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CD4$^+$ and CD8$^+$ T-cell responses in bone marrow to fatty acids in high-fat diets

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Abstract

Obesity is associated with disruptions in the adaptive immune system; however, dietary fatty acids in high-fat diets (HFDs) that induce obesity have consequences that are currently unclear regarding T-cell maintenance in bone marrow (BM). C57BL/6J mice were randomly assigned to isocaloric HFDs formulated with dietary fats rich in saturated fatty acids (SFAs), monounsaturated fatty acids (MUFAs), or MUFAs supplemented with eicosapentaenoic and docosahexaenoic acids for 20 weeks, followed by an analysis of the immunophenotypic feature of lymphocytes (CD3$^+$) T and their subsets CD4$^+$ and CD8$^+$ T cells in spleen and BM, identification of fatty acids in BM extracellular fluid and analysis of the correspondence between fatty acids with the frequency of T-cell subsets in BM. Splenic CD3$^+$ T cells were reduced irrespective of HFDs. In BM, CD3$^+$ T cells were reduced after HFD-SFAs, while CD4$^+$ T cells were increased after HFDs enriched in MUFAs and CD8$^+$ T cells were reduced irrespective of HFDs. In BM extracellular fluid, the content of palmitic and myristic acids increased after HFD-SFAs and that of oleic acid increased after HFDs enriched in MUFAs. There was a statistical correspondence between HFD-induced changes in fatty acids in BM extracellular fluid and HFD-induced changes in the frequency of CD3$^+$ and CD4$^+$ T cells in BM. These findings reveal an undervalued critical role for dietary fatty acids in the selective acquisition of T-cell subsets in BM, highlighting that oleic acid existing in the surroundings of T-cell niches during HFD-induced obesity could be instrumental in the maintenance of CD4$^+$ T cells.

Keywords: Dietary fatty acids; Oleic acid; Lymphocyte subsets; Obesity; Palmitic acid.

1. Introduction

Obesity is a complex multifactorial disease that is characterized by an excessive or abnormal accumulation of intra-abdominal body fat and has become an epidemic worldwide, including in some low-income and transitional countries [1,2]. While the immune response to an insult can impair metabolic status, the bone marrow (BM) progressively loses its homeostatic balance during obesity leading to a decreased consistency of the immune system [3]. Probably due to the chronic low-grade inflammation state, the increase in the number of circulating classically activated monocytes and neutrophils and the decrease in the number of circulating natural killer cells are key events in the onset of obesity in humans [4]. Less conclusive is the impact of obesity on hematopoietic stem cells and intermediate progenitors in the BM of mice, with data supporting the myeloid cell lineage or arguing the impairment on canonical properties of primitive hematopoietic cells [5]. In parallel to these avenues of research, emerging evidence suggests a link

Abbreviations: HFDs, High-fat diets; BM, Bone marrow; SFAs, Saturated fatty acids; MUFAs, Monounsaturated fatty acids; PUFAs, Polyunsaturated fatty acids; EPA, Eicosapentaenoic acid; DHA, Docosahexaenoic acid; LFD, low-fat diet; HFD-SFAs, High-fat diet enriched in saturated fatty acids; HFD-MUFAs, High-fat diet enriched in monounsaturated fatty acids; HFD-MUFAs+EPA+DHA, High-fat diet enriched in monounsaturated fatty acids and supplemented with eicosapentaenoic and docosahexaenoic acids; 7-AAD, 7-Amino-actinomycin D; SPE, Solid-phase extraction; FABP4, Fatty acid binding protein 4; FABP5, Fatty acid binding protein 5; PPARγ, Peroxisome proliferator-activated receptor gamma; GPR84, G protein coupled receptor 84.

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between obesity and vulnerability to infectious diseases [6,7]; several hypotheses have been delineated, among which dysregulation of the adaptive immune system by depletion of $CD4^+$ and $CD8^+$ T cells may be an important contributing factor [8].

The consumption of high-fat diets (HFDs) has been well established to increase weight and adipose tissue and cause obesity [9], and the predominant dietary fatty acids present in HFDs have relevant differences on the development (saturated fatty acids, SFAs) or prevention (monounsaturated fatty acids, MUFAs) of obesity-associated comorbidities [10,11]. Long-chain ω-3 polyunsaturated fatty acids (PUFAs), including eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), have also been documented to normalize metabolic abnormalities in individuals with obesity [12] and in mice with a constant HFD [13].

The interaction of dietary fatty acids and the adaptive immune system during obesity is a very complex subject that requires further research. The fitness of T lymphocytes has been recently documented to be harmed in the spleen of mice fed HFDs, regardless of predominant dietary fatty acids [14]. Interestingly, this effect also occurred in the BM of mice but only when they were fed a HFD enriched in SFAs and not with HFDs enriched in MUFAs, which might suggest that dietary fatty acids might differentially regulate the occurrence of T lymphocytes in the BM pool. However, variability of T-cell populations has been chiefly investigated in the spleen and BM of mice with HFD-induced obesity, and no studies have clearly assessed the impact of different dietary fatty acids in HFDs. Accordingly, we aimed to determine the effects of three isocaloric HFDs enriched in anhydrous butter (a source of SFAs, mainly palmitic acid), olive oil (a source of MUFAs, mainly oleic acid) or olive oil with a dose of EPA and DHA (a source of MUFAs supplemented with long-chain ω-3 PUFAs) after 20 weeks on T-cell populations in spleen and BM of wild-type C57BL/6J mice. Additionally, we investigated whether the frequency of T-cell populations in BM could correlate with selective fatty acids and lipid indexes that were influenced by HFDs in the mouse BM extracellular fluid.

2. Materials and methods

2.1. Mice, diets, and ethics statement

The animal study was designed following the ARRIVE guidelines for the randomization, execution of experiments, and reporting of data. Six-week-old wild-type C57BL/6J male mice (Charles River Laboratoire, Ecully, France) were used for this study. Mice were maintained in 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 low-fat diet (LFD) (10% energy as fat from soybean oil) (Table 1) *ad libitum* and had free access to drinking water for 2 weeks to settle-in at the Mouse Facility of the Centro Andaluz de Biologia del Desarrollo (CABD, Seville). Then, animals were divided in four experimental groups (n=6/group) and fed the LFD (control) or HFDs based on the LFD with additional 43% energy as fat from anhydrous butter (HFD-SFAs), olive oil (HFD-MUFAs) or olive oil (40% energy) plus EPA+DHA (3% energy) in the form of ethyl esters (HFD-MUFAs+EPA+DHA) (Table 1) for 20 weeks. Doses of anhydrous butter and olive oil 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 [15]. The dose of EPA+DHA was equivalent to that used in clinical trials [16,17]. 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 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 2) was determined via lipid extraction, saponification and capillary GC [18]. Carbohydrate was used to adjust the total energy content. All diets had low cholesterol (0.01%), were prepared by SAFE (Augy, France) and presented as pellets to the animals. Body mass, food, and water intake were recorded weekly. At the end of the experiments, animals were euthanized by administration of a lethal dose of anesthesia (sodium thiopental, thiobarbital) 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, and spleen) were then removed. Femurs and tibiae were harvested into PBS supplemented with 0.1% BSA, while spleens in cold PBS with 1% BSA. Except if mentioned otherwise, samples were frozen at −80°C for further analysis. For *ex vivo* experiments, 6-week-old wild-type C57BL/6J male mice (Charles River Laboratoire) were used. Mice were maintained in a controlled room under specific pathogen-free conditions, fed a -LFD- *ad libitum* and had free access to drinking water for 6 weeks (from here referred to as lean mice). Animals were euthanized and tissues (femur, tibia and spleen) were then removed and handled as above. 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).

2.2. 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 the isolation, then centrifuged upon 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 place 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.

2.3. Flow cytometric cell staining

Single-cell suspensions of spleen (2×10^5 cells per condition) or BM (3×10^6 cells per condition) were obtained as described above. To identify the different populations, cells were stained with surface antibodies [CD45-APC (BD Biosciences, CN 559864), CD3-PerCP-Cy5 (BD Pharmingen, CN 551163), CD4-FITC (Immunostep, CN M4F-05MG), and CD8-PE (Immunostep, CN M8APE-02MG)] in PBS with 0.1% BSA for 30 min in the dark at RT. 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. For *ex vivo* experiments, spleen cells were stained with CD45-APC, CD3-FITC (Invitrogen, CN 11-0031-82), CD4-APC-H7 (BD Biosciences, CN 560158), and CD8-V500 (BD Biosciences, CN 560776); and BM cells with CD3-APC (Immunostep, CN MO3A), CD4-BV510 (BD Horizon, CN 563106), and CD8-FITC (BD Biosciences, CN 553031). Fluorescence intensity was acquired on a FACSCanto II Cell Analyzer (BD). The analysis was performed

Table 1
Basal composition of experimental diets.

	Low-fat diet (LFD)	High-fat diets (HFDs)
Pregelatinized corn-starch (g/kg diet)	465.7	205.7
Maltodextrin (g/kg diet)	155.0	155.0
Casein (g/kg diet)	140.0	140.0
Sucrose (g/kg diet)	100.0	100.0
Crude cellulose (g/kg diet)	50.0	50.0
Pre-mixture of minerals AIN 93M/G 3.5% (g/kg diet)	35.0	35.0
Pre-mixture of vitamins AIN 93M/G 1% (g/kg diet)	10.0	10.0
Choline bitartrate (g/kg diet)	2.5	2.5
L-cysteine (g/kg diet)	1.8	1.8
Soybean oil (g/kg diet)	40.0	40.0
Experimental fats (g/kg diet)	-	260.0
Energy from proteins %	13.6	9.7
Energy from fats %	10.7	53.6
Energy from carbohydrates %	75.6	36.8

Diets were formulated on the basis of the American Institute of Nutrition (AIN) standard reference diet. The experimental fats were anhydrous butter, olive oil, or olive oil plus EPA and DHA, as indicated.
Abbreviations: DHA, docosahexaenoic acid (C22:6ω-3); EPA, eicosapentaenoic acid (C20:5ω-3).

Table 2
Fatty acid composition of experimental diets

		High-fat diets (HFDs)		
	Low-fat diet (LFD)	Anhydrous butter(HFD-SFAs)	Olive oil(HFD-MUFAs)	Olive oil+EPA+DHA(HFD-MUFAs+EPA+DHA)
Fatty acids		g/100 g total fatty acids		
C4:0	$<0.1^{a}$	0.7 ± 0.3^{b}	$<0.1^{a}$	$<0.1^{a}$
C6:0	$<0.1^{a}$	0.9 ± 0.5^{b}	$<0.1^{a}$	$<0.1^{a}$
C8:0	$<0.1^{a}$	0.7 ± 0.3^{b}	$<0.1^{a}$	$<0.1^{a}$
C10:0	$<0.1^{a}$	1.4 ± 0.6^{b}	$<0.1^{a}$	$<0.1^{a}$
C12:0	$<0.1^{a}$	3.0 ± 0.5^{b}	$<0.1^{a}$	$<0.1^{a}$
C14:0	0.1 ± 0.0^{a}	9.5 ± 1.1^{b}	$<0.1^{c}$	$<0.1^{c}$
C16:0	10.7 ± 0.1^{a}	30.5 ± 1.8^{b}	10.0 ± 0.2^{a}	9.9 ± 0.2^{a}
C16:1ω-7	0.1 ± 0.0^{a}	1.7 ± 0.1^{b}	0.6 ± 0.0^{c}	0.5 ± 0.1^{c}
C18:0	3.8 ± 0.2^{a}	10.4 ± 0.3^{b}	3.4 ± 0.1^{c}	3.4 ± 0.0^{c}
C18:1ω-9	25.6 ± 0.4^{a}	28.7 ± 0.8^{b}	70.9 ± 0.2^{c}	68.6 ± 0.3^{d}
C18:2ω-6	52.5 ± 1.3^{a}	9.8 ± 1.4^{b}	12.4 ± 0.1^{c}	12.7 ± 0.4^{c}
C18:3ω-3	6.1 ± 0.2^{a}	1.3 ± 0.1^{b}	1.4 ± 0.1^{b}	1.4 ± 0.0^{b}
C20:5ω-3	$<0.1^{a}$	$<0.1^{a}$	$<0.1^{a}$	1.3 ± 0.1^{b}
C22:6ω-3	$<0.1^{a}$	$<0.1^{a}$	$<0.1^{a}$	1.0 ± 0.1^{b}
Others	1.1±0.2	1.4±0.4	1.3±0.4	1.2±0.2

Values are expressed as means ± SD (n=3) and those marked with different lowercase letter in the same row are statistically different ($P<.05$).
Abbreviations: DHA, docosahexaenoic acid (C22:6ω-3); EPA, eicosapentaenoic acid (C20:5ω-3); MUFAs, monounsaturated fatty acids; SFAs, saturated fatty acids.

with FACSDiva or FlowJo software (BD). Fluorescence spectral overlap compensations were continuously validated and the panel for CD45, CD3, CD4, and CD8 was always optimized to avoid spillover effects. There was no need for fluorescence-minus-one controls.

2.4. BM extracellular fluid, spleen, lipid extraction, and fatty acid analysis

Following euthanasia, both femurs and tibiae were removed, and cleaned of muscle and tendon. The ends of femurs and tibiae were cut off to expose the marrow cavity and were placed in a 0.5-mL microcentrifuge tube that was cut open at the bottom. BM extracellular fluid was separated from bones by centrifugation (3,000×g at 4°C for 2 min), collected into 1.5-mL microcentrifuge tube and kept at −80°C until analysis. Fatty acids in the BM extracellular fluid can be the result of BM adipocyte release [19]. Lipid extraction of BM extracellular fluid was performed following essentially the procedure outlined previously [20]. To briefly describe, lipids were extracted from the freeze-dried samples using 0.1 mL of 2.5 M sulfuric acid and 5 mL of diethyl ether/heptane (1:1, vol/vol). After vortexing and centrifuging (3,000×g for 5 min), the lipid phase was collected, evaporated with a stream of N_2 gas and dissolved in 500 mL of heptane. Solid-phase extraction (SPE) (Supelclean LC-NH_2 SPE column, Sigma-Aldrich) was applied for the separation of free fatty acids. SPE column was preconditioned with heptane and 5 mL of 2% formic acid/diethyl

ether (1:1, vol/vol) as used as eluent. Aliquots of 100 μL were lyophilized and stored at −80°C until analysis. A solution composed of 2.64 mL of methanol:toluene:dimethoxypropane:sulfuric acid (16.5:5:1:1, vol/vol/vol/vol) and heptane was added on the lyophilized residue. After shaking, and incubating the mixture at 80°C for 1 h, the upper phase was transferred to another vial and dried with a stream of N_2 gas and stored at −80°C until analysis. Spleen lipids were extracted and methylated as previously described [21] with minor modifications. Briefly, chloroform–methanol (2:1, vol/vol) mixture was added to the spleen cell suspension, vortexed well and centrifuged at 16,100×*g* and 4°C for 20 min. The lower organic phase (containing lipids) was transferred into a fresh tube and was dried with a stream of N_2 gas. The lipids were resuspended in a minimal volume of heptane and a solution composed of 2 mL of methanol:sulfuric acid:toluene (34:1:6, vol/vol/vol) was added to the tube. The mixture was vortexed, and heated at 80°C for 1 h. After this, 1,000 μL of heptane was added, vortexed, and the heptane phase collected and partially evaporated with a stream of N_2 gas. Aliquots of 100 μL were lyophilized and stored at −80°C until analysis. The resulting lipid extract either from BM extracellular fluid or spleen was dissolved in heptane, and fatty acid methyl esters were analyzed using a gas chromatography system (HP-5890, Hewlett-Packard) equipped with flame ionization detector and a SP-2380 capillary column (Supelco, 30 m × 0.32 mm) packed with cyanopropylsiloxane (0.25 mm). The initial column temperature was 165°C, which was held for 10 min, then programmed from 165°C to 200°C at 1.5°C/min. Injector and detector temperature were 250°C, with the carrier gas H_2.

2.5. Cell cultures and fatty acid treatments

Single-cell suspensions of BM or spleen from lean mice were cultured at a density of 0.5 × 10^6 cells/mL in RPMI 1640 medium supplemented with 1% antibiotics (100 U/mL penicillin, 100 mg/mL streptomycin) and treated with either low-endotoxin fatty acid-free BSA control (Cayman Chemical), BSA-palmitic acid complex (Cayman Chemical) (25–300 μM), or BSA-oleic acid complex (Sigma) (25–300 μM) for 18 h. The concentration ranges of palmitic and oleic acids by forming complexes with BSA were chosen to reflect physiological concentrations based on previous studies in humans [22,23] and mice [24–26]. Cells without any treatment were used as the negative controls. Samples were washed with PBS and collected for flow cytometric cell staining. Cells were considered viable when 7-AAD$^-$ and Annexin V$^-$ and dead when 7-AAD$^+$. Longer incubation times than 18 h led to a sudden decrease of cell viability unless BSA and certain cytokines was added to the medium, suggesting that longevity may not be an intrinsic capacity of BM and spleen cells after their isolation and that their natural environment could play an important role in the persistence of these cells in mice (unpublished results). As BSA would interfere with fatty acid uptake, only 18 h was used to establish *ex vivo* the cytotoxic, but not the proliferative potential of palmitic and oleic acids.

2.6. Statistical analysis

Results are expressed as means with error bars and individual data points reflecting SD. *n* represents the number of animals per experiment. Spearman correlations were used to determine the statistical significance as indicated in the figure legends. Levene's test was used to check the homogeneities of the variances of the different variables. Differences between groups in variables with similar variances were assessed by one-way ANOVA with Tukey's *post hoc* test. Otherwise, differences between groups were assessed by Kruskal–Wallis with Bonferroni correction. *P* values less than .05 were considered significant.

3. Results

3.1. The supply of fatty acids in HFDs and the obese phenotype

As shown in Table 2, palmitic acid (C16:0) was the principal fatty acid in HFD-SFAs, while HFD-MUFAs and HFD-MUFAs+EPA+DHA were primarily composed of oleic acid (C18:1ω-9). HFD-MUFAs+EPA+DHA also contained 1.3% EPA (C20:5ω-3) and 1% DHA (C22:6ω-3). We found no changes in feeding habits, with similar food and water intake in mice on either the LFD or a HFD (data not shown). Compared to the mice on the LFD, all of the mice with HFDs had larger increases in body mass after HFD-SFAs (+76%) than after HFD-MUFAs (+37%) and HFD-MUFAs+EPA+DHA (+27%) at the end of 20-week dietary regimens. Mice fed HFDs also developed dyslipidemia due to an increase in the levels of total cholesterol (+33%, +16%, and +17% after HFD-SFAs, HFD-MUFAs, and HFD-MUFAs+EPA+DHA, respectively) and triglycerides (+42%, +18%, and +20% after HFD-SFAs, HFD-MUFAs, and HFD-MUFAs+EPA+DHA, respectively). The magnitude of these effects was as follows: HFD-SFAs>HFD-MUFAs=HFD-MUFAs+EPA+DHA (P<.05). These findings were in line with those reported in a previous study [14].

3.2. The impact of fatty acids in HFDs on fatty acids in spleen

The fatty acid composition in the spleen partially mirrored the fatty acid composition of HFDs (Supplemental Table 1). Compared to the LFD, the HFD-SFAs increased the content of palmitic and myristic (C14:0) acids; the HFD-MUFAs increased the content of oleic acid and α-linolenic acid (C18:3ω-3); and the HFD-MUFAs+EPA+DHA increased the content of palmitoleic acid (C16:1ω-7), oleic acid, α-linolenic acid, EPA, and DHA in the mouse spleen. A decrease in the content of stearic acid (C18:0) was observed after HFDs. The HFD-SFAs also led to a decrease of oleic acid and linoleic acid (C18:2ω-6), while both HFDs enriched in MUFAs decreased the content of arachidonic acid (C20:4ω-6), but more markedly by the HFD-MUFAs+EPA+DHA. As a result of these fatty acid changes, the spleen of mice fed with the HFD-SFAs was enriched in SFAs and depleted in MUFAs. The opposite effects were observed with HFDs enriched in MUFAs, but more markedly by the HFD-MUFAs than by the HFD-MUFAs+EPA+DHA.

3.3. The status of T lymphocytes in spleen and BM after HFDs

Our previous work in a HFD-induced obesity mouse model demonstrated that HFDs, irrespective of their fatty acid composition, lead to a decrease in splenic T cells, while this effect is only evident after HFD-SFAs in BM [14]. Here, we assessed whether HFDs enriched in SFAs, MUFAs or MUFAs supplemented with long-chain ω-3 PUFAs caused differences in the status of CD3$^+$ and naïve CD4$^+$ and CD8$^+$ T cells both in the spleen and BM of mice fed these HFDs for 20 weeks. This study was carried out by the expression of CD45 on CD3$^+$ cells and of CD45CD3 on CD4$^+$ and CD8$^+$ cells with only live cells (Supplemental Fig. 1). Cell clumps and cellular debris were first excluded by analysis of cell size on a forward-side scatter plot. Then, doublets and dead cells were also excluded. Compared to the LFD, approximately 12.4% of splenic CD45$^+$ cells were excluded as dead cells after the HFD-SFAs, 9.3% after the HFD-MUFAs and 9.6% after the HFD-MUFAs+EPA+DHA. These mean values were approximately 3.2% of marrow CD45$^+$ cells excluded as dead cells after the HFD-SFAs, 0.8% after the HFD-MUFAs and 0.6% after the HFD-MUFAs+EPA+DHA.

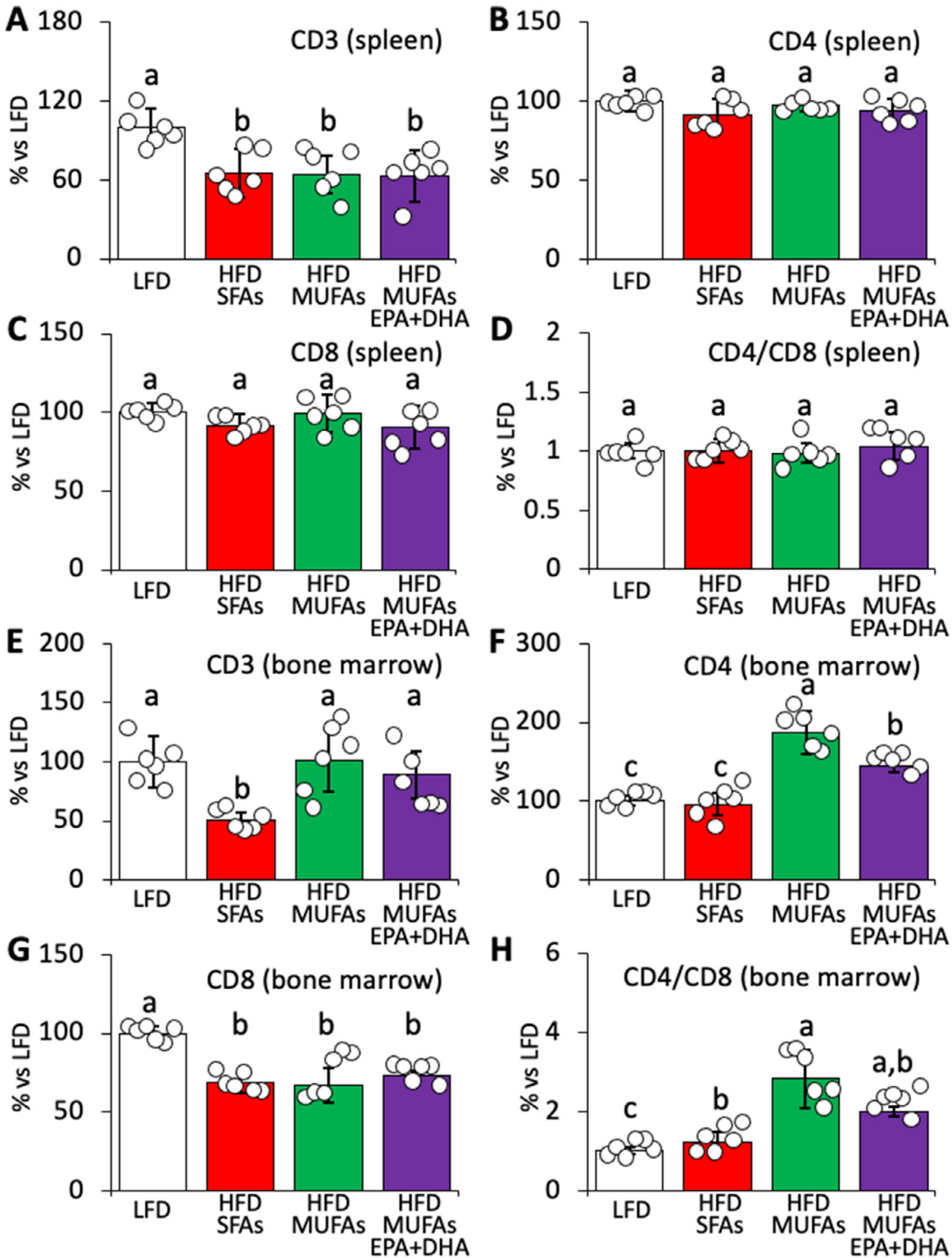

Fig. 1. High-fat diets enriched in SFAs (anhydrous butter, HFD-SFAs), MUFAs (olive oil, HFD-MUFAs) or MUFAs (olive oil) supplemented with EPA and DHA (HFD-MUFAs+EPA+DHA) modulate the fitness of T cells in spleen and bone marrow (BM) of wild-type C57BL/6J mice. Spleen: The percentage relative to the control low-fat diet (LFD) of $CD3^+$ T cells (A), $CD4^+$ T-cell subset (B), $CD8^+$ T-cell subset (C), and $CD4^+/CD8^+$ ratio (D) at the end of 20-week dietary regimens. BM: The percentage relative to the control low-fat diet (LFD) of $CD3^+$ T cells (E), $CD4^+$ T-cell subset (F), $CD8^+$ T-cell subset (G), and $CD4^+/CD8^+$ ratio (H) at the end of 20-week dietary regimens. The data are presented as the mean (bars) and individual points±SD values (n=6), and those marked with different letters are significantly different (P<.05). Differences between groups were assessed by one-way ANOVA with Tukey's *post hoc* test or, in the case of variance heterogeneity, by Kruskal–Wallis with Bonferroni correction. CD, cluster of differentiation; DHA, docosahexaenoic acid (C22:6ω-3); EPA, eicosapentaenoic acid (C20:5ω-3); MUFAs, monounsaturated fatty acids; SFAs, saturated fatty acids.

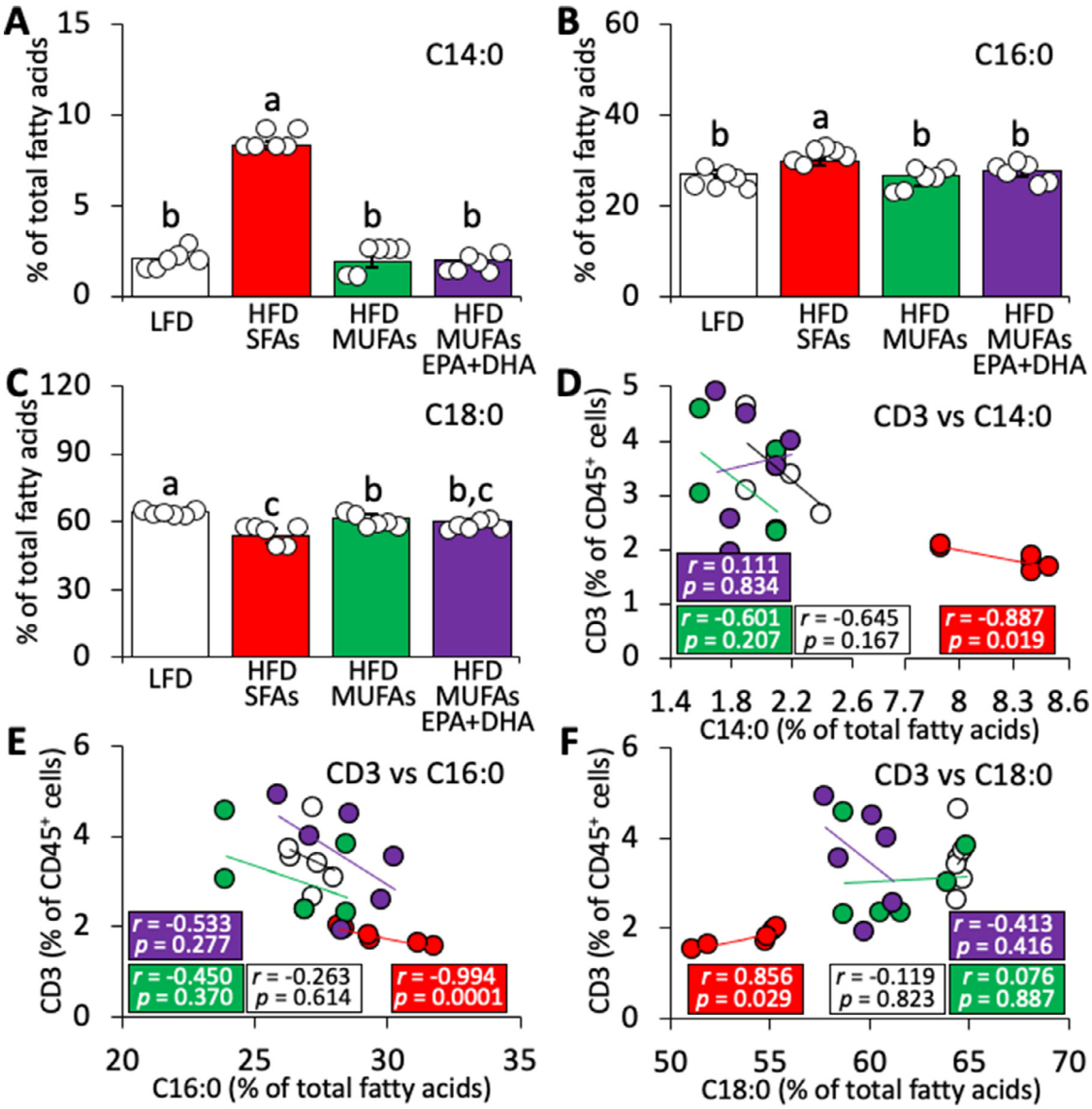

Fig. 2. High-fat diets enriched in SFAs (anhydrous butter, HFD-SFAs), MUFAs (olive oil, HFD-MUFAs) or MUFAs (olive oil) supplemented with EPA and DHA (HFD-MUFAs+EPA+DHA) on SFAs in bone marrow (BM) extracellular fluid and correspondence of these fatty acids with $CD3^+$ T cells in BM of wild-type C57BL/6J mice. The percentage relative to total fatty acids in BM extracellular fluid of myristic acid (C14:0) (A), palmitic acid (C16:0) (B), and stearic acid (C18:0) (C) at the end of 20-week dietary regimens. The data are presented as the mean (bars) and individual points±SD values (n=6), and those marked with different letters are significantly different (P<.05). Differences between groups were assessed by one-way ANOVA with Tukey's *post hoc* test or, in the case of variance heterogeneity, by Kruskal–Wallis with Bonferroni correction. Correlations between the content of myristic acid (D), palmitic acid (E), and stearic acid (F) in BM extracellular fluid and the frequency of $CD3^+$ T cells in BM. CD, cluster of differentiation; DHA, docosahexaenoic acid (C22:6ω-3); EPA, eicosapentaenoic acid (C20:5ω-3); LFD, low-fat diet; MUFAs, monounsaturated fatty acids; SFAs, saturated fatty acids.

$CD3^+$ T cells comprised approximately 15% of the spleen leucocyte population and approximately 3–4% of the BM leucocyte population in mice on the control LFD; the spleen contained proportionally more $CD4^+$ T cells than BM, and the ratio of $CD4^+$ to $CD8^+$ T cell subsets was above 1 in the spleen and below 1 in the BM (Table 3). These observations were in line with previous studies on the analysis of T-cell subsets in spleen and BM from mice of the C57BL/6J genetic background fed a control chow diet [27,28]. The influence of HFDs was examined in detail, and the results, expressed as percentages of the LFD, are outlined below. All HFDs decreased similarly the population of $CD3^+$ T cells in the spleen (Fig. 1A). In addition, we found that the population of $CD4^+$ (Fig. 1B) and $CD8^+$ (Fig. 1C) subsets of T cells and the ratio of $CD4^+/CD8^+$ cells (Fig. 1D) in spleens were not significantly altered in response to any HFD compared to LFD. The population of $CD3^+$ T cells in BM decreased with obesity only after HFD-SFAs (Fig. 1E). Notably, a sharp increase in the $CD4^+$ subset of T cells in BM was observed after HFDs were enriched in MUFAs (Fig. 1F), while the population of $CD8^+$ cells in BM similarly decreased after all HFDs (Fig. 1G). Furthermore, the ratio of $CD4^+/CD8^+$ cells was also increased after HFDs enriched in MUFAs compared to LFD (Fig. 1H).

3.4. Ex vivo *effects of palmitic acid and oleic acid on T lymphocytes of spleen*

After selection of relevant live cell populations through an iterative gating strategy (Supplemental Fig. 2), the percentage of $CD3^+$ T cells was decreased when spleen cells of lean mice were treated

Table 3
$CD3^+$ T cells and $CD4^+$ and $CD8^+$ T-cell subsets in spleen and bone marrow of wild-type C57BL/6J mice at the end of 20-week dietary regimens.

	Low-fat diet (LFD)	High-fat diets (HFDs)		
		Anhydrous butter(HFD-SFAs)	Olive oil(HFD-MUFAs)	Olive oil+EPA+DHA(HFD-MUFAs+EPA+DHA)
Spleen				
$CD3^+$ T[1]	15.2±2.0[a]	10.6±3.0[b]	10.2±2.3[b]	9.7±2.7[b]
$CD4^+$ T[2]	54.0±3.1[a]	49.4±4.9[a]	52.1±2.0[a]	50.8±3.7[a]
$CD8^+$ T[2]	40.0±2.3[a]	36.3±2.7[a]	38.8±4.7[a]	35.8±4.8[a]
Bone marrow				
$CD3^+$ T[1]	3.5±0.8[a]	1.7±0.2[b]	3.5±1.3[a]	3.1±1.0[a]
$CD4^+$ T2	24.1±1.5[a]	23.1±3.3[a]	44.9±6.4[c]	34.8±2.0[b]
$CD8^+$ T[2]	49.8±2.1[a]	34.2±3.0[b]	34.5±5.5[b]	36.5±1.6[b]

[1] As percentage of $CD45^+$ cells.
[2] As percentage of $CD3^+$ cells. Values are expressed as means ± SD (n=6) and those marked with different lowercase letter in the same row are statistically different (P<.05).
Abbreviations: CD, cluster of differentiation; DHA, docosahexaenoic acid (C22:6ω-3); EPA, eicosapentaenoic acid (C20:5ω-3); MUFAs, monounsaturated fatty acids; SFAs, saturated fatty acids.

with palmitic acid (200 and 300 μM) (Supplemental Fig. 3A) or oleic acid (300 μM) (Supplemental Fig. 3B) for 18 h. However, these effects showed no significant differences between tested fatty acids (Supplemental Fig. 3C). Furthermore, neither palmitic acid (Supplemental Fig. 3D) nor oleic acid (Supplemental Fig. 3E) had an effect on the percentage of $CD4^+$ T cells as compared to the BSA control. As expected, the effect of palmitic acid did not differ from that of oleic acid in this respect (Supplemental Fig. 3F). Similar findings as for $CD4^+$ T cells were observed for $CD8^+$ T cells (Supplemental Fig. 4A–C).

3.5. The impact of fatty acids in HFDs on major fatty acid families and lipid indexes in BM extracellular fluid and their correlations with T lymphocytes in BM

Myristic acid (Fig. 2A) and palmitic acid (Fig. 2B) were increased in BM extracellular fluid after HFD-SFAs. Stearic acid was decreased after all HFDs, but with lower intensity after HFDs enriched in MUFAs than after the HFD-SFAs (Fig. 2C). Interestingly, we found that $CD3^+$ T cells in BM were significantly correlated in a negative manner with the content of myristic acid (Fig. 2D; r=–0.887, P=.019) and palmitic acid (Fig. 2E; r=–0.994, P=.0001) in BM extracellular fluid only after the HFD-SFAs. Consistently, $CD3^+$ T cells in BM positively correlated with the content of stearic acid in BM extracellular fluid only after the HFD-SFAs (Fig. 2F; r=0.856, P=.029).

Oleic acid was increased in BM extracellular fluid after HFDs enriched in MUFAs (Fig. 3A), while no alterations in linoleic acid (Fig. 3B) or arachidonic acid (Fig. 3C) were perceived after any diet. The cluster composed of these unsaturated fatty acids and the above SFAs (6 fatty acids in sum) accounted for approximately 96% of the total fatty acids in BM extracellular fluid. EPA and DHA were not detected. Of note, $CD4^+$ T cells in BM positively correlated with the content of oleic acid in BM extracellular fluid after the HFD-MUFAs (r=0.853, P=.030), albeit not reaching statistical significance after the HFD-MUFAs+EPA+DHA (r=0.779, P=.067) (Fig. 3D).

Notably, the content of total SFAs (Fig. 4A) and PUFAs (Fig. 4B) did not change in BM extracellular fluid after the different regimens. However, total MUFAs were increased in BM extracellular fluid after HFDs enriched in MUFAs (Fig. 4C); similar changes were observed for the lipid indexes of oleic acid to palmitic acid (Fig. 4D, left panel) and of MUFAs to SFAs (Fig. 4D, right panel). $CD4^+$ T cells in BM positively correlated with the content of total MUFAs (Fig. 4E; r=0.856, P=.026 after HFD-MUFAs; r=0.941, P=.005 after HFD-MUFAs+EPA+DHA) and with the values of oleic acid to palmitic acid ratio (Fig. 4F; r=0.854, P=.031 after HFD-MUFAs; r = 0.954, P=.003 after HFD-MUFAs+EPA+DHA) and of MUFAs to SFAs ratio (Fig. 4G; r=0.840, P=.036 after HFD-MUFAs; r=0.949, P=.004 after HFD-MUFAs+EPA+DHA). None of the fatty acids measured or lipid indexes calculated in BM extracellular fluid had any correlation with $CD8^+$ T cells in BM (data not shown).

3.6. Ex vivo effects of palmitic acid and oleic acid on T lymphocytes of BM

The percentage of $CD3^+$ T cells was decreased when BM cells of lean mice were treated with palmitic acid from 50 μM to 300 μM (Supplemental Fig. 4D) for 18 h. No change was observed with oleic acid at any concentration (Supplemental Fig. 4E). Palmitic acid significantly reduced the percentage of $CD3^+$ T cells compared to oleic acid at 300 μM (Supplemental Fig. 4F). In addition, bot palmitic acid (Supplemental Fig. 5A) and oleic acid (Supplemental Fig. 5B) had no effect on the percentage of $CD4^+$ T cells at 300 μM. As expected, the effect of palmitic acid did not differ from that of oleic acid in this respect (Supplemental Fig. 5C). However, the percentage of $CD8^+$ T cells was decreased with 300 μM palmitic acid (Supplemental Fig. 5D) or 300 μM oleic acid (Supplemental Fig. 5E). There was no significant difference between the effects of palmitic and oleic acids at that concentration (Supplemental Fig. 5F). For $CD4^+$ and $CD8^+$ T subsets in BM cells, other concentrations lower than 300 μM could not be tested.

4. Discussion

In our lab, we recently reported that HFDs containing MUFAs, in sharp contrast to dietary SFAs, favor the healthy expansion of visceral adipose tissue in a mouse model (leptin/LDLr double knockout) of metabolic syndrome [29] and the fitness of T cells apart from distinct impacts within multilineage progeny of myeloid cells in the BM of wild-type mice that continued to consume HFDs [14]. Thus, MUFAs are fatty acids playing an important role in maintaining adipose homeostasis and safe niches for marrow cells during obesity. Now, we showed that T-cell subsets in murine BM vary in response to dietary fatty acids in HFDs once obesity is established. These findings might be relevant because BM is one of the

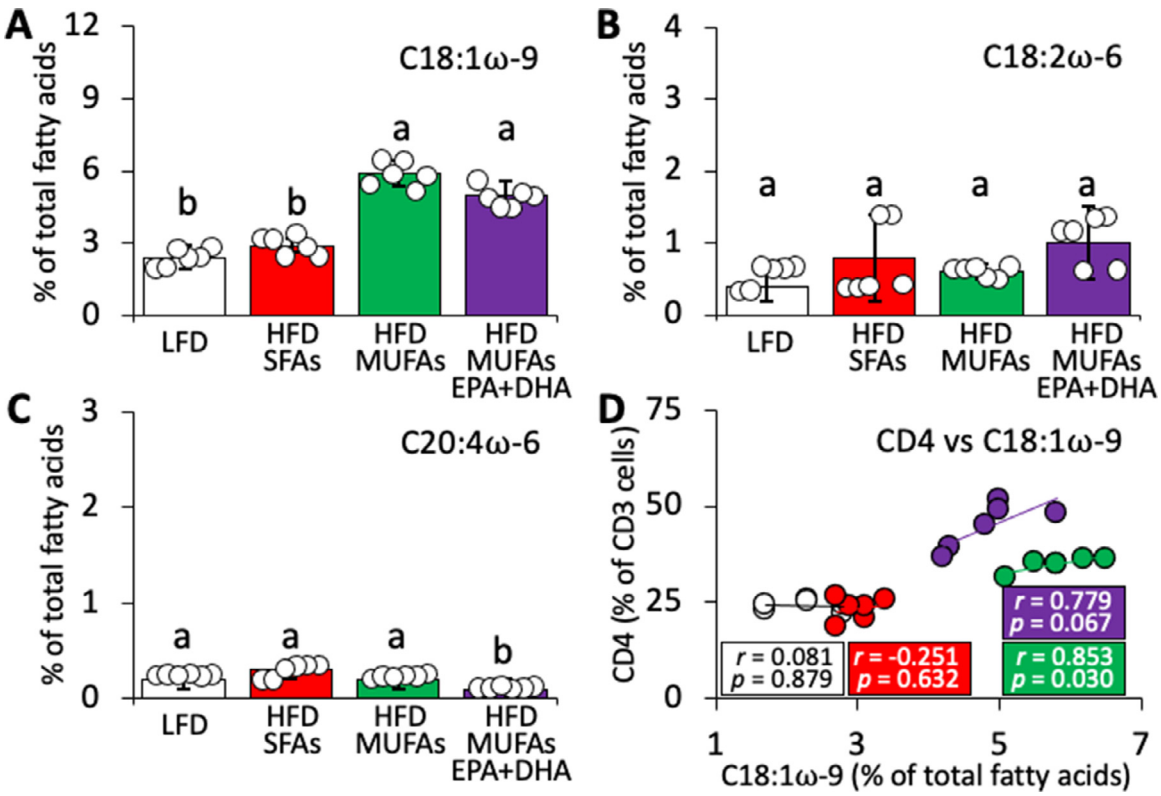

Fig. 3. High-fat diets enriched in SFAs (anhydrous butter, HFD-SFAs), MUFAs (olive oil, HFD-MUFAs) or MUFAs (olive oil) supplemented with EPA and DHA (HFD-MUFAs+EPA+DHA) on unsaturated fatty acids in bone marrow (BM) extracellular fluid and correspondence of oleic acid (C18:1ω-9) with CD4$^+$ T cells in BM of wild-type C57BL/6J mice. The percentage relative to total fatty acids in BM extracellular fluid of oleic acid (A), linoleic acid (C18:2ω-6) (B), and arachidonic acid (C20:4ω-6) (C) at the end of 20-week dietary regimens. The data are presented as the mean (bars) and individual points±SD values (n=6), and those marked with different letters are significantly different (P<.05). Differences between groups were assessed by one-way ANOVA with Tukey's *post hoc* test or, in the case of variance heterogeneity, by Kruskal–Wallis with Bonferroni correction. Correlations between the content of oleic acid (D) in BM extracellular fluid and the frequency of CD4$^+$ T cells in BM. CD, cluster of differentiation; DHA, docosahexaenoic acid (C22:6ω-3); EPA, eicosapentaenoic acid (C20:5ω-3); LFD, low-fat diet; MUFAs, monounsaturated fatty acids; SFAs, saturated fatty acids.

major reservoirs of resting memory T cells but remains largely unexplored during obesity induced by HFDs in which SFAs or MUFAs are the predominant dietary fatty acids. Indeed, the lack of CD4$^+$ T cells lodging into the BM has been documented to dramatically reduce the generation of high-affinity memory B cells and long-lived plasma cells and the maintenance and secondary expansion of CD8$^+$ T cells [30,31]. Therefore, the enrichment of BM in CD4$^+$ T cells could be key to conferring long-lasting immunological memory [32].

Given that adaptive immunity is thought to be dependent on the ability of lymphocytes to circulate between secondary lymphoid organs, among which the spleen is the greatest contributor to this process [33], our initial aim was to explore the splenic T-cell repertoire. In previous studies, obesity induced by a HFD or by hyperphagia of a chow diet through ablation of the melanocortin 4 receptor led to a reduction in CD4$^+$ and CD8$^+$ T cells in the spleen of mice after 28 weeks of feeding [34]. In the present study, we also observed a decrease in pan (CD3$^+$) T cells in the spleen of mice after the different isocaloric HFDs, although their effects on the relative proportions of CD4$^+$ and CD8$^+$ T cells remained invariable; however, the fatty acid composition of HFDs was reflected on the fatty acid composition of spleens, and both palmitic and oleic acids similarly reduced *ex vivo* the integrity of CD3$^+$ T cells, but not of CD4$^+$ or CD8$^+$ T cells, in the spleen of lean mice. Accordingly, we reasoned that the erosion of the integrity of the splenic T-cell niche could be a consequence of lipid stress by high-fat overfeeding and was not specific to dietary fatty acids in HFDs. Increased lipid load in T cells of mice with HFD-induced obesity has been recently reported to inhibit autophagy as a mechanism that may contribute, at least in part, to the loss of control of T-cell activation, proliferation and survival, which can be restored when mice with obesity have a chow diet and lose weight [35].

Several important findings emerged from fatty acids found in BM extracellular fluid and the correspondence of these fatty acids with the frequency of CD3$^+$ and CD4$^+$ T cells in BM. The most striking observation was the interstitial redistribution of SFAs, as shown by a decrease in stearic acid after any of the HFDs and by an increase in palmitic acid and notably myristic acid (remaining palmitic acid the most abundant) after HFD-SFAs, which has the potential to advance our understanding of the regulation of BM fatty acid metabolism [36]. Another clear difference between CD4$^+$ and CD8$^+$ T cells was the manner by which CD4$^+$ T cells were uniquely sensitive to oleic acid from BM extracellular fluid. This dichotomy may represent metabolic differences between CD4$^+$ *versus* CD8$^+$ T cells, as CD4$^+$ T cells take advantage of fatty acids as preferred metabolic substrates for their development in the BM [37] and they could thereby be able to discriminate between both quantity and quality of fatty acids located in their vicinity. T cells can uptake fatty acids through lipid chaperone proteins, such as fatty acid binding protein 4 (FABP4) and 5 (FABP5), under the influence of the peroxisome proliferator-activated receptor gamma (PPARγ), facilitating the entry of fatty acids in mitochondrial oxidative metabolism to support metabolic programming for long-term survival [38]. Interestingly, FABP5 has no preference for any class of fatty acids, SFAs, MUFAs, or PUFAs [39]; and PPARγ, which can be preferentially activated by MUFAs and PUFAs, is a positive

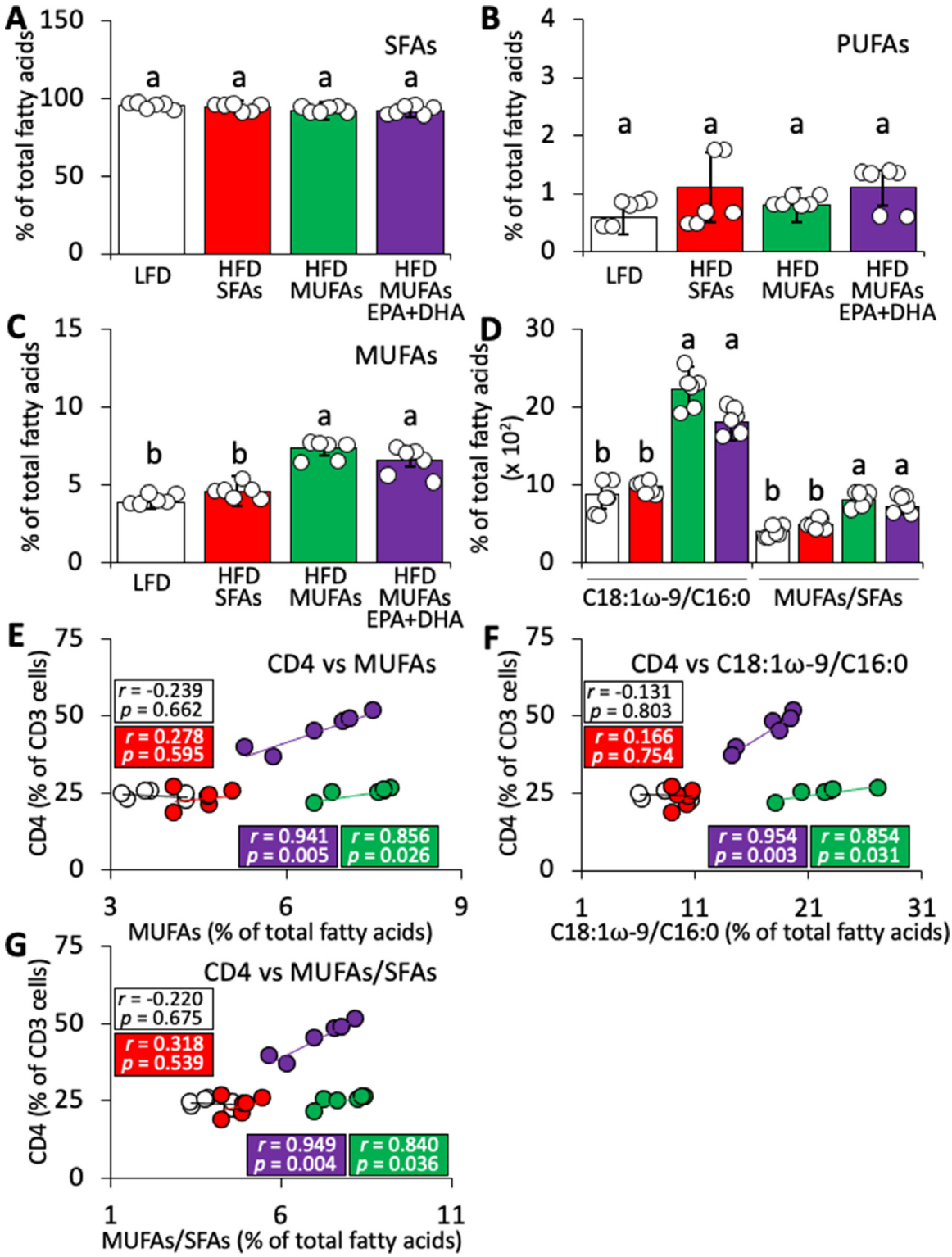

Fig. 4. High-fat diets enriched in SFAs (anhydrous butter, HFD-SFAs), MUFAs (olive oil, HFD-MUFAs) or MUFAs (olive oil) supplemented with EPA and DHA (HFD-MUFAs+EPA+DHA) on total SFAs, MUFAs, and PUFAs in bone marrow (BM) extracellular fluid and correspondence of MUFAs and lipid indexes with $CD4^+$ T cells in BM of wild-type C57BL/6J mice. The percentage relative to total fatty acids in BM extracellular fluid of total SFAs (A), MUFAs (B), PUFAs (C), and oleic acid/palmitic acid (C18:1ω-9/C16:0) and MUFAs/SFAs indexes (D) at the end of 20-week dietary regimens. The data are presented as the mean (bars) and individual points±SD values (n=6), and those marked with different letters are significantly different (P<.05). Differences between groups were assessed by one-way ANOVA with Tukey's *post hoc* test or, in the case of variance heterogeneity, by Kruskal–Wallis with Bonferroni correction. Correlations between the content of total MUFAs (E), C18:1ω-9/C16:0 index (F), and MUFAs/SFAs index (G) in BM extracellular fluid and the frequency of $CD4^+$ T cells in BM. CD, cluster of differentiation; DHA, docosahexaenoic acid (C22:6ω-3); EPA, eicosapentaenoic acid (C20:5ω-3); LFD, low-fat diet; MUFAs, monounsaturated fatty acids; PUFAs, polyunsaturated fatty acids; SFAs, saturated fatty acids.

regulator of FABP4 [37]. Therefore, whether differences in the expression levels and functions of FABP4, FABP5, and PPARγ in T-cell subsets account for the observed differences in $CD4^+$ and $CD8^+$ T-cell responsiveness to HFDs that reshape the fatty acid composition in the bone immune milieu should be further investigated. Contrarily to suppressive effects of palmitoleic acid (C18:1ω-7), oleic acid has been reported to exert stimulatory effects on proliferation in human circulating $CD4^+$ T cells, supporting the high metabolic specificity of the MUFA oleic acid for these cells; however, evidence of $CD4^+$ T-cell exhaustion was observed with palmitic acid [40]. *In vitro*, recent studies have documented the harmful effects of palmitic acid on human BM mesenchymal stem cells, while oleic acid exerting protective effects against this palmitic-acid-induced cytotoxicity [41]. Contrasting effects of oleic acid and palmitic acid on *in vitro* viability of human circulating T cells have also been reported [42]. This could occur in an analogous and a cell-specific manner in BM T-cell subsets of our mice fed with HFDs, as we found that palmitic acid reduced *ex vivo* the integrity of $CD3^+$ while both palmitic and oleic acids reduced that of $CD8^+$ T cells in the BM of lean mice; however, both palmitic and oleic acids were found to be well tolerated by $CD4^+$ T cells. Because T cells in BM are also equipped with the G protein coupled receptor 84 (GPR84), which specific binds medium-chain SFAs from lengths of 9 to 14 carbon atoms [42,43], the role of GPR84 in the expansion of $CD4^+$ and $CD8^+$ T cells within the BM when the interstitial microenvironment is dominated by myristic acid (C14:0) or even shorter SFAs would also be worth investigating. Recent studies have pinpointed to the role of acetate (C2:0) in rescuing the effector function of glucose-restricted $CD8^+$ T cells from mouse spleen [44]. Taken together, our data support *in vivo* and *ex vivo* the general notion demonstrated *in vitro* that MUFAs, compared to SFAs, may have protective properties for T cells [38,45], particularly for BM $CD4^+$ T cells, which could be relevant for the disrupted adaptive immune response seen with the increase in body weight and adiposity [8,34,45]. It is tentative to speculate that accumulated oleic acid in BM may lead to an immunologic adaptation based on increased availability of $CD4^+$ T cells for proper function and coordination of the host adaptive and innate immune responses [46] and even for combat the array of metabolic abnormalities caused by obesity [47].

While advancing our understanding of the relationship between immunological and fatty acid metabolic landscapes in BM in response to HFDs, it was remarkable that the positive correspondence of the pool of stearic acid declined in the BM extracellular fluid after HFD-SFAs with $CD3^+$ T cells, which was not evident when the pool of stearic acid declined after HFDs enriched in MUFAs. To explain this paradox, it is likely that the BM space of different stearic acid compartments were affected differently by HFD-SFAs (probably as a result of an inhibition of the elongation step of its precursor, palmitic acid) and HFDs enriched in MUFAs (probably as a result of its own desaturation to oleic acid being inhibited) to manage the huge affluence of (mainly) palmitic acid or oleic acid from these respective HFDs in the complex ecosystem and anatomy of BM. This notion has been previously shown to occur in the visceral adipose tissue of $Lep^{ob/ob}LDLR^{-/-}$ mice fed with HFDs enriched in MUFAs or in SFAs [29]. The current paradigm is that BM is packed with niches to support the entire lineage of hematopoietic and mesenchymal cells [48,49] and accommodates specific sites within niches for exchanging local metabolites to meet cellular metabolic demands [50,51]. Further studies have suggested that T-cell subtypes do not occupy the same regions [52] and can be separately compartmentalized [53] in the architecture of primary lymphoid tissues. This sophisticated interplay of T cells with the marrow microenvironment could make clear the association between the high frequency of $CD4^+$ T cells and the increase in any lipid index in which the numerator was the content of oleic acid or MUFAs in the BM extracellular fluid. These findings explain the metabolic dependency of T lymphocytes and particularly the $CD4^+$ subset of T cells for oleic acid that can be exploited to avoid BM T-cell dysregulation in the context of HFD-induced obesity. From a mechanistic point of view, fatty acids have been shown to have a direct impact on topography of the plasma lipid bilayer membrane in T cells, which could alter signaling pathways involved in their expansion or maintenance [42,54]. It has been also demonstrated that fatty acids modulate effector functions that are necessary for the stability of T cells by metabolic reprogramming [55] and epigenetic modifications [56].

HFD-MUFAs and HFD-MUFAs+EPA+DHA had similar effects on mice. EPA and DHA were not detected in BM extracellular fluid, although the doses of EPA and DHA administered to mice were consistent with previous studies [16,17]. Other studies have also shown that EPA and DHA supplementation of a HFD enriched in olive oil did not further protect from white fat dysfunction in metabolic syndrome [29] or against obesity-induced bias of hematopoietic cell lineages [14] and that long-chain ω-6 and ω-3 PUFAs have a low ability to regulate the function of skeletal cells in BM cultures [57]. However, in extramedullary sites (lymph nodes and spleen), EPA and DHA have been shown to influence on rearrangement of cytoskeletal proteins that murine $CD4^+$ T cells harness to regulate motility [58]. Together, these findings might emphasize that the role of EPA and DHA in the repository of mature and progenitor cells in the marrow space is limited in the context of obesogenic diets. It is also likely that the impact of EPA and DHA on BM T cells during obesity is incompletely understood, and new experimental approaches will require more specific refinements [59], particularly when dietary MUFAs are also included in the regimen.

There are several limitations of this study that will require further research in the future. One limitation is that these analyses were performed on the bulk of $CD4^+$ and $CD8^+$ T cells. However, upon activation, $CD4^+$ and $CD8^+$ T cells can differentiate into several subsets, which could exhibit differences in the metabolic uptake of fatty acids [60]. Another possible limitation was that derivatives of fatty acids were not determined in the BM, which, at least in the case of those generated from DHA, have been shown to modify the functional state and number of B-cell subsets [61]. Nevertheless, the BM microenvironment can become highly competitive, because BM myeloid cells are known to have a powerful demand for ω-3 PUFAs [62]. This could be also the reason why EPA and DHA were not detected in the BM extracellular fluid of our mice. An added limitation was that, in addition to fatty acids in BM extracellular fluid, we have no identified other factors in the BM microenvironment that could also influence on $CD3^+$ cells and $CD4^+$ and $CD8^+$ T-cell subsets. Also, a potential limitation is that some concentrations of palmitic and oleic acids in our *ex vivo* study could be supraphysiological in relation to the local concentration of fatty acids in the mouse BM.

Overall, we report an undervalued role of dietary fatty acids in the selective acquisition of T-cell subsets into the BM (Fig. 5). The most exciting finding was that oleic acid (the most common MUFA) in the surroundings of marrow T-cell niches during HFD-induced obesity could induce fatty acid metabolism reprogramming for the specific maintenance of $CD4^+$ T cells. Future studies will be needed to further clarify the molecular and functional link between changes in the homeostasis of T-cell subsets in BM that are induced by different dietary fatty acids in the course of obesity. It could also pave the way for research on pathophysiological conditions associated with a decline or exhaustion of $CD4^+$ T cells [63,64] or conditions that require an extra supply of $CD4^+$ T cells

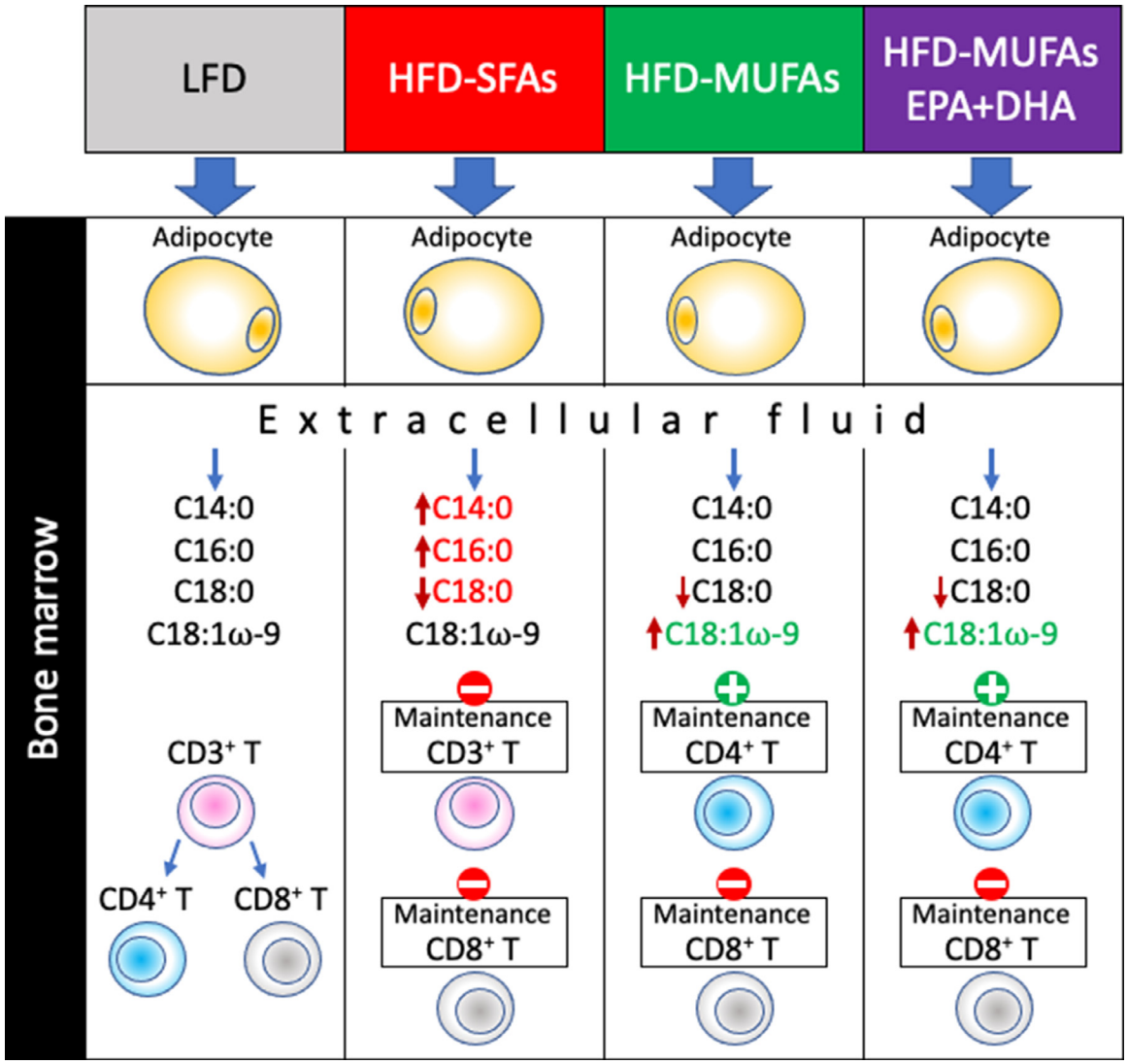

Fig. 5. Overall effects of fatty acids in HFDs on T cells in BM during obesity. Mice fed a low-fat diet (LFD) or high-fat diets enriched in SFAs (anhydrous butter, HFD-SFAs), MUFAs (olive oil, HFD-MUFAs), or MUFAs (olive oil) supplemented with EPA and DHA (HFD-MUFAs+EPA+DHA) for 20 weeks may have a bone marrow (BM) adipose tissue with a fatty acid composition reshaped by fatty acids in the above diets. In BM niches for T cells, $CD4^+$ and $CD8^+$ T cells originate from $CD3^+$ T cells. Released fatty acids from marrow adipocytes in BM extracellular fluid [19] can regulate the maintenance of T cells: the increase of myristic acid (C14:0) and palmitic acid (C16:0) and the decrease of stearic acid (C18:0) reduce the maintenance of $CD3^+$ T cells after HFD-SFAs; the increase of oleic acid (C18:1ω-9) promotes the maintenance of $CD4^+$ T cells after HFD-MUFAs or HFD-MUFAs+EPA+DHA; the maintenance of $CD8^+$ T cells is reduced irrespective of any HFD.

[65,66] through targeting the metabolism of fatty acids and mechanisms regulating their utilization.

Author contributions

Francisco J.G. Muriana and Rocio Abia were principal authors that contributed to the study conception and design. Material preparation, data collection, and analysis were performed by all authors. The first draft of the manuscript was written by Francisco J.G. Muriana and Rocio Abia, and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Declaration of competing interests

The authors declare that there are no conflicts of interest.

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CRediT authorship contribution statement

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Sara Jaramillo: Methodology, Formal analysis, Investigation, Writing – review & editing. **Jose A. Perez-Simon:** Methodology, Formal analysis, Investigation, Writing – review & editing. **Francisco J.G. Muriana:** Conceptualization, Writing – original draft, Supervision, Data curation, Writing – original draft, Writing – review & editing, Funding acquisition. **Rocio Abia:** Conceptualization, Writing – original draft, Methodology, Formal analysis, Investigation, Writing – review & editing, Supervision, Data curation, Writing – original draft, Writing – review & editing, Funding acquisition.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jnutbio.2022.109057.

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