Molecular Nutrition & Food Research
www.mnf-journal.com

MUFAs in High-Fat Diets Protect against Obesity-Induced Bias of Hematopoietic Cell Lineages

*Ana Lemus-Conejo, Mayte Medrano, Sergio Lopez, Maria C. Millan-Linares, Maria A. Rosillo, Jose A. Perez-Simon, Francisco J. G. Muriana, and Rocio Abia**

Scope: The role of dietary fatty acids in the generation of bone marrow (BM) immune cells and their trafficking to extramedullary compartments in the obesity is not yet fully understood.

Methods and Results: C57BL/6J mice are randomly assigned to isocaloric high-fat diets (HFDs) formulate with dietary fats rich in saturated fatty acids (SFAs), monounsaturated fatty acids (MUFAs) or MUFAs fortified with eicosapentaenoic and docosahexaenoic acids for 20 weeks, followed by profiling of the obese metabolic phenotype and immunophenotypic features of immune cells in blood, spleen, and BM. All HFDs induce an obese phenotype, but it becomes largely less disruptive after the HFDs are enriched in MUFAs, which also induce signs of granulopoiesis and an expansion of long-term hematopoietic stem and granulocyte-macrophage progenitor cells in BM. In contrast, a HFD enriched in SFAs disturbs the fitness of medullary lymphocytes and promotes monopoiesis in favor of pro-inflammatory activated subsets.

Conclusion: The reshaping of the fatty acid pools with MUFAs from the diet serves to manipulate the generation and trafficking of immune cells that are biased during obesity. These findings reveal a novel strategy by which dietary MUFAs may be instrumental in combating HFD-induced dysfunctional immune systems.

1. Introduction

The increase in weight and adipose tissue is known as a main driver of overweight and obesity, which result from societal factors, among the most common being the consumption of high-fat diets (HFDs) that are typically rich in saturated fatty acids (SFAs).[1] The high risk of accompanying cardiovascular comorbid disorders remains a major concern for patients who suffer from overweight or obesity.[2] In a provocative way, unlike focusing on the quantitative reduction in energy intake to induce weight loss, growing evidence supports that qualitative changes without energy restriction in a HFD may be an additional choice in modern times in which high-fat foods are readily available and accessible and are preferred foods in many communities. An illustrative example is the European randomized controlled trial, the PREDIMED (Prevención con Dieta Mediterránea) study, showing that a dietary pattern with an intake that reaches and even exceeds 40% of energy from fats can decrease the occurrence of myocardial infarction, stroke, and cardiovascular death.[3] In this trial, the Mediterranean-style diet was supplemented with additional olive oil as the main source of fat, which agrees with the potential relevance of the monounsaturated fatty acid (MUFA) oleic acid to prevent the development of cardiovascular risk[4] and to manage obesity, as shown in a recent systematic review.[5] Moreover, long-chain ω-3 polyunsaturated fatty acids (PUFAs), including eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), have been

Dr. A. Lemus-Conejo, Dr. S. Lopez, Dr. M. A. Rosillo, Dr. F. J. G. Muriana, Dr. R. Abia
Laboratory of Cellular and Molecular Nutrition
Instituto de la Grasa
The Spanish National Research Council (CSIC)
Seville 41013, Spain
E-mail: abia@ig.csic.es

The ORCID identification number(s) for the author(s) of this article can be found under https://doi.org/10.1002/mnfr.202001203

DOI: 10.1002/mnfr.202001203

Dr. M. Medrano, Dr. J. A. Perez-Simon
Department of Haematology
Instituto de Biomedicina de Sevilla (IBiS/CSIC/CIBERONC)
Hospital Universitario Virgen del Rocio
University of Seville
Seville 41012, Spain

Dr. S. Lopez
Department of Cell Biology
Faculty of Biology
University of Seville
Seville 41012, Spain

Dr. S. Lopez
Instituto de Biomedicina de Sevilla (IBiS/CSIC)
Hospital Universitario Virgen del Rocio
University of Seville
Seville 41012, Spain

Dr. M. C. Millan-Linares
Cell Biology Unit
Instituto de la Grasa
CSIC
Seville 41013, Spain

documented to revert cardiovascular and metabolic abnormalities in overweight and obese humans[6] and in mice that continued to consume a HFD.[7]

Cardiovascular complications due to inflammation and cellular immunity abnormalities in obesity are different sides of the same coin. Obesity is indeed linked with changes in the bone marrow (BM), leading to imperfect innate and adaptive immune responses.[8] In BM, immune cells arise from hematopoietic stem cells (HSCs), giving rise to hematopoiesis in all blood cell types. Marginal pools of immune cells capable of mediating innate and adaptive immunity can also be found in extramedullary tissues such as the spleen.[9] The homeostasis of HSCs in the BM niche is essential for normal long-term hematopoiesis and the regular abundance of selective mature hematopoietic populations in blood and spleen, the disruption of which may lead to loss of health.[10] An important overlooked point is that most of the HFDs that have been used to model the immune system during HFD-induced obesity were largely formulated with lard and occasionally supplemented with relatively high amounts of cholesterol [Refs. [11–21] give some examples]. On the one hand, lard is often confused as being high in saturated fat, but it is not exempt from variability and frequently contains a balanced composition of SFAs and MUFAs and even of PUFAs.[22–24] On the other hand, cholesterol interferes with normal hematopoiesis.[25] Therefore, it makes it difficult to discern the role of dietary SFAs, MUFAs, and PUFAs on the BM and the versatile production of cells in the obesity, which is an issue to be clarified.

In this study, we investigated the impact of three isocaloric HFDs enriched in anhydrous butter (a source of SFAs, mainly palmitic acid) (HFD-SFAs), olive oil (a source of MUFAs, mainly oleic acid) (HFD-MUFAs) or olive oil plus a dose of EPA and DHA (a source of MUFAs fortified with long-chain ω-3 PUFAs) (HFD-MUFAs+EPA+DHA) on HSC maturation in the BM niche and the abundance of mature hematopoietic populations in the blood and spleen in wild-type C57BL/6J mice. All the HFDs contained less than 0.01% cholesterol. Our findings illustrate a contrasting landscape of hematopoietic cellular architecture in these compartments, with HFDs enriched in MUFAs triggering a profile characteristic of an expansion of long-term hematopoietic stem cells (LT-HSCs) and granulocyte-macrophage progenitor (GMP) cells in BM and signs of granulopoiesis, in contrast with the HFD enriched in SFAs that disturbed the fitness of lymphocytes in the BM and promoted monopoiesis in favor of pro-inflammatory activated subsets.

2. Results

2.1. Effects of Fatty Acids in HFDs on the Obese Phenotype

As shown in Table S2, Supporting Information, the principal fatty acid in HFD-SFAs was palmitic acid (16:0, >30% of total fatty acids), and the percentage of total SFAs was close to 60%. HFD-MUFAs and HFD-MUFAs+EPA+DHA were primarily composed of oleic acid (18:1ω-9, by ≈70%), but the latter also contained 1.3% EPA (20:5ω-3) and 1% DHA (22:6ω-3). We found no changes in feeding habits, with similar food and water intakes in mice on either the low-fat diet (LFD) or HFDs (data not shown). Compared to the LFD, all of the HFDs induced an increase ($p < 0.001$ vs LFD) in body mass, but this was higher ($p < 0.001$) after HFD-SFAs (+81%) than after HFD-MUFAs (+31%) and HFD-MUFAs+EPA+DHA (+24%) at the end of the 20-week dietary regimen (Table S5, Supporting Information). In a similar fashion, the mass of eWAT, which is predictive of obesity-related co-morbidities[26] and low bone mass,[27] was increased in mice upon exposure to HFDs, and it was higher after HFD-SFAs than after HFD-MUFAs and HFD-MUFAs+EPA+DHA (Table S5, Supporting Information). Mice fed HFDs also developed dyslipidaemia characterized by an increase in the levels of total cholesterol and triglycerides, with HFD-SFAs again exerting the strongest effects (Table S5, Supporting Information). In eWAT, we detected the mRNA expression of genes involved in inflammation (interleukin 6 [*Il6*] in the infiltration of monocyte-derived macrophages[28]; neutrophil elastase [*Elane*] as a marker of neutrophil recruitment[29]; and CCAAT/enhancer-binding protein beta [*Cebpb*] as a regulator of adipocyte differentiation but also of dietary fatty acid-mediated inflammation[30] in adipose depots), adipogenesis (peroxisome proliferator-activated receptor gamma [*Pparg*][31]) and thermogenesis (uncoupling protein 1 [*Ucp1*] and iodothyronine deiodinase 2 [*Dio2*] as markers of "beige" adipocytes[32,33]) by real-time PCR. Mice fed HFDs had increased expression levels of the *Il6* gene, but these levels were lower after feeding HFDs enriched in MUFAs (**Figure 1**A). The *Elane* gene was dramatically repressed after HFDs enriched in MUFAs (Figure 1B). The *Cebpb* gene was also upregulated after HFD-SFAs but downregulated after HFDs enriched in MUFAs compared to the LFD (Figure 1C). Mice fed HFDs had decreased expression levels of the *Pparg* (Figure 1D) and *Ucp1* (Figure 1E) genes, which were lower after HFD-SFAs than after HFDs enriched in MUFAs. The *Dio2* gene was also downregulated after HFD-SFAs, but its expression remained unchanged after HFDs were enriched in MUFAs (Figure 1F). No significant differences were observed between HFD-MUFAs and HFD-MUFAs+EPA+DHA. Therefore, long-term administration of HFDs may induce obesity, adipose inflammation, and dyslipidaemia in wild-type C57BL/6J mice; however, this obese phenotype is characterized by significant, minor metabolic perturbations if the HFD is enriched in MUFAs in place of SFAs.

2.2. Effects of Fatty Acids in HFDs on the Principal Composition of Free Fatty Acids in BM Extracellular Fluid

As shown in Figure S2, Supporting Information, total MUFAs were increased in BM extracellular fluid of mice on the HFD-MUFAs and HFD-MUFAs+EPA+DHA, with no differences between these groups, while the proportion of total SFAs and PUFAs remained similar after any diet. The ratios of palmitic acid to oleic acid and SFAs to MUFAs in this compartment were lower after HFD-MUFAs and HFD-MUFAs+EPA+DHA than after HFD-SFAs or LFD. EPA and DHA were not detected. Details of the protocol can be found in the Supplemental Material.

2.3. Effects of Fatty Acids in HFDs on Mature Hematopoietic Lineage Cells

A panel of antibodies and the gating strategy illustrated in **Figure 2** were used for immunophenotyping blood lymphoid and myeloid subsets by flow cytometry. It was first

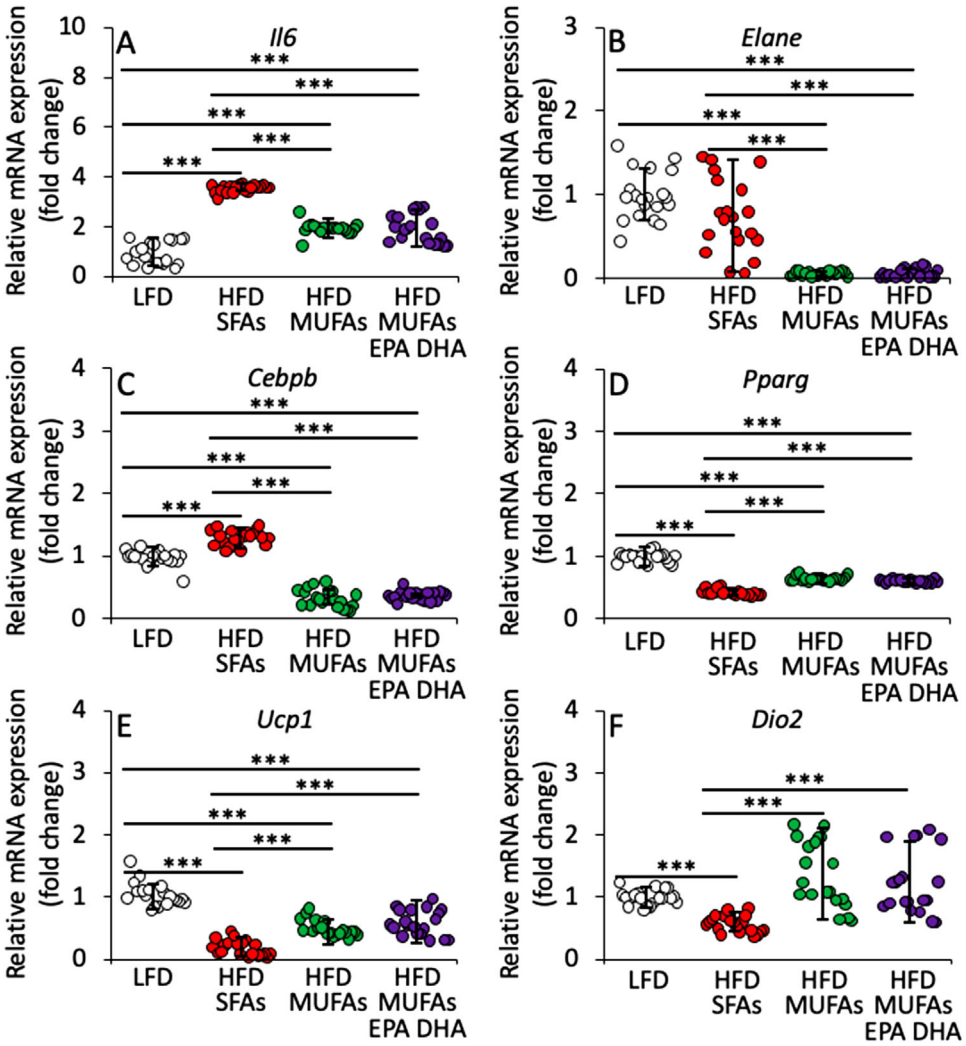

Figure 1. High-fat diets enriched in SFAs (anhydrous butter, HFD-SFAs), MUFAs (olive oil, HFD-MUFAs), or MUFAs (olive oil) fortified with EPA and DHA (HFD-MUFAs+EPA+DHA) on obese phenotype in wild-type C57BL/6J mice. Reverse transcription-quantitative PCR analysis of key hub genes (A) *Il6*, (B) *Elane*, and (C) *Cebpb* involved in inflammation, (D) *Pparg* involved in adipogenesis, (E) *Ucp1*, and (F) *Dio2* involved in thermogenesis in epididymal white adipose tissue (eWAT) at the end of dietary regimens. Values are individual data points ±SD; n = 20/group. Differences between groups were assessed by two-way ANOVA with Tukey's post hoc test. ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$ (compared to all other study groups and as indicated).

demonstrated by blood count assays that HFDs enriched in MUFAs, with no difference between HFD-MUFAs and HFD-MUFAs+EPA+DHA, increased the numbers of white blood cells (**Figure 3**A) and neutrophils (Figure 3B), while the numbers of lymphocytes (Figure 3C) and monocytes (Figure 3D) remained unchanged in the peripheral blood of all four groups of animals. It was also found that HFDs increased the subpopulation of B cells ($CD45^+B220^+$ cells) (Figure 3E) and that only HFD-SFAs reduced the subpopulation of T cells ($CD45^+CD3e^+$ cells) (Figure 3F). In addition, the fraction of $Ly6C^{Hi}$ monocytes ($CD45^+B220^-CD3e^-CD11b^+Ly6G^-Ly6C^{Hi}$ cells) was increased with HFD-SFAs but decreased with HFD-MUFAs+EPA+DHA (Figure 3G), and the fraction of $Ly6C^{Lo}$ monocytes ($CD45^+B220^-CD3e^-CD11b^+Ly6G^-Ly6C^{Lo}$ cells) remained unaffected (Figure 3H) by flow cytometry analysis. None of the HFD regimens induced significant changes compared with LFD on the numbers of red blood cells (Figure S3A, Supporting Information), hemoglobin (Figure S3B, Supporting Information), hematocrit (Figure S3C, Supporting Information), mean corpuscular volume (Figure S3D, Supporting Information), mean corpuscular hemoglobin (Figure S3E, Supporting Information), red cell distribution width (Figure S3F, Supporting Information), platelets (Figure S3G, Supporting Information), or mean platelet volume (Figure S3H, Supporting Information).

By differential expression of CD45 to identify the infiltration of BM cells into the spleen as a major extramedullary reservoir for storage and deployment of mature hematopoietic lineage cells[9] and following a similar gating strategy to that used for blood (Figure S4, Supporting Information), no significant differences in live $CD45^+$ cells in the spleen of mice fed HFDs were observed, but these values were lower than those in the spleen

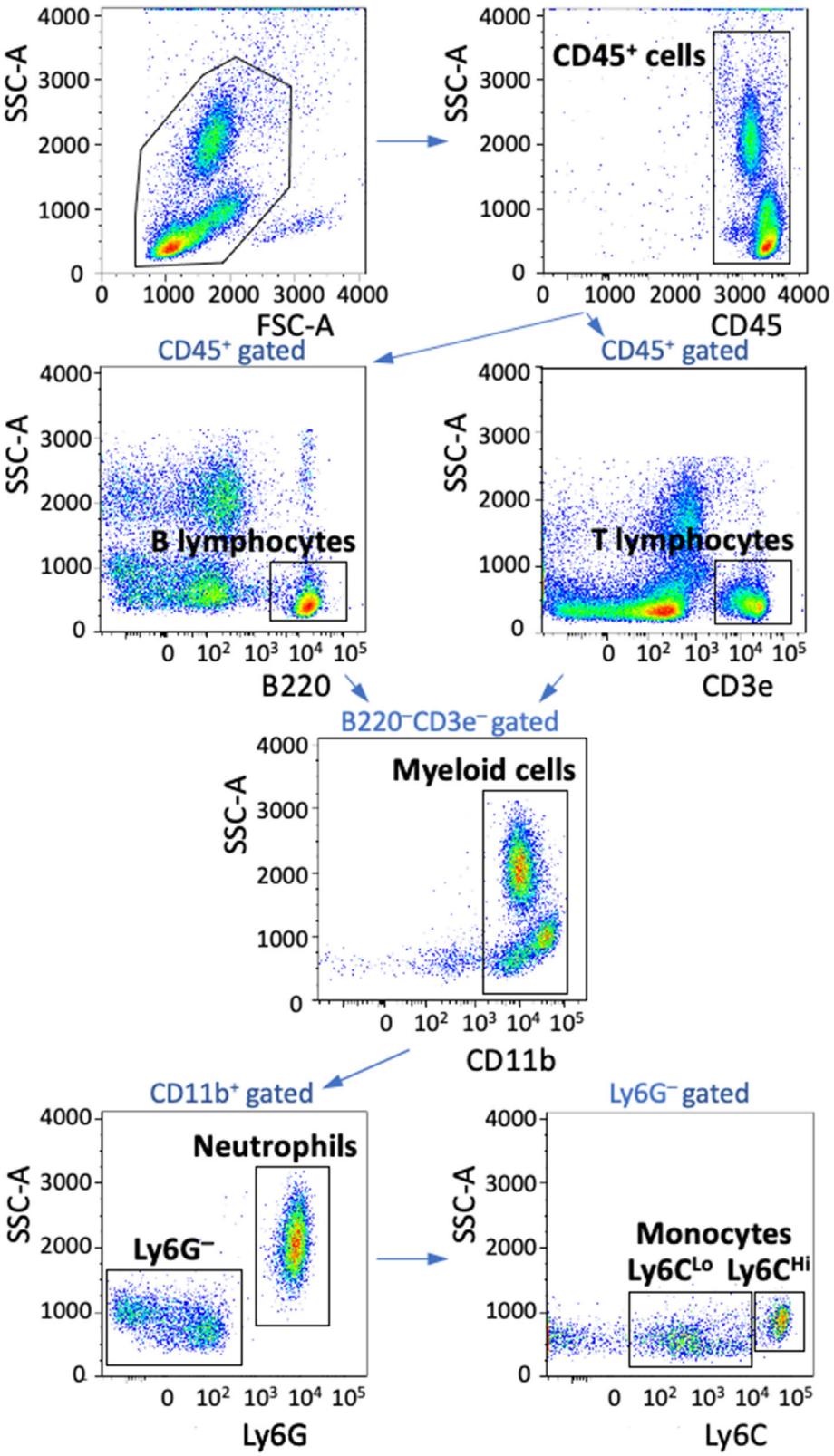

Figure 2. An example of the gating strategy used to identify B ($CD45^+B220^+$) and T ($CD45^+CD3e^+$) cells, and $Ly6C^{Hi}$ ($CD45^+B220^-CD3e^-CD11b^+Ly6G^-Ly6C^{Hi}$) and $Ly6C^{Lo}$ ($CD45^+B220^-CD3e^-CD11b^+Ly6G^-Ly6C^{Lo}$) monocytes in peripheral blood of wild-type C57BL/6J mice. $CD45^+$ viable cells were first individualized by crossing the singlet gate. CD, cluster of differentiation; FSC-A, forward scatter area; SSC-A, side scatter area.

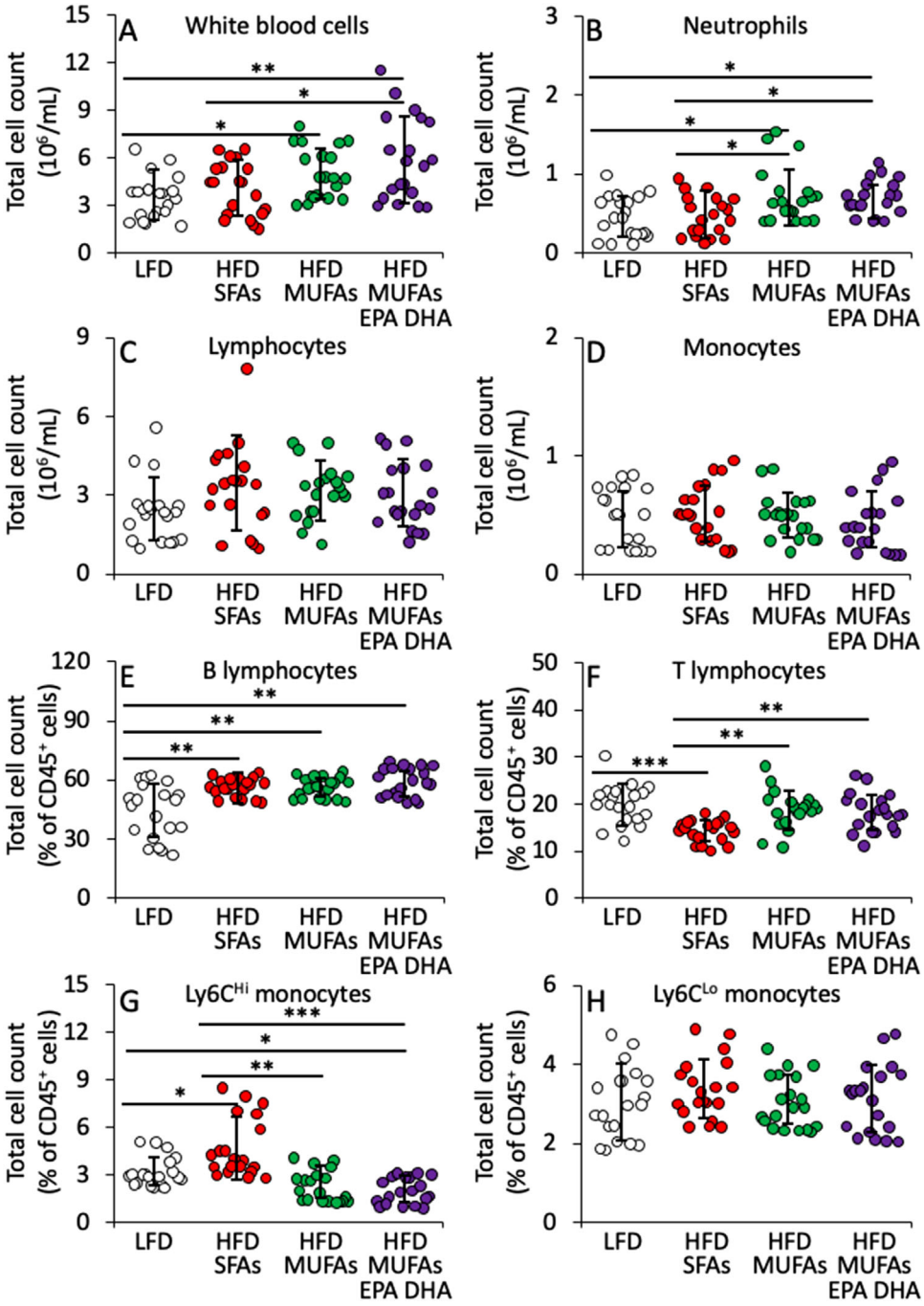

Figure 3. High-fat diets enriched in SFAs (anhydrous butter, HFD-SFAs), MUFAs (olive oil, HFD-MUFAs), or MUFAs (olive oil) fortified with EPA and DHA (HFD-MUFAs+EPA+DHA) on mature hematopoietic lineage cells in peripheral blood of wild-type C57BL/6J mice. The number of (A) white blood cells, (B) neutrophils, (C) lymphocytes, and (D) monocytes by blood count assays, and the percentages of (E) B lymphocytes, (F) T lymphocytes, (G) Ly6C^{Hi} monocytes, and (H) Ly6C^{Lo} monocytes by flow cytometry at the end of 20-week dietary regimens. Viable cells (7-AAD$^-$Annexin V$^-$) were only considered. Values are individual data points ± SD; n = 20/group. Differences between groups were assessed by two-way ANOVA with Tukey's post hoc test. ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$ (compared to all other study groups and as indicated).

of mice fed the LFD (**Figure 4**A). HFDs enriched in MUFAs increased splenic neutrophils (Figure 4B) and Ly6C^{Hi} monocytes (Figure 4C), while HFD-SFAs decreased splenic B cells (Figure 4D), and all HFDs decreased splenic T cells (Figure 4E) relative to CD45$^+$ cells. This change in the cellular composition seen in the spleen of mice on HFD-SFAs was associated with increased levels of total cell death (+14%, $p < 0.05$). Furthermore, the spleen weights of mice on HFD-SFAs were higher than those of mice on the LFD or the HFDs enriched in MUFAs (Figure S5A, Supporting Information). The increase in spleen

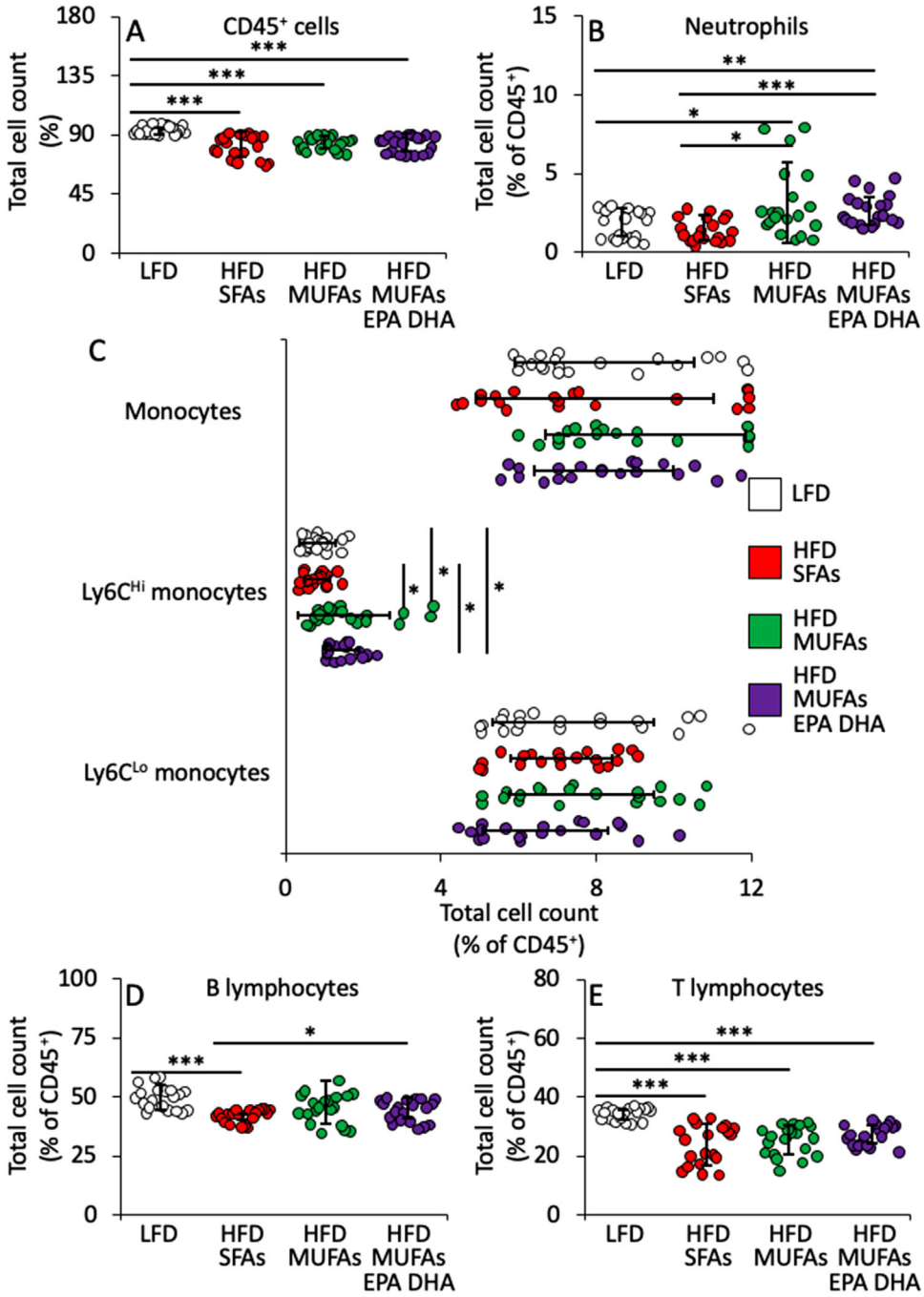

Figure 4. High-fat diets enriched in SFAs (anhydrous butter, HFD-SFAs), MUFAs (olive oil, HFD-MUFAs) or MUFAs (olive oil) fortified with EPA and DHA (HFD-MUFAs+EPA+DHA) on mature hematopoietic lineage cells in spleen of wild-type C57BL/6J mice. The percentages of (A) $CD45^+$ cells and those relative to $CD45^+$ cells of (B) neutrophils, (C) monocytes and $Ly6C^{Hi}$ and $Ly6C^{Lo}$ monocyte subsets, (D) B lymphocytes, and (E) T lymphocytes at the end of 20-week dietary regimens. Viable cells (7-AAD$^-$Annexin V$^-$) were only considered. Values are individual data points ± SD; n = 20/group. Differences between groups were assessed by two-way ANOVA with Tukey's post hoc test. ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$ (compared to all other study groups and as indicated).

enlargement was previously shown to occur in mice after HFDs enriched in SFAs.[34] However, when the spleen weight was normalized to body weight (Figure S5B, Supporting Information), the spleen-to-body-weight ratios remained the same for all four groups of animals (Figure S5C, Supporting Information).

These findings prompted us to further explore mature lymphoid and myeloid cells in BM. The gating strategy, similar to that used for blood and spleen, is shown in Figure S6, Supporting Information. It was first observed that the occurrence of total B and T cells (as a percentage of all BM cells) was similar among

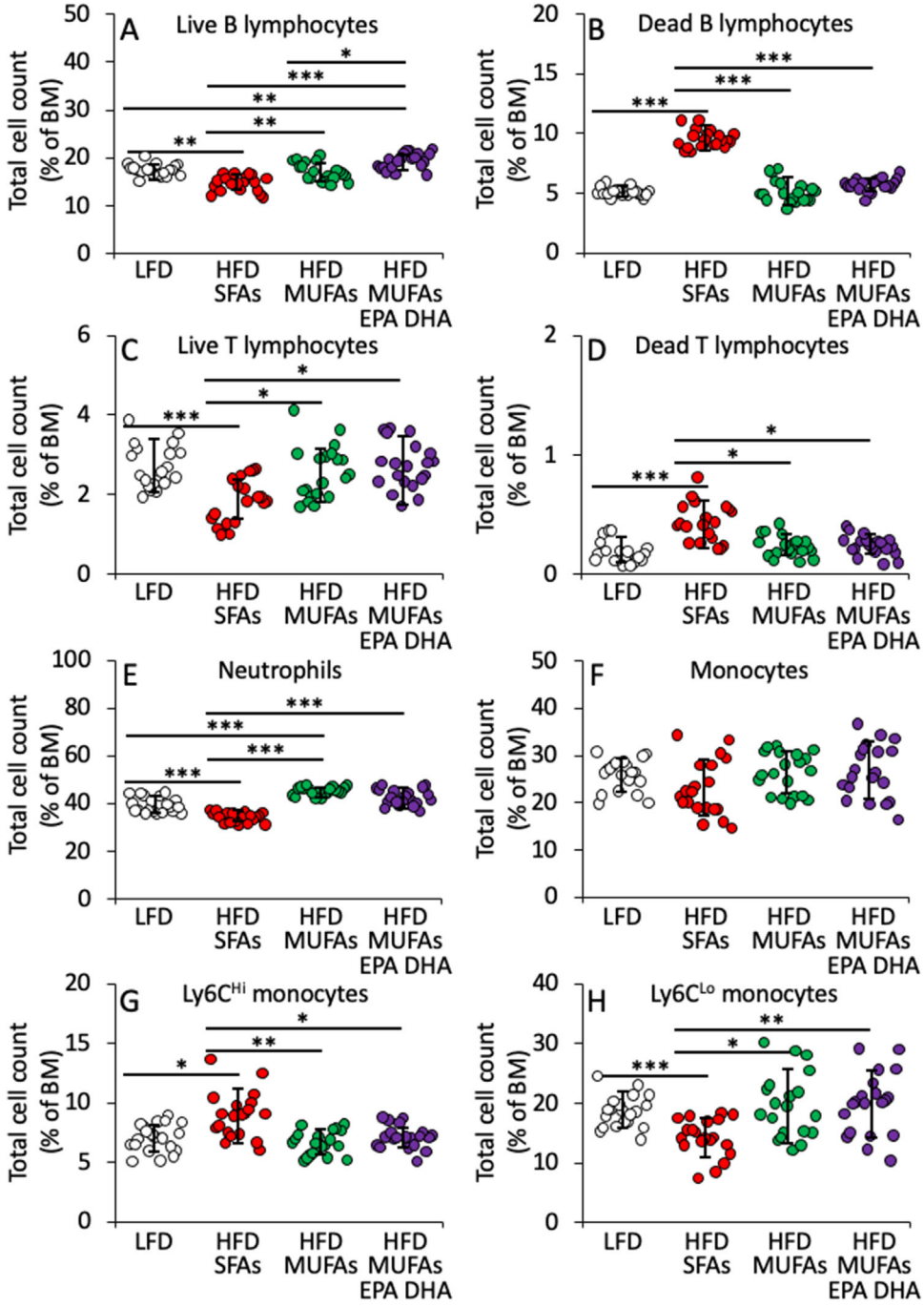

Figure 5. High-fat diets enriched in SFAs (anhydrous butter, HFD-SFAs), MUFAs (olive oil, HFD-MUFAs) or MUFAs (olive oil) fortified with EPA and DHA (HFD-MUFAs+EPA+DHA) on mature hematopoietic lineage cells in bone marrow of wild-type C57BL/6J mice. The percentages of (A) live B lymphocytes, (B) dead B lymphocytes, (C) live T lymphocytes, (D) dead T lymphocytes, (E) neutrophils, (F) monocytes, (G) Ly6C^{Hi}, and (H) Ly6C^{Lo} monocyte subsets at the end of 20-week dietary regimens. Except where otherwise noted, viable cells (7-AAD$^-$Annexin V$^-$) were only considered. Dead cells were 7-AAD$^+$. Values are individual data points ± SD; n = 20/group. Differences between groups were assessed by two-way ANOVA with Tukey's post hoc test. ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$ (compared to all other study groups and as indicated).

the BM samples from any of the study groups. The frequency of live B cells in BM was not significantly different between mice after HFD-MUFAs or LFD; however, that found in the BM of mice after HFD-MUFAs+EPA+DHA and HFD-SFAs was increased and decreased, respectively (**Figure 5**A). HFD-SFAs also increased dead B cells in BM (Figure 5B). Likewise, the frequency of live (Figure 5C) and dead (Figure 5D) T cells was unaltered in the BM of mice after both HFDs enriched in MUFAs compared to the LFD, which was in contrast with the pattern of live (decreasing) and dead (increasing) T cells in the BM of mice after

HFD-SFAs. With respect to myeloid cells, the viability of neutrophils and monocytes in the BM was comparable in all groups (<8% of dead cells). HFD-MUFAs increased BM neutrophils, while HFD-SFAs induced the opposite effect (Figure 5E). Even though the frequency of BM monocytes remained constant after HFDs when compared to LFD (Figure 5F), HFD-SFAs, but not HFDs enriched in MUFAs, disturbed the relative proportion of the two principal monocyte subsets: Ly6C^{Hi} monocytes were increased (Figure 5G), and Ly6C^{Lo} monocytes were decreased (Figure 5H).

2.4. Effects of Fatty Acids in HFDs on Hematopoietic Stem and Progenitor Cells

The relationship between an HSC and its progenies as part of the hematopoietic hierarchy in the mouse system is depicted in **Figure 6**A. By using different antibody combination schemes, we first identified BM cells positive for the markers receptor tyrosine kinase, c-Kit (CD117) and stem cell antigen-1 (Sca-1), and negative for the selection markers of mature hematopoietic cell lineages such as B220, CD3, CD4, CD8, CD11b, CD11c, Gr-1, and Ter-119. This pool of LKS (Lin$^-$cKit$^+$Sca1$^+$) cells that contained HSCs and progenitor (HPCs) cells[10] was refined into self-renewal with extended lifespan long-term hematopoietic stem cells (LT-HSCs) as Lin$^-$cKit$^+$Sca1$^+$CD34$^-$Flt3 (FMS-like tyrosine kinase 3)$^-$ cells (Figure 6B). This strategy included the identification of short-term HSCs (ST-HSCs) as Lin$^-$cKit$^+$Sca1$^+$CD34$^+$Flt3$^-$ cells and multipotent progenitor (MPP) cells as Lin$^-$cKit$^+$Sca1$^+$CD34$^+$Flt3$^+$ cells, which represent cell types of BM compartments immediately downstream of LT-HSCs. Within the LK (Lin$^-$cKit$^+$Sca1$^-$) pool, committed progenitor populations of oligopotent common myeloid progenitor (CMP) cells (Lin$^-$cKit$^+$Sca1$^-$CD34$^+$CD16/32$^-$) and of bivalent granulocyte-macrophage progenitor (GMP) cells (Lin$^-$cKit$^+$Sca1$^-$CD34$^+$CD16/32$^+$) and megakaryocyte-erythroid progenitor (MEP) cells (Lin$^-$cKit$^+$Sca1$^-$CD34$^-$CD16/32$^-$) and of common lymphoid progenitor (CLP) cells (Lin$^-$cKit$^{+/Lo}$Sca1$^-$Flt3$^+$CD127$^+$) were also pinpointed. The proportion of primitive LKS cells in the BM remained unaffected by HFDs compared to LFD (**Figure 7**A). However, HFDs enriched in MUFAs increased the proportion of LT-HSCs (Figure 7B) and decreased that of MPP cells (Figure 7C). The fraction of ST-HSCs remained the same after any diet (Figure S6, Supporting Information). All HFDs led to a decline in the proportion of LK cells, and this effect was larger after HFD-SFAs (Figure 7D). There were no significant differences in the relative abundance of CMP cells when fed any diet (Figure 7E) or in the proportion of MEP cells (Figure 7F). In contrast, HFDs enriched in MUFAs increased the proportion of GMP cells (Figure 7G) and decreased that of CLP cells (Figure 7H). No significant differences were observed between HFD-MUFAs and HFD-MUFAs+EPA+DHA.

3. Discussion

Fatty acids are essential regulators of bone metabolism[35] and skeletal homeostasis.[36] When fatty acids are incorporated to a high extent into a diet (HFD), they impact the overall number of circulating[37] and marginal pools[19] of mature hematopoietic cells in mice. From these data, it is tempting to speculate that HFDs may also result in the regulation of HSC maturation in the BM niche. If it existed, we further hypothesized that this effect would be associated with the type of predominant fatty acids in the HFD.

Building from previous research reporting that MUFAs in HFDs, in sharp contrast to SFAs, favor the homeostasis and a healthy expansion of eWAT in a mouse model of metabolic syndrome,[38] we now show that HFDs enriched in MUFAs or SFAs have distinct impacts within multilineage progeny of myeloid and lymphoid cells in a polygenic developmental model of HFD-induced obesity. All HFDs tested induced weight gain, increased adiposity, promoted dyslipidemia, and led to dysregulation of key hub genes involved in inflammation and energy balance in eWAT. However, such an obese phenotype became largely less disruptive after HFDs enriched in MUFAs than after HFD-SFAs. For example, by indirect evidence of *Ucp1* and *Dio2* gene expression, it may be suggested that MUFAs could pave the way to restrict the fall in energy expenditure in white adipocytes. The expression levels of the *Cebpb* gene may also suggest that adipose tissue inflammation could not be required at all for the increase in adiposity after HFDs enriched in MUFAs. Furthermore, while HFD-SFAs resulted in a decrease in circulating T cells in parallel with an increase in circulating Ly6C^{Hi} monocytes, HFDs enriched in MUFAs increased circulating neutrophils and the HFD with added EPA and DHA also reduced circulating Ly6C^{Hi} monocytes below the values observed with LFD. Decreased circulating T cells have been documented with the progression of obesity induced by lard-based HFDs.[11] The SFA palmitic acid has been reported to stimulate a pro-inflammatory phenotype in T cells and an aberrant T cell infiltration of non-lymphoid tissues both in humans[39] and mice[40] and to be detrimental for cultured T cell survival.[41] Contrary to these effects, the MUFA oleic acid has been shown to promote the formation of lipid droplets in a constant state of flux, resulting from an active cycle of lipolysis and re-esterification to provide fatty acids when needed for T cell development.[42] Recent studies on mice fed a chow diet supplemented with salmon oil for 3 weeks following allogeneic immunization have emphasized the role of EPA and DHA in decreasing the trafficking of pro-inflammatory T cells to fat depots.[43] Therefore, our study may support the hypothesis that dietary MUFAs, EPA, and DHA together are helpful in creating a homeostatic environment for sustaining T cells in the circulation and for suppressing the pro-inflammatory programming of monocytes even under a chronic HFD challenge.

A number of studies have revealed that the circulating pool of neutrophils may be increased in conditions that do not promote explicit priming stimuli.[44] Contrarily to some predictions that a high number of neutrophils in the blood may be regarded as a sign of inflammation or infection, emerging evidence has revealed the ability of menhaden oil that is generously incorporated (38% of total calories) into a HFD to increase survival and decrease renal bacterial load in a mouse model of experimental sepsis through the selective enhancement of neutrophil frequency in blood and BM[45] and in spleen[19] prior to infection and to any evidence of inflammation. Similar findings were reported when menhaden oil was replaced by a commercial product containing

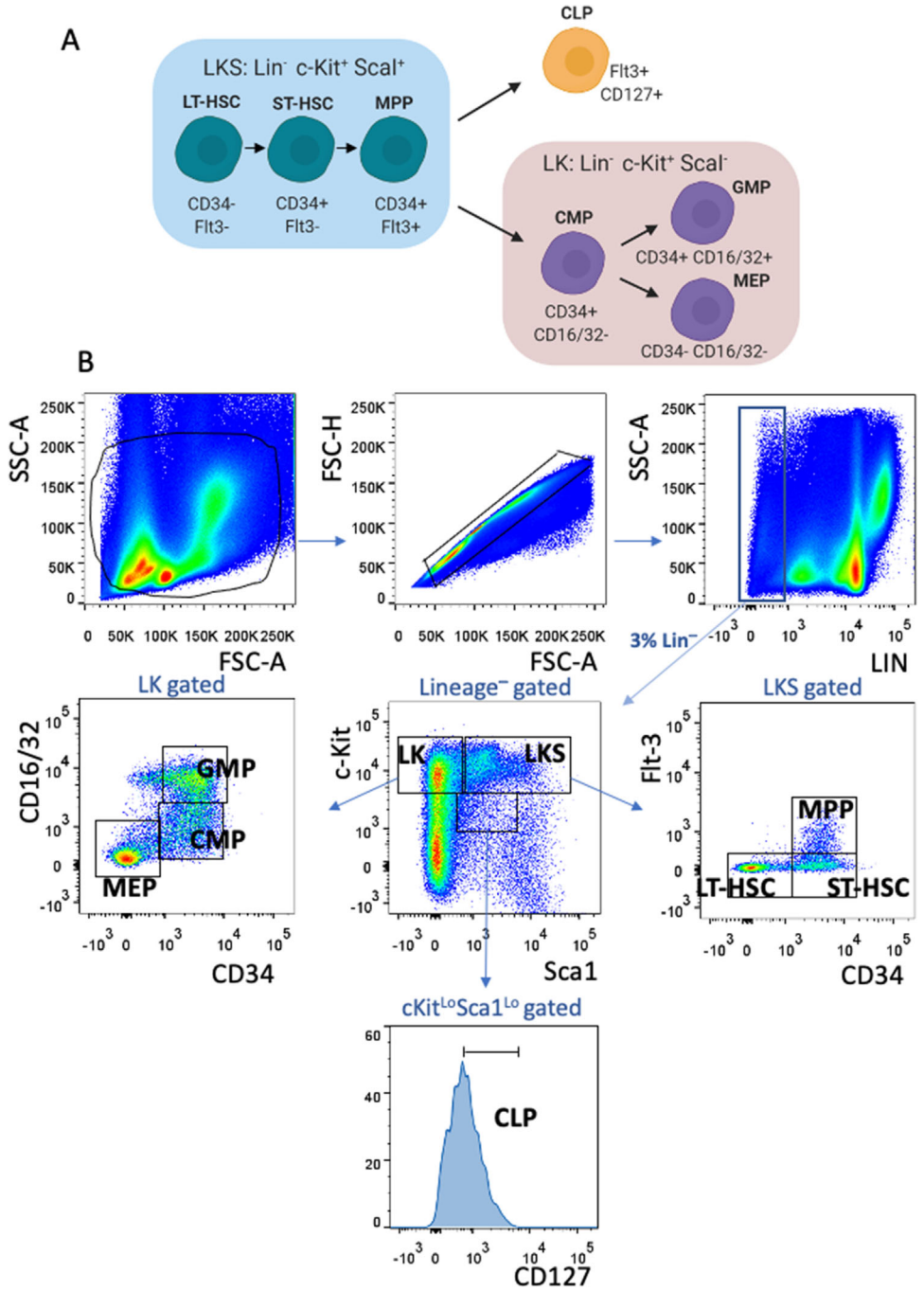

Figure 6. Analysis of hematopoietic stem and progenitor cells in bone marrow of wild-type C57BL/6J mice. (A) Diagram showing the cell types differentiated within LKS ($Lin^{-}cKit^{+}Sca1^{+}$) and LK ($Lin^{-}cKit^{+}Sca1^{-}$) populations as well as their immunophenotypes based on the expression of CD34, Flt3, CD16/32, and CD127 markers. (B) An example of the gating strategy used to identify LT-HSC ($Lin^{-}cKit^{+}Sca1^{+}CD34^{-}Flt3^{-}$), ST-HSC ($Lin^{-}cKit^{+}Sca1^{+}CD34^{+}Flt3^{-}$), MPP ($Lin^{-}cKit^{+}Sca1^{+}CD34^{+}Flt3^{+}$), CMP ($Lin^{-}cKit^{+}Sca1^{-}CD34^{+}CD16/32^{-}$), GMP ($Lin^{-}cKit^{+}Sca1^{-}CD34^{+}CD16/32^{+}$), MEP ($Lin^{-}cKit^{+}Sca1^{-}CD34^{-}CD16/32^{-}$) and CLP ($Lin^{-}cKit^{+/Lo}Sca1^{-}Flt3^{+}CD127^{+}$) cells in bone marrow. Debris (SSC-A vs FSC-A) and doublets (FSC-H vs FSC-A) were excluded. Frequency of cells in some sub-gates is expressed as percentage of live cells. CD, cluster of differentiation; FSC-A, forward scatter area; FSC-H, forward scatter height; SSC-A, side scatter area.

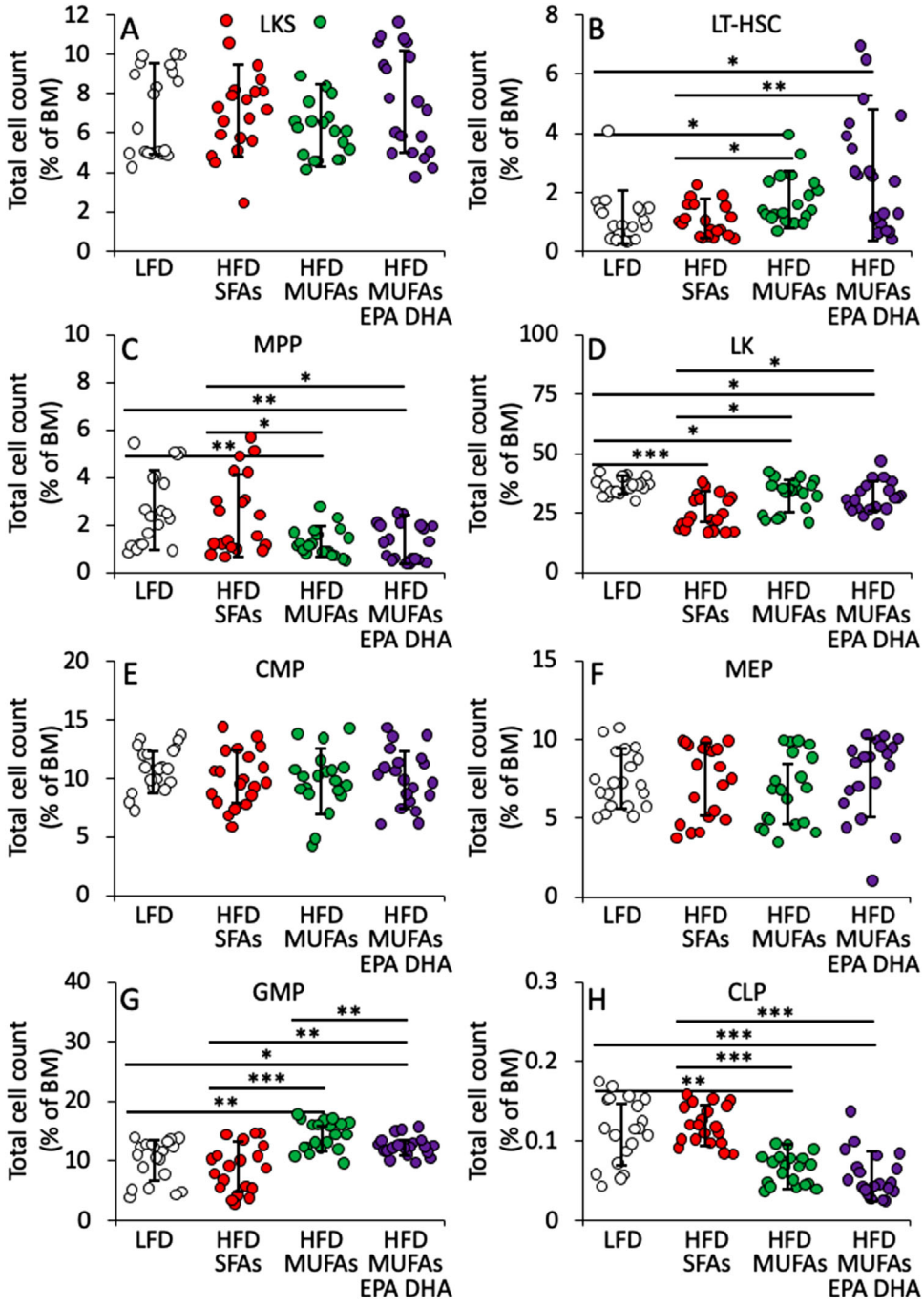

Figure 7. High-fat diets enriched in SFAs (anhydrous butter, HFD-SFAs), MUFAs (olive oil, HFD-MUFAs) or MUFAs (olive oil) fortified with EPA and DHA (HFD-MUFAs+EPA+DHA) on hematopoietic stem and progenitor cells in bone marrow of wild-type C57BL/6J mice. The percentages of (A) LKS, (B) LT-HSC, (C) MPP, (D) LK, (E) CMP, (F) MEP, (G) GMP, and (H) CLP cells at the end of 20-week dietary regimens. Viable cells (7-AAD$^-$Annexin V$^-$) were only considered. Values are individual data points ± SD; n = 20/group. Differences between groups were assessed by two-way ANOVA with Tukey's post hoc test. ***, $P<0.001$; **, $P<0.01$; *, $P<0.05$ (compared to all other study groups and as indicated). CLP, common lymphoid progenitor; CMP, common myeloid progenitor; GMP, granulocyte-macrophage progenitor; LK, Lin$^-$cKit$^+$Sca1$^-$ cell; LKS, Lin$^-$cKit$^+$Sca1$^+$ cell; LT-HSC, long-term hematopoietic stem cell; MPP, multipotent progenitor; MEP, megakaryocyte-erythroid progenitor.

high amounts of EPA and DHA ethyl esters in a HFD.[46] Different from these overfeeding studies, we have observed that the addition of a more realistic dose of EPA and DHA to a HFD formulated with olive oil as the main source of fat had little if any impact on neutrophil frequency in blood, probably unveiling the yet unknown role of dietary MUFAs in supporting or optimizing immune function and surveillance in this compartment during obesity, even in the presence of EPA and DHA. The mediators that can alert the host defense system in a fatty acid-dependent manner and that are not committed to major inflammatory responses remain to be elucidated. These findings evoke the biological process of trained immunity,[47] which is under study in our laboratory.

The observation of increased circulating neutrophil number after HFDs enriched in MUFAs was also found in the spleen. Moreover, HFDs enriched in MUFAs increased myeloid-derived splenic Ly6C^{Hi} monocytes, thus explaining the concomitant reduction in this subset in the blood, particularly after HFD-MUFAs fortified with EPA and DHA. In previous studies, the spleen and most notably its peri-marginal zone areas have been shown to be colonized by neutrophils for homeostatic reasons in healthy mammals, including humans, monkeys, and mice.[48] More recently, the increased accumulation of neutrophils in the peri-marginal zone areas of the spleen has been reported to occur in mice fed a HFD enriched with high levels of menhaden oil after 4 weeks.[19] The spleen is also a compartmental reservoir of extramedullary monocytes. Interestingly, although Ly6C^{Hi} monocytes often synthesize and secrete inflammatory mediators, they can accumulate in the spleen in the absence of demands for rapid-onset inflammation, and when this occurs, they exit the spleen accompanied by Ly6C^{Lo} monocytes with healing properties to inflamed tissue; this pattern is independent of that of splenic neutrophils.[49] If the existence of neutrophils and Ly6C^{Hi} monocytes within the spleen has been proposed to be part of the innate immune mechanisms for early protection,[49] we reasoned that MUFAs in HFDs could have a beneficial impact on immunity in obesity by targeting the recruitment and accretion of professional phagocytic cells into splenic niches, probably for preventive purposes. Remarkably, the precise compartmentalization of neutrophils, Ly6C^{Hi} monocytes and B cells in marginal and peri-marginal zone areas of the mouse spleen[48] could take advantage of common metabolic control mediated by dietary MUFAs, EPA, and DHA in white pulp areas.[50] It is conceivable that these observations may also result as a direct consequence of the inflammatory profile triggered by HFD-SFAs on the mobilization of splenic lymphoid cells (mainly B cells) to adipose depots, which was a previously described mechanism of the adaptive response for reducing inflammation in the eWAT of obese mice.[15] This mechanism would not be relevant, despite the role of macrophages and particularly dendritic cells as potent antigen presenting cells in adipose tissue,[51] as it has been demonstrated that HFDs enriched in MUFAs promote a surrounding lipid environment dominated by oleic acid and the prevalence of alternatively activated macrophages in eWAT[38] and that triglyceride-rich lipoproteins isolated from volunteers after the ingestion of a high-fat meal rich in olive oil impede maturation and pro-inflammatory activation of monocyte-derived dendritic cells.[52] In fact, the repression of the *Elane* gene in the visceral adipose depot may suggest that HFDs enriched in MUFAs did not induce the recruitment of neutrophils into eWAT. The lower cell death background in the spleen after HFDs enriched in MUFAs compared to HFD-SFAs, which may be another proof of decreased inflammatory tone,[20] could have contributed to maintaining the survival of immune cells within this organ. Complementary to this view, HFD-induced neutrophil accumulation in the mouse spleen has been hypothesized to be a mechanism for enhancing antigenic presentation to B cells,[19] a process that may help avoid an impaired splenic B cell response during dysfunctional adipose expansion, particularly in the obese elderly.[53]

When looking at the BM, an interesting fact was observed wherein the values of the palmitic acid/oleic acid and SFA/MUFA ratios were decreased in the BM extracellular fluid of mice on HFDs enriched in MUFAs, presumably because the composition of the fatty acid pool in this compartment was sensitive to fatty acids from the HFDs. We also found a disruption concerning an increase in B and T cell death only after HFD-SFAs, which may suggest that SFAs but not MUFAs in HFDs are a potential source of long-term stress for BM.[54] None of our HFDs resulted in a major loss of myeloid cell viability, hinting at the resistance of neutrophils and monocytes to dietary fatty acids within their local environment in the BM parenchyma during HFD-induced obesity. However, there were large shifts in the percentage of neutrophils and monocytes, with HFD-SFAs decreasing neutrophils and disturbing the ratio of Ly6C^{Hi} to Ly6C^{Lo} monocytes in favor of the Ly6C^{Hi} subset, while HFDs enriched in MUFAs only increased neutrophils relative to the values observed with the LFD. In previous studies with mice fed a 45% lard-based HFD for different periods up to 6 weeks, a progressive increase in the number of lymphocytes in BM was noticed, without signs of inflammation in this compartment.[12] These discrepancies can be explained, at least in part, by the greater fat content in our HFDs (53% of calories) and by the longer feeding time (20 weeks) in our protocol. More in line with our own observations, recent studies in mice fed a 60% lard-based HFD for different feeding periods, even longer than 20 weeks, have reported disturbance in the hematopoietic system giving rise to defective lymphocyte populations[14] and to myeloid progenitors that sustain the production of Ly6C^{Hi} monocytes poised for innate immune activation,[13] as well as a null pro-inflammatory phenotype in the BM and altered stemness and differentiation in the BM stroma.[16] Hence, it is tentatively speculated that HFD-induced myelopoiesis may be dissected by the composition of fatty acids in HFDs, with SFAs involved in the guidance of monopoiesis and MUFAs in the orchestration of granulopoiesis. Intriguingly, both cell fate decisions are known to be mutually exclusive.[55] Recruitment of classical monocytes during chronic inflammation in adipose depots, which is commonly seen in the obesity induced by HFDs enriched in SFAs,[38] has been proposed to be a determinant for BM monocyte output on demand.[56] Hypercholesterolemia, which is provoked in most cases by HFDs enriched in SFAs,[57] has also been shown to be associated with peripheral Ly6C^{Hi} monocytosis.[58] With the aim of gaining insight into the role of SFAs and MUFAs on lineage commitment in BM during obesity, we found that HFD-SFAs decreased the percentage of LK cells when compared to the effects of a LFD and HFDs enriched in MUFAs, which may suggest that SFAs can significantly block the survival or expansion of these cells during obesity. A recent description of a feed-forward mechanism in lard-based

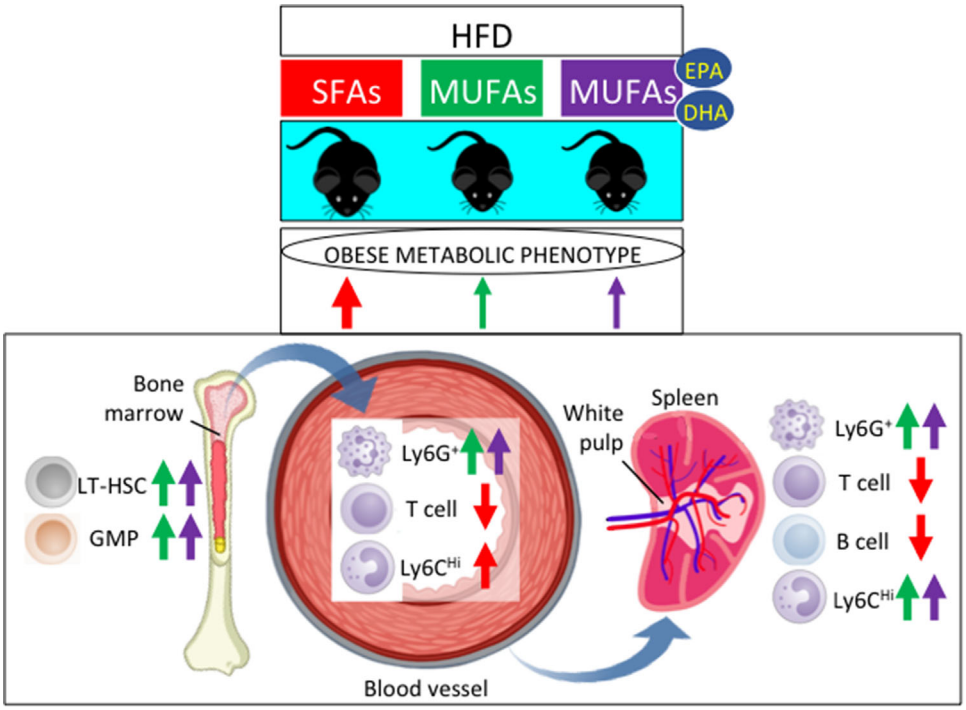

Figure 8. A summary of hematopoietic cells mainly altered in bone marrow, blood, and spleen during the obesity induced by high-fat diets enriched in SFAs (anhydrous butter, HFD-SFAs), MUFAs (olive oil, HFD-MUFAs), or MUFAs (olive oil) fortified with EPA and DHA (HFD-MUFAs+EPA+DHA) in wild-type C57BL/6J mice.

HFD-induced obesity such that inflamed adipose tissue macrophages continuously instigate the production of monocytes could be behind the possible exhaustion of the LK cell pool.[18] The selective reduction of LK cells has been observed in the BM of mice lacking the ability of long term multilineage engraftment,[59] consistent with LK cells also being involved in the immune reconstitution of damaged BM.[60] In addition, MUFAs in HFDs targeted at the root of the branching family tree, where the most immature pool of HSCs is endowed with the highest self-renewal potential (LT-HSCs and MPP cells) and the most restricted progenitors are in charge of myelopoiesis (GMP cells) and lymphopoiesis (CLP cells), increase the percentage of LT-HSCs and bivalent GMP cells and decrease that of MPP and CLP cells. These findings emphasize the regulatory role of MUFAs in the balance between LT-HSC self-renewal and differentiation during obesity, probably promoting a shift towards self-renewing divisions of LT-HSCs and an adhesive environment. Whether this overrepresentation of LT-HSCs led to a depletion of MPP and CLP cells as a feedback compensatory mechanism has yet to be seen. The observed phenotypic signature of LT-HSC and MPP cell populations representative of mice fed HFDs enriched in MUFAs could be associated with the fitness of the HSC compartment.[17] This is an interesting issue, as a decrease in the number of LT-HSCs in the BM is common in the obesity induced by lard-based HFDs[18] and in aged mice,[61] which heightens the vulnerability of LT-HSCs to DNA-damaging processes.[62] Therefore, the effect of enhanced retention and preservation of LT-HSCs in the BM may be crucial to sustain life-long hematopoiesis in obesity if the HFD is mainly composed of MUFAs instead of SFAs.

Additionally, oleic acid but not another MUFA (palmitoleic acid) or PUFA has been recently shown to be a requirement for the induction of innate immunity and resistance to infection in the nematode *Caenorhabditis elegans*,[63] which could reveal robust conservation of the role of oleic acid in the heavy interplay with the immune system among model organisms in the animal kingdom.

Despite EPA and DHA were used to fortify the HFD based on olive oil, many of the selected outcomes of this study did not differ between mice that received the HFD-MUFAs and those that received the HFD-MUFAs+EPA+DHA. We do not rule out a hierarchical usefulness of oleic acid from olive oil over EPA and DHA in a physiological context in which HFDs exhibited canonical proportions of SFAs, MUFAs, and PUFAs. The dose of EPA and DHA was consistent with previous studies,[64] however our understanding of the events underpinning the impact of these long-chain omega-3 fatty acids on hematopoietic cells during obesity is far from complete and novel experimental strategies will require further refinement.

In the face of these promising results, some limitations of our study should be mentioned. First, our exploratory analysis does not inform whether dietary fatty acids ingested at lower doses than those employed in HFDs, that is, in the context of a non-obesogenic diet, have the potential to influence the hematopoietic system. Second, because hematopoiesis is a multi-regulated process, the levels at which dietary fatty acids exert their effects remain unclear; for example, whether the regulation occurs via direct interaction of dietary fatty acids with hematopoietic cells or with selective BM niches or indirectly in an autocrine or a paracrine manner by way of fatty-acid-mediated generation of

specific hormones or cytokines within the medullary tissue or in extramedullary sites remains unclear. Third, quantification of absolute cell numbers per sample volume was not achieved in spleen and BM, and thus, it remains unclear whether the overall cellularity of these tissues was affected by fatty acids in HFDs. Further studies are needed to clarify these points.

In conclusion, these studies delineate a functional role for fatty acids in HFDs to guide HSC maturation in the BM niche and the abundance of mature hematopoietic populations in the blood and spleen. Interestingly, these effects were traced to the type of predominant fatty acids in HFDs. These data support a complex model in which the reshaping of fatty acid pools with MUFAs from the diet serves to manipulate the generation and trafficking of immune cells biased during obesity (**Figure 8**). This perception of fatty acids in sites for hematopoiesis could have significant implications for developing novel strategies targeting the diverse metabolism of dietary fatty acids in the BM and extramedullary compartments to combat the HFD-induced dysfunctional immune system.

4. Experimental Section

Mice and Diets: Six-week-old wild-type C57BL/6J male mice (Charles River Laboratoire, Ecully, France) were used for this study. Mice were maintained in a light/dark- (12-h light/12-h dark), temperature- (22 ± 1 °C), and humidity- (50–60% relative humidity) controlled room under specific pathogen-free conditions, fed a LFD (10% of energy as fat from soybean oil) (Table S1, Supporting Information) ad libitum and had free access to drinking water for 2 weeks to adapt to the Mouse Facility of the Centro Andaluz de Biologia del Desarrollo (CABD, Seville). Then, animals were divided into four experimental groups (n = 20/group) and fed the LFD (control) or HFDs based on the LFD with an additional 43% of energy as fat from anhydrous butter (HFD-SFAs), olive oil (HFD-MUFAs), or olive oil (40% of energy) plus EPA + DHA (3% of energy) in the form of ethyl esters (HFD-MUFAs+EPA+DHA) (Table S1, Supporting Information) for 20 weeks. Diets and drinking water were refreshed every week. Olive oil was devoid of minor constituents as obtained by physical refining of virgin olive oil in a discontinuous deodorizer that used nitrogen as the stripping gas at the Core Facilities for Oil Extraction and Refining of the Instituto de la Grasa (Seville). The fatty acid composition of the diets (Table S2, Supporting Information) was determined via lipid extraction, saponification, and capillary GC.[65] Carbohydrate was used to adjust the total energy content. All diets had low cholesterol (0.01%), were prepared by SAFE (Augy, France) and were presented as pellets to the animals. Body mass and food and water intake were recorded weekly. At the end of the experiments, animals were euthanized at the beginning of the light cycle, blood was collected by cardiac puncture using heparinized syringes and tubes (MiniCollect 1 mL K_3EDTA, Grenier Bio-One, Kremsmünster, Austria), and tissues (femur, tibia, epididymal adipose tissue [eWAT] and spleen) were then removed (Figure S1, Supporting Information). The weights of the eWAT and spleen were determined. Femurs and tibiae were harvested in PBS supplemented with 0.1% BSA, while spleens were harvested in cold PBS supplemented with 1% BSA. eWAT samples were immediately frozen in liquid nitrogen. Peripheral blood samples were analyzed by blood count assays on an automatic Mythic 18 Vet device (Orphee, Geneva, Switzerland), a fully automated veterinary hematology analyzer with capacity to count different mice blood populations, according to manufacturer instructions. Serum samples were used to measure total cholesterol and triglycerides by colorimetric assay kits (Bio-science-medical, Madrid, Spain). Unless mentioned otherwise, samples were frozen at –80 °C for further analysis. All animal protocols received appropriate institutional approval by the corresponding ethics committees of the University of Pablo de Olavide and CSIC (application 03/02/2017/030) and were performed according to the official rules formulated in the Spanish law and European legislation on the care and use of experimental animals (RD 53/2013; UE Directive of 2012: 2012/707/UE).

Dosage Information: Doses of anhydrous butter and olive oil (Tables S1 and S2, Supporting Information) were chosen to represent reasonable amounts of SFAs and MUFAs expected to be present in human diets: SFA-rich Western diet and MUFA-rich Mediterranean diet. The dose of EPA + DHA was equivalent to that used in clinical trials.[64]

Cell Preparations: BM suspensions were obtained from the femur and tibia of mice by flushing the bone cavities with cold PBS. A homogeneous cell suspension was obtained by passing the BM through a needle followed by passage through a 40-µm cell strainer (BD, Madrid, Spain). The BM was kept on ice during isolation and then centrifuged, after which the red blood cells were lysed with ammonium chloride potassium lysis buffer (155 mM NH_4Cl, 10 mM $KHCO_3$, and 0.1 mM EDTA). Cells were centrifuged and resuspended in PBS with 0.1% BSA before staining for FACS analysis.

Spleen cell suspensions were prepared by dicing spleens with a razor blade. Tissue pieces were placed onto a 40-µm cell strainer (BD) and mashed with the plunger end of the syringe. Cells were washed with cold PBS with 1% BSA and centrifuged. Red blood cell lysis was performed using ammonium chloride potassium lysis buffer.

Blood samples were directly treated with FACS Lysing solution (BD) for red blood cell lysis. The remaining white blood cells were washed three times with PBS.

Flow Cytometric Cell Staining: Single-cell suspensions of desired tissues were obtained as described above. For analysis by flow cytometry, cells were incubated with unlabeled anti-CD16/32 (Mouse BD FcBlock, C.N. 553 142) to block non-specific staining (unless anti-CD16/32 was in the staining panel) and were then stained with surface antibodies in PBS with 0.1% BSA for 30 min in the dark at room temperature. A complete list of antibodies used in this study can be found in Table S3, Supporting Information. Cells were considered viable when 7-amino-actinomycin D (7-AAD)$^-$ and Annexin V$^-$ and dead when 7-AAD$^+$. All gating was performed off of live cell populations. The lineage-negative (Lin$^-$) cell fraction in BM was identified as events negative for all the following markers: B220, cluster of differentiation 3 (CD3), CD4, CD8, CD11b, CD11c, granulocyte antigen-1 (Gr-1), and tumor-inducible erythroblast-like-119 (Ter-119), all of which were conjugated to FITC. The rest of the cell types were identified as indicated in the figures. The fluorescence intensity was acquired on a FACSCanto II Cell Analyzer (BD). The analysis was performed with FACSDiva or FlowJo software (BD).

RNA Isolation and qRT-PCR Analysis: eWAT tissue samples were crushed with liquid nitrogen in a pre-chilled mortar and pestle to recover a fine powder. The fine powder was collected in Eppendorf tubes and further processed. Total RNA was then extracted using Trisure Reagent (Bioline, London, UK) and purified through spin columns of the Direct-zol MiniPrep Kit (Zymo Research, Irvine, CA, USA) according to the manufacturer's recommendation. RNA was quantified in a NanoDrop ND-1000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA). RNA quality was assessed using the QIAxcel Advanced System (QIAGEN) and QIAxcel ScreenGel software. The RIS number for all RNA preparations was 7. RNA (1 µg) was subjected to reverse transcription (iScript, Bio-Rad, Madrid, Spain). An amount of 20 ng of the resulting cDNA was used as a template for real-time PCR amplification. The mRNA levels of specific genes were determined in a CFX96 system (Bio-Rad). For each PCR, cDNA template was added to Brilliant SYBR green QPCR Supermix (Bio-Rad) containing the primer pairs for the corresponding gene. The glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*) housekeeping gene was used for normalization. Sequences for qRT-PCR primers can be found in Table S4, Supporting Information. All amplification reactions were performed in triplicate, and average threshold cycle (Ct) numbers of the triplicates were used to calculate the relative mRNA expression of candidate genes. The magnitude of change in mRNA expression for candidate genes was calculated by using the standard $2^{-(\Delta\Delta Ct)}$ method. All data were normalized to the content of housekeeping gene and expressed as a percentage of the control.

Statistics: All results are expressed as the means with error bars reflecting SD. *n* represents the number of animals per experiment.

Differences between groups were assessed by two-way ANOVA with Tukey's post hoc test.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

Acknowledgements

This work was supported by a grant from The Spanish Ministry of Science, Innovation and Universities (AGL2016-80852-R). A.L.-C. had a contract associated with AGL2016-80852-R during the experimental part of this work. S.L. acknowledges financial support by a contract from the "V Own Research Plan" of the University of Seville (VPPI-US) cofounded by the European Social Fund. M.A.R. acknowledges financial support from CSIC/Juan de la Cierva (FJCI-2017-33132). The authors thank Core Facilities for Oil Extraction and Refining and Cell Biology Unit at the Instituto de la Grasa (CSIC, Seville) and Mouse Facility at the CABD (UPO/Junta de Andalucia/CSIC, Seville) for their assistance during the fulfilment of this study.

Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

A.L.-C. and M.M. authors contributed equally to this work. F.J.G.M. and R.A. designed research; A.L., M.M., S.L., M.C.M., M.A.R., J.A.P.S., and R.A. performed experiments, and F.J.G.M. and R.A. wrote the main manuscript. All authors analyzed and discussed the data, and read and approved the final manuscript.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Keywords

dietary fatty acids, hematopoiesis, MUFAs, obesity, progenitor cells, stem cells

Received: December 21, 2020
Revised: May 27, 2021
Published online: June 24, 2021

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