erythroblasts could also be a significant reservoir of lipid mediators.[18] Given that all these cells are packed at high density in the marrow, they may actively exchange many lipid mediators to stimulate each other. This unique situation in BM makes it difficult to precisely evaluate lipid mediators in BM by flushing it with phosphate-buffered saline (PBS) or following pipetting, which immediately changes lipid metabolic cascades. We have developed a new procedure for sampling BM by flushing it directly with -20°C 100% methanol and preparing it for liquid chromatography-tandem mass spectrometry (LC-MS/MS) through which stable and precise evaluation of PGE_2 in BM was achieved.[11]

In this study, we have applied this original method for the comprehensive analysis of marrow fat components, including not only ω6-fatty acids/proinflammatory lipid mediators such as PGE_2 but also ω3-fatty acids during G-CSF-induced mobilization. We found that mobilization efficiency can be enhanced by fat restriction in food. It also appeared that BM has a strong demand for certain ingested ω3-fatty acids, which function as ligands for peroxisome proliferator-activated receptor δ (PPARδ) in BM mature/immature neutrophils to suppress mobilization, at least partially, by regulating BM vascular permeability.

Methods

Mice

Mice were cared for in the Institute for Experimental Animals, Kobe University Graduate School of Medicine. PPARδ$^{-/-}$ mice were generated on a C57BL/6 background as described in the *Online Supplementary Methods*. Because all PPARδ$^{-/-}$ mice died *in utero*, PPARδ$^{+/+}$ and PPARδ$^{+/-}$ littermates at ages 6 to 8 weeks were used as transplant donors to generate chimeric mice. C57BL/6-CD45.1 congenic mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA) and used at ages 6 to 8 weeks. Wild-type (WT) C57BL/6 mice at ages 6 to 8 weeks were purchased from CLEA Japan (Chiba, Japan) and used for experiments after 2 weeks of acclimatization unless otherwise indicated. Male mice were used in all experiments. Mice were fed with a normal diet (ND; CE-2, CLEA Japan) consisting, on average, of 4.69% fat, 24.90% protein, and 51.00% carbohydrates, yielding a total calorie content of 3.45 kcal/g, except for fat-free diet (FFD; CLEA Japan) experiments. The FFD consisted of 0.72% fat, 17.60% protein, and 63.49% carbohydrates by weight, yielding the same total calorie value as the ND, and was started at ages 8 to 10 weeks after 2 weeks of acclimatization. Animals were maintained under specific pathogen-free conditions and on a 12 h light/12 h dark cycle. All animal studies were approved by the Animal Care and Use Committee of Kobe University.

Statistical analysis

All data were pooled from at least three independent experiments. All values were reported as the mean ± standard error of the mean (SEM). The statistical analyses were conducted using a two-tailed unpaired Student *t*-test, the Mann-Whitney U test, a one-way analysis of variance (ANOVA) test with the Tukey *post-hoc* procedure, and Pearson correlation coefficient. No samples or animals were excluded from the analyses. Animals were randomly assigned to groups. Statistical significance was assessed with Prism (GraphPad Software, San Diego, CA, USA) and defined as $P<0.05$.

Detailed descriptions of the methods for the other procedures are provided in the *Online Supplementary Methods*.

Results

A short-term fat-free diet enhances mobilization efficiency

To examine the effect of insufficient fat intake on mobilization, WT C57BL/6 male mice were fed with a FFD, containing sufficient calories with protein and all known vitamins but without fat, or a ND for 2 weeks. The body weight of mice fed with the FFD for 2 weeks was comparable to that of the mice fed with the ND (24.71±0.32 and 24.14±0.35 g, respectively; n=11). The administration of either G-CSF (8 divided doses, every 12 h, 125 μg/kg/dose, s.c.) or vehicle (PBS/bovine serum albumin [BSA]) was followed after this period with the continuation of the same diet. This period of fat restriction was reported to be safe with regard to sequelae associated with deficiency of essential fatty acids.[19,20] This simple regimen had a dramatic effect, with the number of hematopoietic progenitor cells (HPC) being increased in the circulation compared to that in mice fed with a ND, as assessed by lineage$^-$Sca-1$^+$c-kit$^+$ (LSK) cells and colony-forming units in culture (CFU-C) (Figure 1A and B; *Online Supplementary Figure S1A*) with no alteration in BM HPC (*Online Supplementary Figure S1B*). Enhanced mobilization was also confirmed in hematopoietic stem cells (HSC), as assessed by long-term competitive repopulation for 6 months (Figure 1C). Thus, a short-term deficit in fat intake is a promising method to enhance mobilization efficiency.

Marrow PPARδ expression correlates with mobilization efficiency

Enhanced mobilization was unlikely due to the alteration of known key players in BM microenvironment for mobilization, such as osteolineage cell activity and a chemokine, because mRNA levels of Runx2, osteocalcin, and CXCL12 in BM cells after G-CSF treatment were comparable between animals fed the FFD or the ND (*Online Supplementary Figure S2*). According to this observation, we hypothesized that some lipid mediators from food intake might play a role in the BM to inhibit mobilization, and that a FFD led to a lack of these BM lipid mediators.

We first searched for a possible receptor that could induce this inhibitory signal. The PPAR family consists of fatty acid ligand-activated transcription factors.[21] Among all three PPAR, α, γ, and δ (β/δ), in BM cells, PPARα mRNA was unchanged. Consistent with the previously reported suppression of PPARγ in CXCL12-abundant reticular cells by G-CSF,[22] PPARγ mRNA was significantly suppressed after G-CSF treatment (Figure 2A). Meanwhile, PPARδ mRNA displayed the highest expression in the steady state and increased dramatically after G-CSF mobilization

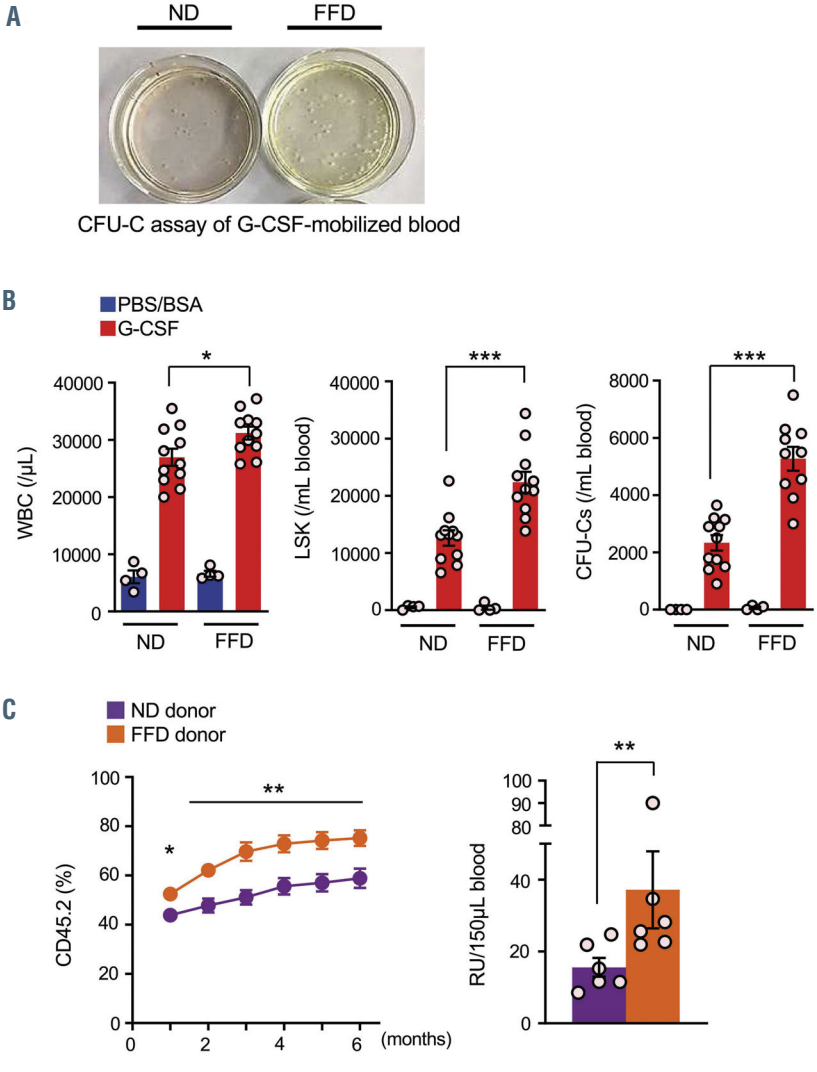

Figure 1. Short-term fat restriction enhanced hematopoietic stem/progenitor cell mobilization by granulocyte colony-stimulating factor. (A) Macrophotographs of culture dishes (35 mm) showing the results of the colony-forming units in culture (CFU-C) assay for mobilization of cells into the peripheral blood (20 μL) following eight doses of granulocyte colony-stimulating factor (G-CSF). ND: normal diet, FFD: fat-free diet. (B) Mobilization efficiency assessed by the presence of white blood cells (WBC), lineage$^-$Sca-1$^+$c-kit$^+$ (LSK) cells, and CFU-C in the blood (n=4 for group treated with phosphate-buffered saline (PBS)/bovine serum albumin (BSA) and n=10-11 for the group treated with G-CSF). (C) Hematopoietic stem cell activity assessed by a long-term competitive repopulating assay of mobilized blood. Repopulating units (RU) were evaluated at 6 months after competitive transplantation (n=6). Representative pictures or combined data from at least three independent experiments are shown. Data are mean ± standard error of mean. *P<0.05, **P<0.01, ***P<0.001 (Student t test and Mann-Whitney U test).

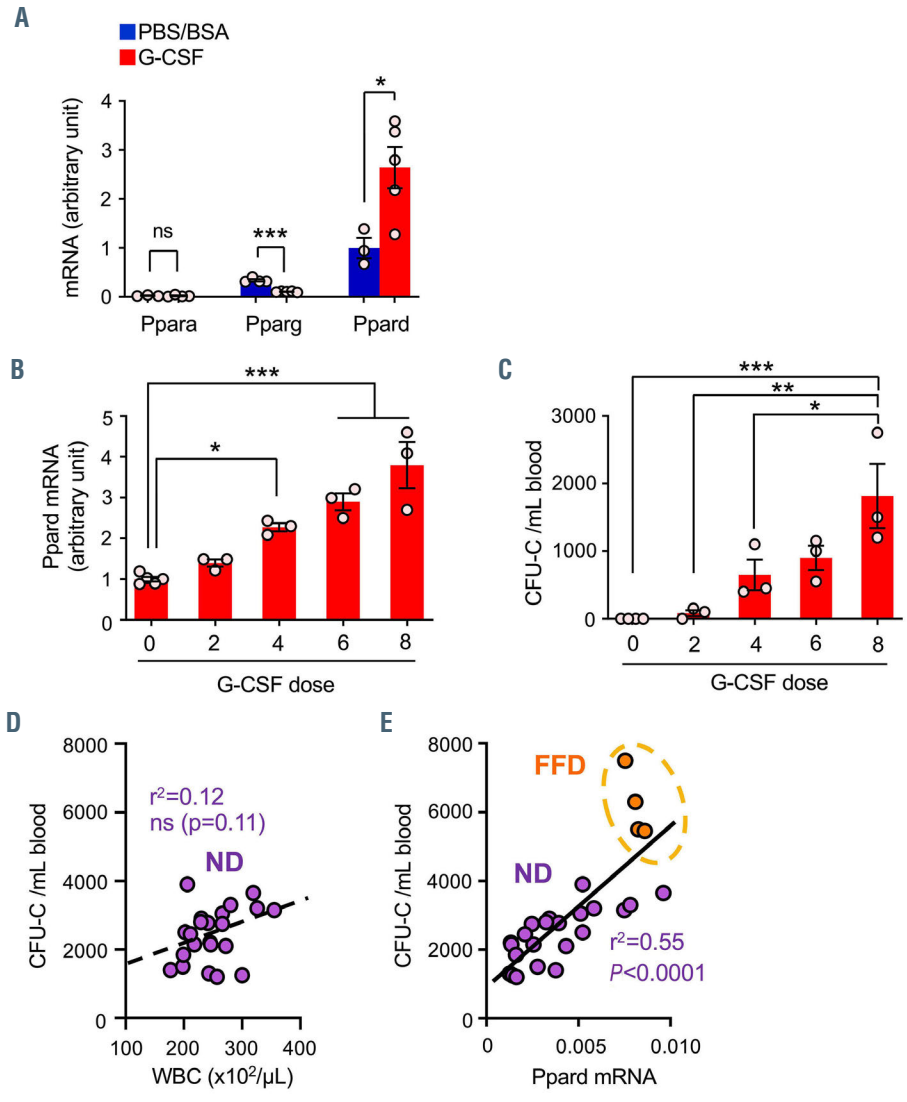

Figure 2. Strong correlation between bone marrow PPARδ mRNA and mobilization efficacy. (A) Alteration of PPAR family mRNA in bone marrow (BM) cells during granulocyte colony-stimulating factor (G-CSF) mobilization (n=3-5). (B, C) Stepwise increase along with increasing G-CSF doses in (B) PPARδ mRNA in BM cells and (C) colony-forming units in culture (CFU-C) in the blood (n=3-5). (D, E) Correlation of mobilization efficiency of CFU-C with (D) white blood cell (WBC) in the blood and (E) PPARδ mRNA in BM cells in mice fed with a normal diet (ND, violet dots; n=22) or a fat-free diet (FFD, orange dots; n=4). ns: not significant. Combined data from at least three independent experiments are shown. Data are mean±standard error of mean. *P<0.05, **P<0.01, ***P<0.001 (Student t test, analysis of variance, and Pearson correlation coefficient).

(Figure 2A). The increase in the expression of BM PPARδ and mobilized HPC in the blood was dependent on the number of G-CSF doses (Figure 2B and C). Based on these data, we analyzed the correlation between mobilization efficiency and BM PPARδ mRNA expression in a subset of C57BL/6 male mice fed with a ND after eight doses of G-CSF. The number of mobilized CFU-C varied greatly (range, 1200-3900/mL blood), and white blood cell count showed only a correlation trend (Figure 2D). Although the correlation between mobilized LSK cells and BM PPARδ mRNA was weak and not statistisically significant (*Online Supplementary Figure S3)*, mobilization efficiency by CFU-C correlated strongly with BM PPARδ mRNA (Figure 2E, violet dots). We also performed the same analysis in a subset of mice fed with the FFD. Consistent with this correlation, both mobilization efficiency and BM PPARδ mRNA were higher than those of the best mobilizer mice fed the ND (Figure 2E, orange dots). Thus, in G-CSF mobilization, higher expression of BM PPARδ is itself a marker of better mobilization. More importantly, this higher mobilization efficiency was likely due to the lack of signaling of this fatty acid ligand-activated transcription factor as a result of the insufficient supply of fat in the BM.

Next, we tried to identify the cell types that express PPARδ protein in BM. Immunohistochemical staining revealed clearly increased PPARδ expression after eight doses of G-CSF treatment. Morphologically, myeloid lineage cells, which were relatively large with various segmental shaped nuclei, were positive, whereas small round lymphocytes with little cytoplasm were negative for PPARδ (Figure 3A). PPARδ protein and mRNA expression was also evaluated in sorted myeloid cell fractions. Flow cytometric analysis revealed that all three major myeloid populations in the BM, i.e., mature neutrophils ($CD11b^{+}Ly6G^{high}F4/80^{low}$) immature neutrophils ($CD11b^{+}Ly6G^{dull}F4/80^{low}$) and monocytes/macrophages ($CD11b^{+}Ly6G^{dull}F4/80^{high}$), showed high expression in steady-state, and both mature and immature neutrophils displayed a significant increase in PPARδ protein and mRNA following G-CSF treatment (Figure 3B–D). In contrast, PPARδ protein expression in these three myeloid fractions in peripheral blood was observed in only minor populations and it was not increased by G-CSF treatment (*Online Supplementary Figure S4*), indicating the marrow-specific role of PPARδ.

Next, the alteration of BM PPARδ mRNA expression by the depletion of mature neutrophils was examined using the anti-Ly6G antibody, 1A8 (*Online Supplementary Figures*

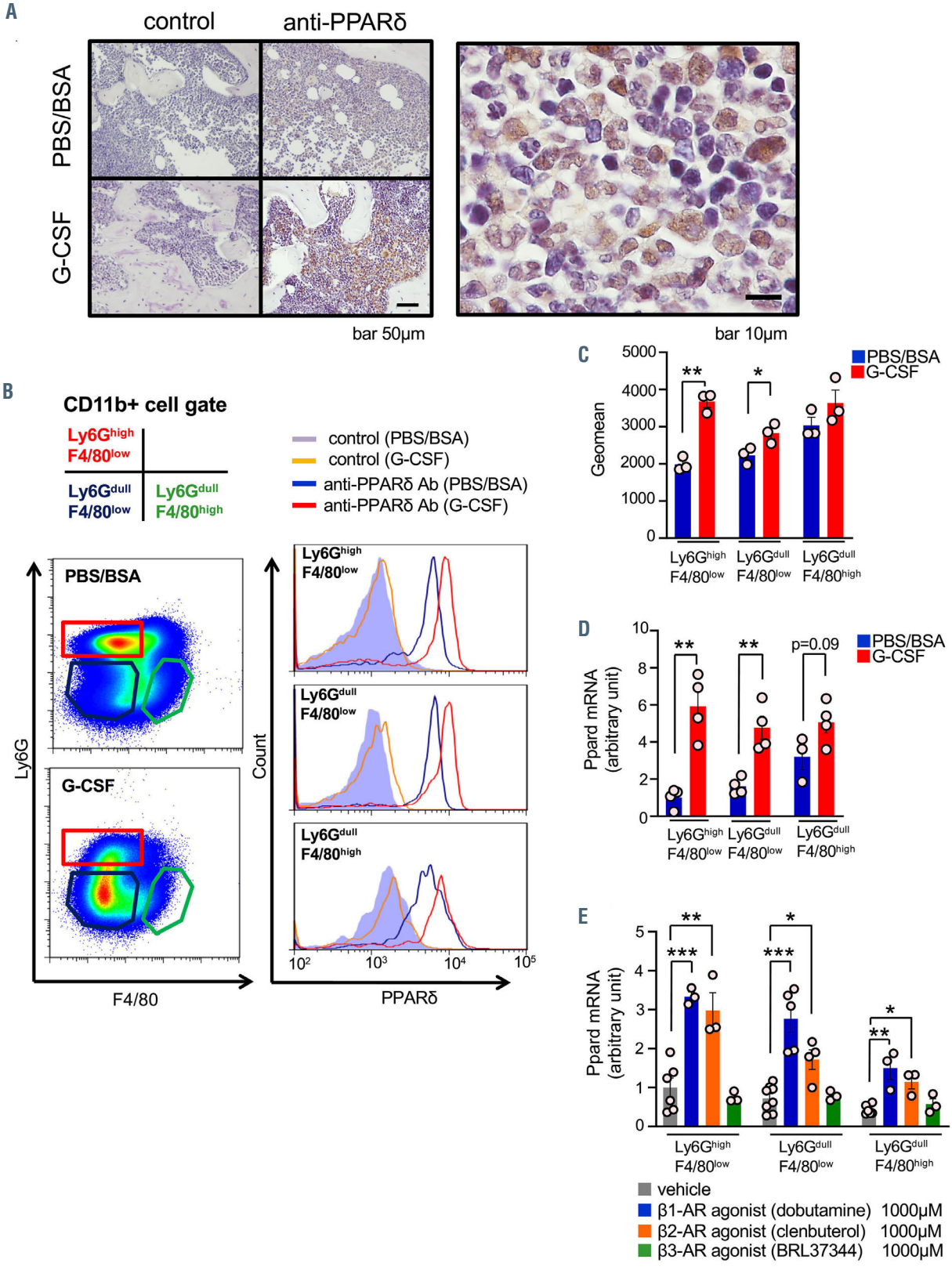

Figure 3. PPARδ expression in bone marrow myeloid cells. (A) Immunohistochemical staining for PPARδ in bone marrow (BM) sections from mice after treatment with eight doses of phosphate-buffered saline (PBS)/bovine serum albumin (BSA) or granulocyte colony-stimulating factor (G-CSF). Right panel shows BM PPARδ staining after G-CSF at a higher magnification. (B, C) Flow cytometric analysis of PPARδ in BM major myeloid populations (dot plot profiles) after treatment with eight doses of PBS/BSA or G-CSF shown in (B) as representative histograms and in (C) as geometric mean values (n=3). (D) PPARδ mRNA expression in sorted BM major myeloid populations after treatment with eight doses of PBS/BSA or G-CSF (n=3-4). (E) Alteration of PPARδ mRNA expression in sorted major myeloid populations from steady-state BM after *in vitro* treatment with selective agonists for each β-adrenergic receptor (β-AR) (dobutamine, clenbuterol, and BRL37344 for β_1-, β_2-, and β_3-AR, respectively; n=3-8). Representative pictures or combined data from at least three independent experiments are shown. Data are mean ± standard error of mean. **P*<0.05, ***P*<0.01, ****P*<0.001 (Student *t* test and analysis of variance).

S5 and *S6*). Without depletion, the absolute numbers of mature and immature neutrophils were comparable in steady-state BM, while the number of immature neutrophils was greatly increased and the number of mature neutrophils decreased by G-CSF treatment (*Online Supplementary Figure S6A*). The vast majority of increased neutrophils in peripheral blood following G-CSF treatment were also immature neutrophils (*Online Supplementary Figure S6B*). With selective depletion of mature neutrophils, the number of immature neutrophils in the BM was greatly increased without G-CSF treatment and the mobilization of both immature neutrophils and HPC (LSK cells and CFU-C) by G-CSF was slightly decreased (*Online Supplementary Figure S6A-H*), with no significant alteration of BM PPARδ mRNA (*Online Supplementary Figure S6I*). Thus, the cell population that mediated the increase of BM PPARδ mRNA in response to G-CSF was not restricted to mature neutrophils.

In addition to myeloid cell fractions, the upregulation of PPARδ mRNA was assessed in sorted BM $CD45^-Ter119^-$ cells (non-hematopoietic [stromal] cells), LSK cells, $B220^+$ B lymphocytes, and $CD3^+$ T lymphocytes. These investigations suggested that some nonmyeloid cell fractions, such as stromal cells and T cells, might contribute partially to the increase of BM PPARδ mRNA by G-CSF treatment (*Online Supplementary Figure S7*).

We assessed the signals that increase PPARδ expression *in vitro* using the neutrophil precursor cell line 32D. As reported previously, BM is richly innervated with sympathetic nerves that regulate mobilization via suppression of the osteoblastic microenvironment through β_2-AR stimulation by catecholamines and a marrow lipid mediator from mature neutrophils through β_3-AR stimulation.[7-11] The pan-β-AR agonist isoproterenol, but not G-CSF, was an inducer of PPARδ mRNA (*Online Supplementary Figure S8A*). Among all three β-AR (β_1, β_2, and β_3-AR) agonists, the β_1-AR agonist dobutamine recapitulated the effect of isoproterenol, significantly increasing PPARδ mRNA, and the β_2-AR agonist clenbuterol also showed a trend to induce PPARδ mRNA, albeit to a lesser extent (*Online Supplementary Figure S8B*). This observation was further confirmed at the protein level by flow cytometry (*Online Supplementary Figure S8C*). The increase of PPARδ mRNA by β_1/β_2-AR agonists was also confirmed in sorted BM mature/immature neutrophils and monocytes/macrophages (Figure 3E).

Thus, marrow PPARδ expression strongly correlates with mobilization efficiency and is enhanced mainly in myeloid cells, particularly in neutrophil lineage cells, by G-CSF-induced high sympathetic tone, likely through β_1/β_2-AR.

Marrow PPARδ signaling negatively regulates mobilization efficiency

Because FFD-G-CSF resulted in the upregulation of both BM PPARδ expression and mobilization efficiency (Figure 2E, orange dots), greater mobilization was likely achieved via reduced PPARδ activity due to the lack of natural fat ligands in the BM. In other words, marrow PPARδ signaling might be a negative regulator of mobilization. We next sought to explore whether the modulation of PPARδ signaling regulates HPC mobilization. The administration of the PPARδ agonist GW501516 inhibited G-CSF-induced mobilization with no alteration in BM HPC (Figure 4A; *Online Supplementary Figure S9A*). In G-CSF-treated mice, mRNA expression of major downstream genes of PPARδ signaling such as carnitine palmitoyltransferase-1α (Cpt1α) and angiopoietin-like protein 4 (Angptl4) in BM was significantly increased by GW501516, suggesting that the PPARδ agonist worked directly in BM cells (Figure 4B). Conversely, the administration of the PPARδ antagonist GSK3787 enhanced G-CSF-induced mobilization through the inhibition of PPARδ signaling in BM cells (Figure 4C and D; *Online Supplementary Figure S9B*). Furthermore, chimeric mice generated by the transplantation of BM cells from PPARδ heterozygous deficient mice into lethally irradiated WT mice showed significantly increased mobilization and lower mRNA expression of Cpt1α and Angptl4 in BM cells (Figure 4E and F; *Online Supplementary Figure S9C*). GW501516 also significantly inhibited the enhanced mobilization of CFU-C by the FFD (Figure 4G; *Online Supplementary Figure S9D*). These results suggest that PPARδ signaling in BM cells is indeed a negative regulator of mobilization.

Certain ω3-fatty acids are PPARδ ligands

We have previously reported an original method of sampling BM in which lipids in the marrow can be stably and precisely evaluated.[11] Using this method combined with LC-MS/MS, a series of ω3- and ω6-polyunsaturated fatty acids (PUFA) in BM were enumerated in mice fed with the ND or FFD in G-CSF mobilization. In Figure 5A, ω3-PUFA, such as eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), and their derivatives, were drastically decreased by eight doses of G-CSF and/or FFD, whereas ω6-PUFA, including arachidonic acid and associated pro-inflammatory lipid mediators, were unchanged (Figure 5B). These observations suggest that BM requires a continuous supply of ω3-fatty acids from diet, and that G-CSF treatment likely triggers strong consumption of ω3-fatty acids in BM. Indeed, similarly to the PPARδ agonist GW501516, EPA- and DHA-induced PPARδ signaling in 32D cells upregulated Cpt1α and Angptl4 mRNA expression (*Online Supplementary Figure S10A* and *B*). This effect, particularly with EPA, was significantly inhibited by the PPARδ antagonist GSK3787 (*Online Supplementary Figure S10C*). Among sorted BM myeloid cells, EPA, but not DHA, significantly upregulated PPARδ mRNA expression in mature/immature neutrophils *in vitro* (*Online Supplementary Figure S10D*). EPA, and to a lesser extent also DHA, upregulated Cpt1α and Angptl4 mRNA expression in these cells, and this effect was inhibited by GSK3787 (Figure 6A). These results suggest that EPA (and/or its metabolites) may be a functional fatty acid ligand for PPARδ in neutrophils and their precursors.

In concordance, EPA administration *in vivo* to normal mice partially attenuated the enhanced mobilization induced by a FFD (Figure 6B; *Online Supplementary Figure S11A*). We repeated the same experiment in chimeric mice with $PPARδ^{+/+}$ or $PPARδ^{+/-}$ BM. Consistently, in $PPARδ^{+/+}$ BM chimera, EPA administration showed a trend to partial reduction in CFU-C mobilization (Figure 6C; *Online Supplementary Figure S11B*). In $PPARδ^{+/-}$ BM chimera, however, mobilization efficiency in the FFD condition was further enhanced, and this effect was greatly inhibited by EPA (Figure 6C; *Online Supplementary Figure S11B*). These results suggest that BM in the FFD condition still contains lipid mediators that function as PPARδ ligands, and that EPA may also use pathways other than PPARδ to inhibit mobilization.

Thus, a certain ω3-fatty acid, partially as a natural ligand

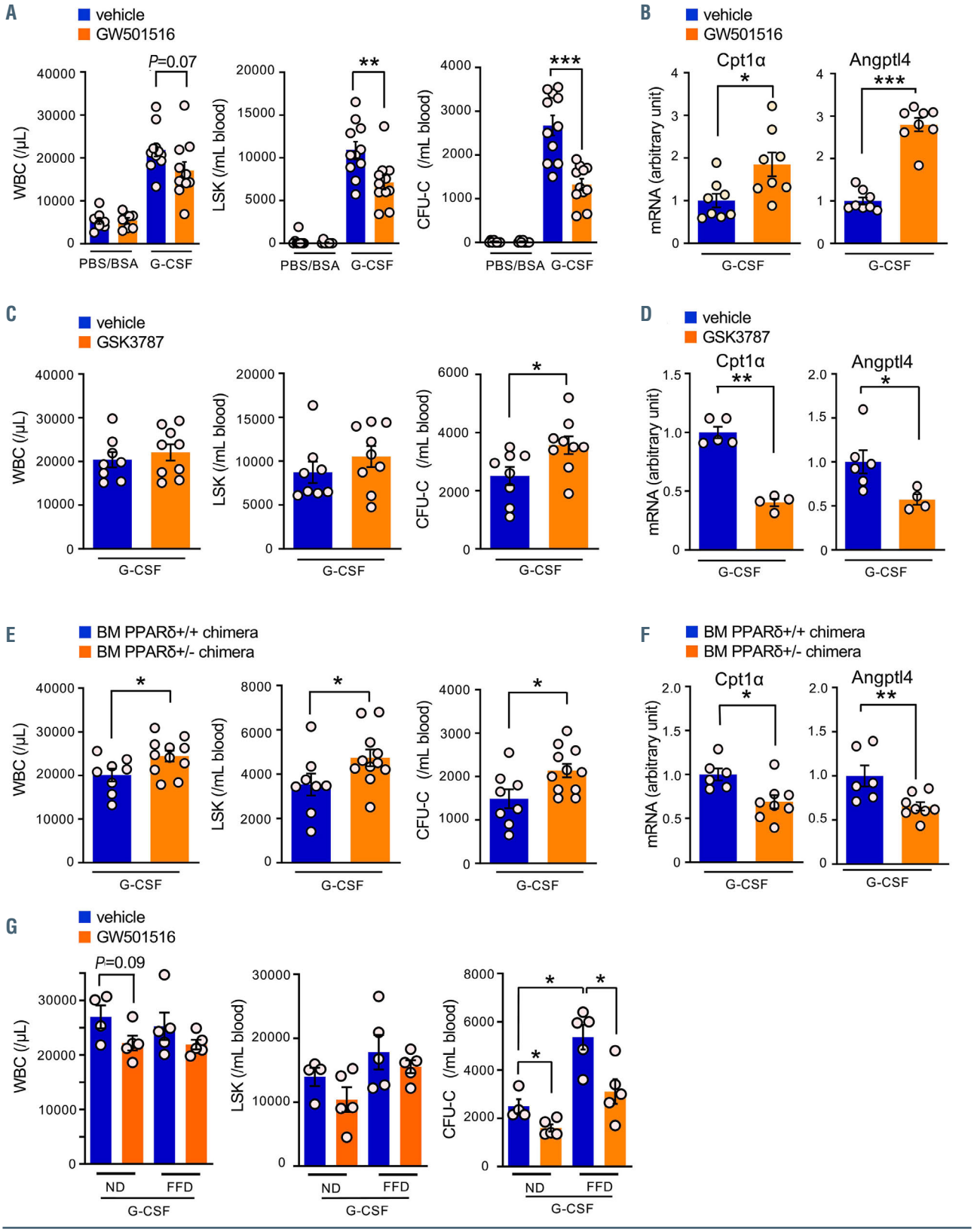

Figure 4. Regulation of granulocyte colony-stimulating factor-induced mobilization by PPARδ signaling. (A, B) Mobilization efficiency modulated by the PPARδ agonist (GW501516) of (A) white blood cells (WBC), lineage$^-$Sca-1$^+$c-kit$^+$ (LSK) cells, and colony-forming units in culture (CFU-C) in the blood (n=8 for the group treated with phosphate-buffered saline [PBS]/bovine serum albumin [BSA] and n=11 for the group treated with granulocyte colony-stimulating factor (G-CSF) and (B) mRNA expression of Cpt1α and Angptl4 in bone marrow (BM) cells (n=8). (C, D) Mobilization efficiency modulated by the PPARδ antagonist (GSK3787) in (C) WBC, LSK, and CFU-C in the blood (n=8-9) and (D) mRNA expression of Cpt1α and Angptl4 in BM cells (n=4-6). (E, F) Mobilization efficiency in BM PPARδ$^{+/-}$ chimeric mice in (E) WBC, LSK, and CFU-C in the blood (n=8-11) and (F) mRNA expression of Cpt1α and Angptl4 in BM cells (n=6-8). (G) Modulation of mobilization (WBC, LSK, and CFU-C) by the PPARδ agonist (GW501516) in mice fed with a normal diet (ND) or a fat-free diet (FFD) (n=4-5). Combined data from at least three independent experiments are shown. Data are mean ± standard error of mean. *P<0.05, **P<0.01, ***P<0.001 (Student t test, analysis of variance, and Mann-Whitney U test).

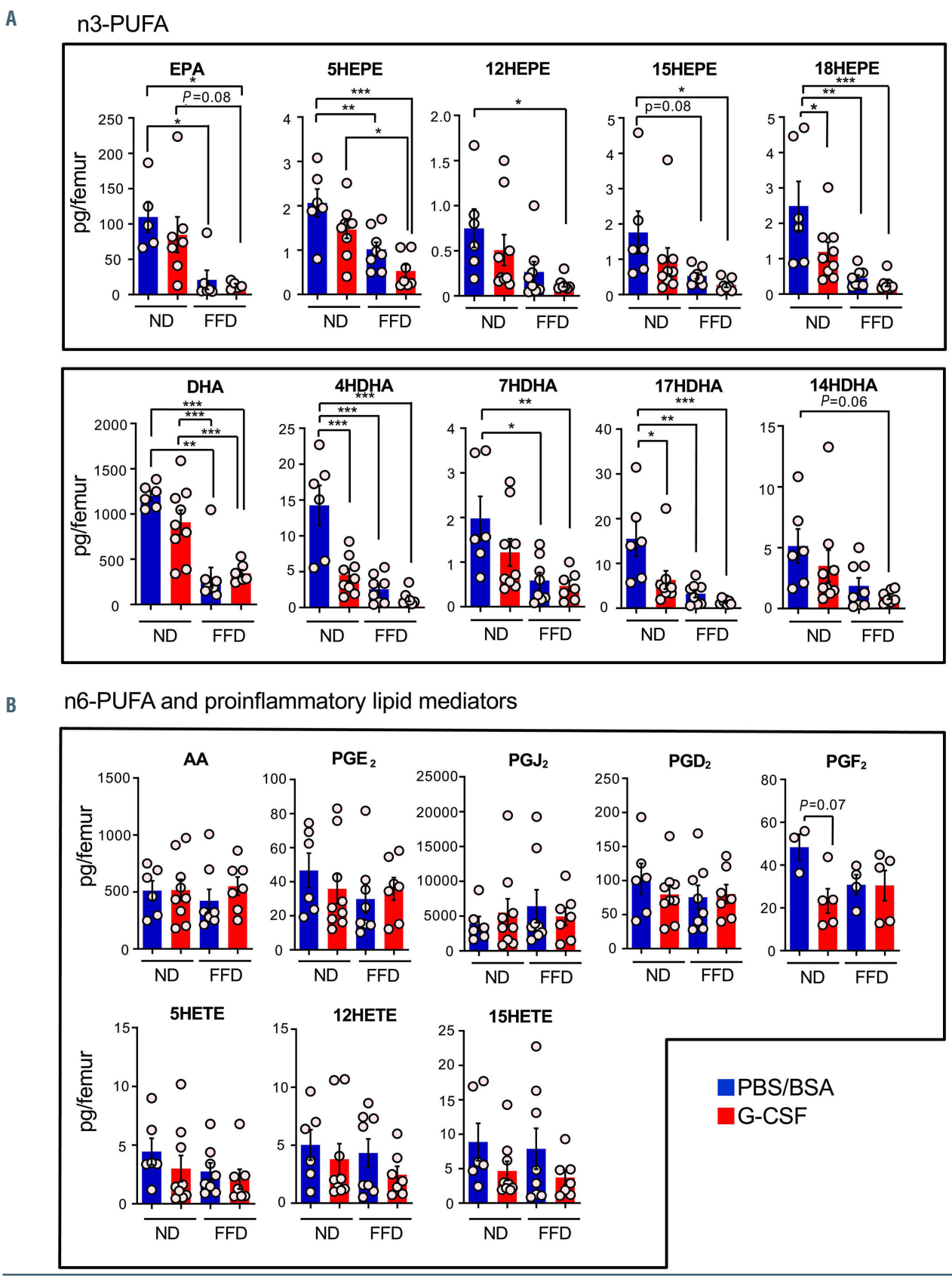

Figure 5. Lipid composition of whole bone marrow during mobilization. Analysis of lipid mediators in whole BM sampled at -20°C with 100% methanol by liquid chromatography and tandem mass spectroscopy after eight doses of phosphate-buffered saline (PBS)/bovine serum albumin (BSA) or granulocyte colony-stimulating factor (G-CSF) in mice fed a normal diet (ND) or fat-free diet (FFD) for representative (A) ω3-fatty acids and (B) ω6-fatty acids (n=3-9). Combined data from at least three independent experiments are shown. Data are mean ± standard error of mean. *P<0.05, **P<0.01, ***P<0.001 (analysis of variance). PUFA: polyunsaturated fatty acids; EPA: eicosapentaenoic acid; HEPE: hydroxyeicosapentaenoic acid; DHA: docosahexaenoic acid; HDHA: hydroxydocosahexaenoic acid; AA: arachidonic acid; PG: prostaglandin, HETE: hydroxy-eicosatetraenoic acid

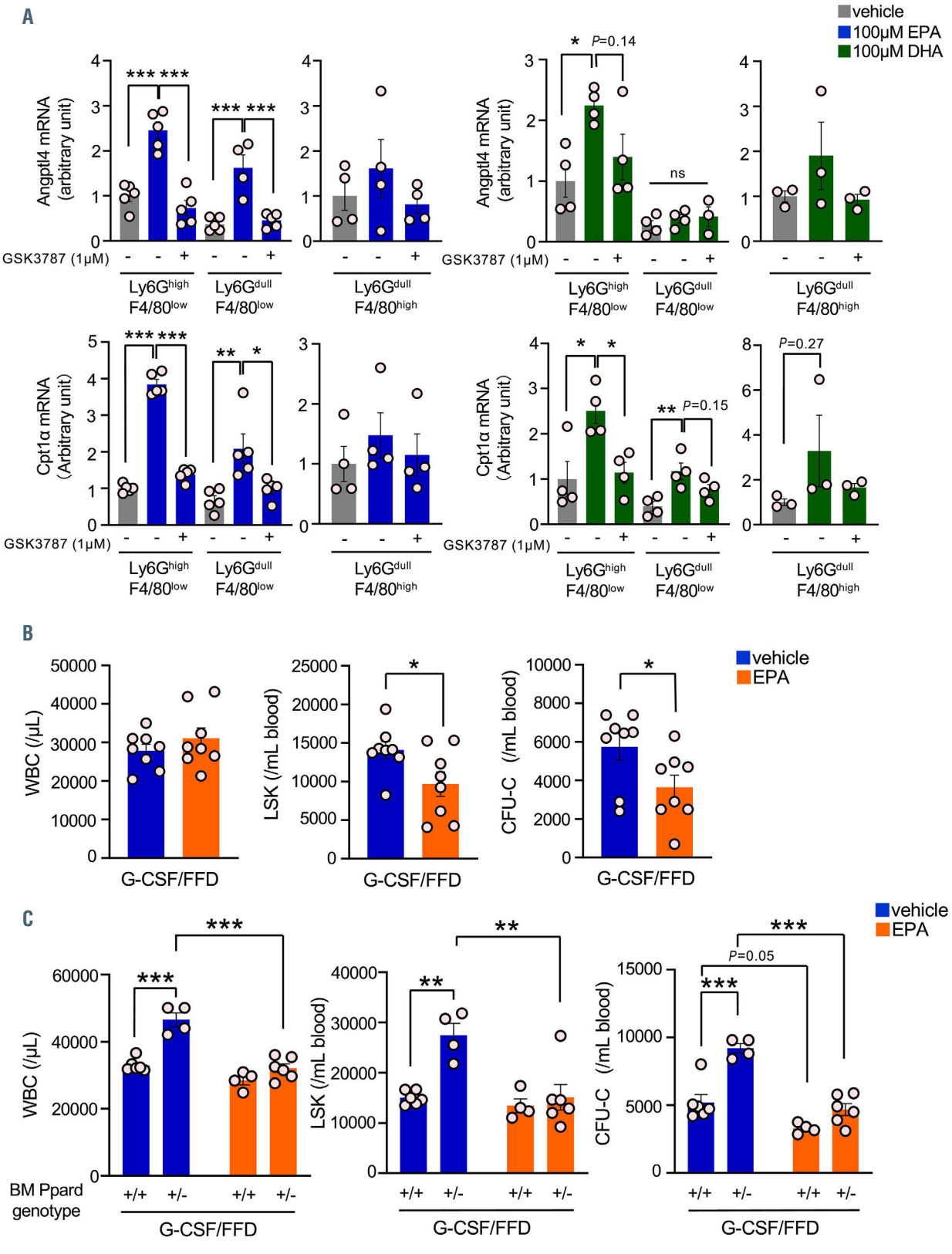

Figure 6. Function of ω3-fatty acids as PPARδ ligands *in vitro* and *in vivo*. (A) Alteration of PPARδ downstream genes by eicosapentaenoic acid (EPA)/docosahexaenoic acid (DHA) in sorted major myeloid populations from steady-state bone marrow (BM) in vitro and its interference by the PPARδ antagonist GSK3787 (n=3-5). (B) Modulation of granulocyte colony-stimulating factor (G-CSF)-induced mobilization by EPA administration in normal mice fed a fat-free diet (FFD) as assessed by white blood cells (WBC), lineage$^-$Sca-1$^+$c-kit$^+$ (LSK) cells and colony-forming units in culture (CFU-C) in the blood (n=8). (C) Modulation of G-CSF-induced mobilization by EPA administration in chimeric mice with PPARδ$^{+/+}$ or PPARδ$^{+/-}$ BM fed a FFD as assessed by WBC, LSK and CFU-C in the blood (n=4-6). The FFD was started 8 weeks after BM transplantation and G-CSF was started 2 weeks after the initiation of the FFD. Combined data from at least three independent experiments are shown. Data are mean ± standard error of mean. *P<0.05, **P<0.01, ***P<0.001 (Student t test and analysis of variance).

for BM PPARδ, is a dietary component able to suppress mobilization.

PPARδ-induced Angptl4 suppresses mobilization

As a downstream molecule of PPARδ signaling, Angptl4 regulates blood vessel permeability leading to the modulation of cell migration, such as tumor metastasis.[23,24] We first confirmed that G-CSF upregulated the level of Angptl4 protein in BM extracellular fluid, which showed a trend of further enhancement following treatment with the PPARδ agonist GW501516 (Figure 7A). In contrast, the level of Angptl4 protein in the blood was not changed by G-CSF treatment (*Online Supplementary Figure S12A*). G-CSF treatment together with GW501516 significantly increased Angptl4 mRNA expression in BM myeloid cells (*Online Supplementary Figure S12B*). The analysis of BM samples from mice used in mobilization experiments with the FFD and GW501516, as shown in Figure 4G, revealed that Angptl4 and Cpt1α mRNA levels in BM after G-CSF were decreased by the FFD but greatly increased by GW501516 treatment (*Online Supplementary Figure S12C*). Thus, the induction and suppression of Angptl4 mRNA expression in BM were likely associated with the suppression and enhancement of mobilization, respectively.

Indeed, this increase in Angptl4 protein in BM caused by G-CSF inhibited mobilization, because administration of the anti-Angptl4 neutralizing antibody (3F4F5)[25] significantly increased mobilization efficiency, as assessed by CFU-C, with a similar, but not statistically significant, trend of increased LSK cell mobilization (Figure 7B; *Online Supplementary Figure S12D*). BM vascular permeability, as assessed by Evans blue dye incorporation in BM, was decreased by GW501516 and/or G-CSF (*Online Supplementary Figure S13*), and significantly enhanced by the addition of the anti-Angptl4 antibody to G-CSF (Figure 7C), suggesting that Angptl4 may inhibit mobilization by, at least partially, suppressing BM vascular permeability. Therefore, these results suggest that Angptl4, produced mainly by BM neutrophils and their precursors via PPARδ signaling, inhibits G-CSF mobilization.

Discussion

The functions of BM as a reservoir and consumer of orally ingested fat have not been thoroughly studied. In this study, we have demonstrated that BM fat is strongly influenced by diet. In particular, ω3-PUFA and their derivatives are almost exhausted by a 2-week restriction of fat contents in food. BM myeloid cells such as neutrophils and their precursors have a strong demand for ω3-PUFA, including EPA, which acts, at least partially, as a PPARδ ligand to suppress HSPC mobilization via Angptl4 production. A widely variable mobilization efficiency in response to G-CSF in healty individuals, including a certain percentage of poor mobilizers, might partially originate from the BM fat profile in association with oral fat intake. Although it is not clear whether these findings in mice are applicable to mobilization in humans, the modulation of dietary fat might be a potential strategy to reduce the risk of poor mobilizers which could be examined in a future clinical study.

In this study, we demonstrated that neutrophils and their precursors, which are the major populations in BM, are strong consumers of ω3-PUFA, particularly after G-CSF treatment. It was reported that dietary ω3-PUFA are rapidly incorporated into phospholipids, such as phosphatidylethanolamine and phosphatidylcholine, of human neutrophils[26] and inhibit these cells' inflammatory responses, such as leukotriene B_4 production and chemotaxis.[27,28] However, the signaling receptor for ω3-PUFA in this pathway is unclear. It was reported that certain ω6-PUFA, 15d-PGJ_2, acted as ligands for PPARγ to inhibit neutrophil chemotaxis by upregulating the sepsis-induced cytokines tumor necrosis factor-α and interleukin-4.[29] Interestingly, a biochemical study has shown that 15d-PGJ_2 can also stimulate PPARδ to a similar magnitude as EPA.[21] Based on our study in BM neutrophils and the reported strong interaction of EPA with PPARδ,[21,30] neutrophils in circulation may also partially utilize ω3-PUFA as PPARδ ligands to diminish inflammation. EPA is also reported to prevent neutrophil migration across the endothelium as a supplier of PGD_3, which antagonizes PGD receptor DP-1 on neutrophils.[31] This pathway might be one of the PPARδ-independent EPA functions in the suppression of mobilization. In our current study, BM lipid mediators were assessed after eight doses of G-CSF, and the transition during the G-CSF treatment was not evaluated. Although no change was observed in BM ω6-PUFA after eight doses of G-CSF, we have previously reported that the level of BM PGE_2 was increased after four doses.[11] These data are consistent with the transition of body temperature during G-CSF treatment, which increased after four doses and returned to normal levels at eight doses.[11] Thus, the contribution of BM ω6-PUFA in mobilization cannot be excluded from the current study.

The signaling partners of the various PPAR are retinoid X receptors (RXR). PPAR-RXR are permissive heterodimers that can be activated by either PPAR ligands or RXR ligands.[32] It was reported that RXR is activated during G-CSF-induced granulopoiesis. The synthetic RXR agonist bexarotene enhanced G-CSF-induced mobilization of neutrophils and CFU-C, but not of LSK cells, in circulation.[20] In contrast, PPARδ ligands in our study suppressed the mobilization of both LSK cells and CFU-C. This difference may be because apo-PPARδ, i.e., the absence of ligand, has been shown to reside on DNA or function as a transrepressor, unlike RXR.[33] It is also possible that RXR may not be a major signaling partner of PPARδ in BM neutrophils and their precursors with ω3-PUFA as PPARδ ligands. Indeed, the promyelocytic leukemia-PPARδ signaling pathway is important for HSC maintenance through the regulation of fatty acid oxidation and asymmetric division.[34] Although the contribution of PPAR is not clear, a very high level of fatty acids is the critical component for the *ex vivo* maintenance of HSC.[35] Thus, a continuous supply of fatty acids from the food is critically important for the maintenance of BM hematopoiesis in several different ways. BM in patients with anorexia nervosa commonly displays hypoplasia with gelatinous transformation.[36,37] This may be partially due to the lack of oral intake of fatty acids, including ω3-PUFA as PPARδ ligands.

Hematopoietic Angptl4 deficiency in hyperlipidemic mice causes leukocytosis,[38] which suggests a potential role of Angptl4 from hematopoietic cells in the cells' intravasation from the BM cavity into the circulation. Angptl4 is known to have two major distinct roles. First, the N-terminal coiled-coil region (nAngptl4) regulates lipoprotein lipase leading to the control of lipid metabolism, insulin

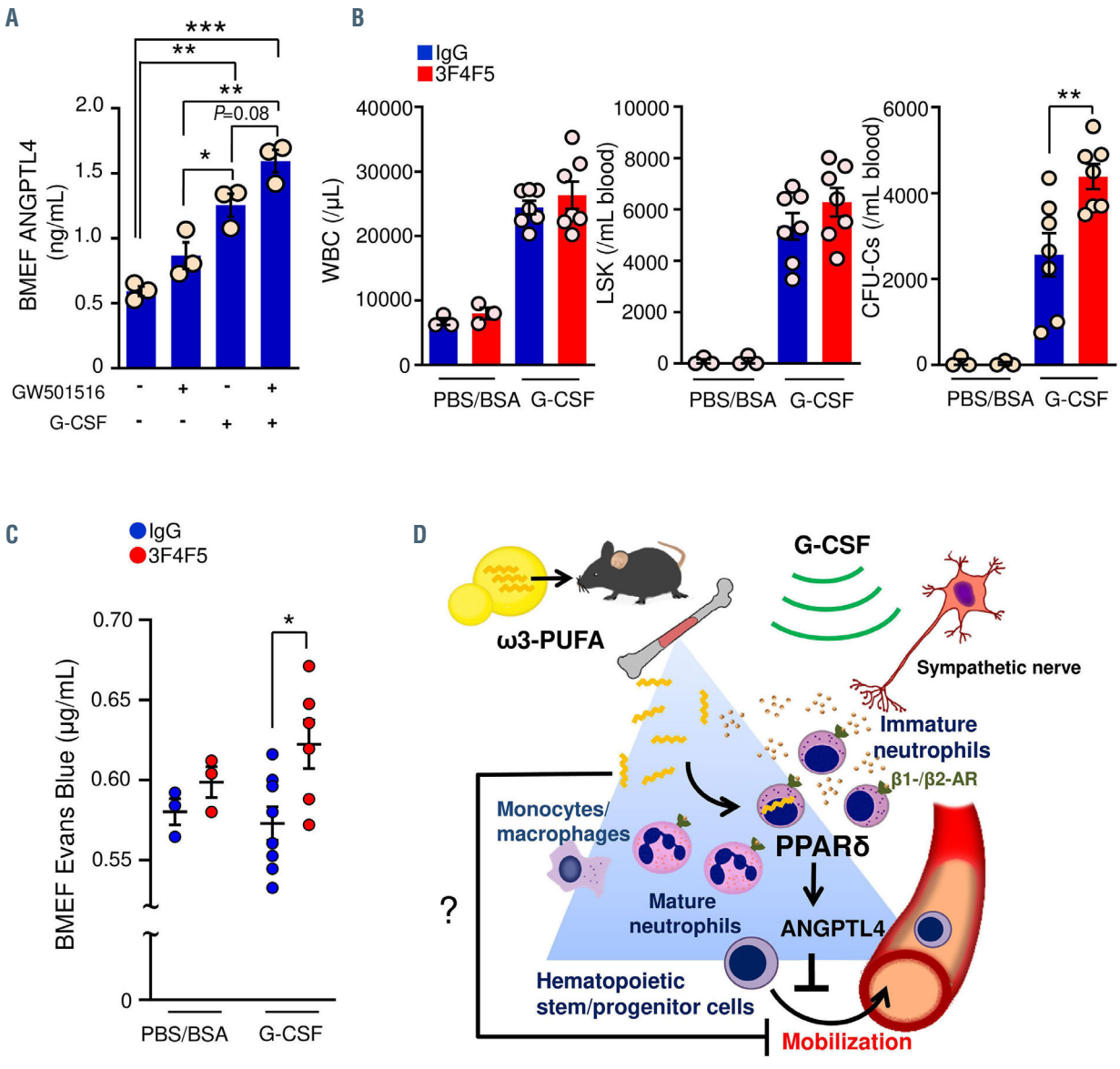

Figure 7. Angptl4 in bone marrow as a negative regulator of mobilization. (A) Angptl4 protein level in bone marrow (BM) extracellular fluid (BMEF) during granulocyte colony-stimulating factor (G-CSF)-induced mobilization with or without the PPARδ agonist GW501516 (n=3). (B) G-CSF-induced mobilization in mice treated with the anti-Angptl4 antibody as assessed by white blood cells (WBC), lineage$^-$Sca-1$^+$c-kit$^+$ (LSK) cells and colony-forming units in culture (CFU-C) in the blood (n=3 for group treated with phosphate-buffered saline [PBS]/bovine serum albumin [BSA] and n=7 for group treated with G-CSF). (C) BM vascular permeability assessed by Evans blue dye concentration in BMEF during G-CSF mobilization with or without the anti-Angptl4 antibody (n=3-8). Combined data from at least three independent experiments are shown. Data are mean ± standard error of mean. *P<0.05, **P<0.01, ***P<0.001 (Student t test and analysis of variance). (D) Schematic representation of the proposed role of dietary fat and PPARδ in G-CSF-induced mobilization. High sympathetic tone induced by G-CSF stimulates β_1/β_2-adrenergic receptors (β_1/β_2 AR) to upregulate PPARδ expression in BM myeloid cells. Dietary fatty acid ligands, such as ω3-poyunsaturated fatty acids (PUFA), bind to PPARδ and promote Angptl4 expression to suppress the mobilization via, at least partially, inhibition of BM vascular permeability. The PPARδ-independent pathway of ω3-PUFA to inhibit mobilization may also exist.

sensitivity, and glucose homeostasis. Second, the regulation of angiogenesis and vascular permeability is mediated by the COOH-terminal fibrinogen-like domain (cAngptl4).[23,24] Among these effects, the regulation of vascular permeability seems to be the most relevant with respect to G-CSF-induced mobilization. The role of Angptl4 in regulating vascular permeability is context-dependent. Early studies suggested that Angptl4, although it was not shown whether cAngptl4 was used, decreased the leak of dye or extravasation of melanoma cells.[39,40] In contrast, the promotion of vascular permeability and tumor metastasis by Angptl4 was reported in a breast tumor model.[41] Mechanistically, cAngptl4 was shown to activate $\alpha_5\beta_1$ integrin and subsequently decluster VE-cadherin and claudin-5 in primary human microvascular endothelial cells, leading to the induction of vascular leakiness and metastasis in a melanoma model.[42] Angptl4-mediated increased vascular leakiness was also reported in nontumor pathological models such as influenza pneumonia and diabetic macular edema.[25,43] It was also reported that altered post-translational modification, such as decreased sialylation, can augment the leakiness of the kidney glomerular epithelium.[44] In our study, Angptl4 inhibition led to increased BM vascular permeability and

increased trafficking of HPC from the BM cavity into the circulation. In addition to the consequences of using different models, proteolytic processing and post-translational modifications of Angptl4 may occur differently in each organ and each type of producer and effector cell, resulting in widely variable results.

BM is tightly regulated by the SNS, and a major step for HSPC mobilization by G-CSF is the strong suppression of osteolineage cells, such as osteoblasts and osteocytes, via β_2-AR stimulation by catecholamines.[7-9] G-CSF stimulation of sympathetic nerves inhibits the reuptake of released catecholamines at the synapse,[10] leading to hypersympathetic tone in the BM. We have previously shown that BM neutrophils express all β_1-, β_2-, and β_3-AR and that the selective β_3-AR agonist activates the arachidonic acid cascade to increase PGE_2 production to protect osteoblast function.[11] In this study, induction of PPARδ mRNA and protein by SNS signals was mainly through β_1/β_2-AR in mature/immature neutrophils. The β_1/β_2-AR-PPARδ/ω3-PUFA-Angptl4 pathway in BM myeloid cells counteracts the alteration of the BM microenvironment and suppresses mobilization upon G-CSF-induced marrow inflammation (Figure 7D). Our study has shed light on oral fat as an important regulator of interorgan communication between the nervous and hematopoietic systems.

Disclosures

No conflicts of interest to disclose.

Contributions

TS performed all the experiments and wrote the manuscript; SI, YKaw, KW, HK, AS, and KM helped with animal maintenance and tissue sample preparation; MS and TF performed the bone marrow lipid analysis; MH and ST supervised the study of PPARδ-deficient mice; NST supervised the study with anti-Angptl4 antibody; TM supervised all experiments; and YKat supervised all experiments and wrote the manuscript.

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