The anti-inflammatory and bioregulatory effects of habitual exercise in high-fat diet-induced obesity involve crown-like structures and MCP-1 in white adipose tissue.

Isabel Gálvez[1,2], María Dolores Hinchado[1,3*], Leticia Martín-Cordero[1,4], Francisco Javier Morán-Plata[3], Gerry Graham[5], Javier Francisco-Morcillo[6], Eduardo Ortega[1,3*]

[1] Immunophyisiology Research Group, Instituto Universitario de Investigación Biosanitaria de Extremadura (INUBE), 06071 Badajoz, Spain
[2] Immunophysiology Research Group, Nursing Department, Faculty of Medicine and Health Sciences, University of Extremadura, 06071 Badajoz, Spain.
[3] Immunophysiology Research Group, Physiology Department, Faculty of Sciences, University of Extremadura, 06071 Badajoz, Spain
[4] Immunophysiology Research Group, Nursing Department, University Center of Plasencia, University of Extremadura, 10600 Plasencia, Spain
[5] Chemokine Research Group; Institute of Infection, Immunity and Inflammation; College of Medical, Veterinary and Life Sciences, University of Glasgow, Glasgow G12 8TT, UK
[6] Cell Biology Area; Anatomy, Cell Biology and Zoology Department, Faculty of Sciences, University of Extremadura, 06071 Badajoz, Spain

ABSTRACT

Macrophage accumulation in the adipose tissue and changes in their inflammatory phenotype is a hallmark of obesity-induced inflammation, notably forming inflammatory structures known as "crown-like structures (CLS)". Exercise can be a key strategy to improve inflammation-related complications, but it is crucial to consider that, although exercise generally exerts systemic and local anti-inflammatory effects, this depends on the basal inflammatory status and exercise modality. In this context, the "bioregulatory effect of exercise" implies to achieve the reduction or prevention of an excessive inflammatory response and also the preservation or stimulation of the innate response. In the present work, our aim was to evaluate the effect of regular exercise on adipose tissue inflammation in high-fat diet-induced obesity in mice, as reflected by macrophage infiltration and phenotype, and CLS formation, together with a potential role for the chemokine MCP-1 in this process. Results showed that obesity is associated with greater MCP-1 expression ($p<0.05$), macrophage accumulation ($p<0.05$), and CLS presence ($p<0.001$). Regular exercise reduced macrophage accumulation ($p<0.05$), MCP-1 expression ($p<0.01$), and CLS presence ($p<0.05$) in obese mice; while it increased macrophage and CLS presence ($p<0.01$), MCP-1 expression ($p<0.05$), and M2 polarization ($p<0.05$) in lean mice. MCP-1 was associated with the proliferation of CLS, showing the first image demonstrating a potential role of this chemokine in the development of these structures. Altogether, these results confirm, for the first time, the "bioregulatory effect of exercise" in the adipose tissue: reducing inflammation in individuals with an elevated inflammatory set-point, but stimulating this response of the immune system in healthy individuals.

Key words: Inflammation. Macrophages. CCL2. CLS. Phenotype.

INTRODUCTION

Obesity and its related comorbidities (insulin resistance, type 2 diabetes, atherosclerosis, cardiovascular disease, cancer) represent one of the greatest health burdens of our time. It is well-known that obesity induces a state of chronic low-grade inflammation, characterized by high circulating levels of inflammatory cytokines and other inflammatory mediators (10,19). These anomalies in the immune and inflammatory activities in obesity are also strongly associated to altered neuroendocrine responses, dysregulated feedback mechanisms between the immune and stress responses (32, 33, 40), and abnormal metabolic homeostasis, all of which can lead to a cluster of chronic metabolic disorders or metabolic syndrome (10, 19).

Obesity-induced low-grade inflammation is also present locally, particularly in the white adipose tissue (WAT), as characterized by the increased cytokine/chemokine/adipokine expression and immune cell activation and infiltration (8, 26). In fact, WAT is now recognized as the main inflammatory source that mediates obesity-induced inflammation and metabolic alterations (24) and has also been proposed to be the main site where low-grade systemic inflammation begins (53). Under metabolic stress, adipocytes produce inflammatory mediators and chemoattractant molecules that can both activate and recruit resident and non-resident immune cells, namely macrophages

*Correspondence to:
E. Ortega (orincon@unex.es), M.D. Hinchado (mhinsan@unex.es): Immunophysiology Research Group, Physiology Department, Faculty of Sciences; and Instituto Universitario de Investigación Biosanitaria de Extremadura (INUBE), University of Extremadura, 06071 Badajoz, Spain.

and T-lymphocytes, that perpetuate WAT inflammation (43). Macrophages are key mediators in obesity-induced low-grade inflammation, producing the majority of cytokines in the adipose tissue, particularly those responsible for the onset of insulin resistance, such as TNF-α (26, 36, 53, 57). In this context, macrophage accumulation in the WAT is recognized as a hallmark of obesity-induced inflammation (53). Although the initial inflammatory triggers in WAT are still almost unknown, it has been proposed that obesity-induced adipose tissue remodelling gives rise to signals (lipids accumulation, mechanical stress, adipocyte hyperplasia and hypertrophy and subsequent hypoxia and cell death) capable of initiating an inflammatory response by promoting macrophage recruitment and activation, which in turn promotes the progression of systemic insulin resistance (9, 24, 28, 36, 45, 48, 60). Moreover, several chemotactic factors have been implicated in the process of macrophage infiltration, with CCL2/MCP-1 playing a crucial but controversial role in the recruitment of inflammatory monocytes and macrophages into WAT (20, 21, 22, 29, 52, 58).

In this way, a notable feature of macrophage infiltration in the WAT of obese subjects is the characteristic formation of clusters of macrophages that surround hypertrophied, dead adipocytes. These unique morphological elements are known as "crown-like structures" (CLS) and represent a major characteristic of WAT inflammation (9, 36). In CLS, macrophages form syncytia that sequester and ingest adipocyte debris as an initially protective response that later becomes a persistent site of macrophage activation (9), with increased expression of TNF-α and IL-6 (50). In obesity, the vast majority of WAT macrophages are aggregated in CLS (9).

Macrophages change not only their number during obesity but also their location and inflammatory phenotype (28, 29, 53). They are typically divided into two groups based on whether they are classically (pro-inflammatory, M1) or alternatively activated (anti-inflammatory, M2). M1 macrophages are linked to cellular immunity and microbicidal activity as well as production of pro-inflammatory mediators that recruit other immune cells into the local inflammatory sites and polarize and activate other immune cells; whereas M2 macrophages are associated with production of anti-inflammatory mediators, tissue repair and remodeling processes, humoral immunity (8, 14, 26, 34), and maintenance of insulin sensitivity and glucose tolerance (37, 38). Hence, macrophages can play various roles in the initiation, progression, and resolution of inflammation (26). Obesity is associated with dramatic increases in M1 macrophages numbers, particularly in the WAT (14, 28) and specially in CLS (28, 29, 54). However, it is not clear yet how obesity increases this subpopulation: by a phenotypic switch of resident M2 macrophages to an M1 state, by recruitment and infiltration of pro-inflammatory monocytes (29), or by local proliferation (1, 59, 62). Conversely, it has also been shown that obesity induces the polarization of M2 macrophages (5, 61), which might suggest that the balance between these two subpopulations shapes obesity-induced inflammation (26).

Bearing all this in mind, exercise can be a key strategy to modulate obesity-induced low-grade inflammation, particularly WAT inflammation (7, 17, 51). The beneficial effects of moderate regular exercise are mediated through several anti-inflammatory mechanisms, such as the increased production and release of anti-inflammatory cytokines and the inhibition of the production of pro-inflammatory cytokines and the expression of co-stimulatory molecules (17). Another important mechanism can be the shift in WAT toward an anti-inflammatory phenotype with a reduction in macrophage infiltration, and an M1 phenotype switch (23). Nevertheless, the exact mechanisms by which exercise reduces WAT inflammation are still not completely understood (43). Moreover, although it is accepted that, in general, exercise exerts systemic and local anti-inflammatory effects (17), this depends on exercise modality and the basal inflammatory state or inflammatory set-point (15, 42), with a special focus on the presence of inflammatory pathologies in which basal inflammatory state is altered (11). Exercise-induced activation of the innate and/or inflammatory responses in healthy people reflects an immunophysiological adaptation in a situation of exercise-induced stress and vulnerability for the organism, being crucial for the defense against potential pathogenic attacks; while the anti-inflammatory effects induced by exercise in subjects with a pro-inflammatory set-point can be explained by the homeostatic adjustment preventing an excessive inflammatory response (42). In this context, whether this phenomenon also occurs in WAT is yet to be elucidated.

In addition, despite these advances, we have yet to elucidate many aspects of WAT macrophage function, infiltration, and polarization in lean and obese states, as well as the biology of cell interplay within CLS. Moreover, the means by which physiological and stress conditions influence adipose tissue immunity are largely unknown. It is important to note that most studies have been carried out in genetically obese animals, and that since CLS can be only determined by microscopical techniques, their complete characterization, including the effects of different anti-inflammatory strategies on these inflammatory elements, remains limited at present. In this work, we aim to study the effect of regular exercise on WAT inflammation in high-fat diet-induced obesity with a new approach to characterize macrophage infiltration, by using macrophage marker F4/80 (with the presence or absence of CD206 as inflammatory phenotype marker), and CLS formation; together with a potential role for the chemokine MCP-1 in this process. Moreover, once demonstrated if MCP-1 is involved in CLS formation and in the anti-inflammatory effect of regular exercise in the WAT of obese individuals, our second objective was to investigate whether these effects are the same or are different in the WAT of lean individuals, since they can be dependent on the basal set-point of inflammation, as proposed by the "bioregulatory effect of exercise" (42).

MATERIAL AND METHODS

Experimental Design

Thirty-four C57BL/6J mice (Envigo, Huntingdon, UK) were randomly allocated to one of two diets at 8 weeks of age, until sacrifice 18 weeks later. To obtain an experimental model of obesity, one group (n = 16) (obese group) was placed on a high-fat diet (HFD) (260HF diet; SAFE, Augy, France) containing 36% fat (58.8% of the energy from fat), which is optimal for the study of obesity and its complications in mice (15). The other group (n = 18) constituted the healthy control group (lean group) and was placed on standard laboratory rodent chow (SD) (A04 diet; SAFE, Augy, France), containing 3.1% fat (8.4% of the energy from fat). Each group was randomly divided into two sub-groups, sedentary and trained groups.

Mice had free access to food and water and were housed

individually, in a temperature- and humidity-controlled room (22 ± 1 °C; 60 ± 5%) with a 12 h light/12 h dark cycle (23:00–11:00 h light; 11:00–23:00 h dark). Olfactory and visual contact between mice was possible all along the study to avoid potential harmful effects of isolation.

After 10 weeks of the diet protocol, the group of obese trained mice (n = 8) and the group of lean trained mice (n = 8) were subjected to a protocol of habitual exercise for 8 weeks. After 12 h fasting and 72 h of rest for the trained groups, blood samples and visceral WAT were collected from anaesthetized animals (comparison obese vs. lean in sedentary and trained groups).

The study was approved by the Bioethics Committee for Animal Experimentation of the University of Extremadura (registry numbers 115/2015 for project DEP2015-66093-R; 70/2018 for project IB18011), in accordance with the National and European legislation for the protection of animals used for research.

Exercise Protocol

The protocol of regular exercise began at approximately 18 weeks of age, after 10 weeks of the diet protocol. Exercise training was carried out 3 days per week for 8 weeks, always at the same time in the active period (dark 11:00–23:00 h). Animals performed treadmill running (model 800, IITC Life Science Inc., Los Angeles, CA, USA) with no slope, with intensity and duration progression from 10 m/min for 10 min in the first week to 18 m/min for 45 min in the last week. A manual prodding technique instead of the traditional electrical shock was used to stimulate running, in order to avoid additional physiological stress. This protocol of moderate regular exercise is accepted to be able to induce physiological adaptations in mice (16, 44). Samples were obtained 72 h after the last training session to avoid the evaluation of the acute effects of exercise.

Body Measurements and Collection of Biological Samples

Body weight, nose-to-anus length, and food consumption measurements started the first week of the protocol and continued weekly for the entire lifespan of each mouse. Fasted animals were gas anaesthetized with isoflurane, by standard procedure (starting dose 3–5% isoflurane, maintenance dose 1.5–3% isoflurane). Biological samples were obtained from live, anaesthetized animals. Whole blood was drawn by cardiac puncture. Fasting blood glucose concentration and lipid profile including total cholesterol, high-density lipoprotein cholesterol (HDL-C), calculated low-density lipoprotein cholesterol (cLDL-C) and triglycerides (TG) were measured in whole blood (LUX®, Biochemical Systems International Srl, Arezzo, Italy) (15, 16, 31). Visceral WAT was carefully dissected via laparotomy. It was embedded in optimal cutting temperature (OCT) compound (Tissue-Tek®, Sakura Finetek Europe, Netherlands) and snap frozen in liquid nitrogen. Until further analysis, samples were stored at −80 °C.

Immunohistochemistry

WAT was cut into 14 µm sections in a cryostat (Microm, HM550, Thermo Fisher Scientific, Waltham, MA, USA) at −35 °C and mounted on Superfrost® Plus microscope slides (Thermo Fisher Scientific). Samples were stored at −20 °C until immunostaining procedure. A hydrophobic barrier pen (Vector Laboratories, Burlingame, CA, USA) was used to encircle the sections.

First, sections were fixed with 4% paraformaldehyde (PFA) in phosphate-buffer solution (PBS) (0.1 M, pH 7.4) for 5 minutes and then washed twice in 0.5% Triton® X-100 (Sigma-Aldrich, Saint Louis, MO, USA) in PBS solution for 10 minutes. A blocking buffer consisting of 10% bovine serum albumin (BSA, Sigma-Aldrich, Saint Louis, MO, USA) and 0.3% Triton® X-100 in PBS was applied for 1 hour in a humidified, light-protected chamber, at room temperature. Afterwards, one section was incubated with different combinations of conjugated antibodies against CD206 (CD206-Alexa Fluor® 594, BioLegend, San Diego, CA, USA; 1:250 dilution), F4/80 (F4/80-FITC, BioLegend, 1:250 dilution), and MCP-1 (CCL2/MCP-1-Alexa Fluor® 647, Novus Biologicals, Centennial, CO, USA, 1:250 dilution) diluted in antibody buffer (0.3% Triton® X-100, 1% BSA) and left overnight at 4 °C in the dark. Optimal concentrations of each antibody were determined after titration. Another section was incubated with the respective isotype control for each antibody, and a third section was a negative control, in order to ensure antibody specificity and lack of strong background signal.

The following day, samples were washed in PBS three times for 5 minutes and subsequently mounted in ProLong™ Gold Antifade Mountant with DAPI (4′,6-diamidino-2-phenylindole) (Invitrogen, Carlsbad, CA, USA). Slides were then stored at 4 °C in humidified, light-protected chambers until observation in the microscope.

Fluorescent images were acquired using a Zeiss Axio Imager M2 microscope at 40x magnification, with Zen software (Oberkochen, Germany). Representative snapshots were taken from each sample. For quantitative analysis, 10 random fields were photographed in each blinded sample. Macrophages were determined by F4/80+ cells, obtaining the mean number of macrophages per field. M2 macrophages were determined by CD206+ cells, obtaining the mean number of M2 macrophages per field. MCP-1 expression was assessed through mean fluorescent intensity (mfi) in 10 fields. Furthermore, samples were doubly stained with F4/80 and MCP-1 for morphological and immunohistochemical characterization. Mean number of crown-like structures, as determined by characteristic morphology and F4/80+ macrophage accumulation, was also counted in 10 random fields. Moreover, mean adipocyte size in 10 random fields (at least 5 adipocytes counted in each field) was measured in bright-field microscopy after standard haematoxylin and eosin staining in the frozen sections, using ImageJ software (Fiji).

Statistical Analysis

Values are expressed as mean ± standard error of the mean (SEM). The variables were normally distributed (tested by the Kolmogorov–Smirnov normality test). Statistical analyses were performed with GraphPad Prism 7.0 (GraphPad Software Inc., San Diego, CA, USA). Student's t-test was used for comparisons between the pairs of groups (paired or non-paired samples). Two-way analysis of variance (ANOVA) test was used to evaluate the interaction between obesity and training on each parameter. Minimum significance level was set at $p < 0.05$.

RESULTS

Body and metabolic parameters

Table 1 shows the results regarding body and metabolic parameters in lean and obese mice, both sedentary and exercised. First, as expected, we confirmed that mice in the obese group presented higher fasting glucose concentrations and elevated levels of TG, total cholesterol, HDL-C, and cLDL-C. The protocol of habitual exercise decreased TG, and cLDL-C levels; and increased HDL-C levels in obese and lean animals, as previously shown in previous works in the same mice strain (16). Moreover, adipocyte size was greater in obese animals than in lean ones and after the exercise protocol, adipocyte size significantly decreased in both lean and obese mice. All of these responses confirm that our model of HFD-induced obesity and our model of exercise training are appropriate for evaluating physiological and metabolic effects in obese mice.

Table 1: Body weight and metabolic profile in obese and lean mice (sedentary and trained).

	Lean		Obese	
	Sedentary	Trained	Sedentary	Trained
Body weight (g)	30.2±1.3	24.9±1.1 **	42.6±1.5 •••	35.9±2.9 *
Adipocyte size (μm)	88.6±2.28	39.9±0.89 ***	118.2±3.16 •••	91.24±1.74 **
Fasting glucose (mg/dL)	203.5±14.5	196.4±25	286.7±21 ••	282.5±28
Total cholesterol	105.1±2.7	107±3	167.7±20 •••	178±25
HDL-C	45.2±2.3	54.1±3.5 *	58.2±5 •	79.5±1 **
cLDL-C	50.7±3.4	39.4±1.7 **	92.6±17 •	38.5±1*
TG (mg/dL)	86.6±2	77.1±0.5 ***	93.1±1.4 •	80.6±1 ***

Each value represents the mean ± SEM of the determinations (one per independent animal) in duplicate. * p<0.05, *** p<0.001 with respect to the corresponding sedentary group value; • p<0.05, •• p<0.01, ••• p<0.001 with respect to the lean sedentary group values. HDL-C high-density lipoprotein cholesterol; cLDL-C calculated low-density lipoprotein cholesterol; TG triglycerides.

Macrophage infiltration and their inflammatory phenotype in white adipose tissue

Both density of infiltrated macrophages and their inflammatory phenotype have been accepted as WAT inflammation biomarkers but have been scarcely evaluated directly in histological images in the context of obesity and exercise. We initially assessed the frequency and distribution of macrophages in the WAT in each group of animals, considering both representative histological images and more importantly, objective statistical data acquired from those images (Figure 1). As expected, number of F4/80+ cells (macrophages) were higher (p<0.05) in obese sedentary animals than in lean ones (Figure 1: graph E) as can be visually observed in the representative image A (lean) versus the representative image C in Figure 1. Obese mice that underwent the habitual exercise protocol presented a lower number of F4/80+ cells (p<0.05) than their sedentary controls (Figure 1: images C and D, graph E), while lean mice showed a higher number of F4/80+ cells (p<0.01) than in sedentary condition (Figure 1: images A and B, graph E). This differential behaviour in response to exercise in lean and obese mice was statistically significant (p<0.01) when compared by a two-way ANOVA test (Figure 1E). It is well known that macrophage infiltration in the adipose tissue in obese individuals is clearly related to increased body fat and adipocyte size (9, 36). In this context, although the lower macrophage infiltration in trained obese mice can be explained by the reduction in body weight and adipocyte size (Table 1), results clearly suggest that the influence of exercise on macrophage infiltration can be independent (or not only dependent) of fat mass, since in both groups body weight and adipocyte size decreased significantly and macrophage infiltration showed an opposite behaviour between both groups in response to exercise. In fact, adipocyte size in obese trained mice is still more than 100% higher than in lean trained mice (Table 1) and macrophage infiltration was significantly lower (p<0.01 in obese trained mice vs. lean trained mice; Student's t-test).

Results obtained regarding the inflammatory phenotype are shown in Figure 2. Number of cells expressing the CD206 marker (M2 macrophages) was similar in lean and obese sedentary mice (Figure 2: images A and C, graph E), but after the protocol of habitual exercise, the presence of these cells was increased only in lean mice (Figure 2: images A and B, graph E; p<0.05). Thus, the decrease in F4/80+ cells after exercise in obese animals (Figure 1) was not accompanied by a decrease in the M2 phenotype of macrophages. This effect of exercise was also significantly different in obese and lean mice (Figure 2E; p<0.05) when evaluated by a two-way ANOVA test.

MCP-1 expression in white adipose tissue

Figure 3 shows MCP-1 expression levels dispersed throughout the adipose tissue in the different groups. Expression levels (mfi) were higher in sedentary obese animals than in lean ones (Figure 3: images A and C, graph E; p<0.05); but in the obese mice, after the regular exercise protocol, this expression was lower than in sedentary condition (Figure 3: images C and D, graph E; p<0.01). However, MCP-1 expression levels increased in lean mice after the exercise protocol (Figure 3: images A and B, graph E; p<0.05). This differential effect of exercise in lean and obese mice was statistically significant (Figure 3E; p<0.01) when evaluated by a two-way ANOVA test, and reflected in general the same response to obesity and exercise than that observed in the number of infiltrated macrophages (Figure 1).

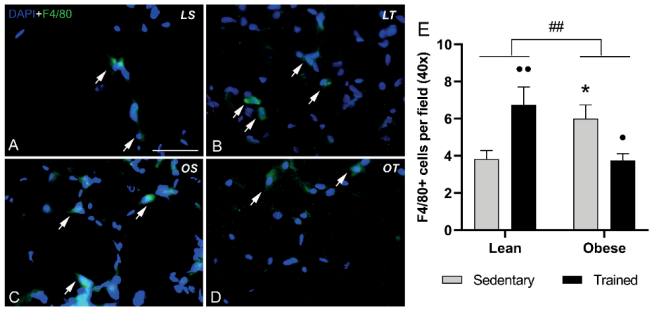

Figure 1: Representative images of F4/80+ (green) cells (macrophages, arrows) in white adipose tissue of lean sedentary mice (A), lean trained mice (B), obese sedentary mice (C) and obese trained mice (D). Nuclei were stained with DAPI (blue). Scale bar: 100 μm. Quantitative analysis showing the number of F4/80+ cells (E) in lean and obese mice (sedentary and trained groups). Each column shows the mean ± SEM of determinations in 10 random fields, performed in 9 animals from each sedentary group and 7 animals from each trained group. * p<0.05 vs. lean sedentary values; • p <0.05, •• p <0.01 vs. the corresponding sedentary values (Student's t-test); ## p<0.01 (two-way ANOVA). Scale bar: 100 μm. Abbreviations: LS, lean sedentary; LT, lean trained; OS, obese sedentary; OT, obese trained.

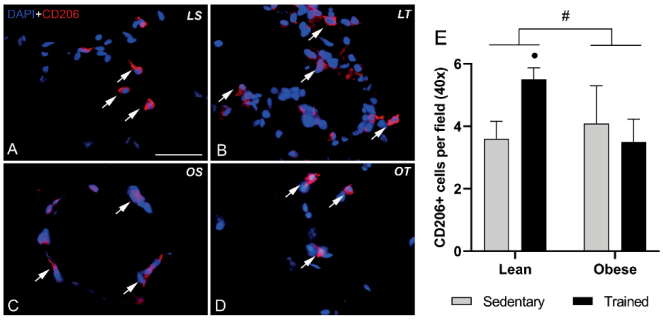

Figure 2: Representative images of CD206+ (red) cells (M2 macrophages, arrows) in white adipose tissue of lean sedentary mice (A), lean trained mice (B), obese sedentary mice (C) and obese trained mice (D). Nuclei were stained with DAPI (blue). Scale bar: 100 µm. Quantitative analysis showing the number of CD206+ cells (E) in lean and obese mice (sedentary and trained groups). Each column shows the mean ± SEM of determinations in 10 random fields, performed in 9 animals from each sedentary group and 7 animals from each trained group. • p <0.05 vs. the corresponding sedentary values (Student's t-test); # p<0.05 (two-way ANOVA). Scale bar: 100 µm. Abbreviations: LS, lean sedentary; LT, lean trained; OS, obese sedentary; OT, obese trained..

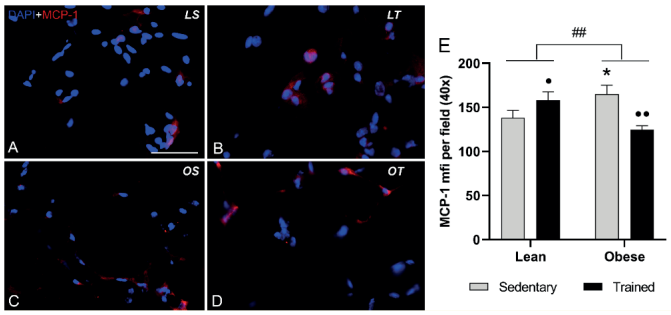

Figure 3: Representative images of MCP-1+ (red) expression in white adipose tissue of lean sedentary mice (A), lean trained mice (B), obese sedentary mice (C) and obese trained mice (D). Nuclei were stained with DAPI (blue). Scale bar: 100 µm. Quantitative analysis showing MCP-1 expression (E) in lean and obese mice (sedentary and trained groups). Each column shows the mean ± SEM of determinations in 10 random fields, performed in 9 animals from each sedentary group and 7 animals from each trained group. * p<0.05 vs. lean sedentary values; • p <0.05, •• p <0.01 vs. the corresponding sedentary values (Student's t-test); ## p<0.01 (two-way ANOVA). Scale bar: 100 µm. Abbreviations: LS, lean sedentary; LT, lean trained; OS, obese sedentary; OT, obese trained.

Crown-like structures in white adipose tissue

Notably, expression of MCP-1 in WAT was also localized to CLS (aggregated macrophages, surrounding individual adipocytes) in close relationship with F4/80 immunoreactive macrophages (as can be seen in novel immunochemistry images depicted in Figure 4A-D), both in lean and obese mice. Number of CLS was higher in sedentary obese animals vs. lean animals (p<0.001) (CLS formation was a rare event in lean sedentary mice) (Figure 4E). After the protocol of habitual exercise, a statistically significant different behaviour was observed between lean and obese groups (p<0.01): the presence of these structures was reduced in obese mice (p<0.05), while it was increased in lean mice (p<0.01) (Figure 4E), confirming that inflammatory responses can differ according to the individual's basal inflammatory set-point (42). Figures 4B, C and D show a representative CLS co-expressing the chemokine MCP-1 in close relationship with F4/80 immunoreactive macrophages. Another important feature in CLS was the presence of CD206+ cells (Figure 2) in lean and obese mice. These novel images reinforce the idea that CLS assessment constitutes an important approach for evaluating WAT inflammation involving both macrophage infiltration and MCP-1, as well as the pro- or anti-inflammatory effects of exercise in the context of their bioregulatory effects.

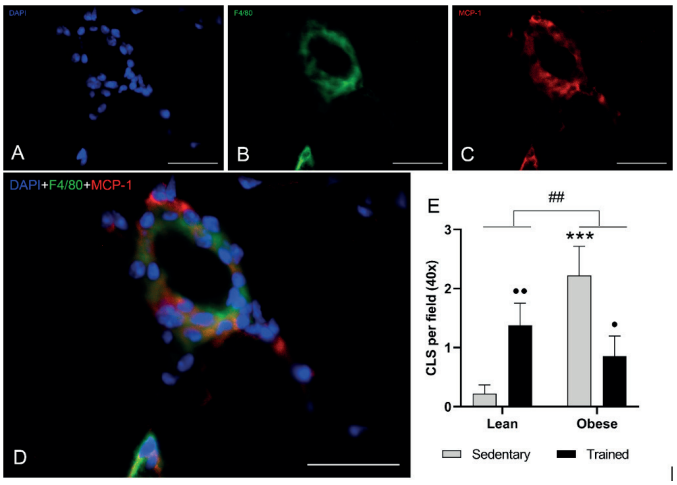

Figure 4: Novel, representative image of a crown-like structure (CLS) (D): F4/80+ (green) cells (macrophages) (B) surrounding an adipocyte in an obese sedentary mouse. This structure co-expressed the chemokine MCP-1 (red) (C). Nuclei were stained with DAPI (blue) (A). Scale bar: 50 µm. Quantitative analysis showing the number of crown-like structures (E) in lean and obese mice (sedentary and trained groups). Each column shows the mean ± SEM of determinations in 10 random fields, performed in 9 animals from each sedentary group and 7 animals from each trained group. *** p<0.01 vs. lean sedentary values; • p <0.05, •• p <0.01 vs. the corresponding sedentary values (Student's t-test); ## p<0.01 (two-way ANOVA). Scale bar: 50 µm

DISCUSSION

Physical exercise presents pleiotropic activity, affecting every physiological system, including the immune system. Although exercise strongly modulates all aspects of the immune response (41), it is particularly important in the modulation of the innate/inflammatory response (42), and the beneficial effects of moderate regular exercise on the inflammatory response are widely recognized to be mediated through anti-inflammatory mechanisms. Moderate regular exercise also has clear benefits in other aspects of the immune function, such as T cell function, antibody production, and phagocyte responses (e.g. phagocytic process) and therefore, it is associated with decreased susceptibility to infection (3, 11, 39). However, it is important to bear in mind that physical exercise does not cause a fixed and unvarying response, since it is a stress situation that causes an appropriate homeostatic response or adaptation to the changes that occur in each organism. This response depends on each individual's immunophysiological characteristics, with a special focus on the presence of inflammatory pathologies in which the basal inflammatory status is altered (11). In the same animal model of obesity used in the present study, results from our group have demonstrated that circulating monocytes from obese mice present a basal pro-inflammatory status (15) and that regular exercise elicits opposite responses in lean and obese mice: an

anti-inflammatory response in monocytes from obese mice and an overall pro-inflammatory effect in monocytes from lean mice (16). We have also found a differential immunophysiological behavior induced by exercise (pro-inflammatory in healthy individuals and anti-inflammatory in pro-inflammatory conditions) in the genetically obese Zucker rat (32) and in fibromyalgia—another inflammatory and stress-related pathology (4, 39).

Thus, inflammatory responses to exercise clearly differ according to the basal status or "inflammatory set-point". Exercise-induced activation of the innate and/or inflammatory responses in healthy people represents an immunophysiological adaptation in a situation of exercise-induced stress and vulnerability for the organism, being crucial for the defense against pathogenic attacks; while the anti-inflammatory effects induced by exercise in subjects with a pro-inflammatory set-point can be explained by the homeostatic adjustment preventing an excessive inflammatory response (42). This phenomenon has been coined "the bioregulatory effect of exercise", and it implies the regulation by exercise of the altered inflammatory and stress status depending on each individual's basal set-point, being anti-inflammatory mainly (or only) in the case of an elevated inflammatory status. This concept is defined as the reduction or prevention of an excessive inflammatory response together with the preservation or stimulation of the innate response (with optimal phagocytic and microbicidal activities) and the achievement of beneficial transitions between proinflammatory and classical monocytes and between M1 and M2 macrophages (42).

The present study examines the bioregulatory effects of exercise on the inflammatory response in the WAT of lean and obese animals, notably analyzing the effect of exercise on the formation of CLS, whose behaviour has shown them to be crucial for the assessment of the effects of exercise on the inflammatory response in the adipose tissue, confirming that inflammatory responses can differ according to the individual's basal inflammatory set-point. To this end, our approach involved the quantitative and qualitative evaluation of macrophage infiltration by using the antibodies F4/80, which identifies macrophages in the rodent tissue (47), and CD206, which has been recently proposed as the ideal marker to assess macrophage phenotype in WAT (37), since it has been shown by flow cytometry analysis that almost all CD206+ cells in WAT are macrophages, and not cells of other lineages, thus constituting exclusively M2 macrophage populations. Moreover, two clearly distinct cell subsets are found in the WAT macrophage population: most of the CD11c+ cells are CD206- (M1), whereas most of the CD11c- cells are CD206+ (M2) (14, 37, 48). Thus, by using this approach, we found that the effects of regular exercise on the inflammatory response observed locally in the WAT were congruent with the bioregulatory effects of exercise previously reported on systemic inflammatory responses. In this context, obese animals, which presented higher macrophage infiltration, showed significant reduction in these cells after the exercise protocol. Conversely, lean mice presented greater macrophage infiltration after the same exercise training protocol. Now, the next question was: what is the effect of exercise on CLS formation? Is it the same in obese and in lean individuals? The response clearly was that CLS results were in line with those regarding macrophage infiltration; that is obese sedentary mice presented a higher number of CLS than lean mice, and, remarkably, after the regular exercise protocol, CLS were also reduced in obese mice, whereas the presence of these structures increased in lean animals. In addition, another key finding was that the same pattern was observed in MCP-1 expression, with a reduction only in obese trained animals, which initially presented elevated expression levels of this chemokine at baseline. In fact, it is important to note that although MCP-1 is present throughout the WAT, it was fundamentally located in CLS. Therefore, these results altogether suggest that the effects of regular exercise on the local inflammatory response in the WAT seem to be anti-inflammatory only in obese subjects who present an elevated inflammatory status at baseline, while pro-inflammatory effects are observed in lean animals, in accordance with the bioregulatory effects of exercise. This novel bioregulatory effect in the adipose tissue involves CLS, representing a critical immunophysiological finding. CLS results are particularly relevant in supporting the bioregulatory effect of exercise, which is crucial in the potential exercise-induced modulation in the context of macrophage-mediated immunometabolism in the adipose tissue, such as macrophage recruitment to CLS (46, 55). To the best of our knowledge, this is the first time that bioregulatory effects of exercise have been reported in the adipose tissue, involving macrophages, MCP-1, and CLS. Most studies evaluating the influence of obesity or the effects of exercise on WAT inflammation do not evaluate structures or protein expression, nor do they use histological techniques and direct observation. Instead, flow cytometry in tissue lysates and mRNA detection methods are more commonly used. Furthermore, it is critical to take into account that CLS formation seems to be a better marker of WAT inflammation than indirect approaches or macrophage infiltration alone, since proliferation seems to occur predominantly in these structures (18). In fact, it has already been reported that 90% of macrophages in the adipose tissue of obese subjects are aggregated forming CLS (9, 36), thus constituting a major hallmark of inflammation in obesity. Additionally, the technique used in this work allowed us to observe the marked expression of MCP-1 in CLS, suggesting for the first time a potential role of this chemokine in the development of these structures, constituting a signal for macrophage recruitment or resident macrophage proliferation. Interestingly, MCP-1 is a promising drug target, although so far modulation of MCP-1 has not yet resulted in pharmacotherapies (12). Altogether, these findings provide a novel framework for understanding the physiological mechanisms that are involved in macrophage recruitment and function in WAT and for elucidating the underlying causes of obesity-associated WAT inflammation, including the influence of exercise on these aspects.

Apart from the bioregulatory effects of exercise, our findings regarding the influence of high-fat diet-induced obesity on macrophage accumulation and MCP-1 expression could be explained due to an infiltration of circulating monocytes into WAT and their subsequent differentiation into mature F4/80-expressing macrophages (53). Moreover, a major fraction of WAT macrophages undergoes cell division locally in obesity, leading to a local proliferation 'in situ' (1). Adipocytes and infiltrated macrophages can release signals such as MCP-1, causing increased monocyte influx (22, 53). MCP-1 has been extensively described as a potent chemokine attracting macrophages to the WAT in obese mice (22, 52), thus playing a very important role in promoting adipose tissue inflammation and insulin resistance (12). Moreover, in situ proliferation is partially driven by MCP-1 as well, also contributing to macrophage accumulation in the adipose tissue in obesity, in addition to blood monocyte recruitment. This suggests a positive correlation between MCP-1 expression and WAT macrophage accumulation in mice (1),

as corroborated by the present study, both by quantitative data analysis and by the novel image in the context of MCP-1 and CLS. Indeed, after exercise, MCP-1 expression and macrophage numbers decrease in obese mice and increase in lean mice. This is in accordance with previous studies showing that a high-fat diet increased MCP-1 gene or mRNA expression in the adipose tissue, with an exercise-induced reduction in MCP-1 (25, 27) and F4/80 gene or mRNA expression (51, 23, 27). However, other works could not find a reduction in MCP-1 mRNA expression in obese mice caused by exercise (23).

Thus, exercise is a crucial strategy in the management of low-grade inflammation in obesity. Controlling adipose tissue macrophage infiltration and proliferation has been proposed as a therapeutic target in the management of obesity, particularly to improve inflammation-related effects such as insulin resistance (35). However, controversial results on the modulation of glucose metabolism after the reduction of tissue macrophages have also been reported. Some studies have shown that a markedly reduced number of adipose tissue macrophages in MCP-1 knockout mice result in an amelioration of total insulin resistance (22), while mice overexpressing MCP-1 have increased numbers of infiltrating macrophages along with mildly increased insulin resistance (21, 22). Conversely, other studies have shown that, surprisingly, MCP-1 knockout mice presented increased glucose intolerance, even though these animals have lower adipose tissue macrophage proliferation (1, 20). This could be explained by the potential beneficial role of macrophages in increasing lipid storage or clearance of dead cells in adipose tissue (36). This does not happen in the present work (fasting glucose is not impaired after the exercise-induced macrophage numbers reduction in obesity), suggesting that an exercise-induced physiological reduction in macrophage infiltration is able to maintain glucose homeostasis, as opposed to genetic or pharmacological approaches aimed at reducing macrophage numbers to non-physiological levels that might negatively affect the physiological immune functions of macrophages in tissues (35). However, at least in our protocol of exercise, the exercise-induced reduction in macrophage accumulation does not seem to be enough to achieve a decrease in fasting glucose levels, although it does not increase them either, as mentioned before. It is important to highlight that, surprisingly, the pro-inflammatory effects in trained lean mice does not seem to be accompanied by an impaired metabolic response (i.e. glycemic and lipid profile), reinforcing the potential bioregulatory physiological role in stimulating the immune response, without detrimental metabolic effects. In fact, several parameters such as HDL-C, cLDL-C and triglycerides significantly improve in lean mice after exercise, and the same parameters also improve in trained obese mice. Thus, exercise-induced inflammatory changes in the WAT are accompanied by metabolic improvement, or at least, lack of impairment.

After establishing all the aforementioned findings regarding macrophage infiltration, CLS formation, and MCP-1 expression in our animal model and in response to the protocol of regular exercise, our next question was whether macrophage inflammatory phenotype (evaluated through CD206 expression) could be altered both in obesity and in response to exercise. No baseline differences in isolated CD206+ macrophages were found between lean and obese mice in the present study. It is well-known that the WAT of obese animals presents a preferential recruitment of M1-type macrophages and/or a phenotypic switch of adipose tissue macrophages towards the M1 phenotype, increasing the proportion of M1 to M2 macrophages; while WAT of lean individuals contains fewer macrophages, which present mainly a M2 phenotype (6, 29). Several studies show differing results and should be examined closely for the way cell numbers and functions are expressed, especially when immune cell numbers are determined by flow cytometric analyses or gene expression. Some studies suggest that obesity only appears to increase the numbers of the M1 population while M2 numbers remain unchanged, thus the obesity-induced changes in macrophage phenotypes are mainly due to increases in the M1 subpopulation (49, 26), which is in accordance with our results showing similar numbers of M2 macrophages in lean and obese mice. Other authors have suggested that, considering the dramatic increase of infiltrated macrophages in obesity, the absolute number of both M1- and M2-polarized macrophages increases (6), while others show an increase in M1 and a decrease in M2 macrophages (23). In any case, very few studies have specifically characterized WAT immune cell phenotypes following exercise training (56). Gleeson et al. (2011) proposed that the anti-inflammatory effects of exercise rely on several mechanisms, including the inhibition of monocyte and macrophage infiltration and the phenotypic switching of macrophages within WAT (17). In the seminal work by Kawanishi et al. (2010), exercise training reduced the elevated CD11c (M1) mRNA expression, but increased the low CD163 (M2) mRNA expression in obese mice, suggesting that exercise was associated, not only with a reduction in total WAT macrophage content, but also with an M1–M2 phenotype switch in obesity (23). A recent study found similar results, proposing that exercise increased M2 macrophage polarization (2). Conversely, other observations in obese animals showed that regular exercise suppressed the elevated CD11c gene expression, and also attenuated the elevated CD206 and arginase-1 mRNA expression (27). However, no significant changes were found in lean exercised animals in any of these studies. In the present paper, we focused on M2 macrophages since they can positively alter the outcome of obesity due to their beneficial function in apoptotic cell clearance, tissue repair, and remodeling in the WAT (6). We observed that the population of CD206+ macrophages increased only in lean mice after exercise. This might occur in response to the post-exercise increase in macrophages in lean animals, as a way to counteract the potential pro-inflammatory effects due to higher macrophage accumulation. Thus, although the presence of infiltrating or proliferating macrophages in the adipose tissue of trained lean mice increases, macrophages with an anti-inflammatory profile increase as well. On the other hand, in trained obese animals the number of macrophages decreases while the number of CD206+ macrophages is maintained. This might reflect a reduction of macrophage accumulation mainly at the expense of pro-inflammatory M1 macrophages, in accordance with the anti-inflammatory effects of exercise observed in this group of obese animals.

It is important to note that circulating monocytes from this animal model of obesity have shown a pro-inflammatory profile, including cytokine expression and phenotype markers (15), while peritoneal macrophages did not seem to present a higher inflammatory profile (30). It can be hypothesized that greater adipose tissue infiltration of macrophages with a pro-inflammatory profile leaves less activated cells in the peritoneal cavity (as non-infiltrated macrophages). In fact, it has already been reported by Forner et al. (1994) that peritoneal macrophages from old animals (in which chronic, low-grade inflammation develops due

to advanced age, "inflammaging"), present increased adherence capacity and reduced chemotaxis capacity (13). Nevertheless, when evaluating macrophage infiltration and their inflammatory profiles, it is crucial to consider that WAT inflammation depends not only on immune cell infiltration per se, but also on the complex immune cell-adipocyte interactions (43), thus the importance of direct observation of major inflammatory structures such as CLS and their association with inflammatory cells and mediators that might have a key role in the development of adipose tissue disfunction in obesity. Therefore, the evaluation of CLS on the context of anti-inflammatory strategies such as exercise seems fundamental.

Since the phenotype study was limited to M2 macrophage analysis, it was not possible to assess M1 macrophages. Future studies should focus on the analysis of the bioregulatory effects of exercise on this macrophage population, together with other chemokines, inflammatory mediators, and innate immune function in the WAT. Moreover, further studies are needed to elucidate the influence of obesity and exercise on the mechanisms by which different macrophage subtypes and inflammatory mediators regulate the cell interplay, tissue remodeling, and metabolic functions within the adipose tissue. Notwithstanding these limitations, our study clearly contributes to the understanding of the effects of exercise on the inflammatory response in the WAT, both in healthy and pro-inflammatory conditions from a novel approach.

CONCLUSIONS

High-fat diet-induced obesity is associated with greater MCP-1 expression, macrophage accumulation, and crown-like structure presence. Regular exercise reduced macrophage accumulation, MCP-1 expression, and crown-like structure presence in obese mice; while it increased macrophage and crown-like structure presence, MCP-1 expression, and M2 polarization in lean mice. A novel finding was that MCP-1 was associated with the proliferation of crown-like structures. Altogether, these results confirm, for the first time, the "bioregulatory effect of exercise" in the adipose tissue, with the particular immunophysiological relevance of crown-like structure formation: reducing inflammation in individuals with an elevated inflammatory set-point, but stimulating this response of the immune system in healthy individuals. The present results contribute to our understanding of adipose tissue inflammation biology and may lead to novel therapeutic strategies and specific targets for potential intervention to prevent or treat obesity-induced adipose tissue inflammation.

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