Macrophage immunophenotype but not anti-inflammatory profile is modulated by peroxisome proliferator-activated receptor gamma (PPARγ) in exercised obese mice

Loreana Sanches Silveira[1,2] | Luana Amorim Biondo[2] | Alexandre Abílio de Souza Teixeira[2] | Edson Alves de Lima Junior[2] |Angela Castoldi[3] | Niels Olsen Saraiva Câmara[3] | Willian T. Festuccia[4] | José Cesar Rosa-Neto[2] | Fábio Santos Lira[1]

[1] Exercise and Immunometabolism Research Group, Postgraduation Program in Movement Sciences, Department of Physical Education, Universidade Estadual Paulista (UNESP), Presidente Prudente, São Paulo, Brazil.
[2] Department of Cell and Developmental Biology, Institute of Biomedical Sciences, University of São Paulo (USP), São Paulo, Brazil
[3] Department of Immunology, Institute of Biomedical Sciences, University of São Paulo, São Paulo, Brazil
[4] Department of Physiology and Biophysics, Institute of Biomedical Sciences, University of São Paulo, São Paulo, Brazil

ABSTRACT

Moderate aerobic training may be therapeutic for chronic low-grade inflammatory diseases due to the associated anti-inflammatory response that is mediated by immune cells. The peroxisome proliferator-activated receptor gamma (PPARγ) regulates the M1 (pro-inflammatory) and M2 (anti-inflammatory) polarization, as well as the immunometabolic response of macrophages. Against this background, the present study seeks to clarify whether the conditional deletion of PPARγ in macrophages would have any effect on the anti-inflammatory role of moderate aerobic training. To test this hypothesis, two mice strains were used: PPARγ LyzCre+/+ (KO) and littermates control animals (WT). Each genotype was divided into 1) sedentary high-fat diet (HF) and 2) high-fat diet and moderate aerobic training (HFT) (n = 5–8 per group). The experimental protocol lasted for 12 weeks, comprising 4 weeks of HF diet only and 8 weeks of HF diet and aerobic training (5 times/week, 50–60 minutes/day at 60% of maximum speed). Metabolic analyses were carried out on the serum glucose homeostase, adipose tissue morphology and cytokine content, and macrophage cytokine production. Immunophenotyping and gene expression were also performed. KO male mice were more prone to hypertrophy in the subcutaneous adipose tissue, though only the IL-1β ($p = 0.0049$) was higher compared to the values observed in WT animals. Peritoneal macrophages from KO animals exhibited a marked inflammatory environment with an increase in TNF-α ($p = 0.0008$), IL-1β ($p = 0.0017$), and IL-6 ($p < 0.0001$) after lipopolysaccharide stimulation. The moderate aerobic training protected both genotypes from weight gain and reduced the caloric intake in the KO animals. Despite the attenuation of the M2 marker CD206 ($p < 0.001$) in the absence of PPAR-γ, the aerobic training modulated cytokine production in LPS stimulated peritoneal macrophages from both genotypes, reducing proinflammatory cytokines such as TNF-α ($p = 0.0002$) and IL-6 ($p < 0.0001$). Overall, our findings demonstrate the essential role of PPARγ in macrophage immunophenotypes. However, the deletion of PPARγ did not inhibit the exercise-mediated anti-inflammatory effect, underscoring the important role of exercise in modulating inflammation.

Keywords: adipose tissue, cytokines, immune system, moderate aerobic training, transcriptional factor

INTRODUCTION

Obesity is a global public health concern with a rising prevalence since the industrial revolution due to the increase in caloric intake (1). The obese adipose tissue is characterized by hypertrophy and subsequent higher lipolysis, which can cause ectopic lipid accumulations and impairments of insulin sensitivity (2). Together with the accumulation of fat depots and an increased risk of cardiovascular diseases, obesity may be a trigger to the inflammatory response characterized by a low-grade but chronic (3-4).

The long-term stimulation of innate immune cells exacerbates the release of cytokines, adipokines, and acute phase proteins (3, 5). One of the first studies describing obesity as a trigger of low-grade inflammation was conducted by Hotamisligil (6), in which the tumour necrosis factor alpha (TNF-α), a potent proinflammatory cytokine from visceral adipose tissue, was considered the link between inflammation and insulin resistance.

Although macrophages are considered the central players in local inflammation within adipose tissues, other immune cells play relevant roles in the origin and maintenance of low-grade inflammation. These include different subsets of T and B lymphocytes, eosinophils, and NK cells (7). However, of all the cells from the innate immune system, macrophages are the majority in the adipose tissue stromal vascular fraction (8). Classically activated macrophages (M1) exhibit a more glycolitic metabolism and a higher secretion of TNF-α, IL-6, and IL-1β from obese adipose tissues (9). In contrast, lean adipose

Correspondence:
José Cesar Rosa-Neto, Department of Cell and Developmental Biology, Institute of Biomedical Sciences, University of São Paulo (USP), Av. Lineu Prestes, 524-lab.435, São Paulo, SP 05508-000, Brazil. Email: josecesar23@hotmail.com

tissues not only recruit immune cells with anti-inflammatory characteristics, but also release cytokines such as adiponectin, which induces the polarization of alternatively activated macrophages (M2) (8).

Macrophages recognize pathogens and some fatty acids by a transmembrane protein called Toll like receptors (TLRs). TLR-4 recognizes lipopolysaccharides (LPS) (10) and triggers a pathway that leads to the dissociation of an inhibitory subunit from the nuclear factor kappa B (NF-κB) complex and its subsequent translocation to the nucleus, leading to the transcription of pro-inflammatory genes (11). Diet-derived fatty acids modulate monocytes/macrophages In addition to TLRs expression patterns, saturated fatty acids are believed to activate TLR4-mediated signalling pathways (12). In contrast, some studies have shown that polyunsaturated fatty acids such as eicosapentaenoic acid and docosahexaenoic acid have anti-inflammatory properties due to the ability to activate the peroxisome proliferator activated receptors (PPARs) (13).

PPARs are a family of transcriptional factors that regulate several genes related to lipid metabolism and lipogenesis. Three members from the PPARs family have been identified (α, β/δ, and γ), with diversified tissue distribution (14). PPARα is considerably expressed in tissues with high metabolic rates, such as liver, heart, muscle, brown adipose tissue, and some immune cells. Its main role is the regulation of fatty acid metabolism (15). PPAR β/δ is involved in the transcription of genes responsible for fatty acid uptake, beta-oxidation, and energy decoupling. This fosters the reduction of lipotoxicity and the improvement of insulin sensitivity (16). PPARγ is considered the main regulator of adipogenesis. It has metabolic functions such as adipocyte differentiation, lipid storage, and glucose metabolism, as well as anti-inflammatory functions that are expressed essentially via macrophages. Some of the PPARγ target genes are lipoprotein lipase, CD36, and adiponectin (2). Two anti-inflammatory mechanisms of PPARγ have been previously identified. One is the suppression of proinflammatory transcription factors, including STAT, NF-κB, and AP-1, via competition for the same PPAR responsive element (PPRE) (17). The other mechanism is the non-removal of corepressor complexes from inflammatory genes, keeping them in a suppressed state (18).

For PPARγ binding to specific DNA sequences to occur, heterodimerization with a second member of the nuclear receptor family, the retinoic X receptor (RXR), is required. In the absence of ligands, PPARγ silencing occurs by transcriptional corepressor complexes. Alternatively, binding of agonists ligands induces conformational changes in PPARγ structure. This leads to the trapping of transcriptional coativators (19). Previous human studies have shown that PPARγ activation leads monocytes into the anti-inflammatory profile by alternative M2 macrophage polarization (20). Additionally, pre-treatment with proinflammatory cytokines down-regulates PPARγ gene expression in bone marrow-derived macrophages. In addition, the pre-treatment of adipocytes with arachidonic acid hinders PPARγ gene and protein expression (21).

In 2010, Kawanishi et al. showed that moderate aerobic training (16 weeks on the treadmill) was able to increase M2 (CD163) mRNA expression of surface markers and reduce M1 (CD11c) in obese mice with a concomitant reduction of TLR-4 mRNA. Besides this, exercise has been considered an important PPARγ activator by different mechanisms such as gene expression (22), DNA-binding (23), or ligands generation (24). Our group has previously showed that PPARα plays an important anti-inflammatory role in acute exercise (25). PPARγ also plays an essential role in the control plasma lipids (26) and in the alternative activation of monocytes in human (27) and animal models (28) in moderate-intensity aerobic training.

Due to the need for therapeutic strategies for chronic inflammatory diseases, such as diabetes, we chose not to use a therapeutic target already well-established in the literature, PPARγ. Rather, we chose to apply an acknowledged treatment, physical exercise, based on the hypothesis that the absence of PPARγ would impair the well-known anti-inflammatory effect of exercise. As such, in the context of obesity the over-activation of inflammatory TLR4 pathway, we seek to investigate whether macrophages PPARγ influences the exercise-mediated anti-inflammatory effect. To evaluate this, we analyzed alterations in the metabolic and inflammatory parameters of PPARγ CreLox mice induced to obesity by a high-fat diet and subsequently submitted to an 8-week moderate-intensity aerobic training.

MATERIALS AND METHODS

Animals and diet

The Animal Care Committee of the Institute of Biomedical Sciences approved all the experimental protocols (University of São Paulo, Brazil, Protocol 112/13CEUA). Mice with a selective deletion of PPARγ in the myeloid lineage (PPARγ Cre+) were produced by crossing PPARy Lox/P mice (The Jackson Laboratory, stock no. 004584) with Lys Mcre mice (The Jackson Laboratory, stock no. 004781). The animals were selected by genotyping using primers and protocols indicated by the manufacturer Figure S1. The mice in the study were male 8–10-week-old mice maintained at 22 ± 2°C in a 12-hour light/dark cycle.

The mice were fed with a high-fat diet (HF, 59% of calories from fat, 15% from proteins, and 26% from carbohydrate) consisting of a modified AIN-93 diet with an increased lipid content and a reduced carbohydrate content (Table S1) (29). The basic composition of the high-fat diet were: hydrogenated vegetable fat, sucrose, casein, dextrinized corn starch, corn starch, microfine cellulose, sunflower oil [omega 6], mineral mix, vitamin mix, L-cystine, and choline bitartrate. Two or three mice were kept in each cage and both water and the diet were supplied *ad libitum*. The food consumption pattern was recorded weekly and the caloric intake was calculated (Figure 1C).

Experimental groups

The mice were subdivided into four groups (8–10 mice each): wild type sedentary (WT HF) [21.6 ± 1.12g], knockout sedentary (KO HF) [21.41 ± 1.33g], wild type trained (WT HFT) [20.51 ± 1.54g], and knockout trained (KO HFT) [19.65 ± 1.15g]. All the animals were adapted to a treadmill for 1 week (10 min—10 m/min) and performed a maximum test speed in three moments (week 1/8, week 4/8, and week 8/8). Only trained groups were submitted to the exercise training protocol (5 days/week, 1 hr/day 55–65% of maximum speed

test) (30-31). After 72 hours of resting from the last maximum speed test, the animals were euthanized (6hr fasting).

Analysis of serum lipid concentrations

The mice were euthanized and blood was collected from the same site of decaptation, and the serum was obtained by centrifugation at 3000rpm for 15 minutes at 4°C. The non-esterified fatty acids (NEFA; HR Series NEFA HR; Wako Pure Chemical Industries, Ltd., Richmond, VA), glucose, triacylglycerol (TAG), and total cholesterol (TC) were analyzed in the serum by a colorimetric method (Labtest, Lagoa Santa, Brazil). All the assays were performed in duplicates.

Glucose (GTT) and insulin (ITT) tolerance tests

The mice received an intraperitoneal injection of D-glucose (2 $g.kg^{-1}$ body weight) or insulin (0.375 $U.kg^{-1}$ body weight) after a 4-hour fast. For the glucose tolerance test (GTT), blood drops were collected from the tail vein before and at 15, 30, 60, and 90 minutes after the glucose injection. The glucose concentrations at each time point were used to calculate the area under the curve (AUC) (32). For the insulin tolerance test (ITT), blood samples were collected from the tail vein before and at 5,10,15,20, 25, and 30 minutes after insulin injection. The constant for serum glucose disappearance (KITT) was calculated by linear regression of glycemic levels measured between 5 and 15 minutes after insulin injection according to Bonora (1989) (33). An interval of 72 hours between the two tests was observed. Accu-Chek Performa (Roche, São Paulo, SP, Brazil) was used to assess the levels of glucose.

Citrate Synthase

The activity of citrate synthase was determined using 5. 5'-dithiobis- (2-nitrobenzoic acid DTNB) using muscle (soleus) homogenates in 100 mM Tris, pH 8.0, 0.1 mM acetyl-CoA, 0.1 mM 5,5'-dithiobis- (2-nitrobenzoic acid), 0.1% triton X-100. 2 µg of supernatant protein were used for the reaction that was started with 0.2 mM oxaloacetate and monitored at 412 nm for 3 minutes at 25 ° C.

Enzyme-linked immunosorbent assay (ELISA)

Subcutaneous (inguinal) adipose tissue (80–100 mg) and adipocytes extracted from the same depot were homogenized in radioimmunoprecipitation assay buffer (0.625% Nonidet P-40, 0.625% sodium deoxycholate, 6.25 mM sodium phosphate, and 1 mM ethylenediaminetetraacetic acid [EDTA] at pH 7.4) containing 10$\mu g.ml^{-1}$ of protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO). After centrifugation, the supernatant was used to determine the protein concentration by the Bradford assay (Bio-Rad, Hercules, CA). Insulin from the serum and cytokines from the supernatant of cultured macrophages and from the adipose tissue were assessed in duplicates by ELISA (DuoSet ELISA; R&D Systems, Minneapolis, MN).

Peritoneal macrophage isolation and culture

Macrophages were obtained by washing the peritoneal cavity with Roswell Park Memorial Institute (RPMI) culture medium (Sigma-Aldrich), enriched with 2% fetal bovine serum (FBS), and antibiotics (1%). The medium collected was centrifuged, the supernatant aspirated, and the cell pellet resuspended in 1 ml of complete medium The cells were counted in a haemocytometer using trypan blue (0.4%) exclusion and 5x105 viable macrophages were cultured in medium (RPMI) supplement with fetal bovine serum (10%), and antibiotics solutions in standard conditions (37°C in humidified 95% air; 5% CO2). After 2 hours, nonadherent cells were removed by washing with RPMI. Adherent cells were then collected using cold phosphate-buffered saline (PBS) 2% simulated body fluid for further flow cytometry analysis or incubated with 2.5µg.ml−1 of lipopolysaccharide (LPS; Escherichia coli, serotype 0111:B4; Sigma Aldrich) for 24 hours (34). The medium was collected for the determination of interleukin-6 (IL-6), IL-10, IL-1β, monocyte chemoattractant protein-1 (MCP-1), IL-1ra, and TNF-α by ELISA (R&D System) according to the manufacturer's instructions.

Adipose tissue-macrophages and peritoneal macrophages flow cytometry

Adipose tissue was digested with collagenase type II (2 $mg.ml^{-1}$; Sigma- Aldrich) in PBS with EDTA (2 mM; Sigma-Aldrich) and 0.5% FBS for 60 minutes at 37°C in a shaker. The suspension was filtered using a cell strainer 100 µm and subsequently centrifuged at 400 rpm for 10 min at 4°C. The supernatant was decanted and the pellet was resuspended in 3 ml lysis buffer (17 mM Tris–HCl + 0.144M de cloreto de amônia, pH 7.2) for 2 min for purification of leukocytes. After centrifugation at 3,000 rpm for 10 minutes at 4°C, the cells were washed with PBS, and stained with the following antibody panel: Anti-CD11b, F4/80, CD11c, CD206, diluted 1:100 (BioLegend, San Diego, CA). M1 macrophages were characterized by the expression of CD11c in adipose tissue-resident macrophages and CD86 in peritoneal macrophages concomitant with F4/80 and CD11b markers and M2 macrophages by the expression of CD206 concomitant with F4/80 and CD11b markers (Table S2). The characterization of subpopulations of leukocytes was performed on the FACS CANTOII machine (Beckton Dickson, Franklin Lakes, NJ) and data analysis was performed with FlowJo 9.5.3 software Tree Star (Ashland, OR).

Real-time reverse transcription PCR

Macrophage total RNA was extracted with TRIzol reagent (Invitrogen Life Technologies, Grand Island, NY), following the method described by Chomczynski and Sacchi (35). Reverse transcription to complementary DNA (cDNA) was performed using the high capacity cDNA kit (Applied Biosystems, Foster, CA). Gene expression was evaluated by real time PCR according to Higuchi et al. (36), using SYBR Green Master Mix (Applied Biosystems) as a fluorescent dye. Primer sequences are shown in Table S3. Quantification of gene expression was carried out using the RPLP0 gene as an internal control, as previously described by Liu and Saint (37).

Histological sections

Tissues were removed, fixed in 4% formaldehyde (phosphate buffer) at pH 7.4, for a period of approximately 24 hours at room temperature. After fixation, the tissues were dehydrated in ethanol, clarified in xylol and embedded in paraffin at 60°C. Subsequently, the paraffin blocks containing the tissues were cut into a microtome (Leica®, Germany),

resulting in cuts of 5 µm in thickness. The sections were stained with hematoxylin and eosin (H & E). For histological investigation. An image analysis system consisting of a digital camera (Evolution, Media Cybernetics, Inc., Bethesda, MD) attached to a light microscope (Eclipse 400, Nikon) was used. High quality images (2048 × 1536 pixels) were captured with ImagePro Plus 4.5.1 (Media Cybernetics) and quantified areas of adipocyte perimeters and diameters by Fiji Software (ImageJ) Madison, Wisconsin, USA - Adiposoft (38).

Statistical analysis

All the groups were first tested for the distribution normality (Shapiro Wilk test) and subsequently compared in terms of the effects of genotype (WT HF x KO HF) and exercise (sedentary x trained) by using the two-way ANOVA followed by Bonferroni post hoc test. For comparison between two variables, the Student T test was used. The analyses were performed using the software GraphPad Prism version 6.01 (La Jolla, CA). Results are presented as mean ± standard error of mean. The significance level adopted was $p < 0.05$.

RESULTS

Aerobic training avoids exacerbated weight gain in both genotypes and decreases calorie in KO animals

Despite the occurrence of body weight gains in the same proportion (with no statistically significant differences between the WT HF and KO HF) (Figure 1A), the KO HF mice showed a higher calorie intake in the sedentary groups

Table 1. Effect of PPARγ deletion and aerobic training on variables of body composition, lipid profile, and serum adiponectin of obese mice. All values are mean ± standard error of the mean (n = 8–10 / group) (two-way ANOVA followed by Bonferroni post-test $p < 0.05$).

	WT HF	KO HF	WT HFT	KO HFT
Body weight (g)	33.50 ± 4.94[a]	34.94 ± 3.04[b]	28.82 ± 2.30	31.18 ± 4.12
SAT (%)	1.97 ± 0.77	2.47 ± 1.34	1.89 ± 0.63	1.78 ± 1.04
RAT (%)	0.97 ± 0.42	1.10 ± 0.42[b]	0.78 ± 0.60	0.67 ± 0.44
EAT (%)	4.46 ± 1.575	5.39 ± 1.51[b]	3.52 ± 1.53	3.01 ± 1.75
Liver (%)	4.11 ± 0.19	4.20 ± 0.53	3.65 ± 1.01	4.19 ± 0.22
GAST (%)	0.76 ± 0.12[ac]	0.89 ± 0.11	1.01 ± 0.13	0.96 ± 0.077
TC (mg.dL^{-1})	171.01 ± 9.18	159.52 ± 16.64	138.69 ± 8.40	137.87 ± 5.99
TAG (mg.dL^{-1})	136.29 ± 11.88	127.77 ± 5.81	124.05 ± 7.02	113.40 ± 3.42
NEFA (mg.dL^{-1})	0.766 ± 0.04	0.654 ± 0.01	0.712 ± 0.03	0.676 ± 0.03
Adiponectin (ng.mL^{-1})	5.20 ± 2.64	6.26 ± 0.511[b]	5.72 ± 0.32	4.76 ± 0.37

g = grams; % = relative weight; SAT = Subcutaneous adipose tissue; RAT = Retroperitoneal adipose tissue; GAST = Gastrocnemious; EAT = Epididimal adipose tissue; TC = Total Cholesterol; TAG = triacylglycerol; NEFA = free fatty acids; a = different from WT HFT; b = different from KO HFT; c = different from KO HF.

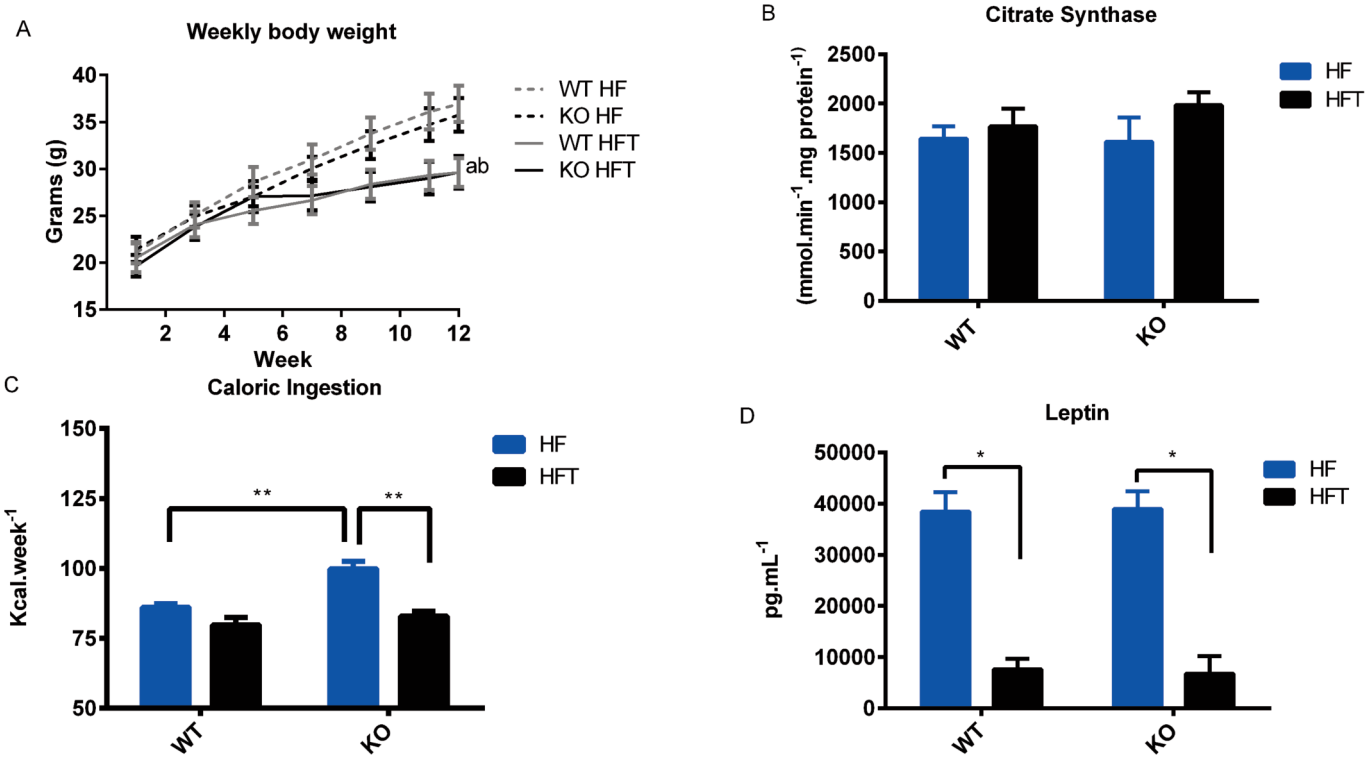

Figure 1. Effect of PPARγ deletion and aerobic training in obese mice. Weekly body weight (A), citrate synthase from the soleus muscle (B), caloric intake (C) and serum leptin concentration (D). All values are mean ± standard error of the mean (n = 8–10 per group). (Two-way ANOVA followed by Bonferroni post-test). a = different from WT HF; b = different from KO HF; * $p < 0.05$; ** $p < 0.01$.

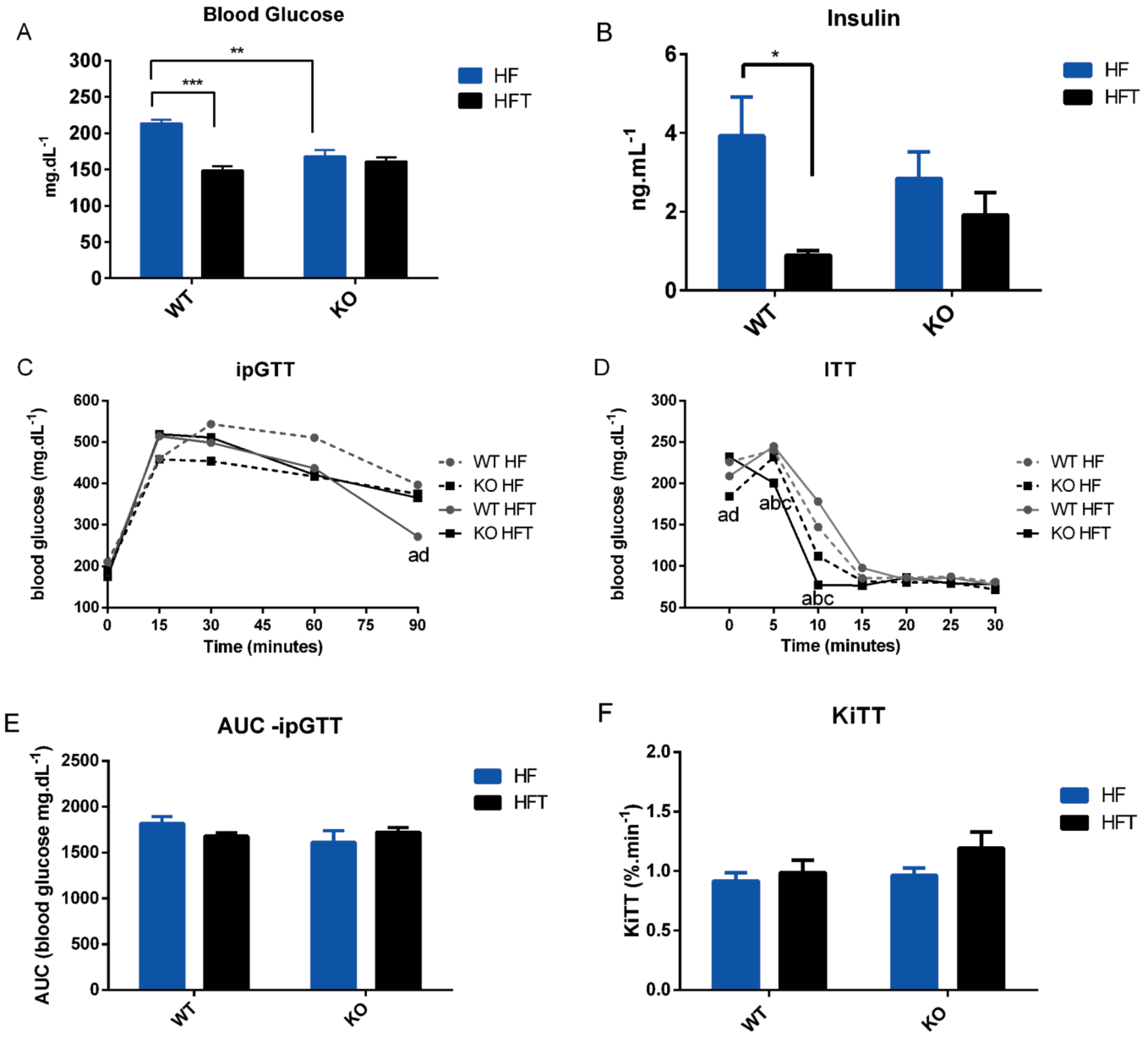

Figure 2. Glucose metabolism of WT and KO animals submitted to a high-fat diet and aerobic training. Glucose (A) and fasting insulin (B), intraperitoneal glucose tolerance test (ipGTT) (C), intraperitoneal insulin tolerance test (ipITT) (D), area under the AUC curve of the tolerance test (E) and glucose decay constant (KiTT) (F). All values are mean ± standard error of the mean (n = 5–6 per group). (ANOVA two-way followed by Bonferroni) a = different from WT HF. b = different from KO HF. c = different from WT HFT; d = different from KO HFT. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

but aerobic training balanced the calorie intake in the genotypes (Figure 1C). In addition, leptin concentrations were equivalent between the genotypes with exercise-mediated decreases in both groups (Figure 1D).

Adipose tissues from the KO animals seems to be more susceptible to weight variances generated by exercise training, especially decreasing the ones located in the central region (retroperitoneal and epididymal) (Table 1). Neither the liver nor brown adipose tissue weights were affected by exercise *(data not shown)*. However, the gastrocnemius relative weight, which was naturally higher in the WT HF, was more enlarged in the training group (Table 1).

Aerobic training reduces fasting blood glucose and insulin in WT animals fed with a high-fat diet

Serum glucose and insulin concentrations in WT HFT animals were lower when compared to their sedentary pairs. In the KO HF, there was no significant effect by aerobic training (Figure 2A and B). With regards to the glucose tolerance and insulin resistance tests, exercise does not alter the area under the curve nor the glucose decay constant after insulin injection in any of the genotypes (Figure 2C and D).

Neither aerobic training nor the genotype affected the serum lipid concentrations (Table 1), however, adiponectin was lower in the KO HFT group when compared to their sedentary pairs (Table 1).

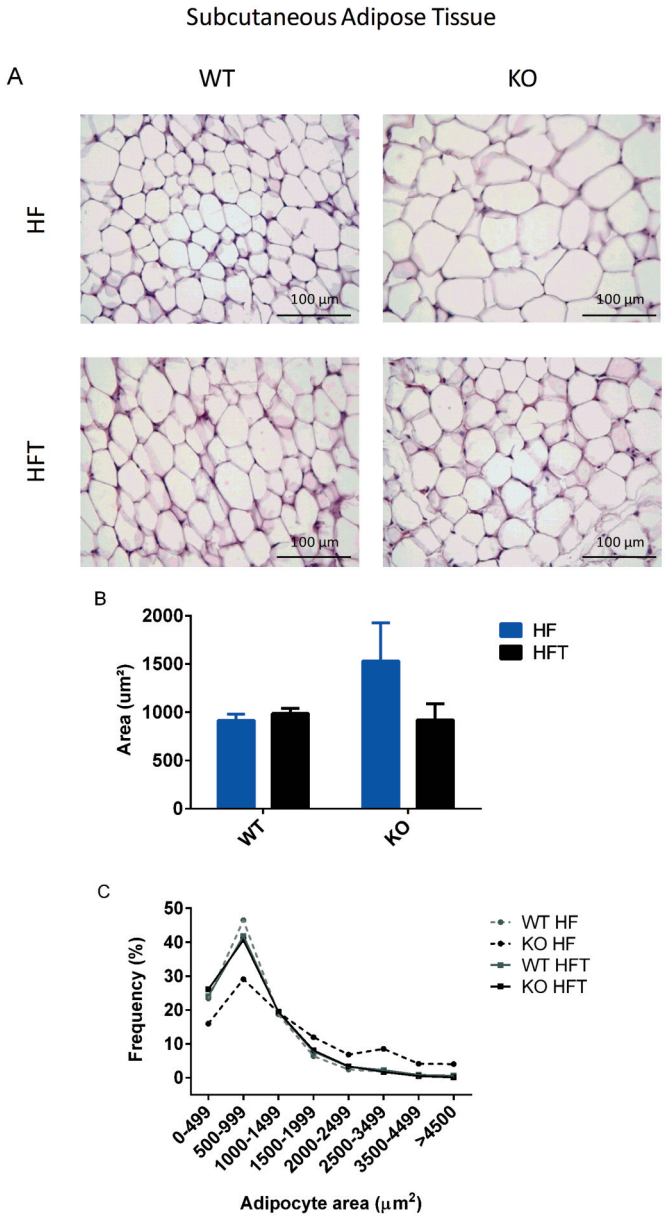

Figure 3. Morphology of adipose tissues of WT and KO animals fed with high-fat diet and submitted to aerobic training. Representative pictures of each group using 40 times amplification (A), mean area (B) and frequency of subcutaneous adipocyte distribution (C). The values were calculated from histological sections images using the ImagePro Plus Fiji version 6.0 program. All values are expressed as the mean ± standard error of the mean (n = 3 per group). (Two-way ANOVA followed by Bonferroni).

Aerobic training attenuates hypertrophy in subcutaneous adipose tissue of KO animals

Although not significant, the KO HF had a slightly higher adipocyte area in the subcutaneous adipose tissues but aerobic training did not prevent the expansion of this exacerbated area (Figure 3B and C).

Although the aerobic training did not contribute to the morphological alteration in the WT subcutaneous adipose tissues, the M1 marker CD11c was significantly reduced. However, even with normalization of subcutaneous adipose tissue area in the KO animals, macrophages maintained their M1/M2 markers balance (Figure 4B).

The IL-6 and adiponectin profiles were similar in both isolated adipocytes and total adipose tissue (adipocytes plus stromal portion) microenvironments. However, TNF-α seems to be essentially produced in the stromal portion of the tissue (Figure 5A and D). In addition, regardless of the non-reduction of M1 marker $CD11c^+$ as observed in the KO HFT (Figure 4B), exercise anti-inflammatory effects such as decreased TNF-α and IL-1β should be highlighted in this genotype (Figure 5D and H).

Aerobic training maintains anti-inflammatory effects in peritoneal macrophages despite of reduced M2 markers CD206 in the absence of PPARγ.

Aerobic training increased mainly M1 markers in both obese groups. In addition, M2 markers $CD206^+$ were lower in cells with PPARγ deletion when compared to WT (Figure 4A). The KO HFT showed a reduction of proinflammatory cytokines production such as TNF-α and IL-6 in 24h-cultured cells with LPS stimulation (Figure 6A and B). IL-10 production after LPS was lower in the KO macrophages of the trained animals compared to WT (Figure 6C). IL-1β showed higher values when LPS stimulated and increased due to the effect of aerobic training on WT macrophages (Figure 6F).

It was observed that LPS significantly increased the TLR-4 gene expression and that exercise prevents this phenomenon regardless of PPARγ (Figure 6H). The hypoxia inducible factor 1 alpha was higher in the KO HF when compared to the WT HF and KO HF LPS (Figure 6G).

DISCUSSION

In this study, different responses in macrophages subpopulation (peritoneal and adipose tissue-associated) were identified as a result of obesity and moderate aerobic training. Furthermore, PPARγ transcription factor has been shown to be necessary for modulating macrophages surface markers, though it plays a lesser degree of importance in the modulation of cytokines production. Since macrophages are plastic cells (39), it is reasonable that exercise can alter their function/secretion without undergoing significant phenotypic changes. This finding suggests the idea that when exercise is involved, not only macrophages phenotype but cell functionality (cytokine production) must be taken into account.

The KO HFT group showed a lower caloric intake despite the decrease in serum leptin concentrations which suggests that such animals may develop resistance to leptin when fed a high-fat diet, phenomenon that is also common in obese humans (40) and associated with hyperinsulinaemia and the risk of type 2 diabetes (41). Reduction in fasting insulin as well as improvement in glucose tolerance test in the WT HFT animals may be explained by an increase in glucose uptake in peripheral tissues such as skeletal muscle by insulin-independent pathways (42-43). In a study conducted simultaneously with the high-fat diet, exercise training improved insulin sensitive in the WT group (44) facilitated by the reduction in both fasting insulin and glucose.

In the knockout model for PPARγ in adipose tissue, high-fat diet did not induce a significant increase in body weight and insulin resistance (45). Compared to the data of the present study, which uses animals with the same gene deletion but

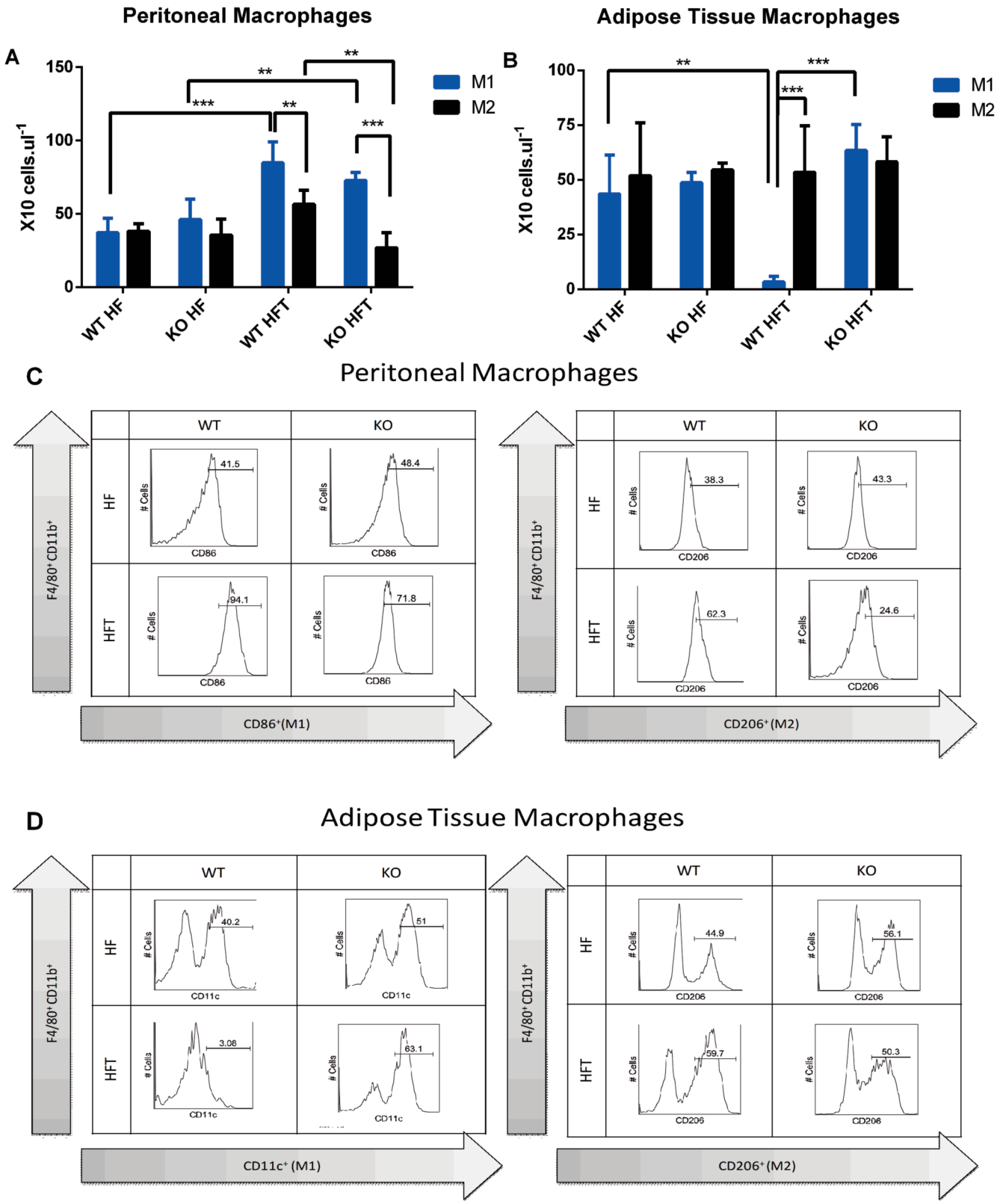

Figure 4. Phenotypic characterization of macrophages by flow cytometry. Effects of high fat-diet and aerobic training on peritoneal (A) and adipose tissue-associated (B) macrophages phenotypic markers in PPARγ conditional knockout (KO) and control (WT) mice. Quantification of markers (A and B) and histogram representation of the groups analysed (C and D). Leukocytes were identified by CD45. A sequential labeling strategy was used to identify populations expressing specific markers ($F4/80^+CD11b^+$), followed by identification of subpopulations with expression patterns: peritoneal macrophages M1 ($CD86^+$), M2 ($CD206^+$) and adipose tissue M1 ($CD11c^+$), M2 ($CD206^+$). (Two-way ANOVA followed by Bonferroni or t test for differences between M1 and M2) * $p < 0.05$; ** p, 0.01; *** $p < 0.001$

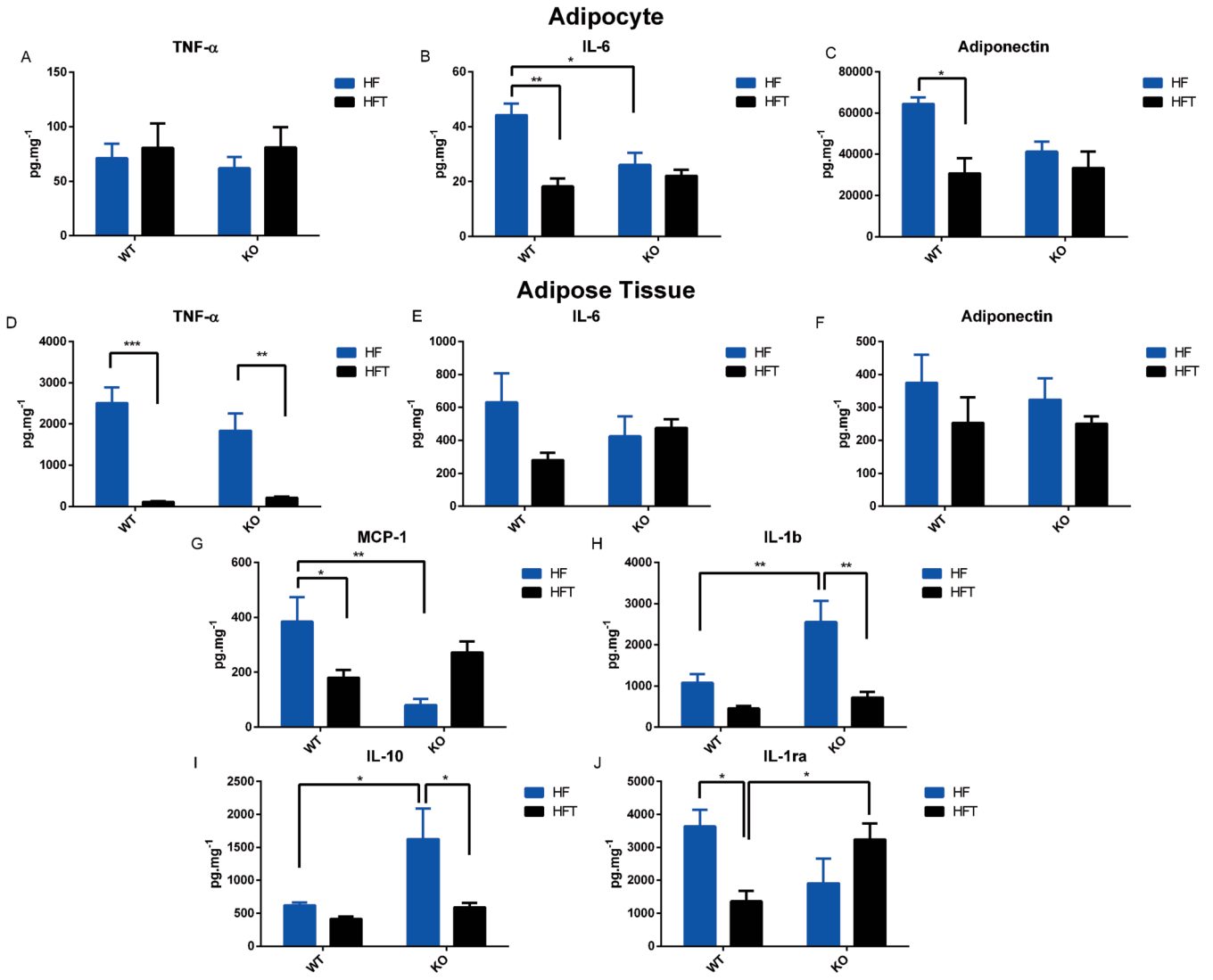

Figure 5. Isolated adipocytes and adipose tissue cytokines content of WT and KO mice fed with a high-fat diet and submitted to aerobic training. Adipocyte isolated from subcutaneous adipose tissue: tumor necrosis factor-α (TNF-α) (A), interleukin-6 (IL-6) (B) and adiponectin (C) and cytokine content in subcutaneous adipose tissue: TNF-α (D), IL-6 (E), adiponectin (F), monocyte chemotactic protein-1 (MCP-) (G), interleukin-1beta (IL-1β) (H), interleukin-10 (IL-10) (I) and interleukin-1 antagonist receptor (IL-1ra) (J). Cytokine concentrations were determined by ELISA and relativized by total proteins (n = 5 per group). Data are presented as mean ± standard error of the mean. (Two-way ANOVA followed by Bonferroni). * $p < 0.05$; ** p, 0.01; *** $p < 0.001$.

in myeloid cell lines, it was observed that the KO HFT animals had a more pronounced reduction in adipose tissue weight in the central regions (epididymal and retroperitoneal) compared to WT. Aerobic training is known to be a useful therapy for reducing visceral adiposity and one of the underlying mechanisms may be an increase in adipocyte triglyceride lipase expression (ATGL) related to an overexpression of PPARγ2 in adipose tissues (46). Other mechanisms that may explain an improvement in insulin sensitivity is the AMPK (Adenosine 5-Monophosphate-Activated Protein Kinase) activation, an energy sensor increases glucose uptake in an insulin-independent manner (47). AMPK activation in macrophages, either via exercise or pharmacological means (48), is also strongly related to an anti-inflammatory phenotype (49).

In relation to the serum variables, adiponectin was lower in KO HFT. A systematic review focused on the effects of exercise on serum adiponectin concentrations concluded that in humans adiponectin was acutely or chronically increased as an effect of aerobic training (50). Although adiponectin is produced in adipose tissues, PPARγ deletion in myeloid cells may have affected its serum concentration. In isolated adipocytes from WT HFT, adiponectin was significantly reduced compared to WT sedentary. This fact suggests that the reduction in WT adipocytes may be due to a decrease in adipose tissue mass, though the lack of PPARγ may also be involved. PPARγ is a potent regulator of adipogenesis and the incorporation of fatty acids by adipose tissues (51). In obesity, there is also an increase in the expression of adipose tissue–associated macrophages in animal (52) and human models (53). Thus, the results of cytokine content (total tissue and isolated adipocytes) as well as the immunophenotying, indicates that these cells may be a relevant way to examine PPARγ interferences.

The exact mechanism by which this inflammatory stimulus initiates insulin resistance and/or metabolic dysfunctions has not been fully elucidated, however, some studies have investigated the alleged low-grade chronic inflammation caused by

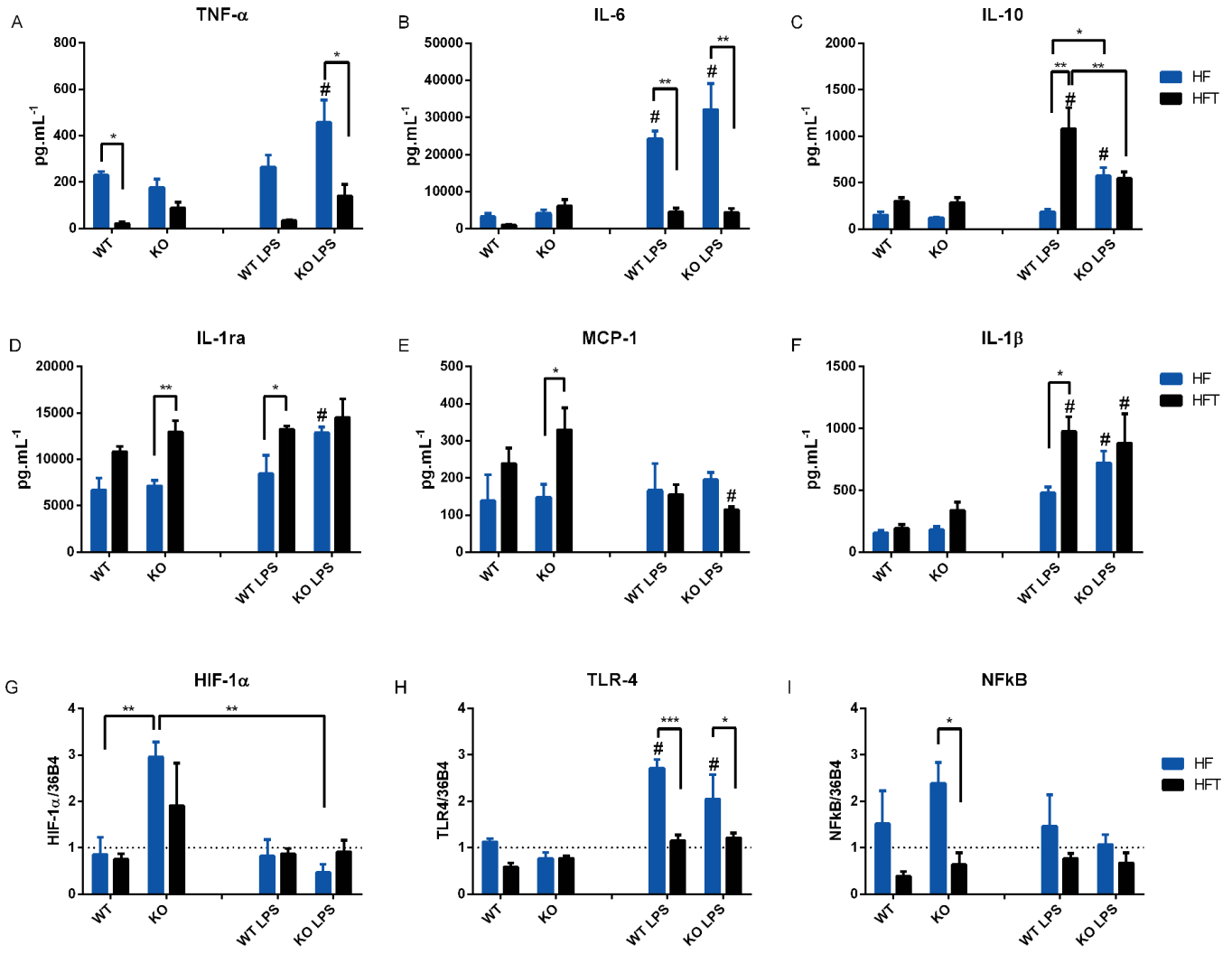

Figure 6. Peritoneal macrophages cytokines production and gene expression from WT and KO obese mice submitted to aerobic training stimulated or not by LPS (2.5 μg.mL^{-1}). Cytokines production: Tumor necrosis factor-α (TNF-α) (A), interleukin-6 (IL-6) (B), interleukin-10 (IL-10) (C), interleukin- -1ra (IL-1ra) (D), monocyte chemoattractant protein-1 (MCP-1) (E) and interleukin-1β (IL-1β) (F) were determined by ELISA in culture medium (n = 8–10 per group) and gene expression: hypoxia inducible factor 1-α (HIF-1α) (G), toll- like receptor 4 (TLR4) (H) and nuclear factor kappa B (NF-κB) (I) were obtained by polymerase chain reaction (n = 5 per group). Data are presented as mean ± standard error of the mean. (ANOVA Two-way followed by Bonferroni). * $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$; # different from the same non-stimulated group.

obesity. Previous research has shown that adipose tissue macrophages from non-obese subjects present an M2 profile (54). In our model, the aerobic training reduced M1 marker CD11c^{+} only in WT HFT, in agreement with Kawanishi et al.'s findings which showed that the increase of CD11c^{+} by a high-fat diet was attenuated by chronic exercise due to the inhibition in M1 surface markers mRNA in adipose tissues of obese mice and TLR4 downregulation (55). In the present study, TLR4 gene expression in adipose tissues was not measured, though TLR4 downregulation was present in LPS stimulated peritoneal macrophages from the trained mice, indicating that the expression of the receptor is a strong regulator of exercise-induced immune response over obesity. One mechanism recently related to this theory is the irisin production, an adipomyokine induced by muscle contraction, and relevant for adipose tissue browning that may be associated with downregulation of downstream pathways of TLR4/MyD88 (56).

An important role has been suggested for proinflammatory cytokines derived from adipose tissue-associated macrophages, especially TNF-α and IL-1β (54). Reflecting what has been previously suggested our data showed that in the KO HF animals there was an increase of IL-1β in comparison with WT HF, which was reverted by the aerobic training. IL-1β plays a very important role in adipose tissues as it is associated not only with insulin resistance but with the secretion of proinflammatory cytokines (57). Secretion of IL-1β is mediated by the NLRP3 inflammatory pathway and the adipose tissue is believed to be a major contributor to serum IL-1β concentration (58). The KO HFT exhibited a lower IL-1β content in the adipose tissues, agreeing with a recent study that observed reduced NLRP3 and IL-1β gene expression via resistive and aerobic training, respectively (59) (Figure 7).

Aerobic training was also effective in lowering inflammatory cytokines such as TNF-α, MCP-1, and a tendency to reduce IL-6 in WT HFT. Surprisingly, there was no excess of MCP-1 in KO HF. However, studies have shown that increased MCP-1 in adipose tissues may be associated with insulin resistance. Since the KO HF animals exhibited a

milder insulin resistance, this may be one of the reasons why MCP-1 was unchanged (60).

Similar to adipose tissue-associated macrophages immunophenotyping, peritoneal macrophages markers were also not unbalanced by the absence of PPARγ or by the high-fat diet, but exercise was a main point to disturb the M1/M2 equilibrium. One assumption may be the constitutive secretion of proinflammatory cytokines previously observed in obese Zucker rats in response to LPS (61) that was possibly increased due to the HF diet (62). In addition, peritoneal macrophages isolated from the trained mice showed higher numbers of the M1 marker CD86 in cells from both genotypes. However, the M2 marker CD206 was lower in the KO HFT compared to WT HFT.

A previous study showed that proinflammatory cytokines such as TNF-α and IL-6 even under LPS stimulation had similar secretion patterns independently of PPARγ macrophages deletion (63), though the LPS dose (0.1ug.mL−1) was lower compared to the present study (2.5ug .mL−1). Chawala et al. have suggested that macrophages PPARγ is involved in inflammation due to the effect of its ligands on lipid metabolism, such as regulation of its target gene, CD36. However, in regards to cytokine production and the inflammatory response, the effects may be independent of PPARγ activation capacity (63).

The LPS-stimulated WT HFT group showed an anti-inflammatory response to aerobic training by reducing TNF-α and IL-6, while increasing IL-10 and IL-1ra. The possibility of a suppression of inflammatory responses may be due to a functional anti-inflammatory phenotype via the activation of AMPK, the negative regulation of IκB degradation and the positive regulation of Akt (49). In this context, a study evaluating the surface markers of monocytes after aerobic training reported that the profile of polarization markers was representative of a M2 subtype of macrophages, the "M2b," characterized by the production of M1-associated cytokines as well as high concentrations of IL-10 (27, 64). Although the same reduction in TNF-α and IL-6 have been observed in HFT KO, no improvement in M2 related cytokines such as IL-10 and IL-1ra was noted in the present study. In humans, studies have shown that low-intensity aerobic training may induce monocyte differentiation into M2 macrophages via PPARγ/PGC-1α/β (65), confirming the harmful effect of the anti-inflammatory action of exercise on the absence of PPARγ.

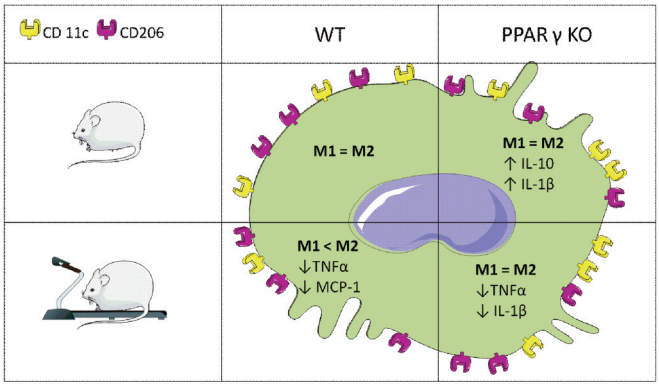

Figure 7. Exercise-mediated effects on subcutaneous adipose tissue of WT and PPARγ KO mice. Adipose tissue macrophages from WT exercised mice showed lower M1 marker (CD11c) compared to KO although the absence of PPARγ did not inhibit proinflammatory cytokines (TNF-α and IL-1β) content reduction. WT = wild type; PPARγ = peroxisome proliferator-activated receptor-gamma; KO = knockout; CD = cluster of differentiation; TNF-α = tumoral necrosis factor alpha; IL-1β =interleukin 1 beta; IL-10 = interleukin 10; MCP-1 = monocyte chemo attractant protein -1.

In endothelial cells, the role of PPARγ in the inhibition of MCP-1 and IFN-γ has been suggested by Murao, et al. (66), but little is known about its effects on macrophages. In the present study, there was a tendency for MCP-1 to decrease in the HFT KO group after stimulation of LPS in culture. One possible reason for this may be that the exercise induced LPS overflow into the circulation, resulting in autoimmunization or LPS tolerance (67). This is because TNF-α and IL- 6 did not increase in the presence of LPS in animals of both genotypes.

Activation of the TLR4/NF-κB pathway results in an increased expression of inflammatory cytokines (68).This increase can be explained by HF diet causing local inflammation, via TLR4/NF-κB, in the gut (69). However, this has not been previously explored in peritoneal macrophages in the context of exercise. Unchanged gene expression of inflammation-related factors such as HIF1α and TLR4 in non-LPS-stimulated peritoneal macrophages may be suggested as a possible explanation for the non-increase in proinflammatory cytokines in the KO HF compared to WT HF. However, the decrease in TLR4 gene expression in LPS-stimulated cells from trained mice, independently of the genotype, suggests that exercise was primordial in TLR4 mRNA downregulation. This is one of the reasons why training may have reduced inflammatory cytokines.

HIF-1α remained unchanged in WT animals and elevated in KO (without LPS), demonstrating that this factor does not appear to have an effect on the secretion of proinflammatory cytokines in peritoneal macrophages. Yet, this increase in HIF-1α expression may be signalling a change in cellular metabolism via a Krebs cycle accumulation of succinate, caused by the decline in the activity of the succinate dehydrogenase enzyme, and generating a subsequent stabilization in the transcription factor HIF-1α (70). Despite being well-known as a key regulator of glycolysis, HIF-1α seems not to be essential for the proinflammatory activation of adipose tissue-associated macrophages (71).

Each phenotype, M1 or M2, has a different metabolic signature. In other words, there is a different activation and metabolic pathways for each profile. While M2 macrophages exhibit high rates of oxidative phosphorylation and fatty acid oxidation, cells with M1 characteristics have predominant glycolytic metabolism (70). It is extremely important that studies and therapies involving macrophages focus on their metabolic reprogramming and the attendant effects on their profile. In fact, similarities among PPARs function may be an experimental dilemma when an isotype knockout is used due to its possible compensation/overexpression by others. In a study conducted by our group, a PPARγ-positive regulation was observed when palmitoleic acid supplementation was used in the culture medium of PPARα knockout peritoneal macrophages (72). Indicating that this regulatory effect could be one of the hypotheses for the anti-inflammatory effect of exercise in the absence of PPARγ macrophages.

Overall, moderate aerobic training protected both genotypes from weight gain, as well as reducing caloric intake

from KO animals. There were different responses in the surface markers of macrophages, depending on the local environment (peritoneum or adipose tissue). Aerobic training was able to support an anti-inflammatory profile in PPARγ knockout peritoneal macrophages with impaired M2 markers, possibly via a reduction in the expression of the TLR-4 gene.

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