Original Article

Impaired Immune Response in Old Mice Suffering from Obesity and Premature Immunosenescence in Adulthood

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Abstract

Obesity and aging share an impaired immune system and oxidative and inflammatory stress. Therefore, the hypothesis of obesity as a possible model of premature immunosenescence has been proposed. In this study, we investigated whether adult obese mice, as a consequence of being fed with a fat-rich diet during their adolescence, showed premature immunosenescence and if this was aggravated with aging. Peritoneal cell suspensions were obtained when ICR/CD1 obese female mice were adults (28 weeks) and old (72 weeks), and several functions and antioxidant defenses were evaluated. The results showed that the chemotaxis of both macrophages and lymphocytes, phagocytosis of macrophages, activity of natural killer cells, proliferative response of lymphocytes, interleukin-1β, tumor necrosis factor-alpha, interleukin-6, interleukin-2, and interleukin-10 released in leukocyte cultures, as well as antioxidant and oxidant capacity were significantly impaired in adult obese mice with respect to adult nonobese mice, with values similar to those in chronologically old mice. When these obese animals grew older, although having been fed with a standard diet, they showed a higher deterioration of their immune functions in comparison with the old control group. In conclusion, these results demonstrate that a high fat intake during adolescence can produce an obesity state in adult age associated with a premature immunosenescence, which is aggravated through aging.

Keywords: Obesity—Immune function—Oxidative stress—Premature immunosenescence

It is widely accepted that nutrition and diet play an important role in maintaining health, as well as in causing certain kinds of diseases. Thus, an excessive consumption of energy dense food, such as dietary fat, has been associated with the current burden of obesity worldwide (1). In particular, adolescence has been described as a critical period for the onset of obesity, and once this condition is established, it is likely to persist into adulthood (2). Furthermore, the early onset of obesity and its related health problems may increase the risk of morbidity and premature mortality (3).

Increasing evidence suggests that the central mechanism underlying obesity and its related diseases are a state of persistent low-grade systemic inflammation together with a deregulation in the inflammation–stress feedback mechanisms (4). This inflammatory condition is possibly generated by the recruitment of immune cells, mostly bone marrow–derived macrophages, into the adipose tissue in response to excess nutrients, hypertrophy, and hypoxia of adipocytes (5). Consequently, these dysfunctional adipocytes contribute to immune activation and to the increased production of several proinflammatory molecules like tumor necrosis factor-alpha (TNF-α), interleukin (IL)-6, and macrophage chemoattractant protein-1 (MCP-1) (6). Moreover, dysfunctional adipocytes seem also to be implicated in the production of oxidative stress through the generation of reactive oxygen species, which could result in further inflammation and tissue damage (7). Although an appropriate immune response requires certain levels of oxidation and inflammation, which are two interlinked processes with many feedback loops (8), several studies have shown that the enhanced inflammatory milieu observed in obesity is not associated with an optimal immune defense. Thus, obese subjects present a poorer immune response to pathogen infection and to vaccines (9,10).
In addition, it is known that the process of aging is accompanied by an impairment of the immune system, which is known as immunosenescence. In this condition, the immune system undergoes a wide range of age-associated restructuring that may result in decreased as well as increased activities of immune cells. For instance, there is a progressive decline in the functional activity of phagocytic cells, natural killer (NK) cells, in lymphoproliferative response, and in mitogen-stimulated cytokine production (11–14). These age-related changes seem to be produced by the oxidative and inflammatory stress situation of the immune cells. In fact, although the production of low levels of oxidants and inflammatory compounds is essential for the normal defensive role of immune cells against infection, when these levels are very high, cellular damage appears. Thus, innate immune cells, such as macrophages, exhibit an age-related over-activated production of oxidative and inflammatory compounds, which occurs mainly in the absence of antigenic stimulus and cannot be neutralized by antioxidant and anti-inflammatory defenses. This leads to an oxidative and inflammatory stress (8,15–17). It is accepted that the process of aging is the result of the accumulation of cellular damage, which is caused by oxidative and inflammation stress throughout the lifetime of an organism. Moreover, it has been recently proposed that the immune system can be involved in these stresses and consequently in the rate of aging (16,17).

Because both obesity and aging share common features, such as an impaired immune system and oxidative and inflammatory stress, the hypothesis of obesity as a possible model of premature immunosenescence, has been proposed (18). Although it is known that the physiological conditions at adult age determine the aging process and that aging exacerbates obesity-induced oxidative stress and inflammation (19), the long-term immune effects of the early onset of obesity and its possible consequences in the process of aging have been scarcely investigated. Therefore, the aim of this study was to confirm whether adult obese mice, as a consequence of being fed with a high-fat diet during their adolescence, presented immune cells and redox values similar to those observed in chronologically old animals, and if this premature immunosenescence is aggravated with aging.

**Methods**

**Animals**

Female ICR/CD1 mice, 8 weeks of age, were purchased from Harlan Interfauna Iberica (Barcelona, Spain). The animals were housed in polyurethane cages (6–10 animals per cage) and maintained under standard laboratory conditions (12:12 hour reversed light/dark cycle; lights on at 8:00 pm in order to avoid circadian interferences, relative humidity at 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 1201/2005 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.

**Experimental Groups**

A group of 20 adolescent mice (14±2 weeks of age) were divided into two groups of similar average body weight and were fed either a standard diet (n = 10) or a high-fat diet (n = 10) for 14 weeks. A group of middle-aged mice (38±2 weeks old) were fed a standard diet (n = 8). The animals had free access to water and food. When the adolescent mice reached adulthood (ie, the adult control and the adult obese mice groups, 28±2 weeks of age) and the middle aged were considered old (ie, the old control mouse group, 72±2 weeks of age), several parameters of function and oxidative state of leukocytes were evaluated. In addition, adult obese and adult control mice were submitted to the evaluation of biochemical parameters and blood pressure measurements. After 14 weeks on the high-fat diet, the adult obese mice returned to a standard diet, whereas the adult control mice maintained the standard diet. All mice were marked for their individual follow-up. When these animals reached old age (72 weeks old) (ie, the old control and the old obese mice groups, n = 9, in each group), biochemical parameters, blood pressure measurements, and peritoneal leukocyte functions were measured again.

The diets used were provided by Harlan Interfauna Iberica and the nutritional compositions were (i) standard diet (Teklad Global 14% Protein Rodent Maintenance Diet): energy 2.9 Kcal/g, protein 20%, carbohydrate 67%, and fat 13%, in which fat was derived from soybean oil (saturated 0.8%, monounsaturated 0.7%, and polyunsaturated fatty acids 2.1%) and (ii) high-fat diet (TD.06414): energy 5.1 Kcal/g, protein 18.4%, carbohydrate 21.3%, and fat 60.3%, in which fat was derived from 31% of lard and 3% of soybean oil (saturated 37%, monounsaturated 47%, and polyunsaturated fatty acids 16%).

**Biochemical Parameters and Arterial Blood Pressure Measurements**

Glucose, cholesterol, and triglyceride levels were measured with Accutrend (Roche Diagnostics, Mannheim, Germany) using blood samples collected from the tail vein of mice. Arterial blood pressure was measured using a noninvasive pressure gauge (Panlab Non-Invasive Blood Pressure System for Rodents, Harvard). Measurements were performed three times in a stress-free environment (without light and background noise), and after subjecting the mouse to a temperature of 30°C–35°C for 20 minutes to calm the animal and avoid stress inside the holder.

**Collection of Peritoneal Leukocytes**

The peritoneal suspensions were obtained between 8:00 am and 10:00 am to minimize circadian variations in the immune system, without sacrificing the animals, which allowed monitoring the life spans of the mice, by a procedure previously described (20,21). Briefly, 3 mL of Hanks’ 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, Germany). The suspensions were adjusted to a final concentration of 5×10⁶ macrophages or lymphocytes/mL in Hanks’ solution or 10⁶ leukocytes/mL in Hanks’ solution or complete medium (Roswell Park Memorial Institute [RPMI] 1640 enriched with l-glutamine (PAA, Pasching, Austria) and supplemented with 10% heat-inactivated (56°C, 30 minutes) fetal calf serum (GIBCO) and gentamicin (100 mg/mL, GIBCO) with or without phenol red). Macrophages and lymphocytes were identified by their morphology. The cellular viability was routinely measured using the trypan-blue (Sigma, St. Louis, MO) exclusion test, and in all cases, it was higher than 98%.

The peritoneal compartment offers the potential to study unfragmented leukocytes, which better preserve the physiological environment surrounding the immune cells in vivo. This is fundamental in studies ex vivo that try to reproduce immune cell response in vivo. In fact, this response may vary or be lost in populations of purified leukocytes (22).
Chemotaxis Assay
Chemotaxis of peritoneal leukocytes were evaluated according to a slight modification of the Boyden’s method (20) consisting basically of the use of chambers with two compartments separated by a filter with a pore diameter of 3 µm (Millipore, Bedford, MA). Aliquots of 300 µL of the peritoneal suspensions, with macrophages or lymphocytes adjusted to 5 x 10^5 cells/mL in Hank’s solution, were deposited into the upper compartment, and aliquots of 400 µL of the chemotaxtractant, formyl-Met-Leu-Phe (10^-6 M) (Sigma) were put into the lower compartment. The chambers were incubated for 3 hours, and then the filters were fixed and stained. The chemotaxis index was determined by counting, using an optical microscope (100x), the total number of macrophages and lymphocytes on one third of the lower face of the filters.

Phagocytosis Assay
Phagocytosis assay of inert particles was carried out following the method described by De la Fuente and colleagues (20). Aliquots of 200 µL of the peritoneal suspensions, adjusted to 5 x 10^5 macrophages/mL in Hanks’ medium, were incubated in migration inhibitory factor plates (Kartell, Noviglio, Italy) for 30 minutes. The adhered monolayer was washed with prewarmed phosphate buffer saline and then 200 µL of Hank’s medium and 20 µL latex beads (1.09 µm diluted to 1% phosphate buffer saline) (Sigma) were added. After 30 minutes of incubation, the plates were washed, fixed, and stained. The number of particles ingested by 100 macrophages was counted using an optical microscope (100x) and expressed as phagocytic index. The percentage of macrophages, which phagocytosed at least one latex bead, was also determined and expressed as phagocytic efficiency.

Superoxide Anion Production Assay
Superoxide anion production was evaluated assessing its capacity to reduce nitroblue tetrazolium (NBT, Sigma), in an equimolecular reaction, following the method described by De la Fuente and colleagues (20). Briefly, aliquots of 250 µL of peritoneal cell suspensions (1 x 10^6 leukocytes/mL, Hank’s medium) were mixed with 250 µL of NBT solution (1 mg/mL in Hank’s solution) (Sigma) and with 50 µL of latex beads (1%) in stimulated samples. After 60 minutes of incubation at 37°C, the reaction was stopped, the samples were centrifuged, and the supernatants were discarded. The reduced NBT was extracted with dioxin (Merck, Darmstadt, FRG), 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).

Natural Killer Assay
An enzymatic colorimetric assay was carried out for cytolytic measurements of target cells (Cytotox 96 TM Promega, Boehringer, Ingelheim) based on the determination of lactate dehydrogenase enzyme using tetrazolium salts, as previously used by us in these kind of samples (21). Aliquots of 100 µL of peritoneal leukocytes, used as effector cells, were seeded in 96-well U-bottom culture plates (Numc, Roskilde, Denmark) adjusted to 10^6 leukocytes per well in RPMI 1640 medium without phenol red. Murine lymphoma YAC-1 cells, used as target cells, were added adjusted to 10^5 cells per well. Thus, the effector/target ratio was 10:1. The plates were centrifuged at 250g for 4 minutes to facilitate cell contacts. After 4 hours of incubation, lactate dehydrogenase enzymatic activity was measured in 50 µL/well of the supernatants by addition of the enzyme substrate and absorbance recording spectrophotometrically at 490 nm. Three kinds of control measurements were performed: a target spontaneous release, a target maximum release, and an effector spontaneous release. The results were expressed as percentage of lysis of target cells. To determine this percentage, the following equation was used: % lysis = ((E-ES-TS) / M-ES-TS) x 100, where E is the mean of absorbances in the presence of effector cells; ES is the mean of absorbances of effector cells incubated alone; TS is the mean of absorbances in target cells incubated with medium alone; and M is the mean of maximum absorbances after incubating target cells with lysis solution.

Lymphoproliferation Assay
Following the method previously described (21), aliquots (200 µL) of peritoneal lymphocytes (10^6 cells/mL complete medium) were seeded in 96-well flat-bottomed microtiter plates (Numc, Roskilde, Denmark) and 20 µL of concanavaline A (ConA 1 µg/mL; Sigma), a T-cell mitogen (lectin), 20 µL of lipopolysaccharide (LPS, Escherichia coli, 053:B5 1 µg/mL; Sigma), a B-cell mitogen, or 20 µL of complete medium (spontaneous proliferation) were added per well. The plates were incubated for 48 hours at 37°C in an atmosphere of 5% CO₂. After this time, 100 µL of culture supernatants were collected for cytokine measurements and the medium was renewed, and 0.5 µCi/H-thymidine (Du Pont, Boston, MA, USA) were added to each well. After 24 hours of incubation, the cells were harvested in a semiautomatic microharvester and retained in filter paper. Thymidine uptake was measured using a beta counter (LKB, Uppsala, Sweden). The results were expressed as 3H-thymidine uptake (cpm).

Cytokine Levels
The levels of the cytokines, including growth factors (IL-2), pro-inflammatory (TNF-α, IL-6, and IL-1β), and anti-inflammatory (IL-10) cytokines, released into the supernatants of leukocyte cultures, after 48 hours of incubation with ConA or LPS, were measured simultaneously using a Luminex xPONENT (Milliplex Mouse Cytokine/Chemokine Panel Catalog MPXMCYT0-70K, Millipore Corp, Billerica, MA). The results were expressed as pg/mL.

Total Glutathione Levels
The total intracellular glutathione levels were measured using spectrophotometry based on a previously described method with some modifications (23). Briefly, aliquots of 1 mL of peritoneal suspension (adjusted to 10^6 leukocytes per mL in Hank’s medium) were centrifuged at 1,200g for 10 minutes at 4°C. The pellet cells were resuspended in a medium containing 5% trichloroacetic acid (TCA, Panreac, Barcelona, Spain) in 0.01 N HCl (previously degassed with helium for a minimum of 10 minutes). This was followed by three cycles of sonication for 10 seconds (with 20 seconds rest between each cycle), keeping the sample cold. Then, the samples were centrifuged at 3,200g for 5 minutes at 4°C. Aliquots of the supernatants of leukocytes samples were measured using the following reaction mixture: 5,5’-dithiobis (2-nitrobenzoic acid) (6 mM, Sigma), β-nicotinamide adenine dinucleotide phosphate, reduced form (β-NADPH, 0.3 mM, Sigma), and glutathione reductase (10 U/mL, Sigma). The reaction was monitored for 240 seconds and measured using spectrophotometry at a wavelength of 412 nm. The results were expressed in nmol/10^6 cells.
Catalase Assay
The activity of catalase (CAT) was determined following the method described by Beers and Sizer, with slight modifications introduced by us (24). The peroxisomal suspension was previously adjusted to 10⁶ leukocytes/mL and aliquots of 1 mL were used to perform the enzymatic assay. The cells were centrifuged at 1,076 g for 10 minutes at 4°C and the pellets were resuspended in 50 mM phosphate buffer. Then, the samples were sonicated and centrifuged at 3,200 g for 20 minutes at 4°C. The enzymatic assay was followed using spectrophotometry for 80 seconds at 240 nm through the decomposition of H₂O₂ (14 mM in phosphate buffer) (Merck, Germany) into H₂O + O₂. The results were expressed as international units (U) of enzymatic activity per 10⁶ cells.

Xanthine Oxidase Assay
To study the activity of xanthine oxidase (XO), we employed the commercial kit “Amplex Red Xanthine/Xanthine Oxidase Assay Kit” (Molecular Probes). The hydrogen peroxide (H₂O₂) produced by XO reacts with horseradish peroxidase present in the reaction mixture and generates a fluorescent oxidation compound resoru- fin whose fluorescence is measured in a plate reader (Fluorostar Optima, BMG Labtech Biomedical, Spain). Briefly, 50 µL of perito-neal suspension adjusted to 10⁶ leukocytes/mL in Hank’s medium were incubated with 50 µL working solution of Amplex Red reagent (100 µM) containing horseradish peroxidase (0.4 U/mL) and xanthine (200 µM). After 30 minutes of incubation at 37°C, measurements of fluorescence were 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 10⁶ cells.

Statistical Analysis
SPSS 10.0 (SPSS, Inc., Chicago, IL) was used for the statistical analysis of the results. The data were expressed as mean ± SD. Each value is the mean of the data from an assay performed in duplicate or triplicate. Normality of the samples was checked by the Kolmogorov-Smirnov test and homogeneity of variances by the Levene test. The results were statistically evaluated by the Student’s t-test for independent samples. p < .05 was statistically significant and .05 < p < .1 was considered as a trend.

Results

Body Weight, Biochemical Parameters and Arterial Blood Measurements
At adulthood (28 weeks), animals fed with a high-fat diet for 14 weeks displayed a significantly higher body weight when compared with animals fed with a standard diet (p < .01). Furthermore, they also presented higher levels of triglycerides (p < .05) and systolic pressure (p < .001), marking them as obese. No significant differences were observed in total glucose, cholesterol, and diastolic pressure levels between the obese and the control group (see Supplementary Table 1). When these adult obese animals reached the age of 72 weeks, they continued to present a higher body weight than the old control group (p < .001). The glucose (p < .01), triglycerides (p < .05), and systolic pressure levels (p < .01) were also significantly increased compared with that of old controls. However, with respect to measurements of total cholesterol and diastolic pressure levels, no differences were found between old control and old obese mice groups in old age (see Supplementary Table 1).

Peritoneal Leukocyte Functions
The migration of peritoneal macrophages, measured by the chemotaxis index (Figure 1A), declined significantly in adult obese and in old control mice compared with that in adult controls (p < .001). In addition, the number of latex beads ingested by macrophages (Figure 1B) and the number of macrophages with phagocytic ability (Figure 1C) were also significantly decreased in adult obese mice and in old controls (p < .001) with respect to adult control animals. The levels of intracellular superoxide anion in stimulated macrophages, an important capacity of phagocytic cells in killing pathogens, showed significantly lower values in adult obese mice (p < .001) and in old mice (p < .05) when compared with that in adult controls (Figure 1D).

Regarding the lymphocyte functions studied, the chemotaxis capacity of peritoneal lymphocytes (Figure 1E) was significantly decreased in adult obese and in old control mice (p < .01) with respect to adult control animals. A decreased activity of NK cells (% lysis) was also observed in both adult obese (p < .05) and old control mice (p < .01) in comparison with adult controls (Figure 1F). The results of the proliferative capacity of peritoneal lymphocytes in response to T-cell mitogen ConA and to B-cell mitogen LPS are shown in Figure 1G and H, respectively. Adult obese mice and old control mice displayed a significantly suppressed mitogen-induced proliferative response compared with that of adult controls (p < .001). However, the basal lymphoproliferation was not significantly different between the three groups of animals, adult obese (1,770 ± 1,361 cpm), old mice (1,248 ± 100 cpm), and adult controls (1,459 ± 697 cpm). The levels of several cytokines (IL-1β, TNF-α, IL-6, IL-2, and IL-10), released in culture supernatants of perito-neal leukocytes after 48 hours of incubation under ConA and LPS-stimulated conditions, are shown in Figure 2. All these cytokines displayed a trend towards decrease or were significantly decreased in adult obese and in old animals in comparison with adult controls. In addition, the cytokines IL-1β and IL-10 under LPS-stimulated conditions (p < .05) and IL-2 under ConA- (p < .01) and LPS-stimulated conditions (p < .001) were significantly lower in adult obese mice as compared with that in old mice. Nevertheless, the levels of IL-6 released in response to ConA and LPS were significantly higher in adult obese mice than in old controls (p < .05).

With aging, impairments of leukocyte functions became more pronounced in old obese mice in comparison with old controls. As shown in Table 1, macrophage functions, such as chemotaxis capacity (p < .01) as well as phagocytosis index (p < .01) and phagocytosis efficacy (p < .001), were significantly diminished in old obese mice when compared with that in old controls. However, with respect to stimulated generation of superoxide anion, there were no significant differences between both groups. In relation to NK cell activity, there was a significant decrease in old obese mice in comparison with old controls (p < .05). The lymphocyte functions (chemotaxis index and proliferation in response to ConA and LPS), which are shown in Table 1, were highly impaired in old obese mice when compared with old controls (p < .001). Even though not statistically significant, there was a tendency of enhanced basal lymphoproliferative response in old obese with respect to old controls (p = .06) (Table 1).

Peritoneal Leukocyte Oxidative Stress Parameters
In order to investigate the oxidative stress status of peritoneal leukocytes in mice, the activity of XO, which is associated with the production of free radicals, and the enzymatic and nonenzymatic antioxidants, such as CAT activity and glutathione levels, were evaluated. The levels of total glutathione (Figure 3A), which have an important defensive role in neutralizing free radicals,
presented decreased values in peritoneal leukocytes in adult obese and old controls as compared with that in adult controls ($p < .001$).

Similarly, CAT activity (Figure 3B), which protects against oxidant compounds, was diminished in leukocytes from adult obese ($p < .001$) and old mice ($p < .01$) with respect to adult control animals. The activity of XO (Figure 3C) was increased in leukocytes from adult obese mice ($p < .001$) and in those from old mice ($p < .01$) when compared with adult controls. When these animals grew older, reaching 72 weeks old, obese mice exhibited significantly lower levels of total glutathione when compared with old controls ($p < .001$). Nevertheless, the activities of XO and CAT did not present statistical differences between old obese and old control animals (Table 1).
The present work shows the negative effects on the immune system of old mice as adults, were obese due to the ingestion of a high-fat diet during adolescence. Obesity was induced in adolescent mice by the administration of a high-fat diet for 14 weeks. These animals progressively and significantly increased their body weights and also presented significantly elevated levels of triglycerides and systolic arterial pressure when compared with the control group. Moreover, these mice showed conditions associated with obesity similar to those obtained in mice of a previous study in which an identical experimental design was followed to generate adult obesity. Thus, the administration of a high-fat diet during adolescence seems to be a good model to induce obesity in adult mice. Additionally, this type of diet and the increased adiposity have also been reported to enhance the levels of oxidation and inflammation and to promote a dysfunctional oxidative state of leukocytes in adulthood. Thus, the adult obese mice, when compared with the controls of the same age, presented several deteriorated immune parameters, such as chemotaxis of both macrophages and lymphocytes, phagocytosis of macrophages, NK cell activity, mitogen-stimulated lymphoproliferation, and mitogen-stimulated release of several cytokines (IL-1β, TNF-α, IL-6, IL-2, and IL-10) derived from leukocytes culture supernatants. These parameters showed values similar to those in chronologically old animals. On the other hand, these adult obese mice with a premature immunosenescence reach old age with a higher deterioration of immune response than those adults showing an appropriate immune response. The present study found, on one hand, that the development of obesity during adolescence promoted a premature immunosenescence and oxidative state of leukocytes in adulthood. Thus, the adult obese mice, when compared with the controls of the same age, presented several deteriorated immune parameters, such as chemotaxis of both macrophages and lymphocytes, phagocytosis of macrophages, NK cell activity, mitogen-stimulated lymphoproliferation, and mitogen-stimulated release of several cytokines (IL-1β, TNF-α, IL-6, IL-2, and IL-10) derived from leukocytes culture supernatants. These parameters showed values similar to those in chronologically old animals. On the other hand, these adult obese mice with a premature immunosenescence reached old age in worsened conditions, although they were fed a standard diet during aging. The parameters of immune function and redox state of peritoneal leukocytes analyzed in the present study have been established as excellent markers of health and rate of aging. Moreover, it has been shown that the age-related changes in these parameters of peritoneal immune cells are similar to those in human blood leukocytes.

With respect to the functions studied in the peritoneal macrophages, which represent the first line of immune response, adult period, could also produce some features of immunosenescence at adult age. Moreover, it is also important to know if individuals that start their aging process at adult age with premature immunosenescence reach old age with a higher deterioration of immune response than those adults showing an appropriate immune response. The present study found, on one hand, that the development of obesity during adolescence promoted a premature immunosenescence and oxidative state of leukocytes in adulthood. Thus, the adult obese mice, when compared with the controls of the same age, presented several deteriorated immune parameters, such as chemotaxis of both macrophages and lymphocytes, phagocytosis of macrophages, NK cell activity, mitogen-stimulated lymphoproliferation, and mitogen-stimulated release of several cytokines (IL-1β, TNF-α, IL-6, IL-2, and IL-10) derived from leukocytes culture supernatants. These parameters showed values similar to those in chronologically old animals. On the other hand, these adult obese mice with a premature immunosenescence reached old age in worsened conditions, although they were fed a standard diet during aging. The parameters of immune function and redox state of peritoneal leukocytes analyzed in the present study have been established as excellent markers of health and rate of aging. Moreover, it has been shown that the age-related changes in these parameters of peritoneal immune cells are similar to those in human blood leukocytes.

Discussion

The present work shows the negative effects on the immune system of old mice as adults, were obese due to the ingestion of a high-fat diet during adolescence.

Obesity was induced in adolescent mice by the administration of a high-fat diet for 14 weeks. These animals progressively and significantly increased their body weights and also presented significantly elevated levels of triglycerides and systolic arterial pressure when compared with the control group. Moreover, these mice showed conditions associated with obesity similar to those obtained in mice of a previous study in which an identical experimental design was followed to generate adult obesity. Thus, the administration of a high-fat diet during adolescence seems to be a good model to induce obesity in adult mice. Additionally, this type of diet and the increased adiposity have also been reported to enhance the levels of oxidation and inflammation and to promote a dysfunctional immune response. Because obesity has been associated with a premature immunosenescence, it seems relevant to evaluate whether obesity generated at adolescence, a critical development period, could also produce some features of immunosenescence at adult age. Moreover, it is also important to know if individuals that start their aging process at adult age with premature immunosenescence reach old age with a higher deterioration of immune response than those adults showing an appropriate immune response. The present study found, on one hand, that the development of obesity during adolescence promoted a premature immunosenescence and oxidative state of leukocytes in adulthood. Thus, the adult obese mice, when compared with the controls of the same age, presented several deteriorated immune parameters, such as chemotaxis of both macrophages and lymphocytes, phagocytosis of macrophages, NK cell activity, mitogen-stimulated lymphoproliferation, and mitogen-stimulated release of several cytokines (IL-1β, TNF-α, IL-6, IL-2, and IL-10) derived from leukocytes culture supernatants. These parameters showed values similar to those in chronologically old animals. On the other hand, these adult obese mice with a premature immunosenescence reached old age in worsened conditions, although they were fed a standard diet during aging. The parameters of immune function and redox state of peritoneal leukocytes analyzed in the present study have been established as excellent markers of health and rate of aging. Moreover, it has been shown that the age-related changes in these parameters of peritoneal immune cells are similar to those in human blood leukocytes.

Table 1. Functions and Oxidative Parameters in Peritoneal Immune Cells From Old Mice. Survival of Mice.

<table>
<thead>
<tr>
<th></th>
<th>Old Control Mice</th>
<th>Old Obese Mice</th>
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<tbody>
<tr>
<td><strong>Macrophage functions</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chemotaxis index</td>
<td>601 ± 50</td>
<td>473 ± 72**</td>
</tr>
<tr>
<td>Phagocytic index</td>
<td>236 ± 59</td>
<td>144 ± 25**</td>
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<tr>
<td>Phagocytic efficacy</td>
<td>64 ± 7</td>
<td>41 ± 9***</td>
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<tr>
<td>Stimulated intracellular superoxide anion (nmol/10⁶ cells)</td>
<td>138 ± 57</td>
<td>86 ± 24</td>
</tr>
<tr>
<td><strong>Lymphocyte functions</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chemotaxis index</td>
<td>660 ± 65</td>
<td>507 ± 66***</td>
</tr>
<tr>
<td>Natural killer cell activity (% lysis)</td>
<td>50 ± 6</td>
<td>36 ± 11*</td>
</tr>
<tr>
<td>Basal lymphoproliferative (cpm)</td>
<td>1,570 ± 269</td>
<td>2,269 ± 873</td>
</tr>
<tr>
<td>Lymphoproliferative response to LPS (cpm)</td>
<td>2,795 ± 1,071</td>
<td>718 ± 241***</td>
</tr>
<tr>
<td>Lymphoproliferative response to ConA (cpm)</td>
<td>2,662 ± 1,062</td>
<td>887 ± 328***</td>
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<tr>
<td><strong>Oxidative stress parameters</strong></td>
<td></td>
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<tr>
<td>Total glutathione levels (nmol/10⁶ cells)</td>
<td>1.8 ± 0.7</td>
<td>0.6 ± 0.2***</td>
</tr>
<tr>
<td>Catalase activity (UI CAT/10⁶ cells)</td>
<td>9 ± 1.2</td>
<td>14 ± 9</td>
</tr>
<tr>
<td>Xanthine oxidase activity (mU XO/10⁶ cells)</td>
<td>2.7 ± 1.2</td>
<td>2.9 ± 0.9</td>
</tr>
<tr>
<td>Survival of mice</td>
<td>117 ± 11</td>
<td>106 ± 13</td>
</tr>
<tr>
<td>Number of weeks</td>
<td>8–10</td>
<td>8–10</td>
</tr>
</tbody>
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Each data represents the mean ± SD of 8–10 values corresponding to that number of animals and each value being the mean of duplicate or triplicate assays.

*p < .05; **p < .01; ***p < .001 with respect to the values of old control mice.

![Figure 3. Oxidative stress parameters. (A) Total glutathione levels (nmol/10⁶ cells). (B) Catalase activity (CAT, U CAT/10⁶ cells). (C) Xanthine oxidase activity (mU XO/10⁶ cells). Each column represents the mean ± SD of 8–10 values corresponding to that number of animals and each value being the mean of duplicate or triplicate assays. ***p < .001; **p < .01; *p < .05 with respect to the values of adult control mice.](https://academic.oup.com/biomedgerontology/article-abstract/71/8/983/2465575)
Obese mice showed diminished chemotaxis, phagocytosis and intracellular levels of superoxide in comparison with adult nonobese control mice. These parameters showed similar values to those in old mice. In fact, in aging individuals, an impaired migration of macrophages to the site of inflammation, a diminished phagocytosis capacity, and decreased levels of intracellular superoxide needed to kill intracellular pathogens, have been observed (12,23,28). Studies from genetic models of obesity corroborate these findings, indicating a decreased macrophage phagocytic capacity and an impaired oxidative burst in obese individuals (29,30). Morrow and colleagues (31) also reported a decreased phagocytosis by peritoneal macrophages in mice fed a high-fat diet. In addition, morbidly obese humans had a decreased bactericidal capacity by neutrophils (32). Nevertheless, another study performed in obese humans found a significantly higher phagocytosis and oxidative burst activity by monocytes and neutrophils (33). These contradictory results could be due to a different assay technique used in this study.

With respect to NK cells, also critical players of innate immunity, the data obtained in the present study showed a decreased cytotoxic capacity in old and in adult obese mice compared with that in adult controls. Previous research has indicated that aged individuals, despite their increased number of NK cells, present depressed NK cytotoxicity (12,14). Obesity also seems to produce an impaired NK cell activity. In agreement with our study, earlier findings have indicated that obese rats fed a cafeteria diet and obese mice fed a high-fat diet, as well as obese human subjects, suffer from a diminished NK cell cytotoxicity (18,27,31,34). Moreover, another study demonstrated that obese mice fed with a high-fat diet exhibited a poor cytotoxicity against influenza infection and increased mortality (35).

In addition, immune functions driven by T and B lymphocytes, such as migration and proliferation, also were affected by obesity. In fact, the present results showed that both adult obese and old mice presented similar deteriorated values in relation to peritoneal lymphocyte functions, including chemotaxis and proliferation in response to T- and B-cell mitogens such as ConA and LPS, respectively, in comparison with adult controls. There is evidence showing that aged individuals exhibit impaired chemotaxis and, especially, proliferative response to mitogens of lymphocytes (12,36). In relation to obese individuals, we did not find studies that evaluated chemotaxis of lymphocytes. Therefore, our study seems to be the first to describe a decrease in this activity in obese mice. In relation to the proliferative response of lymphocytes to mitogen stimulation, studies have indicated a reduced capacity of this parameter in obese individuals. In obese mice, Sato Mitoh and colleagues (37) found a decreased splenocyte proliferative response with T- and B-cell mitogens. Moreover, obese humans also exhibited suppressed stimulated proliferation of T and B lymphocytes (33,38). Increasing evidence suggests that the conditions of aging and obesity are accompanied by overactivation of innate immune cells with increased production of proinflammatory cytokines, such as TNF-α, IL-6, and IL-1β, especially in basal conditions (13,39-42). However, a previous study showed decreased levels of IL-1β, TNF-α, IL-6, IL-2, and IL-10 in peritoneal leukocytes from aged mice under mitogen-stimulated states (13). The data of the present study agree with those results. Thus, following stimulation with mitogens ConA and LPS, the secretion of these cytokines presented a decreased tendency or statistically significant differences in old and adult obese mice in comparison with adult controls. These findings are in agreement with those obtained in obese mice, in which peritoneal macrophages, in response to a bacterial infection, exhibited lower levels of the proinflammatory cytokines TNF-α, IL-1β, and IL-6 (42-44). A decrease in spleen cells of IL-6 under LPS-stimulated conditions (45) and of the IL-2 following mitogen stimulation (46) in obese with respect to lean human individuals have also been shown. Nevertheless, in young-adult overweight human subjects, the generation of IL-2 by mononuclear or lymphocyte cells in response to LPS increased, whereas the levels of IL-10 were similar between obese and lean individuals (47). One possible explanation for these differences could be that individuals were considered mildly obese and thus might not produce significantly impaired immunity. The data of the present study also demonstrate that the release of mitogen-stimulated IL-2 was further impaired in adult obese mice with respect to old controls. Among several factors that modulate the release of cytokines, prostaglandin E2 (PGE2) has been described to inhibit the release of IL-2 (48). Although this inflammatory mediator is increased in both processes of aging and obesity (49,50), a recent study reported that adipocytes from young obese mice displayed higher levels of PGE2 with respect to old mice (51). Therefore, it is possible that the release of IL-2 was more suppressed in adult obese mice by higher levels of PGE2 than in old controls.

Oxidative stress, which is generated by an increase of oxidants and a decrease of antioxidants, has been linked to aging and obesity (16,52,53). In fact, oxidative stress seems to contribute to the pathogenesis of several diseases that are common to both aged and obese individuals, including diabetes mellitus, cardiovascular diseases, and cancer (54). The results demonstrate that old and adult obese mice exhibited elevated XO activity (an enzyme that produces oxidants) and decreased CAT activity and glutathione levels (two relevant antioxidant defenses). Previous studies have confirmed that peritoneal leukocytes from old mice or from prematurely aging mice showed enhanced activity of XO (8,55) and a decrease of enzymatic and nonenzymatic antioxidants, such as CAT activity and glutathione levels (12,16,24). In obese individuals, an increased production of oxidants and a decreased capacity of antioxidants have also been observed. Saiki and colleagues (56) reported increased levels in the blood of serum hypoxanthine and uric acid in obese compared with those in nonobese individuals, whereas Chiney and colleagues (57) found that XO activity was significantly elevated in obese when compared with that in nonobese children. Regarding antioxidant defenses, a decrease of glutathione levels in erythrocytes of obese individuals during their childhood and adulthood was reported (58-60). In addition, CAT activity was significantly lower in erythrocytes of obese women (61). Therefore, these data indicate that obesity might produce a similar oxidative stress to those reported in the elderly, and this condition could accelerate the aging process in obese individuals.

Surprisingly, when obese animals grew older, although they were fed a standard diet during their aging process, they continued to exhibit a significant higher body weight than old controls. Biochemical parameters, such as glucose and triglycerides, as well as systolic blood pressure levels, were also significantly increased in old obese mice. In addition, these animals, which maintained obesity during their aging process, showed an increased deterioration of the immune and oxidative parameters, with respect to old controls. This aggravated immunosenescence was reflected in the life span of mice, although not statistically significant, obese mice tended to exhibit a shorter life span. The consumption of a standard diet across aging was not able to restore functions and the redox state of the immune cells to the normal levels of the corresponding chronological age.

In conclusion, the results of this study provide evidence that a high-fat intake during adolescence can produce an obesity state in
adult age associated with a premature immunosenesence, which is aggravated through aging although individuals ingest a normal diet. It is possible that intervention with lifestyle strategies, such as a caloric restriction diet or the administration of appropriate amounts of antioxidants, as well as physical exercise and environmental enrichment, among others, which are effective in improving immune function in aging (62, 63), could decrease the deterioration of obese mice. Thus, further studies are needed to corroborate how inadequate nutrition at an early age can influence the immune response in adulthood and then accelerate the aging process, as well as if some of the previously mentioned lifestyle strategies could prevent or delay these effects.

**Supplementary material**

Please visit the article online at [http://gerontology.oxfordjournals.org/](http://gerontology.oxfordjournals.org/) to view supplementary material.

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**References**


