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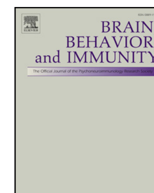
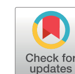


Impacts of the late adulthood diet-induced obesity onset on behavior, immune function, redox state and life span of male and female mice



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ABSTRACT

The aim of the present study was to investigate whether the late onset of diet-induced obesity (DIO) in middle-aged mice affected behavioral, immunological and oxidative stress parameters as well as life span of male and female mice. Also, it was analyzed whether the late DIO onset aggravated immunosenescence in old female mice. Late-adult male and female ICR/CD1 mice (28 weeks old) were fed either a high-fat diet or a standard diet during 14 weeks. After that, in these middle-aged (42 weeks old) diet-induced obese (DIO) and non-DIO controls, behavior as well as functions and redox state of peritoneal leukocytes were evaluated. These same parameters (excepting behavioral tests) were repeated when female mice were old (72 weeks old). The results showed lower exploratory activity and higher anxiety-like behavior in middle-aged male and female DIO than in controls. Moreover, these DIO animals from both sexes exhibited statistically significant impaired immune cell functions, such as chemotaxis of macrophages and lymphocytes, phagocytosis of macrophages, natural killer activity and lymphoproliferation in response to ConA and LPS, as well as an oxidative stress state in comparison with controls. Male DIO mice exhibited higher impairments in a variety of the evaluated parameters and a shorter life span than their female counterparts. In addition, female DIO mice, at old age, showed aggravated immunosenescence. In conclusion, the late DIO onset leads to impairments in behavior as well as in immune system functions of middle-aged male and female mice, males being significantly more affected than females.

1. Introduction

Unhealthy dietary patterns, such as increased consumption of diets high in fat and energy, have been associated with the worldwide obesity epidemic. The prevalence of obesity has been rising at alarming rates in all age groups (from young to older ages) and in both sexes (Johnson et al., 2015; Swinburn et al., 2011).

Obesity has been linked to systemic chronic oxidative stress, low-grade inflammation and dysfunctional immune system (Hunsche et al., 2016; Huttunen and Sryjänen, 2013; Rath and Haller, 2011; Sheridan et al., 2012). This condition seems to be generated and maintained in the adipose tissue by excess of nutrients, reactive oxygen species (ROS) and inflammatory compounds, which in turn promotes increased infiltration of immune cells and results in a vicious cycle of oxidation and inflammation. Both chronic oxidative stress and inflammation, which are two closely related processes (Vida et al., 2014), in adipose tissue, may spread to a systemic level, contributing to the development of obesity-related complications (de Mello et al., 2018). It is largely known that a controlled immune response with adequate production of inflammatory and oxidative compounds is essential for maintaining health and protecting against infections, cancer and damages. However, if the levels of these compounds exceed the capacity of anti-inflammatory and antioxidant defenses of the organism, an imbalance occurs and oxidative and inflammatory stresses are established, which contribute to cellular and tissue damage in all physiological systems, including the nervous and immune systems (Bauer and De la Fuente, 2016; De la Fuente and Miquel, 2009; Vida et al., 2014). Moreover, given that the nervous and immune systems share a bidirectional communication (Vida et al., 2014), it is expected that the dysfunctional immune system associated with obesity, would have an impact on the nervous system. In fact, increasing evidence suggests that obese individuals show increased infiltration of oxidative and inflammatory compounds into the brain, and the neuroinflammation generated has been related with the development of cognitive, motor and behavioral dysfunctions (Pistell et al., 2010; White et al., 2009).

Similar to obesity, the aging process affects functions of all

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physiological systems, including those of the nervous and immune systems (Vida et al., 2014). Thus, there is a progressive deterioration in a variety of brain and behavioral functions, including memory as well as cognitive and motor activity (Rosenzweig and Barnes, 2003; Vandervoort, 2002). In addition, the immune system also suffers age-related alterations (immunosenescence), which includes marked changes of the innate and adaptive immune responses. This immunosenescence results in impaired migration and phagocytosis of macrophages, anti-tumor activity of natural killer (NK) cells and proliferative response of lymphocytes (Martínez de Toda et al., 2016). These impairments seem to be caused mainly by the age-related chronic oxidative and inflammatory stresses of the immune cells, which are also involved in the oxidative and inflammatory state of the organism and, consequently, in the rate of aging (De la Fuente and Miquel, 2009).

Given that obesity and aging are linked to oxidative and inflammatory stresses, and both conditions result in health complications, there is an increasing concern whether obesity could accelerate the process of aging and increases the risk of morbidity and mortality at premature ages (De la Fuente and Castro, 2012; Zamboni et al., 2005). We have recently shown that the early adulthood diet-induced obesity onset resulted in premature features of aging (such as immunosenescence). Thus, adult diet-induced obese (DIO) mice, as a consequence of being fed during their adolescence with a high-fat diet, presented functions and oxidative stress state of peritoneal leukocytes similar to those observed in chronologically old mice. Furthermore, when these adult DIO animals reached old age, they continuous to show higher impairments of immune cell functions, and a shorter life span than old non-DIO animals (Hunsche et al., 2016).

Previous studies indicate that the age of obesity onset (Boitard et al., 2012) and sex (Salinero et al., 2018) have an impact on obesity-related health problems. However, nothing is known about the impacts of the late adulthood diet-induced obesity onset in the nervous (behavior) and immune system (immune functions and redox state of peritoneal leukocytes) of middle-aged mice, as well as if there are differences between males and females. Therefore, in the present study, we investigated whether middle-aged male and female DIO mice, as a consequence of being fed with a high-fat diet during their late adulthood, showed alterations in relevant parameters of behavior, immune and redox state as well as in their life span. Also, it was analyzed whether the late adulthood obesity onset aggravated immune and redox state parameters of old female mice.

2. Materials and methods

2.1. Animals

Male and female ICR/CD1 mice (*Mus musculus*) were purchased from Janvier SAS (Chassal, France) at the age 8 weeks. The animals were housed in polyurethane cages (4–5 animals per cage) and maintained under standard laboratory conditions (12:12 h reversed light/dark cycle; lights on at 8:00 pm, relative humidity of 50–60%, temperature of 22 ± 2 °C and adequate ventilation). The experiments were conducted in accordance with the guidelines and protocols of the Royal Decree 53/2013 regarding the care and use of laboratory animals for experimental procedures, and were approved by the Committee for Animal Experimentation of the Complutense University of Madrid.

2.2. Experimental groups

Late-adult male and female mice, at the age of 28 weeks, were divided into groups (8–10 animals for each group) of similar average body weight and were fed either a standard diet or a high-fat diet for 14 weeks. A table with detailed nutritional content of diets is provided in a previously published paper (Hunsche et al., 2018). Briefly, the diets used were provided by Harlan Interfauna Iberica (Barcelona, Spain) and the nutritional compositions were (i) standard diet (Teklad Global 14% Protein Rodent Maintenance Diet, reference 2014): energy 2.9 Kcal/g, protein 20%, carbohydrate 67%, and fat 13% and (ii) high-fat diet (Teklad Custom Diet, reference TD. 06414): energy 5.1 Kcal/g, protein 18.4%, carbohydrate 21.3%, and fat 60.3%. When male and female mice were diet-induced obese (DIO) and middle-aged (42 weeks of old), a variety of tests and assays were performed in order to evaluate behavior, biochemical parameters as well as functions and redox state of peritoneal leukocytes. After 14 weeks on the high-fat diet, middle-aged male and female DIO mice returned to a standard diet. During the entire study, all animals had free access to water and food. Body weight and food intake were measured every week throughout the study.

Given that mice are capable of remembering previously performed behavioral tests, only biochemical parameters and functional and redox states of peritoneal leukocytes were repeated in old age (72 weeks old). Moreover, since a significant number of male mice did not reach old age (3–6 animals for each group), these parameters were assessed only in female mice at the age of 72 weeks (5–8 animals for each group).

2.3. Total body fat mass

Nuclear magnetic resonance (NMR) was used to evaluate the total fat mass in mice. NMR was performed using magnetic resonance imaging (MRI) technology Biospec BMT 47/40 with a field of 4.7 Tesla. MRI was obtained *in vivo* and analyzed (acquisition, reconstruction, and data processing) using advanced digital technology with imaging software. During the measurements, mice were anaesthetized with isoflurane (Judex et al., 2010).

2.4. Biochemical parameters

Glucose, triglyceride, and total cholesterol levels were measured with Accutrend (Roche Diagnostics, Mannheim, Germany) using blood samples collected from the tail vein of mice.

2.5. Behavioral tests

Behavioral tests were carried out between 8 am and 10 am during three consecutive days, under illumination of fluorescent lamps (20 W). On the first day, a battery of tests (reflexes, corner, T-maze, wood rod and tightrope tests) was performed in mice. On the second day, mice were subjected to the holeboard test, and on the third day mice were assessed in the elevated plus maze. The experimental apparatus was cleaned before the performance of each mouse in order to avoid olfactory trails.

2.5.1. Reflexes

When the mouse was suspended by the tail and lowered toward a solid black surface, the visual placing reflex was evaluated by observing the complete extension of the forelimbs, and the hindlimb reflex was measured by the complete extension of these limbs. The mean response was rated in three trials.

2.5.2. Motor coordination and equilibrium abilities

In order to assess motor coordination and equilibrium, animals were placed in the center of a wood elevated rod for one trial of 60 s (Baeza et al., 2010). Motor coordination and equilibrium were measured by the time (in seconds) to leave the first segment and by the percentage of mice falling off the rod. In addition, the percentages of mice that cover at least 1 segment and that complete the test were also considered as motor coordination and equilibrium abilities.

2.5.3. Muscular vigor and traction

Muscular vigor and traction were evaluated in an elevated horizontal tightrope, using a test trial of 60 s (Baeza et al., 2010). Muscular vigor was assessed as the percentage of mice falling off the rope and that complete the test. Traction was evaluated by analyzing the

different parts of the body that mice used to remain hanged (forelimbs, hindlimbs and tail) and, subsequently, the percentage of mice displaying the maximum traction capacity (using forelimbs, hindlimbs and tail).

2.5.4. Vertical exploratory activity

The vertical exploratory activity (studied as the performance of "rearing", that is, when the mouse stands up on his hindlimbs so that his body becomes more perpendicular to the ground) was evaluated in the corner test and in the holeboard test. The corner test consists in placing the mouse in a new cage with bedding during 30 s (Baeza et al., 2010). In contrast, the holeboard test consists in placing the animal in a box made of wood with matte-painted metallic walls, divided into 36 squares, bearing four equally spaced holes in the central area (all but 20 peripheral squares were considered central) (Baeza et al., 2010). The test was performed during 5 min.

2.5.5. Horizontal exploratory activity

Horizontal exploratory activity was assessed as the number of corners of the cage visited (corner test) and as the peripheral ambulation (number of line crossings in the peripheral area, by the walls) and central ambulation (number of line crossings in the central area) in the holeboard test. Peripheral ambulation was considered lower exploratory activity than central ambulation due to the tendency of mice to remain close to walls (thigmotaxis) (Simon et al., 1994). In addition, horizontal exploratory behavior was also recorded using the T-maze test, which is a wooden apparatus with three enclosed arms (Baeza et al., 2010). The mouse was placed inside the "short" arm of the maze with its head facing the wall. The time (in seconds) to complete the test (exploration of all three maze arms) was assessed as horizontal exploratory activity.

2.5.6. Goal-directed exploratory behavior

The goal-directed exploratory behavior of mice was assessed in the holeboard test. A small plastic object was placed into each of the four holes of the holeboard to attract mice attention. The frequency (percentage and number) of mice that perform head-dips (when the animal places its head into the hole) was considered as a goal-directed exploratory behavior.

2.5.7. Anxiety-like behavior

The anxiety-like behavior was measured in the elevated plus maze, as previously described (Garrido et al., 2018). The test was performed during 5 min. The total numbers of entries (four paws criteria) in open arms and in closed arms were recorded. The percentage of time spent in open and closed arms, and platform was calculated. In addition, the frequency of self-grooming (when mouse cleans, licks or scratches its body) as well as the presence of defecation in the holeboard and in the T-maze tests were also considered as anxiety-like behaviors (Archer, 1973; Sake and Graybiel, 2003).

2.6. Collection of peritoneal leukocytes

The peritoneal suspensions were obtained between 8 am and 10 am to minimize circadian variations in the immune system, by a procedure previously described, without animal sacrifice (Guayerbas et al., 2002a,b), which allowed monitoring the life span of the mice. Briefly, 3 ml of Hank's solution, adjusted to pH 7.4, were injected into the peritoneum, the abdomen was massaged and the peritoneal exudate cells were collected allowing the recovery of 90–95% of the injected volume. The peritoneal leukocytes, consisting of lymphocytes and macrophages, were counted in Neubauer chambers (Blau Brand, Wertheim, Germany). The suspensions were adjusted to a final concentration of 5 × 10^5 macrophages or lymphocytes per ml in Hank's solution or 10^6 leukocytes per ml in Hank's solution or complete medium (RPMI 1640 enriched with L-glutamine (PAA, Pasching, Austria) and supplemented with 10% heat-inactivated (56 °C, 30 min) fetal calf serum (PAA, Pasching, Austria) and gentamicin (100 mg/ml, PAA, Pasching, Austria) with or without phenol red, depending on the type of assay used. Macrophages and lymphocytes were identified by their morphology. The cellular viability was measured using the trypan-blue (Sigma-Aldrich, Madrid, Spain) exclusion test and in all cases was higher than 98%.

The immune functions and redox state parameters studied were performed using unfractionated peritoneal leukocytes, which better preserved the physiological environment surrounding the immune cells *in vivo* (Alonso-Fernández and De la Fuente, 2011).

2.7. Chemotaxis assay

Chemotaxis of peritoneal leukocytes was evaluated according to a slight modification of Boyden's method (Guayerbas et al., 2002a), consisting basically of the use of chambers with two compartments separated by a filter with a pore diameter of 3 μm (Millipore, Bedford, MA, USA). The chemotaxis index (CI) was determined by counting, in an optical microscope (100×), the total number of macrophages and lymphocytes on one third of the lower face of the filters.

2.8. Phagocytosis assay

Phagocytosis assay of inert particles (latex beads) was carried out following a method previously described (Guayerbas et al., 2002a). The number of particles ingested by 100 macrophages was counted using an optical microscope (100×) and expressed as phagocytic index (PI). The percentage of macrophages, which phagocyted at least one latex bead, was also determined and expressed as phagocytic efficiency (PE).

2.9. Natural killer assay

An enzymatic colorimetric assay was carried out to measure the cytolysis of tumor cells (murine lymphoma YAC-1 cells) (Cytotox 96 TM Promega, Boehringer Ingelheim, Germany) based on the determination of lactate dehydrogenase enzyme (LDH), as previously described (Guayerbas et al., 2002a). The results were expressed as percentage of lysis of tumor cells.

2.10. Lymphoproliferation assay

Following the method previously described (Guayerbas et al., 2002b), aliquots (200 μl) of peritoneal leukocytes (10^6 cells/ml complete medium) were seeded in 96 well flat-bottomed microtitre plates (Numc, Roskilde, Denmark) and 20 μl of concanavaline A (ConA 1 μg/ml, Sigma-Aldrich), 20 μl of lipopolysaccharide (LPS, *E. coli*, 055:B5 1 μg/ml, Sigma-Aldrich) or 20 μl of complete medium (spontaneous proliferation) were added per well. After 48 h of incubation at 37 °C in an atmosphere of 5% CO2, 0.5 μCi ^{3}H-thymidine (Du Pont, Boston, MA, USA) were added to each well. The cells were harvested in a semi-automatic microharvester 24 h later. Thymidine uptake was measured using a beta counter (LKB, Uppsala, Sweden). The results were expressed as ^{3}H-thymidine uptake (cpm).

2.11. Catalase activity assay

The activity of catalase (CAT) was determined following a previously described method (Alvarado et al., 2006). The enzymatic assay was followed using spectrophotometry for 80 s at 240 nm. The results were expressed as international units (U) of enzymatic activity per 10^6 cells.

2.12. Glutathione peroxidase activity assay

The glutathione peroxidase activity was determined according to

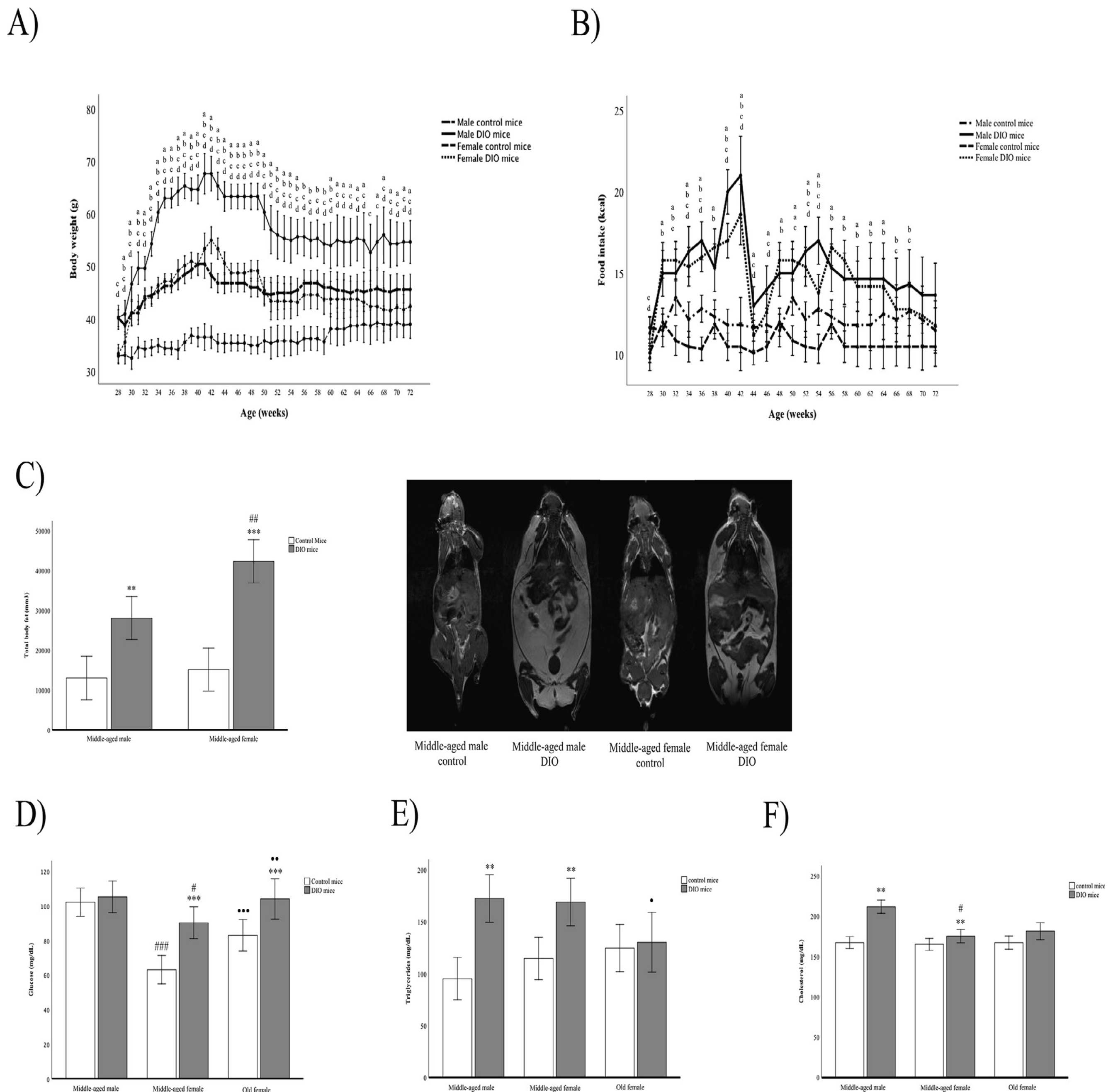

Fig. 1. (A) Body weight (g) of male and female control and diet-induced obese (DIO) mice during high-fat diet feeding (28–42 weeks of age) and aging (43–72 weeks old). (B) Food intake (kcal) of male and female control and DIO mice during high-fat diet feeding (28–42 weeks of age) and aging (43–72 weeks old). Significant differences: a: male control vs male DIO; b: female control vs female DIO; c: male control vs female control; d: male DIO vs female DIO. Data represent mean ± SEM (n = 3–10 animals for each group). (C) Total body fat mass (mm^3). Each column represents mean ± SEM (n = 4 animals for each group). $^{***}P < 0.001$; $^{**}P < 0.01$ with respect to the corresponding values of control mice. $^{\#\#}P < 0.01$ with respect to the corresponding values of middle-aged male DIO mice. (D) Glucose levels (mg/dL). $^{***}P < 0.001$ with respect to the corresponding values of control mice. $^{\#\#\#}P < 0.001$; $^{\#}P < 0.05$ with respect to the corresponding values of middle-aged male control mice and middle-aged male DIO mice. $^{\bullet\bullet\bullet}P < 0.001$; $^{\bullet\bullet}P < 0.01$ with respect to the corresponding values of middle-aged female control mice and middle-aged female DIO mice. Data represent median ± SEM (n = 5–10 animals for each group). (E) Triglyceride levels (mg/dL). $^{**}P < 0.01$ with respect to the corresponding values of control mice. Data represent median (IQR) (n = 5–10 animals for each group). (F) Total cholesterol levels (mg/dL). $^{**}P < 0.01$ with respect to the corresponding values of control mice. $^{\#}P < 0.05$ with respect to the corresponding values of middle-aged male DIO mice. Data represent median (IQR) (n = 5–10 animals for each group).

the method described by Lawrence and Burk (1976) with some modifications (Alvarado et al., 2006). The reaction was followed spectrophotometrically by the decrease of the absorbance at 340 nm. The results were expressed as milliunits (mU) of enzymatic activity per 10^6 cells.

2.13. GSH and GSSG concentrations assay

Both reduced (GSH) and oxidized (GSSG) concentrations of glutathione were measured using a fluorometric method, with some modifications (Garrido et al., 2018). Measurement of fluorescence was performed in a microplate reader using excitation at 350 nm and emission detection at 420 nm. The results were expressed as nmol/10^6

cells. The GSSH/GSH ratio was calculated for each sample.

2.14. Xanthine oxidase activity assay

Xanthine oxidase (XO) activity was measured by fluorescence using a commercial kit "Amplex Red Xanthine/Xanthine Oxidase Assay Kit" (Molecular Probes, Paisley, UK), as previously described (Vida et al., 2011). Measurement of fluorescence was performed in a microplate reader using excitation at 530 nm and emission detection at 595 nm. Data analysis was performed with xanthine standard curves at different concentrations, the results being expressed in international milliunits (mU) of enzymatic activity per 10^6 cells.

2.15. Lipid peroxidation (malondialdehyde) assay

The estimation of malondialdehyde (MDA) concentration in cells was evaluated using the commercial kit "MDA Assay Kit" (Biovision, Mountain View, CA, USA), which measures the reaction of MDA with thiobarbituric acid (TBA) and the MDA-TBA adduct formation, as previously described (Hunsche et al., 2018). The MDA-TBA adduct was measured using a spectrophotometer at 532 nm of absorbance. The results were expressed as nmol/10^6 cells.

2.16. Superoxide anion concentration assay

The superoxide anion concentration was evaluated assessing its capacity to reduce nitroblue tetrazolium (NBT, Sigma-Aldrich, Madrid, Spain), in an equimolecular reaction, following the method previously described (Guayerbas et al., 2002a). The reduced NBT was extracted with dioxin (Merck, Germany) and the absorbance of the supernatants was determined at 525 nm using a spectrophotometer. The data obtained were expressed as nmoles of NBT reduced per 10^6 leukocytes by extrapolating in a standard curve of NBT reduced with 1,4-dithioerythritol (Sigma-Aldrich, Madrid, Spain).

2.17. Statistical analysis

SPSS 25.0 (SPSS, Inc., Chicago, USA) was used for the statistical analysis of the results. The data were tested for normality of distribution (Kolmogorov-Smirnov test) and homogeneity of variance (Levene test). For non-normal distributions and non-homogeneous variances, the non-parametric Kruskal-Wallis and Friedman tests were used, and Mann-Whitney and Wilcoxon Signed Ranks to run pairwise comparisons, respectively. Two-way analysis of variance (ANOVA) with the factors being diet (standard and high-fat) and sex (male and female) was used to analyze results from middle-aged male and female mice. Two-way repeated ANOVA with the factors being diet (standard and high-fat diet) and age (middle-aged and old) was used to analyze results from old and middle-aged female mice. If significant effects were observed, post-hoc comparisons were performed as appropriate, with Bonferroni corrections for multiple comparisons. For comparison of qualitative data in behavioral tests, the chi-square was employed. Finally, a Kaplan-Meir survival analysis was conducted to verify the effects of obesity on the survival of male and female mice. The data were expressed as mean ± standard error of the mean (SEM), or median and interquartile range (IQR), depending upon the normality of data. P value < 0.05 was considered statistically significant.

3. Results

3.1. Body weight, total fat mass, food intake and biochemical parameters

After the period of 14 weeks on a high-fat diet, male and female diet-induced obese (DIO) mice displayed significantly higher body weight than their control counterparts, with males weighing more than females (Fig. 1A; Kruskal-Wallis test $X^2(3) = 31.059$, $P < 0.001$; significant differences: male control vs male DIO, $P < 0.001$; female control vs female DIO, $P < 0.001$; male control vs female control, $P < 0.001$; male DIO vs female DIO, $P < 0.001$, pairwise comparisons with Mann-Whitney). In addition, male and female DIO mice consumed significantly more kilocalories than their controls, with males consuming more kilocalories than females (Fig. 1B; Kruskal-Wallis test $X^2(3) = 29.631$, $P < 0.001$; significant differences: male control vs male DIO, $P < 0.001$; female control vs female DIO, $P < 0.001$; male control vs female control, $P = 0.010$; male DIO vs female DIO, $P = 0.008$, pairwise comparisons with Mann-Whitney). At old age (72 weeks old), male DIO mice continue to exhibit a higher body weight than their respective controls, with males weighing more than females (Fig. 1A; Kruskal-Wallis test $X^2(3) = 13.540$, $P = 0.004$; significant differences: male control vs male DIO, $P = 0.02$; male control vs female control, $P = 0.005$; male DIO vs female DIO, $P = 0.024$, pairwise comparisons with Mann-Whitney).

Male and female DIO mice showed higher total fat mass than the corresponding controls, with female DIO mice showing higher total body fat than male DIO mice (Fig. 1C; two-way ANOVA, significant interaction diet × sex $F(1,12) = 5.889$, $P = 0.032$, main effect of diet $F(1,12) = 71.798$, $P < 0.001$, main effect of sex $F(1,12) = 10.797$, $P = 0.007$; significant differences: male control vs male DIO, $P = 0.001$; female control vs female DIO, $P < 0.001$; male DIO vs female DIO, $P = 0.002$, post-hoc comparisons with Bonferroni corrections).

Female DIO mice showed higher values of glucose than their respective non-DIO counterparts. In addition, males displayed significantly higher levels of glucose than females (Fig. 1D; two-way ANOVA, significant interaction diet × sex $F(1,32) = 6.187$, $P = 0.018$, main effect of diet $F(1,32) = 9.716$, $P = 0.004$, main effect of sex $F(1,32) = 31.178$, $P < 0.001$; significant differences: female control vs female DIO, $P < 0.001$; male control vs female control, $P < 0.001$; male DIO vs female DIO, $P = 0.046$, post-hoc comparisons with Bonferroni corrections). The levels of triglycerides were significantly higher in male and female DIO mice in comparison with their respective non-DIO controls (Fig. 1E; Kruskal-Wallis test $X^2(3) = 25.135$, $P < 0.001$; significant differences: male control vs male DIO, $P = 0.001$; female control vs female DIO, $P = 0.003$, pairwise comparisons with Mann-Whitney). In addition, the levels of total cholesterol were higher in male and female DIO mice than in their respective non-DIO mice, with male DIO mice showing higher cholesterol levels than female DIO mice (Fig. 1F; Kruskal-Wallis test $X^2(3) = 20.255$, $P < 0.001$; significant differences: male control vs male DIO, $P = 0.001$; female control vs female DIO, $P = 0.001$; male DIO vs female DIO, $P = 0.013$, pairwise comparisons with Mann-Whitney).

At old age, when females reached 72 weeks of age, they exhibited higher levels of glucose than middle-aged females (Fig. 1D; two-way repeated ANOVA measures, significant main effect of diet $F(1,11) = 43.099$, $P < 0.001$, main effect of age $F(1,11) = 31.385$, $P < 0.001$; significant differences: old female control vs old female DIO, $P < 0.001$; middle-aged female control vs old female control, $P < 0.001$; middle-aged female DIO vs old female DIO, $P = 0.007$, post-hoc comparisons with Bonferroni corrections). In addition, old female DIO mice showed lower levels of triglycerides than middle-aged female DIO mice (Fig. 1E; Friedman test, $X^2(3) = 9.000$, $P = 0.029$; significant differences: middle-aged female DIO vs old female DIO, $P = 0.043$, pairwise comparisons with Wilcoxon Signed Ranks).

3.2. Behavioral tests

3.2.1. Reflexes

No significant differences were found between middle-aged male and female DIO mice and their respective non-DIO counterparts in reflexes (male control: 100%; male DIO: 100%; female control: 100%; female DIO: 100%).

Table 1
Behavioral tests in middle-aged male and female control and middle-aged male and female DIO (diet-induced obese) mice.

	Middle-aged male		Middle-aged female	
	Control	DIO	Control	DIO
Motor coordination and equilibrium (Wood rod test)				
% of mice falling off	0	10^{**}	0	$0^{\#\#}$
% of mice that cover at least 1 segment	100	80^{***}	100	$100^{\#\#\#}$
% of mice that complete the test	100	80^{***}	100	$100^{\#\#\#}$
Time to leave first segment (s)	4 ± 1	5 ± 1	2 ± 0	4 ± 1
Muscular vigor (Tightrope test)				
% of mice falling off	33	75^{***}	25	$40^{*\#\#\#}$
% of mice that complete test	67	25^{***}	75	$60^{*\#\#\#}$
% of mice that show maximum traction capacity	80	50^{***}	$100^{\#\#\#}$	$80^{***\#\#\#}$
Vertical exploratory activity				
Total number of rearings (Corner test)	8 ± 1	$6 \pm 1^{*}$	8 ± 1	$5 \pm 1^{*}$
Total number of rearings (Holeboard test)	24 ± 2	$15 \pm 2^{***}$	$34 \pm 2^{\#\#\#}$	$27 \pm 2^{**\#\#\#}$
Horizontal exploratory activity				
Total number of corners visited (Corner test)	11 ± 1	9 ± 1	11 ± 1	$8 \pm 1^{*}$
Total number of line crossings (Holeboard test)	354 ± 15	$275 \pm 16^{**}$	296 ± 16	301 ± 15
Number of line crossings in the peripheral area (Holeboard test)	231 ± 11	$188 \pm 13^{*}$	$172 \pm 13^{\#\#}$	198 ± 11
Number of line crossings in the central area (Holeboard test)	123 ± 6	$87 \pm 7^{***}$	125 ± 7	$104 \pm 6^{*}$
Time to complete the test (s) (T-maze test)	17(3)	$56(52)^{***}$	21(6)	$23(6)^{*\#\#}$
Anxiety-like behavior				
Total number of entries in open arms (Elevated plus maze test)	8 ± 1	6 ± 2	13 ± 2	9 ± 1
% of time in open arms (Elevated plus maze test)	36 ± 2	$16 \pm 2^{***}$	39 ± 2	$31 \pm 2^{*\#\#\#}$
Total number of entries in closed arms (Elevated plus maze test)	8 ± 2	14 ± 2	11 ± 2	11 ± 2
% of time in closed arms (Elevated plus maze test)	37 ± 2	$55 \pm 2^{***}$	37 ± 2	$43 \pm 2^{\#\#\#}$
% of time in central platform (Elevated plus maze test)	25 ± 3	30 ± 3	26 ± 3	27 ± 3
Total number of self-grooming (T-maze test)	0 ± 0	0 ± 0	0 ± 0	1 ± 0
Time of self-grooming (s) (Holeboard test)	2(4)	$15(9)^{***}$	2(8)	$7(6)^{*\#}$
Total number of fecal bolei (Holeboard test)	1 ± 0	$4 \pm 0^{***}$	2 ± 0	$4 \pm 0^{***}$
% fecal bolei presence (T-maze test)	0	40^{***}	0	$20^{***\#\#}$

Each value represents the mean ± SEM or median (IQR) (n = 8–10 animals for each group). $^{***}P < 0.001$; $^{**}P < 0.01$; $^{*}P < 0.05$ with respect to the values of the corresponding control mice. $^{\#\#\#}P < 0.001$; $^{\#\#}P < 0.01$; $^{\#}P < 0.05$ with respect to the values of middle-aged male control mice and middle-aged male DIO mice.

3.2.2. Motor coordination and equilibrium

The abilities of motor coordination and equilibrium that were evaluated, using the wood rod test, are shown in Table 1. The percentage of falls from the wood rod was significantly higher in male DIO mice than in their respective non-DIO controls (Table 1; Chi square test, $X^2(1) = 8.526$, $P = 0.004$). In addition, male DIO mice displayed significantly lower percentages of test completion and the covering of one segment in the wood rod test than male non-DIO mice (Table 1; Chi square test, $X^2(1) = 20.056$, $P < 0.001$). No differences were observed in these parameters between female DIO mice and their respective non-DIO controls (Table 1). In the wood rod test, male DIO mice showed a worse performance in the percentage of falls (Table 1; Chi square test, $X^2(1) = 8.526$, $P = 0.004$), on test completion (Table 1; Chi square test, $X^2(1) = 20.056$, $P < 0.001$) and the covering of one segment (Table 1; Chi square test, $X^2(1) = 20.056$, $P < 0.001$) than female DIO mice.

3.2.3. Muscular vigor

The muscular vigor characteristics of mice, which were evaluated by the tightrope test, are shown in Table 1. Male and female DIO mice showed significantly higher percentage of falls from the rope than their respective non-DIO controls (Table 1; Chi square test, $X^2(1) = 33.837$, $P < 0.001$) for male and (Table 1; Chi square test, $X^2(1) = 4.467$, $P = 0.035$) for female. In addition, the percentage of the test completed was significantly lower in male and female DIO mice in comparison with their respective controls (Table 1; Chi square test, $X^2(1) = 33.837$, $P < 0.001$) for male and (Table 1; Chi square test, $X^2(1) = 4.467$, $P = 0.035$) for female. The percentage of mice that used their forelimbs, hindlimbs and tail to remain hanging and consequently showed maximum traction capacity, was significantly lower in male and female DIO mice in comparison with their respective controls (Table 1; Chi square test, $X^2(1) = 18.483$, $P < 0.001$) for male and (Table 1; Chi square test, $X^2(1) = 20.056$, $P < 0.001$) for female. Male DIO mice showed a worse response in the percentage of falls (Table 1; Chi square test, $X^2(1) = 23.652$, $P < 0.001$), test completed (Table 1; Chi square test, $X^2(1) = 23.652$, $P < 0.001$), and traction capacity (Table 1; Chi square test, $X^2(1) = 18.484$, $P < 0.001$) than female DIO mice.

3.2.4. Vertical exploratory activity

The vertical exploratory activity of mice, which was evaluated by the performance of rearing in the corner and holeboard tests, is shown in Table 1. The total number of rearings in the corner test was significantly lower in male and female DIO mice than their respective non-DIO controls (Table 1; two-way ANOVA, significant main effect of diet $F(1,32) = 11.083$, $P = 0.002$; significant differences: male control vs male DIO, $P = 0.027$; female control vs female DIO, $P = 0.023$, post-hoc comparisons with Bonferroni corrections). In addition, male and female DIO mice performed significantly lower number of rearings in the holeboard test than their respective controls, with males showing a lower total of rearings than females (Table 1; two-way ANOVA, significant main effect of diet $F(1,32) = 25.176$, $P < 0.001$, main effect of sex $F(1,32) = 45.773$, $P < 0.001$; significant differences: male control vs male DIO, $P < 0.001$; female control vs female DIO, $P = 0.005$; male control vs female control, $P < 0.001$; male DIO vs female DIO, $P < 0.001$, post-hoc comparisons with Bonferroni corrections).

3.2.5. Horizontal exploratory activity

The horizontal exploratory activity was evaluated by the ambulation of mice in the corner, holeboard and T-maze tests (Table 1). Female DIO mice visited lower number of corners in the corner test than their respective controls (Table 1; two-way ANOVA, significant main effect of diet $F(1,32) = 4.861$, $P = 0.035$; significant differences: female control vs female DIO, $P = 0.049$, post-hoc comparisons with Bonferroni corrections). Male DIO mice performed lower number of total line crossings than their respective controls (Table 1; two-way ANOVA,

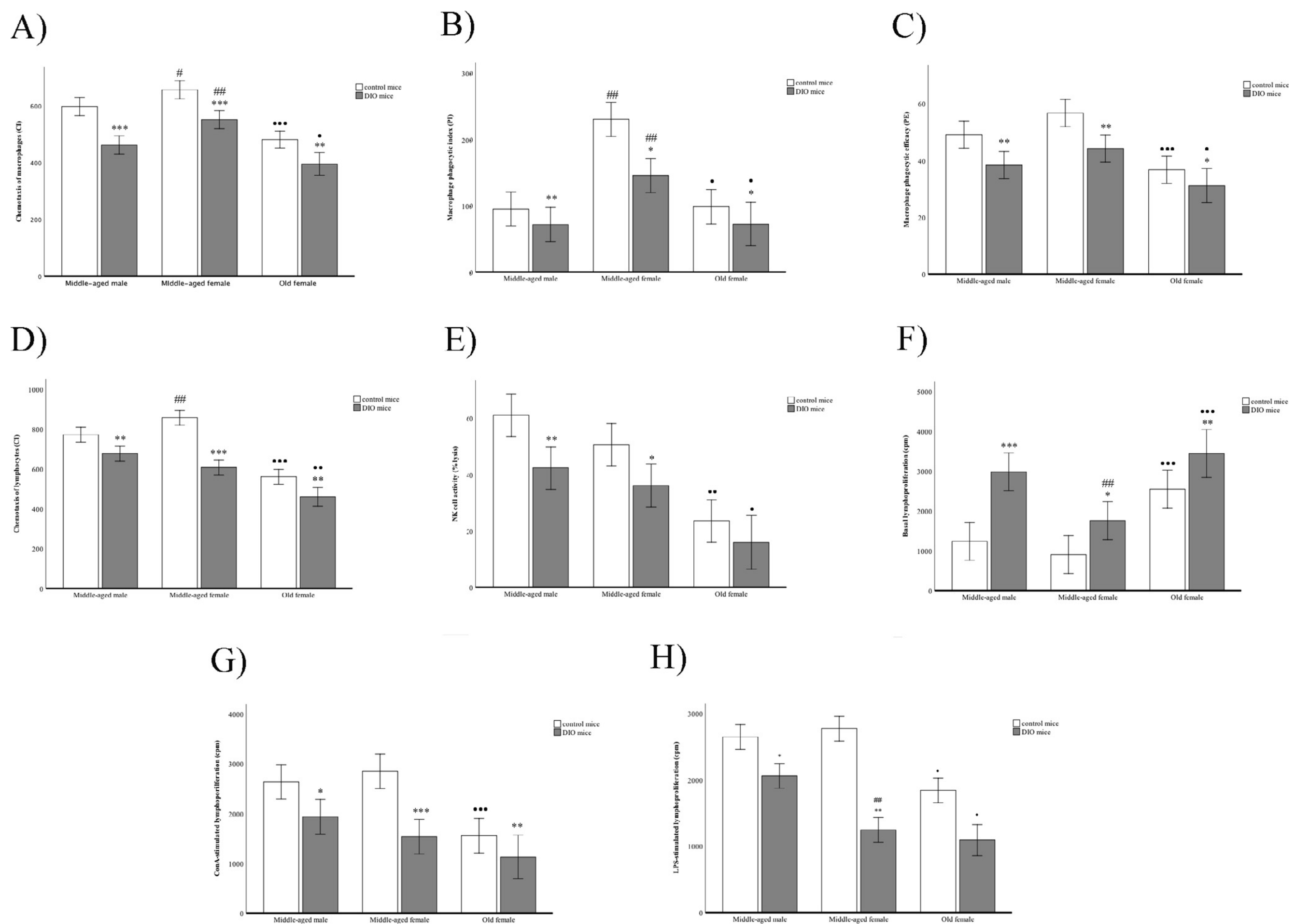

Fig. 2. Immune cell functions. (A) Macrophage chemotaxis index (CI, number of macrophages). (B) Macrophage phagocytic index (PI, number latex beads/100 macrophages). (C) Macrophage phagocytic efficacy (PE, number of phagocyting macrophages/100 macrophages). (D) Lymphocyte chemotaxis index (CI, number of lymphocytes). (E) Natural killer cell activity (% lysis). (F) Basal lymphoproliferation (cpm). (G) Lymphoproliferative response to concanalvaline A (ConA) (cpm). (H) Lymphoproliferative response to lipopolysaccharide (LPS) (cpm). Data represent mean ± SEM or median (IQR) (n = 5–10 animals for each group). $^{***}P < 0.001$; $^{**}P < 0.01$; $^{*}P < 0.05$ with respect to the corresponding values of control mice. $^{\#\#}P < 0.01$; $^{\#}P < 0.05$ with respect to the corresponding values of middle-aged male control mice and middle-aged male DIO mice. $^{\bullet\bullet\bullet}P < 0.001$; $^{\bullet\bullet}P < 0.01$; $^{\bullet}P < 0.05$ with respect to the values of middle-aged female control and DIO mice and middle-aged female DIO mice.

significant main effect of diet F(1,32) = 5.563, $P = 0.025$; significant differences: male control vs male DIO, $P = 0.001$, post-hoc comparisons with Bonferroni corrections). Male DIO mice displayed significantly lower peripheral ambulation (which was measured by the number of line crossings in the peripheral area of the holeboard) than their respective controls. Also, male controls showed significantly higher peripheral ambulation than female controls (Table 1; two-way ANOVA, significant interaction diet × sex F(1,32) = 8.341, $P = 0.007$, main effect of sex F(1,32) = 4.460, $P = 0.043$; significant differences: male control vs male DIO, $P = 0.016$; male control vs female control, $P = 0.001$, post-hoc comparisons with Bonferroni corrections). In addition, male and female DIO mice showed a significant lower central ambulation (which were measured by the number of line crossings in the central area of the holeboard) than their respective non-DIO controls (Table 1; two-way ANOVA, significant main effect of diet F (1,32) = 20.303, $P < 0.001$; significant differences: male control vs male DIO, $P < 0.001$; female control vs female DIO, $P = 0.023$, post-hoc comparisons with Bonferroni corrections).

Male and female DIO mice took a longer time to complete the exploration of the three arms of the T-maze test than their non-DIO counterparts, with male DIO mice showing a longer time to complete the test than female DIO mice (Table 1; Kruskal-Wallis test $X^2(3) = 23.553$, $P < 0.001$; significant differences: male control vs male DIO, $P < 0.001$; female control vs female DIO, $P = 0.044$; male DIO vs female DIO, $P = 0.001$, pairwise comparisons with Mann-Whitney).

3.2.6. Goal-directed exploratory behavior

The goal-directed exploratory behavior, which was evaluated by the percentage and total number of mice that performed head-dips in the holeboard test, was not significantly different between the studied groups for percentage of head-dips (male control: 100%; male DIO: 100%; female control: 100%; female DIO: 100%), and for total number of head-dips (male control: 12 ± 4; male DIO: 10 ± 5; female control: 13 ± 5; female DIO: 10 ± 2).

3.2.7. Anxiety-like behavior

The anxiety-like behavior, which was assessed by the elevated plus maze test, is shown in Table 1. Male and female DIO mice exhibited a lower percentage of time in open arms than their respective non-DIO controls, with male DIO mice showing a lower percentage of time than female DIO mice (Table 1; two-way ANOVA, significant interaction diet × sex F(1,32) = 6.820, $P = 0.014$, main effect of diet F (1,32) = 36.992, $P < 0.001$, main effect of sex F(1,32) = 17.599, $P < 0.001$; significant differences: male control vs male DIO, $P < 0.001$; female control vs female DIO, $P = 0.02$; male DIO vs

female DIO, $P < 0.001$, post-hoc comparisons with Bonferroni corrections). Moreover, the percentage of time in closed arms was higher in male DIO mice in comparison with their respective controls and female counterparts (Table 1; two-way ANOVA, significant interaction diet × sex $F(1,32) = 8.932$, $P = 0.005$, main effect of diet $F(1,32) = 30.330$, $P < 0.001$, main effect of sex $F(1,32) = 7.884$, $P = 0.008$; significant differences: male control vs male DIO, $P < 0.001$; male DIO vs female DIO, $P < 0.001$, post-hoc comparisons with Bonferroni corrections). In addition, the time (in seconds) spent by mice performing self-grooming in the holeboard test was significantly higher in male and female DIO mice than in their respective controls (Table 1; Kruskal-Wallis test $X^2(3) = 21.087$, $P < 0.001$; significant differences: male control vs male DIO, $P < 0.001$; female control vs female DIO, $P = 0.027$; male DIO vs female DIO, $P = 0.027$, pairwise comparisons with Mann-Whitney).

Male and female DIO mice also showed a higher percentage of fecal bolei presence (Table 1; Chi square test, $X^2(1) = 47.531$, $P < 0.001$) for male and ($X^2(1) = 20.056$, $P < 0.001$) for female, with male DIO mice having a higher percentage of fecal bolei presence than female DIO mice (Table 1; Chi square test, $X^2(1) = 8.595$, $P = 0.003$). In addition, the total number of fecal bolei was higher in male and female DIO mice than their respective non-DIO controls (Table 1; Kruskal-Wallis test $X^2(3) = 23.908$, $P < 0.001$; significant differences: male control vs male DIO, $P < 0.001$; female control vs female DIO, $P < 0.001$, pairwise comparisons with Mann-Whitney).

3.3. Peritoneal leukocyte functions

The peritoneal macrophage functions are shown in Fig. 2. The migration of peritoneal macrophages in response to a chemotactic gradient (formylated peptide), which mimics the migration of immune cells towards the infection site, was significantly lower in male and female DIO mice compared with their respective non-DIO controls. Males displayed significantly lower chemotactic index (CI) than females (Fig. 2A; two-way ANOVA, significant main effect of diet $F(1,28) = 51.573$, $P < 0.001$, significant main effect of sex $F(1,28) = 19.477$, $P < 0.001$; significant differences: male control vs male DIO, $P < 0.001$; female control vs female DIO, $P < 0.001$; male control vs female control, $P = 0.019$; male DIO vs female DIO, $P = 0.001$, post-hoc comparisons with Bonferroni corrections). In addition, the number of latex beads ingested by macrophages (the phagocytic index) was lower in male and female DIO mice with respect to the corresponding controls, with males displaying a lower phagocytic index than females (Fig. 2B; Kruskal-Wallis test $X^2(3) = 26.087$, $P < 0.001$; significant differences: male control vs male DIO, $P = 0.002$; female control vs female DIO, $P = 0.016$, male control vs female control, $P = 0.001$; male DIO mice vs female DIO mice, $P = 0.001$, pairwise comparisons with Mann-Whitney). The number of macrophages with phagocytic ability (measured by phagocytic efficacy) were also lower in male and female DIO mice with respect to the corresponding controls (Fig. 2C; two-way ANOVA, significant main effect of diet $F(1,28) = 19.211$, $P < 0.001$; significant differences: male control vs male DIO, $P = 0.008$; female control vs female DIO, $P = 0.002$, post-hoc comparisons with Bonferroni corrections).

With aging, the chemotactic index (CI) was significantly lower in old female DIO mice than in their corresponding non-DIO mice, with old females displaying a lower CI than middle-aged females (Fig. 2A; two-way repeated measures ANOVA, significant main effect of diet $F(1,11) = 30.551$, $P < 0.001$, main effect of age $F(1,11) = 41.213$, $P < 0.001$; significant differences: old female control vs old female DIO, $P = 0.002$; middle-aged female control vs old female control, $P < 0.001$; middle-aged female DIO vs old female DIO, $P = 0.010$, post-hoc comparisons with Bonferroni corrections). In addition, the phagocytic index (PI) was significantly lower in old female DIO mice than in non-DIO mice, with old females displaying a lower PI than middle-aged females (Fig. 1B; Friedman test, $X^2(3) = 13.653$, $P = 0.003$; significant differences: old female control mice vs old female DIO mice, $P = 0.012$, middle-aged female control vs old female control, $P = 0.012$, middle-aged female DIO vs old female DIO, $P = 0.043$, pairwise comparisons with Wilcoxon Signed Ranks). The phagocytic efficacy (PE) was significantly lower in old female DIO mice than in their corresponding controls, with old females displaying a lower PE than middle-aged females (Fig. 2C; two-way repeated measures ANOVA, significant main effect of diet $F(1,11) = 12.892$, $P = 0.004$, main effect of age $F(1,11) = 30.985$, $P < 0.001$; significant differences: old female control vs old female DIO, $P = 0.032$; middle-aged female mice vs old female control, $P < 0.001$; middle-aged female DIO vs old female DIO, $P = 0.015$, post-hoc comparisons with Bonferroni corrections).

The chemotactic index (CI) of peritoneal lymphocytes was significantly lower in male and female DIO mice in comparison with their non-DIO counterparts. Male control mice displayed lower CI than female control mice (Fig. 2D; two-way ANOVA, significant interaction diet × sex $F(1,28) = 17.467$, $P < 0.001$, main effect of diet $F(1,28) = 86.028$, $P < 0.001$; significant differences: male control vs male DIO, $P = 0.001$; female control vs female DIO, $P < 0.001$; male control vs female control, $P = 0.003$, post-hoc comparisons with Bonferroni corrections).

The NK activity was significantly lower in male and female DIO mice in comparison with their non-DIO controls (Fig. 2E; two-way ANOVA, significant interaction diet × sex $F(1,28) = 17.467$, $P < 0.001$, main effect of diet $F(1,28) = 16.420$, $P < 0.001$; significant differences: male control vs male DIO, $P = 0.003$; female control vs female DIO, $P = 0.019$, post-hoc comparisons with Bonferroni corrections).

The basal proliferative capacity of peritoneal lymphocytes as well as in response to T-cell mitogen (ConA) and to B-cell mitogen (LPS) are shown in Fig. 2F, G and H, respectively. Male and female DIO mice displayed significantly higher basal lymphoproliferation in comparison with non-DIO controls, with male DIO mice showing higher basal lymphoproliferation than female DIO mice (Fig. 2F; two-way ANOVA, significant main effect of diet $F(1,28) = 24.967$, $P < 0.001$; main effect of sex $F(1,28) = 8.982$, $P = 0.006$; significant differences: male control vs male DIO, $P < 0.001$; female control vs female DIO, $P = 0.028$; male DIO vs female DIO, $P = 0.002$, post-hoc comparisons with Bonferroni corrections). However, in response to the mitogen ConA, the proliferation of lymphocytes was lower in male and female DIO mice in comparison with their controls (Fig. 2G; two-way ANOVA, significant main effect of diet $F(1,28) = 25.513$, $P < 0.001$; significant differences: male control vs male DIO, $P = 0.019$; female control vs female DIO, $P < 0.001$, post-hoc comparisons with Bonferroni corrections). Male and female DIO mice also showed lower LPS-stimulated proliferation in comparison with their non-DIO mice, with male DIO mice showing higher LPS-stimulated proliferation than female DIO mice (Fig. 2H; Kruskal-Wallis test $X^2(3) = 23.172$, $P < 0.001$; significant differences: male control vs male DIO, $P = 0.021$; female control vs female DIO, $P = 0.001$; male DIO vs female DIO, $P = 0.001$, pairwise comparisons with Mann-Whitney).

With aging, the chemotactic index (CI) of peritoneal lymphocytes in old female DIO mice was significantly lower when compared with their corresponding non-DIO mice, with old females displaying lower CI than middle-aged females (Fig. 2D; two-way repeated measures ANOVA, significant interaction diet × age $F(1,11) = 13.264$, $P = 0.004$, main effect of diet $F(1,11) = 72.556$, $P < 0.001$, main effect of age $F(1,11) = 92.563$, $P < 0.001$; significant differences: old female control vs old female DIO, $P = 0.005$; middle-aged female control vs old female control, $P < 0.001$; middle-aged female DIO vs old female DIO, $P = 0.003$, post-hoc comparisons with Bonferroni corrections). Old females showed significantly lower anti-tumor NK activity than middle-aged females (Fig. 2E; two-way repeated measures ANOVA, significant main effect of age $F(1,11) = 27.653$, $P < 0.001$; significant differences: middle-aged female control vs old female control, $P = 0.001$;

middle-aged female DIO vs old female DIO, $P = 0.011$, post-hoc comparisons with Bonferroni corrections).

The basal proliferation of lymphocytes in old female DIO mice was higher than in their respective controls, with old females displaying higher basal lymphoproliferation than middle-aged females (Fig. 2F; two-way repeated measures ANOVA, significant main effect of diet $F(1,11) = 24.379$, $P < 0.001$, main effect of age $F(1,11) = 150.177$, $P < 0.001$; significant differences: old female control vs old female DIO, $P = 0.006$; middle-aged female control vs old female control, $P < 0.001$; middle-aged female DIO vs old female DIO, $P < 0.001$, post-hoc comparisons with Bonferroni corrections). Nevertheless, old female DIO mice exhibited significantly lower proliferation of lymphocytes in response to ConA mitogen-stimulated condition (Fig. 2G; two-way repeated measures ANOVA, significant main effect of diet $F(1,11) = 46.743$, $P < 0.001$, main effect of age $F(1,11) = 22.975$, $P = 0.001$; significant differences: old female control vs old female DIO, $P = 0.001$; middle-aged female control vs old female control, $P < 0.001$, post-hoc comparisons with Bonferroni corrections). In addition, old females displayed significantly lower LSP-stimulated lymphoproliferation than middle-aged females (Fig. 2H; Friedman test, $X^2(3) = 13.776$, $P = 0.003$; significant differences: middle-aged female control vs old female control, $P = 0.012$; middle-aged female DIO vs old female DIO, $P = 0.043$, pairwise comparisons with Wilcoxon Signed Ranks).

3.4. Peritoneal leukocyte oxidative stress parameters

Fig. 3 shows the results of enzymatic and non-enzymatic antioxidants, such as catalase (CAT) and glutathione peroxidase (GPx) activities as well as reduced glutathione (GSH) concentrations, respectively. The CAT activity was lower in leukocytes of male and female DIO mice in comparison with their corresponding non-DIO controls (Fig. 3A; two-way ANOVA, significant main effect of diet $F(1,28) = 37.361$, $P < 0.001$; significant differences: male control vs male DIO, $P < 0.001$; female control vs female DIO, $P < 0.001$, post-hoc comparisons with Bonferroni corrections). The GPx activity in leukocytes of male and female DIO mice was higher in comparison with their respective controls, with male control mice showing lower GPx activity than female control mice (Fig. 3B; two-way ANOVA, significant main effect of diet $F(1,28) = 33.633$, $P < 0.001$, main effect of sex $F(1,28) = 17.618$, $P < 0.001$; significant differences: male control vs male DIO, $P < 0.001$; female control vs female DIO, $P = 0.003$, male control vs female control, $P = 0.001$, male DIO vs female DIO, $P = 0.043$, post-hoc comparisons with Bonferroni corrections). The GSH concentrations were lower in peritoneal leukocytes of male and female DIO mice in comparison with their respective non-DIO controls (Fig. 3C; two-way ANOVA, significant main effect of diet $F(1,28) = 27.421$, $P < 0.001$; significant differences: male control vs male DIO, $P = 0.004$; female control vs female DIO, $P < 0.001$, post-hoc comparisons with Bonferroni corrections).

With aging, old female control mice displayed significantly lower CAT activity than middle-aged female control mice (Fig. 3A; two-way repeated measures ANOVA, significant main effect of age $F(1,11) = 22.686$, $P = 0.001$; significant differences: middle-aged female control vs old female control, $P < 0.001$, post-hoc comparisons with Bonferroni corrections). Old females also exhibited lower GPx activity than middle-aged females (Fig. 3B; two-way repeated measures ANOVA, significant main effect of age $F(1,11) = 80.339$, $P < 0.001$; significant differences: middle-aged female control vs old female control, $P < 0.001$; middle-aged female DIO vs old female DIO, $P < 0.001$, post-hoc comparisons with Bonferroni corrections). Regarding the GSH concentrations, old female DIO mice displayed lower values than those of old female non-DIO mice, with old females showing lower concentrations of GSH than middle-aged females (Fig. 3C; two-way repeated measures ANOVA, significant main effect of diet $F(1,11) = 9.116$, $P = 0.012$, main effect of age $F(1,11) = 21.591$, $P = 0.001$; significant differences: old female control vs old female DIO, $P = 0.005$, middle-aged female control vs old female control, $P = 0.001$, middle-aged female DIO vs old female DIO, $P = 0.033$, post-hoc comparisons with Bonferroni corrections).

Fig. 4 shows the values of enzymatic and non-enzymatic oxidants, including the activity of xanthine oxidase, the concentrations of MDA and superoxide anion as well as the GSSG/GSH ratios. The activity of xanthine oxidase, which is associated with the production of free radicals, was significantly higher in male and female DIO mice in comparison with their corresponding non-DIO controls, with males showing higher XO activity than females (Fig. 4A; two-way ANOVA, significant main effect of diet $F(1,28) = 58.360$, $P < 0.001$, main effect of sex $F(1,28) = 34.629$, $P < 0.001$; significant differences: male control vs male DIO, $P < 0.001$; female control vs female DIO, $P < 0.001$; male control vs female control, $P < 0.001$; male DIO vs female DIO, $P < 0.001$, post-hoc comparisons with Bonferroni corrections). The MDA concentrations, which are an indicator of lipid oxidation and oxidative damage in cells, were significantly higher in male and female DIO mice with respect to their non-DIO controls, with male DIO mice showing higher MDA concentrations than female DIO mice (Fig. 4B; two-way ANOVA, significant interaction diet × sex $F(1,28) = 8.633$, $P = 0.007$, main effect of diet $F(1,28) = 61.532$, $P < 0.001$, main effect of sex $F(1,28) = 8.475$, $P < 0.001$; significant differences: male control vs male DIO, $P < 0.001$; female control vs female DIO, $P = 0.002$; male DIO vs female DIO, $P < 0.001$, post-hoc comparisons with Bonferroni corrections). The concentrations of superoxide anion were significantly higher in male and female DIO mice with respect to their non-DIO controls (Fig. 4C; two-way ANOVA, significant main effect of diet $F(1,28) = 20.017$, $P < 0.001$; significant differences: male control vs male DIO, $P = 0.001$; female control vs female DIO, $P = 0.017$, post-hoc comparisons with Bonferroni corrections). The GSSG/GSH ratios, an indicator of oxidative stress, were significantly higher in male and female DIO mice than in their respective non-DIO controls (Fig. 4D; two-way ANOVA, significant main effect of diet F

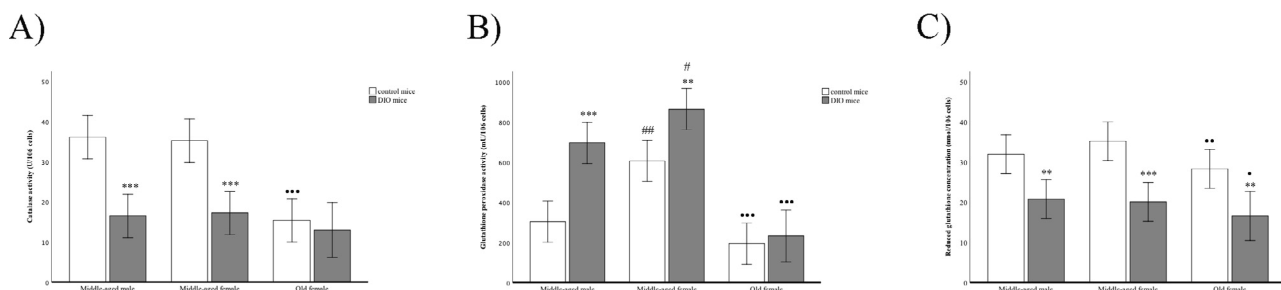

Fig. 3. Antioxidant parameters. (A) Catalase activity (U/10^6 cells). (B) Glutathione peroxidase activity (mU/10^6 cells). (C) Reduced glutathione concentration (GSH) (nmol/10^6 cells). Data represent mean ± SEM (n = 5–10 animals for each group). $^{***}P < 0.001$; $^{**}P < 0.01$ with respect to the corresponding values of control mice. $^{\#\#}P < 0.01$ with respect to the corresponding values of middle-aged male control mice and middle-aged male DIO mice. $^{\bullet\bullet\bullet}P < 0.001$; $^{\bullet\bullet}P < 0.01$; $^{\bullet}P < 0.05$ with respect to the values of middle-aged female control mice and middle-aged female DIO mice.

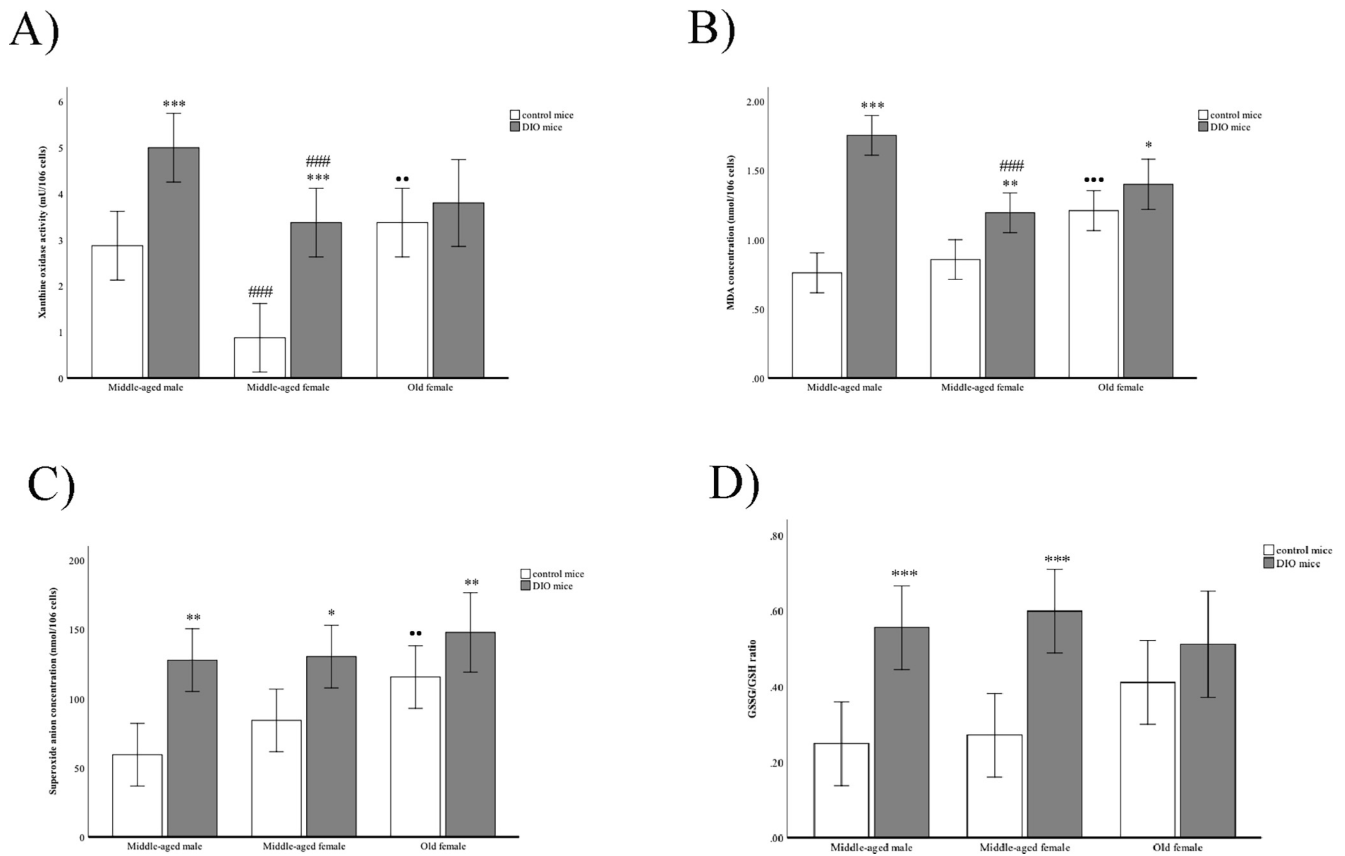

Fig. 4. Oxidative parameters. (A) Xanthine oxidase activity (mU/10^6 cells). (B) MDA concentration (nmol/10^6 cells). (C) Superoxide anion concentration (nmol/10^6 cells). (D) Oxidized glutathione (GSSG)/Reduced glutathione (GSH) ratio. Data represent mean ± SEM (n = 5–10 animals for each group). $^{***}P < 0.001$; $^{**}P < 0.01$; $^{*}P < 0.05$ with respect to the corresponding values of control mice. $^{\#\#\#}P < 0.001$ with respect to the corresponding values of middle-aged male control mice and middle-aged male DIO mice. $^{\bullet\bullet\bullet}P < 0.001$; $^{\bullet\bullet}P < 0.01$ with respect to the values of middle-aged female control mice and middle-aged female DIO mice.

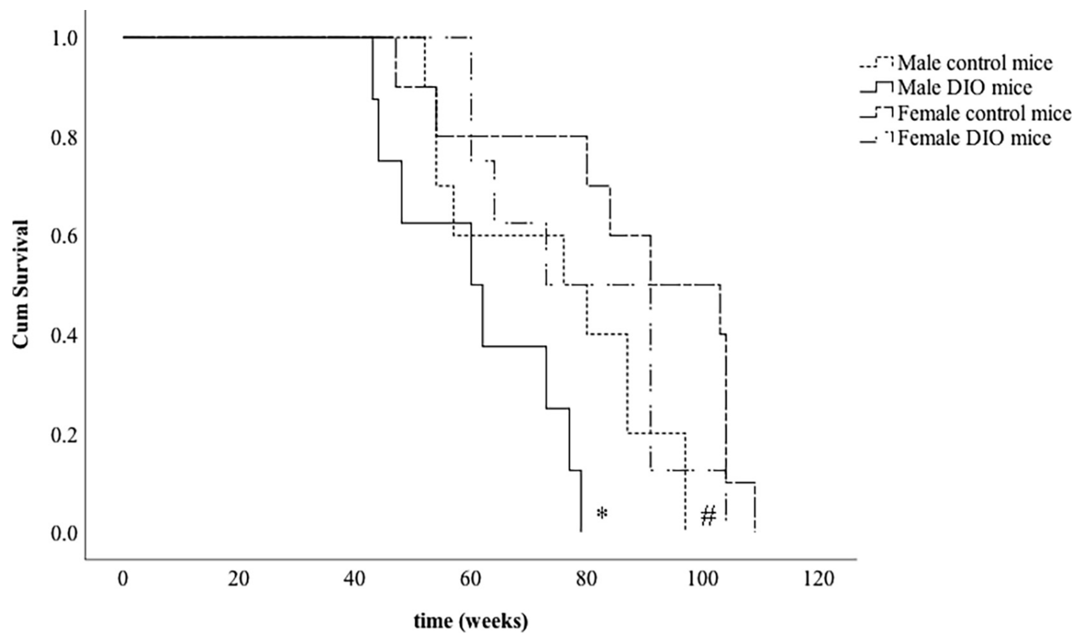

Fig. 5. Mortality records from male and female control mice and from male and female DIO (diet-induced obese) mice. $^{*}P < 0.05$ with respect to the corresponding values of middle-aged control mice. $^{\#}P < 0.05$ with respect to the corresponding values of middle-aged male DIO mice.

(1,28) = 75.625, $P < 0.001$; significant differences: male control vs male DIO, $P < 0.001$; female control vs female DIO, $P < 0.001$, post-hoc comparisons with Bonferroni corrections).

With aging, the xanthine oxidase activity in old female controls was significantly higher than in middle-aged female controls (Fig. 4A; two-way repeated measures ANOVA, significant main effect of age F (1,11) = 8.372, $P = 0.015$; significant differences: middle-aged female control vs old female control, $P = 0.003$, post-hoc comparisons with Bonferroni corrections). The MDA concentrations were significantly higher in old female DIO mice than in their respective controls, with old female controls showing higher MDA concentrations than middle-aged controls (Fig. 4B; two-way repeated measures ANOVA, significant interaction diet × age F(1,11) = 5.573, $P = 0.038$, main effect of diet F (1,11) = 13.696, $P = 0.004$, main effect of age F(1,11) = 27.484, $P < 0.001$; significant differences: old female control vs old female DIO, $P = 0.034$; middle-aged female control vs old female control, $P < 0.001$, post-hoc comparisons with Bonferroni corrections). The superoxide anion concentrations were significantly higher in old female DIO mice in comparison with old female non-DIO controls, with old female controls showing higher superoxide anion amounts than middle-aged controls (Fig. 4C; two-way repeated measures ANOVA, significant main effect of diet F(1,11) = 38.509, $P < 0.001$, main effect of age F (1,11) = 14.439, $P = 0.003$; significant differences: old female control vs old female DIO, $P = 0.003$; middle-aged female control vs old female control, $P = 0.002$, post-hoc comparisons with Bonferroni corrections).

3.5. Life span

Male DIO mice showed a shorter life span than male non-DIO control mice (Fig. 5; Kaplan-Meier survival curve, $P = 0.043$). Male DIO mice also exhibited a shorter life span than female DIO mice (Fig. 5; Kaplan-Meier survival curve, $P = 0.033$). The differences were not statistically significant between female DIO mice and female non-DIO mice (Fig. 5).

4. Discussion

This is the first study, to the best of our knowledge, showing that the late adulthood diet-induced obesity onset led to significant impairments in behavior as well as in functions and redox state of peritoneal leukocytes of middle-aged male and female mice, with males being significantly more affected than females. Thus, middle-aged male DIO mice exhibited greater impairments in a variety of behavioral, immune and redox state parameters, and consequently showed a shorter life span than their female counterparts. In the middle-aged female DIO mice some parameters presented values similar to those in old female animals. These results confirm the state of accelerated immunosenescence as the consequence of the diet-induced obesity of the present study. In addition to obesity-related impairments of the immune system, other factors, such as insulin resistance (Frasca et al., 2017), dysbiosis and increased intestinal permeability (Boulangé et al., 2016), could also contribute to oxidative and inflammatory systemic stresses, and consequently to the process of accelerated aging in obese individuals.

We have previously shown that the high-fat diet intake is a good model to develop obesity in mice (Hunsche et al., 2016, 2018). This experimental animal model of obesity has been demonstrated to be capable of mimicking human obesity, regarding its etiologic aspects (Kanasaki and Koya, 2011). Thus, this is applied to both sexes, since in the present study, middle-aged male and female mice fed on a high-fat diet during 14 weeks of their late-adulthood displayed significantly higher body weight and fat mass than their respective non-DIO counterparts. Moreover, middle-aged male and female DIO mice showed higher levels of triglycerides and total cholesterol, which are well-known common features of obesity (Hunsche et al., 2016, 2018; Klop et al., 2013). Furthermore, the late adulthood diet-induced obesity onset differentially affected the total body fat of middle-aged male and female mice. Thus, female DIO mice showed significantly higher body fat mass in comparison with their male counterparts. In agreement, previous studies reported that females have a higher propensity to gain body weight and consequently to store more fat in the adipose tissue than males (Mauvais-Jarvis, 2015). In general, females, prior to menopause, tend to accumulate more fat in the subcutaneous adipose tissue, while males in the visceral adipose tissue. This visceral accumulation of fat has been associated with worsen metabolic outcomes in this sex (Palmer and Clegg, 2015). In agreement, in the present study, middle-aged male DIO mice showed higher plasma levels of glucose and total cholesterol than their female counterparts.

With respect to the results of behavioral tests, we observed lower muscular vigor, horizontal and vertical exploratory activities as well as higher anxiety in middle-aged male and female DIO mice than in their respective non-DIO controls. Middle-aged male and female DIO mice displayed a significantly lower central ambulation in the holeboard, which indicates a lower exploratory activity and higher anxiety behavior, since mice with anxiety tend to ambulate more in the peripheral area (close the walls) than in the central area (Simon et al., 1994). Moreover, middle-aged male and female DIO mice exhibited a longer time to complete the exploration of the three arms of the T-maze test than their respective non-DIO counterparts, which also indicates a lower exploratory activity. Regarding the elevated plus maze test, middle-aged male and female DIO mice displayed a lower percentage of time in the open arms. It is known that a lower activity in the open arms of the elevated plus maze apparatus indicate higher anxiety behavior (Walf and Frye, 2007). Moreover, the behavior of self-grooming and the presence of defecation, which could be considered as anxiety-related measures (Archer, 1973; Sake and Graybiel, 2003), were also significantly higher in middle-aged male and female DIO mice in comparison with their respective non-DIO controls. Previous studies indicate lower cognitive and exploratory activity as well as higher anxiety in DIO mice. However, in these studies, the high-fat diet was administered soon after weaning (André et al., 2014; Arnold et al., 2014; da Costa et al., 2015; Sharma and Fulton, 2013; Vallodolid-Acebes et al., 2011; Yamada et al., 2011). Studies in humans, including data from younger to older individuals, also observed an association between increased body weight and worse performance on gross and fine motor skills, equilibrium and muscular vigor (Deforche et al., 2009; Gentier et al., 2013; Kumar et al., 2008; Maffiuletti et al., 2007). Accordingly, it can be suggested that obese individuals perform worse gross motor tasks because a greater proportion of excess mass has to be supported or moved against gravity during these tasks. However, the worse performance on fine motor tasks cannot be solely explained by excess fat mass, given that fine motor skills are not directly influenced by the amount of mass (Gentier et al., 2013). In this sense, previous studies suggest that obese individuals show lower neural efficiency and slowed integration and processing of information (Gunning-Dixon and Raz, 2000; Mehta and Shortz, 2014). In addition, an association has been found between increased muscular fat infiltration in obese and aged individuals and lower muscle quality and strength (Marcus et al., 2010; Moore et al., 2014). The results of the present study also showed sex differences in the evaluation of behavioral tests, with males being more affected than females. Thus, middle-aged male DIO mice showed a worse performance than their female counterparts in the tests that evaluated motor coordination, equilibrium, muscular vigor, vertical and horizontal exploratory activities and anxiety-like behavior. However, most of the previous studies on obesity performed in rodents have used a single sex (André et al., 2014; da Costa et al., 2015; Sharma and Fulton, 2013; Vallodolid-Acebes et al., 2011; Yamada et al., 2011), thus there is little information comparing the behavioral effects of obesity in both sexes. Nevertheless, in agreement with our data, a previous study considering sex differences in the performance of behavioral tests, showed males more vulnerable than females to high-fat diet (HFD)-induced impairments, such as learning functions and synaptic plasticity

(Hwang et al., 2010). The mechanisms underlying this higher impact of HFD and obesity in males than in females remain unknown, but some evidence indicates that estrogen could have a positive effect in protecting female mice. In this sense, male mice treated with estradiol showed less adiposity than controls (Salinero et al., 2018).

With respect to the functions studied in the peritoneal macrophages, middle-aged male and female DIO mice showed diminish chemotactic and phagocytic capacities of these cells in comparison with their respective non-DIO counterparts. These results are in agreement with those obtained in our previous study in which obesity in early adulthood was induced during the adolescent period (Hunsche et al., 2016). Thus, both the different ages of obesity onset (early and late adulthoods), resulted in impaired innate immune response. In addition, differently from our previous study, the present study also evaluated the immune effects of obesity in males. The results showed that middle-aged male DIO mice had lower chemotactic and phagocytic indexes than their respective female counterparts. Moreover, sex differences were also observed in non-DIO controls, with males showing lower values than females in some functions. Previous evidence confirms that males display a lower innate immune response against infection in comparison with females (Jaillon et al., 2017). Thus, it is possible that obesity could further exacerbate these impairments found in the innate immunity of males.

Regarding the anti-tumor activity natural killer (NK) of peritoneal leukocytes, middle-aged male and female DIO mice showed a lower response than their non-DIO counterparts, although no sex differences were observed in this function. The migration capacity of lymphocytes to a chemoattractant was significantly lower in middle-aged male and female DIO mice in comparison with their respective non-DIO controls. Similarly, the proliferation of lymphocytes in ConA and LPS-stimulated conditions, two mitogens for T and B lymphocytes, respectively, were also lower in middle-aged male and female DIO mice than in their non-DIO counterparts. These results are in agreement with those obtained in adult DIO female mice that ingested the diet rich in fat during adolescence (Hunsche et al., 2016). Thus, both early and late adulthood obesity onsets resulted in impaired functions of lymphocytes. Although middle-aged male control mice had lower values than middle-aged female control mice in chemotaxis of lymphocytes, male DIO mice presented higher chemotactic index and LPS-stimulated proliferation of lymphocytes than female DIO mice. The higher response of these immune functions in males could be possibly interpreted as a compensatory mechanism to counteract the lower response found in innate immune cell functions (Franceschi et al., 1995), especially in the context of an obesity state.

Oxidative stress, which is generated by an imbalance between oxidants and antioxidant in favor of oxidants, is associated with the development of obesity, aging and health complications (Bauer and De la Fuente, 2016; Marseglia et al., 2014). The current results demonstrated that middle-aged male and female DIO mice showed higher values of oxidants, such as xanthine oxidase activity, lipid peroxidation (MDA) concentrations, and GSSH/GSH ratios as well as lower values of antioxidant defenses (such as catalase activity and GSH concentrations). These results are partly in concordance with our previous experiment in which it was found increased oxidative stress in adult DIO female mice (Hunsche et al., 2016). However, differently from these previous data, the activity of glutathione peroxidase (an antioxidant enzyme) was significantly higher in middle-aged DIO animals in comparison with their respective non-DIO counterparts. In this sense, it is possible that the higher activity of this antioxidant enzyme could be explained by a compensatory mechanism in an attempt to restore the appropriate redox state of DIO animals. In fact, the activity of this enzyme can increase or decrease in oxidative stress situations depending on the moment of their evolution and the amount of peroxides generated (Liu et al., 2004; Yan and Harding, 1997). Sex differences were observed in the redox parameters studied of middle-aged non-DIO animals, with males being more affected than females. Thus, in response to the late age of obesity onset, middle-aged male DIO mice showed higher values of xanthine oxidase and lipid peroxidation (MDA) and lower values of glutathione peroxidase activities in comparison with their female counterparts. Previous studies indicate that males are more susceptible to present higher oxidative stress than females (Baeza et al., 2011); however this condition is still controversial and contradictory data have been reported (Kander et al., 2017). Moreover, there is a lack of evidence about sex differences in the redox state of obese individuals.

At old age, female DIO mice continuous to exhibit some impaired immune functions and redox state parameters than old female non-DIO controls. However, differently from our previous data (in the early adulthood obesity onset) (Hunsche et al., 2016), the late adulthood diet-induced obesity onset resulted in no significant differences in some immune functions (such as anti-tumor NK cell activity and LPS-stimulated proliferation of lymphocytes), redox state (xanthine oxidase activity, GSSG/GSH ratio, catalase and glutathione peroxidase activities) and in the life span between female DIO mice and female non-DIO mice. Thus, the early adulthood obesity onset, in which adolescent mice were exposed to a high-fat diet, had more long-lasting deleterious effects in the immune system and consequently in the life span than a later obesity onset. Increasing evidence suggest that adolescence is a critical period in which the nervous and immune systems are still experiencing developmental changes and thus are more susceptible to stresses, such as an increased consumption of high-fat diet (Boitard et al., 2012; Holder and Blaustein, 2014; Simon et al., 2015; Spear, 2000).

Although several innate and adaptive immune functions were analyzed here, other relevant aspects of immunosenescence should be considered in future studies. Thus, acquisition of the senescence-associated phenotype (SASP), immunophenotyping aging subsets, and intracellular signaling pathways, could confirm the accelerated immunosenescence state of obese individuals.

In conclusion, the results of the current study provide evidence that the late adulthood diet-induced obesity onset leads to impairments in the functions of the nervous (behavior) and immune system (function and redox state of peritoneal leukocytes) of middle-aged male and female mice. Sex differences were found in some of the behavioral, immune function and redox state parameters, being males significantly more affected than females. Since many of these functions have been proposed as markers of health, rate of aging and predictor of longevity (De la Fuente and Miquel, 2009, Martínez de Toda et al., 2016), the results show a worst health status in middle-aged female and especially male DIO mice and, consequently, a lower life span. In addition, the late adulthood obesity partially exacerbated the age-related impairments in immune cell functions and redox state of old female mice.

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Conflict of interest

The authors declare no conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbi.2019.01.010.

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