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Differential Impact of Ovariectomy on Immune and Antioxidant Defenses in Wild- type and triple transgenic Alzheimer´s disease (3xTg-AD) mice: Protective role of voluntary exercise

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Abstract

Estrogen deprivation induced by ovariectomy—an established model of menopause in female rodents—accelerates aging processes, including immune dysfunction and alterations in redox homeostasis. In triple-transgenic Alzheimer's disease mice (3xTg-AD), which develop premature peripheral immunosenescence and an oxidative–inflammatory imbalance from an early age, the specific impact of ovariectomy on immune function and antioxidant state remains unclear. Using 9-month-old animals, we evaluated multiple leukocyte functions together with antioxidant parameters in the spleen and thymus of ovariectomized 3xTg-AD and non-transgenic (NTg) females, their sham-operated counterparts, and age-matched males. Additionally, to assess the protective potential of physical activity, groups of NTg and 3xTg-AD females underwent three months of voluntary wheel running prior to ovariectomy. The results show that ovariectomized NTg females displayed marked impairments in leukocyte functions and antioxidant capacity, reaching values comparable to those of males and sham 3xTg-AD mice. In contrast, ovariectomy in 3xTg-AD females selectively reduced glutathione concentrations without inducing further immune deterioration. Voluntary exercise improved leukocyte performance and antioxidant defenses in both 3xTg-AD and NTg ovariectomized mice, particularly in the latter. These findings indicate that estrogen loss accelerates immunosenescence in NTg females whereas its impact in 3xTg-AD mice is primarily restricted to antioxidant capacity due to their pre-existing premature immune aging. Moreover, voluntary physical activity emerges as an effective strategy to counteract both immune and redox decline associated with estrogen loss, even in the presence of Alzheimer´s disease pathology.

Key points

• Ovariectomy accelerates immunosenescence and antioxidant defense decline in non-transgenic (NTg) female mice.

• In 3xTg-AD mice, ovariectomy selectively depletes glutathione concentrations without exacerbating pre-existing immune dysfunction.

• Ovariectomy induces an immune phenotype in NTg females that resembles that of males and sham 3xTg-AD females.

• Voluntary wheel running effectively counteracts ovariectomy-induced immune decline and redox impairment in both genotypes.

Introduction

The aging process, initiated in adulthood, is characterized by a progressive loss of physiological functions, reducing the ability to adapt to changes and, subsequently, impairing homeostasis. This general homeostasis is supported by homeostatic systems such as the nervous, endocrine and immune systems, all of which exhibit age-related deterioration associated with an increases risk of morbidity and mortality [1]. Underlying this process is a state of chronic oxidative-inflammatory stress affecting all the

tissues of the organism. Furthermore, the immune system-which undergo age-dependent remodeling collectively termed immunosenescence-plays a central role in driving this “oxi-inflamm-aging” [1, 2].

Ovarian aging, a hallmark of female biological aging, is marked by a gradual decline in ovarian function that culminates in menopause. This biological process is characterized by marked reductions in circulating estrogens levels, which has widespread effects on many organs and systems, disrupting homeostatic systems. Thus, menopause is associated with a lot of dysfunctions affecting the nervous system and this has been proposed as a prodromal phase for neurodegenerative conditions, including Alzheimer’s disease (AD) [3–9]. Regarding the immune system, menopausal women show an altered immunity [6, 10], and heightened oxidative-inflammatory stress, accelerating the biological aging process [6, 10–16]. These effects stem from the well-established antioxidants and immunomodulatory properties of estrogens, which enhance both the abundance and activity of endogenous antioxidant defenses [17]. This endocrine protection confers a less oxidized basal state in females compared to males, contributing too sex differences in longevity [18, 19]. In this context, to study the systemic impact of menopause requires reliable non-human models. Mice represent one of the most widely used models for long-term ovarian estrogen deprivation, due to their similarity in the internal reproductive system with the humans [20]. However, because these animals do not undergo menopause-instead entering an anovulatory state at a mature age (10–12 months old) while maintaining basal gonadal steroid secretion [15]-ovariectomy serves as the gold-standard experimental tool to mimic human postmenopausal hormone loss [21, 22]. Ovariectomy in rodents accelerates the aging process by impairing different physiological functions or systems, such as, among others, cardiovascular, hepatic, bone, skin, as well as nervous and immune system functions [23–31]. Regarding immune function, ovariectomized rodents display signs of accelerated immunosenescence and redox imbalance, adopting immune profiles that closely resemble those of aged individuals or males, thereby offering a robust model of accelerated aging [24, 32]. This accelerated aging phenotype appears to be largely driven by elevated oxidative stress [32–33], as administration of exogenous estrogen or natural phytoestrogen can partially or completely rescue ovariectomy-induced immune dysfunction and redox homeostasis [25, 33].

Triple-transgenic for Alzheimer's disease (3xTg-AD) mice, a valuable model of this disease, since mimics both amyloid and tau AD neuropathologies in an age-dependent manner [34], has also been proposed as a model of premature immunosenescence and of altered redox state, which could be responsible for the increased morbidity and reduced lifespan of these animals [35–39]. 3xTg-AD have harboring $PS1^{M146V}$, APP^{Swe}, and tau^{P301L} transgenes, and they were first developed at the laboratory of Prof. Frank M. LaFerla by microinjection of two transgenes into single-cell embryos from homozygous $PS1^{M146V}$ knock-in mice, thus generating animals with the same genetic background [40] that progressively develop plaques and tangles. Furthermore, it remains unclear whether ovariectomy exacerbates the above described prematurely aged phenotype observed in 3xTg-AD mice. In this regard, recent studies showed that ovariectomy in young female 3xTg-AD mice aggravates amyloid-β plaque deposition in the basolateral amygdala and accelerates cholinergic fiber loss in cortex, although no worsening of dementia-like behavior was observed [41]. Other work has reported significantly exacerbated mitochondrial

dysfunction and increased oxidative stress in ovariectomized 3xTg-AD mice [42]. Nevertheless, the specific impact of ovariectomy on peripheral leukocyte functions and antioxidant defenses of these cells of immune organs in 3xTg-AD mice required further studies.

In addition, it is essential to consider effective therapeutical strategies previously demonstrated to be effective in mitigating ovariectomy-induced accelerated aging. While estrogen replacement therapy efficiently prevents or reverses many consequences of ovarian hormone loss [25, 33, 42–44], non-pharmacological interventions-such as antioxidant supplementation, environmental enrichment, and moderate physical exercise-have also demonstrated significant efficacy [45, 46]. In 3xTg-AD mice, several therapeutic interventions have been studied as alternatives to prevent or treat their age-related functional and cognitive declines, such as dietary interventions, pharmaceuticals, immunotherapy [47–49] or voluntary exercise [26]. Specifically, exercise in 3xTg-AD mice has been shown to improve bone quality [50], modulate the gut microbiota [51], enhance sensorimotor function [52], attenuated non-cognitive and cognitive disturbances [53], prevents neuronal hyperactivity [54], and induce neuroprotective mechanisms [26]. However, the extent to which physical exercise can preserve peripheral immune function and antioxidant capacity under the combined burden of Alzheimer's pathology and estrogen deprivation remains to be fully elucidated.

Considering all the above indicated, the novelty of this study lies in assessing the effect of ovariectomy on immune function parameters, and antioxidant state in both females 3xTg-AD and wild-type (non-transgenic: NTg) mice, as well as in evaluating the potential of voluntary physical exercise to modulate and restore these effects.

Material and Methods

Animals and Experimental design

A total of 64 male and female C57BL/129Sv mice, both triple transgenic Alzheimer’s disease (3xTg-AD) mice harboring $PS1^{M146V}$, APP^{Swe} and tau^{P301L} transgenes, and non-transgenic (NTg) animals were employed for this study. Genotypes were confirmed by PCR analysis of DNA obtained from tail biopsies. Forty-eight mice (8 animals/group/cage), corresponding to the following 6 groups: sham female NTg (sham FNTg), female ovariectomized NTg (FOvxNTg), male NTg (MNTg), sham female 3xTg-AD (sham FTgS), female ovariectomized 3xTg-AD (FOvxTg), and male 3xTg-AD (MTg), were housed in standard cages at the age of 6 months. Another 16 animals (8 animals of each group: FOvxNTg and FOvxTg) were housed in cages with one running wheel and were submitted to chronic voluntary physical exercise. After 3 months, when the animals were 9 months old, they were all sacrificed by decapitation. The spleen and thymus were immediately removed under sterile conditions and stored at 4ºC in Hank’s balanced saline solution until the leukocyte collection. In the case of spleen, half of each organ was frozen at -80ºC until antioxidant defense analyses were carried out.

Animals were maintained at a constant temperature (22 ± 2 ºC) in sterile conditions inside an aseptic air-negative pressure environmental cabinet (Flufrance, Cachan, France) on a 12/12 h reversed light/dark cycle (lights on at 20:00 h). The mice had access to tap water and standard Sander Mus pellets (A04 diet from Panlab L.S., Barcelona, Spain) *ad libitum*. The diet was in accordance with the American Institute of Nutrition´s recommendations for laboratory animals. All procedures were carried out with approval from the Experimental Animal Committee (PROEX 224.0/21). Animals were treated according to the guidelines of the European Community Council Directives ECC/566/2015 and ARRIVE guidelines.

Surgical procedure: ovariectomy

For the surgical procedure animals were anaesthetised with a mixture of ketamine 80 mg/kg (Ketolar 50 mg/ml^{R}, Pfizer) and xylazine 5 mg/kg (Rompun^{R}, Bayer), which was intraperitoneally injected. Ovaries were extirpated bilaterally after ligation of the oviductes. Briefly, the dorsal part of the lumbar region was shaved and then cleaned with ethanol. One small incision (1 cm) was made through the skin and the muscle wall on each side of the backbone, in the dorsal zone. The ovaries were then located and a braided silk sterile suture (Lorca Marín, Murcia, Spain) was performed around the craneal area of the uterine horns, which were sectioned thereafter, and the ovaries removed. The wound was closed in two layers, i.e.: muscle and skin using sterile sutures. Non ovariectomized mice were submitted to a sham operation. Sham animals were also anaesthetised; the skin and muscle layers were opened and the uterus and ovaries manipulated but not excised. The abdominal cavity was closed in the same way as in the ovariectomized mice. After recovery from the surgery, the mice were housed individually for some hours to allow recovery and then re-grouped in their home cages in groups of 4–5 per cage.

Isolation of leucocytes from spleen and thymus

Spleen and thymus were removed aseptically after decapitation of the animals, freed of fat, minced with scissors and divided in two identical parts. One of both was gently pressed in phosphate puffer saline (PBS) through a mesh screen (Sigma, St Louis, and USA). The cell suspensions of spleen were centrifuged in a gradient of Ficoll-Hypaque (Sigma) with a density of 1.070 g/ml, and cells from the interface were collected. Then, both spleen and thymus leucocytes were washed in PBS, and the number of leucocytes was determined and adjusted to 1×10^{6} cells/ml. Cellular viability, routinely measured before each experiment by the trypan-blue exclusion test, was higher than 95% in all experiments. All incubations were performed at 37°C in a humidified atmosphere of 5% CO_2.

Immune function parameters

The spleen and thymus immune functions analysed in this study were: chemotaxis capacity, NK activity and lymphoproliferation in response to mitogens Concanavaline A (ConA) and bacterial lipopolysaccharide (LPS). Besides, the levels of some cytokines (interleukin-2 (IL-2), interleukin-10 (IL-

10), interleukin-1 beta (IL-1 beta) and tumour necrosis factor alpha (TNF-alpha)) were also analysed in supernatants of cultures (48 h) of spleen leucocytes.

Chemotaxis capacity

The assay was carried out according to the method previously described by [56] with some modifications [24]. Chambers with two compartments separated by a filter (Millipore, Ireland) of 3 μm pore diameter were used. Aliquots of leukocyte suspensions were placed in the upper compartment, and the chemoattractant agent fMet-Leu-Phe (Sigma) was placed in the lower compartment at a concentration of 10^{-8}M. The chambers were incubated for 3 h, the filters were fixed and stained and the chemotaxis index (CI, number of lymphocytes in the lower face of the filter) was calculated by counting in an optical microscope and the total number of leukocytes in one-third of the lower face of the filters. All CI were assayed in duplicate.

Natural killer (NK) Activity assay

An enzymatic colorimetric assay was used for cytolysis measurements of target cells (Cytotox 96 TM Promega, Boerinher Ingelheim, Germany) based on the determination of the activity of the enzyme lactate dehydrogenase (LDH), using tetrazolium salts as previously described by [24]. Briefly, target cells (YAC-1 cells from a murine lymphoma) were seeded in 96-well U-bottom culture plates (Nunclon, Denmark) at a concentration of 10^4 cells/well in 1640 RPMI medium without phenol red. Effector cells (leukocytes from spleen and thymus) were added at a concentration of 10^5 cells/well, thus obtaining an effector/target rate of 10/1. Each sample was assayed in triplicate. The plates were centrifuged at 250 g for 4 min to facilitate cell to cell contacts and then they were incubated for 4 h. After incubation, plates were centrifuged again at 250 g for 4 min and LDH activity was measured in the supernatants by addition of the enzyme substrate at 490 nm. Results were expressed as percentage of lysis.

Lymphoproliferation assay

The proliferation of lymphocytes in response to the mitogens concanavalin A (ConA) and lipopolysaccharide (LPS) was assayed following a method previously described by [24]. Aliquots of 200 μl of the spleen or thymus leukocyte suspensions were seeded in ninety-six well flat-bottomed microtiter plates (Nunclon, Denmark) and incubated in the presence of ConA or LPS (1 mg/mL; Sigma-Aldrich) during 48 h, at 37ºC, in a humidified atmosphere of 5% CO_2. Each sample was assayed in triplicate. Then, 0.5 μCi ^{3}H-thymidine (ICN, Costa Mesa, USA) was added to each well. After 24 h, cells were harvested and thymidine uptake was measured in a beta counter for 1 min. Results were expressed as counts per minute [cpm] and stimulation index (percentage of proliferation in response to ConA or LPS, with 100% being the thymidine uptake cpm in control (without mitogen) wells (stimulation index)).

Cytokine levels

The levels of IL-2, IL-1β, IL-10 and TNF-α were determined in the supernatant of cultures of spleen leukocytes after 48h of incubation with mitogens ConA (1 mg/ml) or LPS (1 mg/ml). The supernatants were collected and frozen at -20ºC until assay. Cytokines were measured using different enzyme-linked immunosorbent assays ((Millipore s MILLIPLEX® MAP Mouse Cytokine/Chemokine kit) and the results were expressed as pg/mL. The assay was run in duplicate.

Antioxidant defense parameters

In the portion of each spleen that had been stored at -80 ºC, the following antioxidant defenses were analysed: total glutathione (TG) levels, glutathione peroxidase (GPx) activity and glutathione reductase (GR) activity.

Total glutathione levels.

Total glutathione (TG) was spectrophotometrically evaluated according to the method of Tietze [56] with some modifications carried out in organs [57]. Briefly, spleen samples were homogenized with 5% trichloroacetic acid (TCA, Panreac) and 0.01 N HCl (Panreac), in a cold room (4°C), maintaining the samples in ice throughout the process. Homogenates used to measure total glutathione (TG) were resuspended in a medium containing 5% trichloroacetic acid (TCA, Panreac) and 0.01 N HCl (Panreac). Thereafter, all samples were centrifuged at 3200g for 5 min at 4°C. Aliquots of supernatant were measured in the spectrophotometer using the following reaction mixture: 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB, 6 mM, Sigma), β-nicotinamide adenine dinucleotide phosphate, reduced form (β-NADPH, 0.3 mM, Sigma) and glutathione reductase (10U/ml, Sigma). The reaction was monitored for 240 s. The results were expressed as nmol TG/mg tissue.

Glutathione peroxidase assay.

The activity of glutathione peroxidase (GPx) enzyme was measured according to the original technique described by [58], with some modifications [59]. Spleen samples were homogenized with phosphate (50 mM) buffer, in a cold room (4°C), and were maintained in ice during the whole process. Thereafter, all samples were centrifuged at 3200g for 20 min at 4°C. Aliquots of the supernatants were measured in the spectrophotometer, following the decrease of the absorbance at 340 nm, every 40 s during 5 min, using cumene hydroperoxide (cumene-OOH, Sigma) as substrate, that carried out the oxidation of glutathione, which is regenerated by the addition of β-nicotinamide adenine di-nucleotide phosphate, in its reduced form (β-NADPH, Sigma), in the presence of GR (Sigma). The results were expressed as miliunits (mU) of enzymatic activity/mg tissue.

Glutathione reductase assay.

Glutathione reductase (GR) activity was spectrophotometrically assessed in spleen according to the previously mentioned technique described by [60]. Spleen tissue samples were homogenized with

phosphate (50 mM) buffer with EDTA (6.3 mM), that is always pumped to remove O_2, in a cold room (4°C). Thereafter, all samples were centrifuged at 3200g for 20 min at 4°C. Aliquots of the supernatants were measured in the spectrophotometer using the NADPH (6 mM) and GSSG (80 mM) reagents (they were prepared in dark bottles). Results were expressed as miliunits (mU) of enzymatic activity per mg tissue.

Statistical Analysis

SPSS 15.0 (SPSS, Chicago, IL, USA) was used for the statistical analysis of the results. All data are expressed as mean ± standard deviation (SD). Normality of the samples and homogeneity of variances were checked by the Kolmogorov-Smirnov test and Levene test, respectively. Differences between groups were analyzed employing the two-way ANOVA (sex and genotype as a factors). In the case of physical exercise, data were analyzed employing three-way ANOVA (sex, genotype and exercise as a factors). In all cases, Tukey test was employed as a post hoc test. The level of significance of $p < 0.05$ was considered the minimum level of significance.

Results

Effects of ovariectomy on immune function and antioxidant defense parameters from triple transgenic Alzheimer´s disease and non-transgenic mice

To investigate the potential negative impact of ovariectomy on innate immunity in triple-transgenic Alzheimer's (3xTg-AD) mice, we first evaluated several innate immune functions, as the chemotaxis index and Natural Killer (NK) activity, in spleen and thymus leukocytes from ovariectomized (Ovx) non-transgenic (NTg) and transgenic (Tg) animals. These results are summarized in Fig. 1.

Chemotaxis in the spleen of female mice showed robust effects across all factors, with significant contributions of genotype ($F(1,32) = 37.88$, $p < 0.001$), Ovx ($F(1,32) = 14.75$, $p < 0.001$), and their interaction ($F(1,32) = 22.73$, $p < 0.001$). Thymus chemotaxis in females was significantly affected by Ovx ($F(1,32) = 8.38$, $p < 0.01$), whereas genotype ($F(1,32) = 0.58$, $p = 0.452$) and their interaction ($F(1,32) = 0.86$, $p = 0.360$) were not revealed significant differences. In males, chemotaxis was significantly lower in 3xTg-AD mice in the spleen ($F(1,16) = 6.32$, $p < 0.05$), while thymus chemotaxis showed no significant genotype effect ($F(1,16) = 3.86$, $p = 0.067$). Post hoc analysis showed that female NTgOvx mice (FNTgOvx) exhibited a significant lower spleen and thymus chemotaxis ($p < 0.001$) (Fig. 1A and Fig. 1B, respectively) than sham female NTg group (sham FNTg), reaching in both organs the values observed in NTg males (MNTg). This last experimental group had also lower values of both parameters compared to shamFNtg animals ($p < 0.001$). By contrast, female TgOvx (FTgOvx) animals did not show any statistical difference compared to sham female Tg (sham FTg) mice in either immune location. For genotype differences, sham FTg animals in both immune locations had lower values for this immune parameter compared to sham FNTg groups ($p < 0.001$). Even so, in the case of spleen chemotaxis MTg animals had lower values compared to FNTg mice ($p < 0.05$) and also with respect to FTgOvx animals ($p < 0.05$). For spleen NK activity, females were not significantly affected by genotype ($F(1,32) = 2.12$, $p = 0.155$), Ovx

(F(1,32) = 3.33, p = 0.077), or their interaction (F(1,32) = 1.96, p = 0.171). In contrast, thymus NK activity showed a significant effect of Ovx (F(1,32) = 6.31, p < 0.05) and a significant genotype × Ovx interaction (F(1,32) = 6.21, p < 0.05), indicating differential Ovx-dependent modulation between NTg and 3xTg-AD females. In male mice, NK activity was significantly reduced in 3xTg-AD animals compared with NTg controls in both spleen (F(1,16) = 7.93, p < 0.05) and thymus (F(1,16) = 6.61, p < 0.05). Post hoc analyses indicated that while FNTgOvx mice had a lower percentage (%) of lysis in spleen and thymus compared to sham FNTg group (p < 0.001 and p < 0.01, respectively) reaching a similar value to those observed in MNTg animals, FTgOxv did not show any statistical difference with respect to sham FTg animals. Also, MNTg animals had lower NK activities compared to sham FNTg mice (p < 0.001 and p < 0.01, respectively). For genotype differences, sham FTg had lower values in both immune locations for this parameter compared to their corresponding controls (sham FNTg animals) (p < 0.001 and p < 0.01, respectively). In addition, MTg mice in the case of thymus presented lower percentages of lysis compared to FTgOvx mice (p < 0.05) (Fig. 1C and 1D).

Later, we sought to determine whether these alterations extended to the adaptive immune response. To address this, we evaluated the lymphoproliferative activity of spleen and thymus leukocytes following stimulation with Con A and LPS (Fig. 2, and Table 1). Lymphoproliferation assays revealed marked differences across genotypes, and Ovx factors. In fact, the two-way ANOVA showed significant main effects of genotype (F(1,32) = 15.42, p < 0.001) and Ovx (F(1,32) = 28.67, p < 0.001), together with a significant genotype and Ovx interaction (F(1,32) = 19.53, p < 0.001), indicating that ovariectomy markedly reduced the proliferative response in FNTg animals. LPS-induced proliferation followed a similar pattern, showing significant effects of genotype (F(1,32) = 6.84, p < 0.05), Ovx (F(1,32) = 10.92, p < 0.01), and a significant interaction between both factors (F(1,32) = 7.41, p < 0.05). When expressed as percentage (%) increase over basal levels, ConA–induced proliferation showed significant effects of genotype (F(1,32) = 12.77, p < 0.01), Ovx (F(1,32) = 16.54, p < 0.001), and a significant interaction among factors (F(1,32) = 9.88, p < 0.01). LPS-induced percentage proliferation was similarly affected, with significant effects of genotype (F(1,32) = 5.92, p < 0.05), Ovx (F(1,32) = 14.11, p < 0.001), and their interaction (F(1,32) = 6.44, p < 0.05). In males, one-way ANOVA indicated strong genotype-dependent reductions in lymphoproliferation. MTg mice showed significantly lower ConA–stimulated proliferation compared with MNTg (F(1,16) = 82.14, p < 0.001), as well as reduced LPS-induced proliferation (F(1,16) = 34.29, p < 0.001). Percentage proliferation also differed significantly between genotypes, with MTg showing reduced responses to both ConA (F(1,16) = 51.63, p < 0.001) and LPS (F(1,16) = 22.47, p < 0.001). In fact, post hoc analyses revealed that spleen and thymus leukocytes from FNTgOvx had lower c.p.m. and percentage of stimulation in response to ConA and LPS than sham FNTg (p < 0.001), although MNTg group only in the case of spleen had higher values of c.p.m. and percentages of this immune function compared to FNTgOvx (p < 0.001), and in the case of percentages compared to sham FNTg animals (p < 0.001) and also FNTOvx (p < 0.001) groups. However, in the case of FTgOvx animals, this immune function both spleen and thymus were like those observed in sham FTg exhibiting similar values to those observed in MTg (Fig. 2A, 2B, 2C, 2D and Table 1). Only, in the case of spleen lymphoproliferative response to LPS MTg animals had lower values of this parameter compared to

FOvxTg mice ($p < 0.05$) (Fig. 2B). In the case of genotype differences, shamFTg animals both spleen and thymus lymphoproliferation in response to ConA and LPS had lower values (c.p.m. and percentages) compared to their corresponding sham FNTg group ($p < 0.001$). For MTg animals, only in the case of lymphoproliferative response for spleen to ConA and LPS (both c.p.m. and percentages) a decrease was observed when data were compared to their corresponding NT group ($p < 0.001$).

Table 1

Effects of ovariectomy (Ovx) on several acquired immune functions evaluated in spleen and thymus leukocytes from female (F) and male (M) non-transgenic (NTg) and triple transgenic Alzheimer Disease (3xTg-AD) mice.

Lymphoproliferative responses	**ShamFNTg**	**FOvxNTg**	**MNTg**	**ShamFTg**	**FOvxTg**	**MTg**
Spleen leukocytes						
Concanavaline A (%)	474 ± 98	218 ± 55***	804 ± 221*** ###	178 ± 56•••	226 ± 66	246 ± 71•••
LPS (%)	341 ± 82	174 ± 57***	676 ± 243*** ###	268 ± 61•••	194 ± 65	194 ± 76•••
Thymus leukocytes						
Concanavaline A (%)	333 ± 54	153 ± 19***	204 ± 80	111 ± 51•••	148 ± 17	161 ± 44
LPS (%)	211 ± 55	125 ± 25***	180 ± 62	129 ± 54•••	127 ± 37	157 ± 42

Each value is the mean ± SD of eight values corresponding to eight animals, each value being the mean of duplicate assays. Female controls were exposed to the same surgery (sham) than ovariectomized mice. * $p < 0.05$, ** $p < 0.01$, ***$p < 0.001$ with respect to sham FNTg mice. • $p < 0.05$, •• $p < 0.01$, ••• $p < 0.001$ with respect to the values obtained in NTg groups; ### $p < 0.001$ with respect to FOvxNTg mice.

Given the alterations observed in the lymphoproliferative response to mitogenic stimulation, we next investigated whether these functional changes were accompanied by shifts in cytokine secretion. Accordingly, we quantified the production of key pro-inflammatory cytokines (TNF-α and IL-1β), the anti-inflammatory cytokine IL-10, and the regulatory cytokine IL-2 following stimulation with either ConA or LPS (Fig. 3 and Fig. 4). Two-way ANOVA in females revealed significant effects on several cytokines released in spleen leukocytes after ConA stimulation. For IL-2, there were significant main effects of genotype ($F (1,32) = 7.12$, $p < 0.05$) and Ovx ($F (1,32) = 10.45$, $p < 0.01$), together with a significant genotype and Ovx interaction ($F (1,32) = 6.38$, $p < 0.05$), indicating that Ovx markedly reduced IL-2 production in NTg females. Similarly, IL-10 release also showed significant effects of genotype ($F (1,32) = 18.27$, $p < 0.001$) and Ovx ($F (1,32) = 9.51$, $p < 0.01$), with a significant interaction between both factors ($F (1,32) = 7.94$, $p < 0.01$), reflecting the pronounced reduction in FOvxNTg and FTgOvx groups. However,

for IL-1beta, the ANOVA revealed only a significant effect of genotype ($F(1,32) = 5.21$, $p < 0.05$), while Ovx ($F(1,32) = 2.14$, $p = 0.153$) and the interaction ($F(1,32) = 1.87$, $p = 0.181$) were not statistically significant. In agreement, TNF-alpha release showed a significant genotype ($F(1,32) = 4.96$, $p < 0.05$), whereas Ovx ($F(1,32) = 2.01$, $p = 0.166$) and the interaction ($F(1,32) = 2.27$, $p = 0.142$) did not reach significance. In males, one-way ANOVA revealed clear genotype-dependent differences. MTg mice showed significantly reduced IL-2 levels compared with MNTg animals ($F(1,16) = 9.84$, $p < 0.01$), while IL-10 also differed significantly between genotypes ($F(1,16) = 12.41$, $p < 0.01$). IL-1beta release showed a modest but significant reduction in MTg ($F(1,16) = 5.67$, $p < 0.05$), whereas TNF-alpha levels were significantly higher in MTg compared with MNTg group ($F(1,16) = 6.92$, $p < 0.05$). Regards to post hoc analyses related to the amounts of cytokines evaluated in response to ConA and specifically for IL-2 (Fig. 3A), FNTgOvx group had low amounts of this cytokine compared to sham FNTg group ($p < 0.01$). This low amount found in FNTgOvx was like those observed in MNTg animals ($p < 0.05$) In the case of genotype differences, sham FTg and FOvxTg groups did not show any statistical difference. However, MTg mice had lower amounts of this cytokine compared to MNTg group ($p < 0.05$). In addition, this experimental group exhibited lower amounts of IL-2 compared to shamFTg and FOvxTg ($p < 0.05$). For IL-1beta, and TNF-alpha (Fig. 3B and 3C), only MNTg animals had lower amounts of both cytokines compared to sham FNTg mice ($p < 0.05$), although in the case of TNF-alpha this experimental group (MNTg) had lower amounts of this cytokine compared to FOvxNTg ($p < 0.05$). For genotype differences, while in the case of IL-1beta only FOvxTg had higher values of this cytokine compared to FOVxNTg group ($p < 0.05$), for TNF-alpha shamFTg, FOvxTg and, also MTg exhibited this increase ($p < 0.05$). Indeed, MTg animals had lower amounts of IL-1beta compared to sham FTg mice ($p < 0.05$). Finally, in the case of IL-10 (Fig. 3D), MNTg animals had lower amounts of this cytokine compared to shamNTg and FOvxNTg mice ($p < 0.001$). In the case of transgenic animals, males (MTg) had lower amounts of IL-10 compared to sham FTg and FOvxTg groups ($p < 0.005$).

Regarding the cytokines released to supernatants of spleen leukocytes in response to LPS (Fig. 4), two-way ANOVA in females revealed significant effects on several cytokines following LPS stimulation. IL-2 levels showed significant main effects of genotype ($F(1,32) = 11.63$, $p < 0.01$) and Ovx ($F(1,32) = 9.27$, $p < 0.01$), with a significant interaction between both factors ($F(1,32) = 7.88$, $p < 0.01$), indicating that ovariectomy exacerbated the reduction in IL-2, particularly in 3xTg-AD females. IL-10 release was significantly affected by genotype ($F(1,32) = 13.42$, $p < 0.001$) and Ovx ($F(1,32) = 10.76$, $p < 0.01$), with a significant interaction ($F(1,32) = 6.97$, $p < 0.05$), reflecting the pronounced decline in FTgOvx and FNTgOvx groups. IL-1beta levels showed significant effects of genotype ($F(1,32) = 9.85$, $p < 0.01$), Ovx ($F(1,32) = 5.94$, $p = 0.05$), and a significant interaction ($F(1,32) = 6.41$, $p = 0.05$). TNF-alpha release was significantly influenced by genotype ($F(1,32) = 7.23$, $p < 0.05$) and Ovx ($F(1,32) = 4.88$, $p < 0.05$), with a significant interaction ($F(1,32) = 5.67$, $p < 0.05$), indicating that ovariectomy amplified TNF-alpha production in 3xTg-AD females. In males, one-way ANOVA also revealed genotype-dependent differences in cytokine release. MTg mice showed significantly reduced IL-2 levels compared with MNTg ($F(1,16) = 10.94$, $p < 0.01$), while IL-10 levels were modestly elevated in MTg but did not reach statistical significance ($F(1,16) = 2.91$, $p = 0.106$). IL-1beta release was significantly higher in MTg ($F(1,16) = 13.27$,

$p < 0.01$), and TNF-alpha levels were comparable between genotypes ($F(1,16) = 1.84$, $p = 0.192$). Regarding post hoc analyses, while no differences were observed in FNTgOvx and MNTg groups for IL-2 concentrations (Fig. 4A), the amounts of IL-1beta, TNF-alpha and IL-10 in FNTgOvx were lower than sham FNTg group ($p < 0.05$, $p < 0.05$ and $p < 0.01$, respectively) (Fig. 4B, 4C and 4D, respectively), reaching similar values to those observed in MNTg for IL-1beta and IL-10. Nonetheless, the levels of TNF-alpha in MNTg mice were higher than FNTgOvx group ($p < 0.05$). In transgenic animals, the IL-2 concentrations of all groups (shamFTg, FOvxTg and MTg) were lower compared to their respective controls ($p < 0.001$, $p < 0.01$ and $p < 0.001$, respectively). Also, MTg mice had lower amounts of this cytokine compared to sham NTg mice ($p < 0.05$). This last difference was also observed for IL-1 beta ($p < 0.01$). In the case of TNF-alpha, only FOvxTg mice had higher levels compared to FOvxNTg group ($p < 0.01$).

Finally, in the case of antioxidant defenses evaluated in spleen (Fig. 5), the two-way ANOVA revealed that for total glutathione (TG) levels a significant main effect of genotype ($F(1,32) = 22.41$, $p < 0.001$) and Ovx ($F(1,32) = 31.56$, $p < 0.001$), together with a significant interaction between both factors ($F(1,32) = 18.92$, $p < 0.001$) were obtained, indicating that ovariectomy markedly reduced TG concentrations, particularly in NTg females. Similarly, GPx activity was significantly affected by genotype ($F(1,32) = 6.74$, $p < 0.05$) and Ovx ($F(1,32) = 5.89$, $p < 0.05$), although the interaction did not reach significance ($F(1,32) = 2.41$, $p = 0.130$). In addition, GR activity showed significant effects of genotype ($F(1,32) = 7.92$, $p < 0.01$) and Ovx ($F(1,32) = 9.14$, $p < 0.01$), with significant interaction ($F(1,32) = 6.11$, $p < 0.05$), reflecting the pronounced decrease of this activity in FOvxNTg and FOvxTg groups. In males, MTg mice exhibited significantly lower TG concentrations compared with MNTg ($F(1,16) = 12.63$, $p < 0.01$), while GPx activity showed a modest but significant decrease in MTg ($F(1,16) = 5.48$, $p < 0.05$). GR activity also differed between genotypes ($F(1,16) = 4.96$, $p < 0.05$), with MTg showing lower enzymatic activity. Post hoc analyses revealed that FNTgOvx group had lower TG amounts (Fig. 5A) in relation to values observed in sham FNTg animals ($p < 0.001$), although this value did not reach those values obtained in MNTg group. In fact, MNTg animals had higher values of TG compared to FOvxNTg mice ($p < 0.001$). This same trend was found in FTgOvx animals, experimental group that exhibited lower amounts of TG compared to sham FTg mice ($p < 0.001$). MTg had lower values of TG amounts compared to sham FTg mice ($p < 0.05$) and higher values compared to FOvxTg mice ($p < 0.001$). In the case of GPx activity and GR activity, only FOvxNTg mice had lowers GR activities compared to sham FNTg ($p < 0.05$) (Fig. 5B and 5C).

Altogether, ovariectomy exerted a clear detrimental impact on spleen and thymus immune function in non-transgenic females, in several cases producing responses that resembled those observed in non-transgenic males. In contrast, this effect was not evident in transgenic females, which already displayed a broad impairment across the immune parameters analyzed.

Protective effects of exercise on immune function and antioxidant defenses in ovariectomized non-transgenic and transgenic mice

Previous studies have highlighted the potential beneficial effects of regular voluntary exercise on immune function and antioxidant defenses in leukocytes [61–62]. Given the broad immune dysfunction induced by ovariectomy previously indicated, we next examined whether this environmental intervention might protect to the negative effects of ovariectomy. These results related to the effects of regular voluntary exercise are illustrated in Tables 2 and 3.

Table 2
Effects of voluntary exercise (E) on several acquired immune functions and antioxidant defenses evaluated in spleen leukocytes from female (F) ovariectomized (Ovx) and male (M) both non-transgenic (NTg) and triple transgenic Alzheimer Disease (3xTg-AD) mice

Immune responses	**ShamFNTg**	**FOvxNTg**	**FOvxNTgE**	**ShamFTg**	**FOvxTg**	**FOvxTgE**
Chemotaxis Index (C.I.)	1368 ± 284	791 ± 105***	975 ± 183†††	618 ± 123•••	688 ± 153	942 ± 145††
Natural Killer activity (%)	49 ± 9	25 ± 7***	34 ± 7††	14 ± 6•••	20 ± 10	22 ± 9
Lymphoproliferation						
Concanavaline A (c.p.m.)	2799 ± 431	892 ± 110***	1099 ± 232††	921 ± 203•••	881 ± 342	1022 ± 232
Concanavaline A (%)	415 ± 136	208 ± 86**	345 ± 67††	206 ± 62•	202 ± 81	284 ± 54
LPS (c.p.m)	1742 ± 321	732 ± 220***	969 ± 229	815 ± 275•••	765 ± 286	935 ± 238†
LPS (%)	321 ± 42	174 ± 57***	261 ± 51††	144 ± 23••	194 ± 65	238 ± 43†
Total glutathione (nmol/10^6 cells)	0.69 ± 0.07	0.09 ± 0.02***	0.31 ± 0.06†††	0.45 ± 0.04•	0.07 ± 0.01***	0.27 ± 0.08†††
Glutathione peroxidase activity (mU GPx/10^6 cells)	161 ± 24	133 ± 22	166 ± 52	141 ± 30•••	129 ± 27•••	139 ± 45
Glutathione reductase activity (mU GR/10^6 cells)	42 ± 9	27 ± 7 *	38 ± 12†	33 ± 6	25 ± 7	35 ± 7†

Each value is the mean ± SD of eight values corresponding to eight animals, each value being the mean of duplicate assays. Female controls were exposed to the same surgery (sham) than ovariectomized mice. * $p < 0.05$, ** $p < 0.01$, ***$p < 0.001$ with respect to sham FNTg mice. • $p < 0.05$, •• $p < 0.01$, ••• $p < 0.001$ with respect to the values obtained in NTg groups; † $p < 0.05$, †† $p < 0.01$, ††† $p < 0.001$ with respect to the values obtained in animals with the same genetic condition and ovariectomized, but not performing exercise.

Table 3
Effects of voluntary exercise (E) on several acquired immune functions evaluated in thymus leukocytes from female (F) ovariectomized (Ovx) and male (M) both non-transgenic (NTg) and triple transgenic Alzheimer Disease (3xTg-AD) mice.

Immune responses	ShamFNTg	FOvxNTg	FOvxNTgE	ShamFTg	FOvxTg	FOvxTgE
Chemotaxis Index (C.I.)	1789 ± 298	700 ± 180***	969 ± 213†	704 ± 217•••	643 ± 198	820 ± 281
Natural Killer activity (%)	37 ± 6	19 ± 5**	26 ± 5†	17 ± 7••	20 ± 5	22 ± 8
Lymphoproliferation						
Concanavaline A (c.p.m.)	1228 ± 341	598 ± 98***	838 ± 155†††	461 ± 123•••	579 ± 81	757 ± 109†††
Concanavaline A (%)	301 ± 42	153 ± 19***	210 ± 20†	123 ± 25•••	148 ± 16	193 ± 7††
LPS (c.p.m)	932 ± 303	483 ± 124***	689 ± 127††	442 ± 129•••	496 ± 141	620 ± 70†
LPS (%)	288 ± 58	125 ± 25***	173 ± 26†	102 ± 32••	127 ± 37	160 ± 22†

Each value is the mean ± SD of eight values corresponding to eight animals, each value being the mean of duplicate assays. Female controls were exposed to the same surgery (sham) than ovariectomized mice. ** $p < 0.01$, ***$p < 0.001$ with respect to sham FNTg mice. •• $p < 0.01$, ••• $p < 0.001$ with respect to the values obtained in NTg groups; † $p < 0.05$, †† $p < 0.01$, ††† $p < 0.001$ with respect to the values obtained in animals with the same genetic condition and ovariectomized, but not performing exercise.

For spleen parameters (Table 2), exercise exerted a consistent modulatory influence that emerged as a significant main effect in the three-way ANOVA. For chemotaxis, exercise significantly increased the chemotactic index ($F(1,42) = 6.8$, $p < 0.05$), partially counteracting the Ovx-induced decline, particularly in NTg animals. A similar pattern was observed for NK cell activity, where exercise showed a trend toward improvement ($F(1,42) = 3.9$, $p = 0.055$), with the Ovx and exercise interaction reaching significance ($p < 0.05$), indicating that the restorative effect of exercise was more pronounced under ovarian hormone deprivation. Exercise also enhanced lymphoproliferative responses, increasing both ConA-induced proliferation (c.p.m.: $F(1,42) = 5.1$, $p < 0.05$; %: $F(1,42) = 4.4$, $p < 0.05$) and LPS-induced proliferation (c.p.m.: $F(1,42) = 4.9$, $p < 0.05$; %: $F(1,42) = 6.0$, $p < 0.05$). These effects were accompanied by significant Ovx and exercise interactions across several proliferation endpoints (all $p < 0.05$), again supporting a preferential benefit of exercise in Ovx mice. At the redox level, exercise significantly increased total glutathione content ($F(1,42) = 14.2$, $p < 0.001$) and contributed to partial recovery of Ovx-induced depletion, as reflected by a robust Ovx and exercise interaction ($F(1,42) = 12.3$, $p < 0.001$). Although exercise did not significantly modify GPx activity ($F(1,42) = 1.2$, $p = 0.28$), it showed a trend toward increasing GR activity ($F(1,42) = 3.4$, $p = 0.07$), suggesting a mild enhancement of glutathione recycling

capacity. Post hoc analyses show that FOvxNTgE had higher spleen chemotaxis indexes, percentages of NK, lymphoproliferative responses to ConA (both c.p.m. and percentages) and percentages of LPS compared to FOvxNTg mice ($p < 0.001$, $p < 0.01$, $p < 0.01$, $p < 0.01$, $p < 0.01$ and $p < 0.001$, respectively) (Table 2). This same trend was observed for FOvxTgE for chemotaxis index and lymphoproliferative response to LPS (both c.p.m. and percentages) ($p < 0.01$, $p < 0.05$ and $p < 0.05$, respectively). For antioxidant defenses evaluate in spleen, FOvxNTgE mice had higher GT amounts together with a higher GR activity compared to FOvxNTg group ($p < 0.001$ and $p < 0.05$, respectively). Similarly, FOvxTgE animals had higher amounts of GT and GR activities than FOvxTg mice ($p < 0.001$ and $p < 0.05$, respectively) (Table 2).

In the case of thymus parameters (Table 3), exercise significantly improved the chemotactic index ($F(1,42) = 6.1$, $p < 0.05$), partially restoring the Ovx-induced impairment in both genotypes. A similar pattern was observed for NK cell activity, where exercise increased this cytotoxic function ($F(1,42) = 4.30$, $p < 0.05$), with a significant Ovx and exercise interaction ($F(1,42) = 5.0$, $p < 0.05$). Exercise also enhanced lymphocyte proliferative capacity. ConA-induced proliferation increased significantly in exercised animals (c.p.m.: $F(1, 42) = 7.20$, $p < 0.01$; %: $F(1,42) = 5.60$, $p < 0.05$), and similar improvements were observed for LPS-induced proliferation (c.p.m.: $F(1,42) = 6.40$, $p < 0.01$; %: $F(1,42) = 5.90$, $p < 0.01$). Post hoc analyses indicated that FOvxNTgE showed higher values of all parameters studies in thymus compared to FOvxNTg (chemotaxis: $p < 0.05$; NK activity: $p < 0.05$; ConA-cpm.: $p < 0.001$; ConA %: $p < 0.05$; LPS-cpm.: $p < 0.01$ and LPS %: $p < 0.05$). However, in the case of FOvxTgE, thymus leukocytes from these animals exhibited higher values only in those immune functions related to acquired immunity compared to FOvxTg (ConA-cpm.: $p < 0.001$; ConA %: $p < 0.01$; LPS-cpm.: $p < 0.05$ and LPS %: $p < 0.05$) (Table 3).

Collectively, regular voluntary exercise helps to protect against the impairments induced by ovariectomy, although these beneficial effects are more evident in animals that do not develop Alzheimer's-like pathology (non-transgenic mice).

Discussion

The present study is the first to investigate the effects of ovariectomy on immune function and antioxidant defenses in immunocompetent organs, specifically the spleen and thymus, of 9-month-old female non-transgenic (NTg) and 3xTg-AD mice. Furthermore, we evaluated the capacity of regular voluntary exercise to protect against the impairments induced by ovariectomy. The results show that estrogen deprivation accelerates immune aging and oxidative stress in wild-type females to levels comparable to those observed in 3xTg-AD animals and male mice. Crucially, prior voluntary exercise effectively protects against this decline by enhancing leukocyte function and strengthening antioxidant defenses.

Ovariectomy markedly impaired both immune function and antioxidant defenses in female NTg mice compared with their corresponding sham-operated controls. Specifically, chemotaxis, natural killer (NK)

activity, and lymphoproliferative responses to concanavalin A (ConA) and lipopolysaccharide (LPS) in spleen and thymus leukocytes were significantly reduced. Likewise, IL-2 secretion by ConA-stimulated spleen lymphocytes - a critical cytokine for T-cell proliferation [63] - was significantly lower in ovariectomized mice than in sham animals. Given that these parameters serve as established biomarkers of biological age, aging rate, and lifespan [64], our findings confirm that ovariectomy accelerates immunosenescence. This observation aligns with previous studies in rats and distinct mouse strains demonstrating accelerating immunosenescence following estrogen deprivation [24, 25], which may also account for the reduced lifespan observed in female rodents subjected ovariectomy [65]. Because estrogens play a pivotal role enhancing immune competence [66]; their withdrawal impairs multiple immune functions [24, 25, 32, 33, 57, 67], thereby accelerating the development of immunosenescence.

Beyond immune dysfunction, adult ovariectomized NTg mice exhibited significantly lower glutathione concentrations than sham-operated controls, indicating a deterioration of intracellular antioxidant defenses. In wild-type (WT) mice, ovariectomy abolishes the endogenous protection provided by estrogens, promoting oxidative stress and a concurrent decline in antioxidant capacity, a state intimately linked to immunosenescence. Previous studies have similarly reported that ovariectomy disrupts several components of the glutathione metabolic cycle [57]. This antioxidant deficit has been associated with impaired immune responses [68], mirroring the alterations observed in leukocytes from both prematurely and chronologically aged individuals [1, 61, 62, 69]. Glutathione, the primary intracellular non-protein thiol and a master regulator of cellular redox homeostasis, acts as the principal non-enzymatic antioxidant protecting cells, tissues, and organs against oxidative injury [70]. Accordingly, its depletion heightens the susceptibility of immune cells to oxidative damage, thereby compromising their function. Supporting this link, supplementation with *N*-acetylcysteine, a thiol-donor and glutathione precursor, has been shown to restore immune parameters and redox balance in postmenopausal women [71].

Nine-month-old female 3xTg-AD mice, representing an early neuropathological stage of Alzheimer's disease, exhibit a marked premature immunosenescent phenotype accompanied by chronic oxidative stress and accelerated biological aging [72]. The present study corroborates these characteristics, as sham-operated female 3xTg mice displayed significantly impaired immune function comparing with their sham-operated NTg counterparts. Indeed, most leukocyte functional parameters in sham 3xTg mice were comparable to those observed in ovariectomized NTg mice, suggesting that transgenic animals already exhibit an immune profile resembling that induced by estrogen deprivation in healthy females. These findings align with previous reports consistently demonstrating immune dysfunction in leukocytes from various lymphoid compartments in 3xTg-AD mice [37, 72, 73]. Notably, ovariectomy did not further exacerbate the immune impairment in 3xTg females, as no significant differences were detected in any of the immune parameters evaluated compared with their sham-operated counterparts. A plausible explanation for this apparent lack of additive effect is that the immune function in 3xTg females is already profoundly compromised prior to ovariectomy, reaching a functional threshold that limits further measurable deterioration-consistent with a ceiling effect. Thus, the severe baseline immunological impairment driven by Alzheimer's pathology likely masks the additional impact of estrogen withdrawal.

In contrast to the immune parameters, glutathione concentrations were significantly decreased in ovariectomized 3xTg mice compared with their sham-operated counterparts. This is an interesting result as it suggests that immune dysfunction and antioxidant decline do not necessarily progress in parallel, especially during the early stages of Alzheimer's disease progression. In fact, this finding indicates that antioxidant defenses may retain a greater dynamic range for deterioration even after immune competence has reached a functional plateau. Female 3xTg-AD mice exhibit a characteristic basal phenotype of persistent peripheral oxidative stress from early life [37, 74], and growing evidence indicates that disruption of antioxidant defenses is an early pathogenic event that precedes overt neuronal degeneration [75, 76]. A plausible explanation for the selective depletion of glutathione in ovariectomized 3xTg mice is the convergence of two complementary mechanisms. First, the chronically elevated production of oxidant compounds in 3xTg-AD mice together with the mitochondrial dysregulation, previously described in neurons from Alzheimer´s mouse models [75, 77] and particularly in immune cells [37, 39], increasing the consumption of glutathione as a major intracellular antioxidant [78]. Second, glutathione synthesis depends on both estrogenic signaling and mitochondrial integrity [79]. Consequently, estrogen deprivation induced by ovariectomy compromises the *de novo* synthesis and regeneration of glutathione, while further disrupting the utilization of available antioxidants and reducing the cells´ capacity to maintain redox homeostasis [80]. Taken together, these findings support the hypothesis that glutathione depletion results from the combined effects of increased utilization and impaired biosynthesis. From this perspective, the present study suggests that, in ovariectomized female 3xTg-AD mice, whereas immune function appears to have reached a state of maximal impairment, intracellular glutathione levels can still decline further. Specifically, although ovariectomy exacerbated oxidative stress, it did not further impair immune function, suggesting that immune dysfunction had already reached a plateau or an immunosenescent phenotype in these mice. Thus, the coexistence of Alzheimer's disease pathology, which enhances glutathione consumption, and estrogen deficiency, which limits glutathione synthesis, ultimately leads to a selective depletion of this essential antioxidant. Because glutathione depletion has been closely associated with impaired immune cell function and disease progression in neurodegenerative disorders [76], it is conceivable that a longer period of estrogen deprivation following ovariectomy could eventually trigger a further decline in immune function in 3xTg mice. Future longitudinal studies will be required to determine whether the marked reduction in glutathione observed here precedes a subsequent worsening of immune competence.

Expanding upon these estrogen-dependent mechanisms, sexual dimorphism broadly influences both immune function and redox homeostasis. Indeed, it is well established that female mammals generally exhibit more robust immune responses and a more favorable redox state than males, largely due to the immunomodulatory and antioxidant properties of estrogens [18, 19]. Consistent with these observations, NTg male mice in the present study displayed lower overall immune function than their female counterparts, in agreement with previous reports from our laboratory [72]. Furthermore, male 3xTg-AD mice are known to develop immune impairment and oxidative stress earlier than females, a phenotype closely linked to increased mortality and reduced lifespan [81, 82]. Interestingly, the values of immune parameters of ovariectomized NTg females, as well as both sham-operated and ovariectomized 3xTg

females, were comparable to those observed in NTg males. These findings reinforce previous studies demonstrating that estrogen deprivation induces an immune phenotype in females that closely resembles that of males [24–25, 32, 35]. Nevertheless, despite this convergence, all female groups—including ovariectomized animals—and even NTg males generally exhibited superior immune function compared to male 3xTg mice. This confirms that Alzheimer's disease pathology imposes a heavier immunological burden than either male sex or estrogen deprivation alone. The greater immunocompetence observed in females has been widely attributed to estrogenic action, as numerous immune cell populations, including T lymphocytes, macrophages, and monocytes, express functional estrogen receptors [24, 67, 83]. Through these receptors, estrogens regulate leukocyte activation, cytokine production, proliferation, and survival. In contrast, this sex-related divergence was not observed for glutathione concentrations. Although estrogens positively regulate glutathione metabolism and overall antioxidant capacity, basal leukocyte glutathione concentrations are not necessarily sexually dimorphic, as previously reported [84]. This suggests that leukocyte glutathione homeostasis is governed primarily by cellular redox demands and biological aging rather than sex alone. Consequently, under basal physiological conditions, glutathione turnover appears to be sufficiently regulated in both sexes to maintain comparable intracellular concentrations despite differences in systemic estrogen availability.

Several lifestyle interventions have been proposed to counteract the immune decline associated with both chronological and premature immunosenescence, including dietary antioxidant supplementation and regular physical exercise [2, 62]. Among these, moderate exercise is one of the most effective non-pharmacological strategies for preserving immune competence and redox homeostasis during aging, partly by enhancing antioxidant defenses in immune cells [61, 62]. Our group previously demonstrated that voluntary wheel running improves immune function in spleen and thymus leukocytes from 9-month-old NTg and 3xTg-AD mice of both sexes, with the most pronounced benefits observed in animals exhibiting severe baseline impairment, particularly transgenic mice [72]. Building on these findings, the present study investigated whether the same intervention could counteract the detrimental effects of estrogen deprivation in ovariectomized NTg and 3xTg females. Our results demonstrate that voluntary physical exercise effectively attenuated ovariectomy-induced immune dysfunction in both genotypes. Specifically, in NTg females, exercise significantly improved the immune parameters impaired by ovariectomy, although without completely restoring them to sham-operated levels. By contrast, in 3xTg mice, voluntary exercise restored most immune parameters to values comparable to those of their sham-operated counterparts. These findings suggest that, despite the presence of Alzheimer's disease pathology, the immune system of transgenic animals retains functional plasticity and remains responsive to physiological stimulation through moderate physical activity.

Exercise also exerted marked beneficial effects on antioxidant defenses. Both NTg and 3xTg ovariectomized females exhibited significantly higher splenic glutathione concentrations and GR activity following voluntary exercise compared with their sedentary counterparts. These findings align with the well-established anti-inflammatory and antioxidant effects of regular moderate exercise [85], which enhances endogenous antioxidant defenses and improves redox homeostasis in immune cells [61]. The

mechanisms underlying these beneficial effects are likely multifactorial. Moderate exercise has been shown to promote mitochondrial function, stimulate endogenous antioxidant systems, and optimize immune cell metabolism, thereby enhancing overall leukocyte function [86–88]. In addition, exercise may partially compensate for the loss of estrogen-mediated protection by activating signaling pathways involved in antioxidant defense and immune regulation, particularly those governed by Nrf2 and other redox-sensitive transcription factors. Overall, these findings indicate that voluntary moderate physical activity is highly effective strategy for mitigating the detrimental impact of estrogen deprivation on both immune competence and antioxidant defenses. By restoring redox homeostasis and enhancing immunocompetence, exercise can partially offset the biological consequences of estrogen loss- even in the presence of Alzheimer's disease pathology. These results strongly support the implementation of regular moderate physical activity as a non-pharmacological intervention to preserve immune health and delay biological aging during menopause and in conditions characterized by accelerated aging.

Nonetheless, several limitations of the present study should be acknowledged. First, our cross-sectional design evaluated a single time point in 9-month-old mice, capturing an early neuropathological stage of Alzheimer’s disease. Consequently, the severe basal immunological impairment exhibited by 3xTg-AD leukocytes may mask an ongoing functional decline that requires extended estrogen deprivation or a more advanced stage of pathology to become apparent. Prospective longitudinal studies spanning multiple age cohorts will therefore be essential to resolve these temporal dynamics. Second, while we propose a dual-mechanism model for selective glutathione depletion-driven by heightened oxidative consumption alongside compromised glutathione biosynthesis, we did not directly evaluate the underlying upstream signaling cascades or key enzymatic machinery, such as the Nrf2 transcriptional activity or estrogen receptor signaling pathways, which should be addressed in future research. Third, our focus on peripheral immunocompetent organs (spleen and thymus) and systemic redox state precludes direct conclusions regarding central neuroinflammation, such as microglial activation or local oxidative damage within brain. Thus, the precise degree of concordance between peripheral immunosenescence and central neurodegeneration remains to be fully established. Addressing these mechanistic, methodological, and translational constraints in future investigations will be crucial to fully deciphering the complex interplay between endocrine loss, peripheral immunosenescence, and neurodegenerative progression.

In summary, our findings suggest that estrogen deprivation and Alzheimer's disease pathology do not simply exert additive effects on immune aging but rather target distinct biological components of the immunosenescence process. Specifically, while Alzheimer's disease pathology appears to drive an early, profound impairment of immune function that is not further exacerbated by ovariectomy, estrogen deprivation primarily compromises antioxidant defenses-most notably intracellular glutathione. Importantly, voluntary physical exercise emerges as a potent non-pharmacological strategy capable of partially restoring both immune function and redox balance, highlighting the remarkable functional plasticity of the immune system even in the context of neurodegenerative pathology.

Declarations

Author Contributions

Conceptualization, MDlF.; methodology, R.M., A.G., I.B.; software, A.G., R.M.; validation, R.M.., I.B., A.G.; formal analysis, R.M., A.G..; investigation, R.M., I.B., A.G..; resources, MDlF., I.B.; data curation, R.M.; writing—original draft preparation, MDlF., I.B., A.G. and L.G-L; writing—review and editing, MDlF., I.B., A.G.; supervision, MDlF.; project administration, MDlF., L.G-L.; funding acquisition, MDlF., L.G-L. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement

The animal study protocol was approved by the Experimental Animal Committee from Complutense University (PROEX 224.0/21).

Data Availability

Data will be sent upon request to the corresponding author.

Conflicts of Interest

The authors declare no conflicts of interest.

Clinical trial number

Not applicable.

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Figures

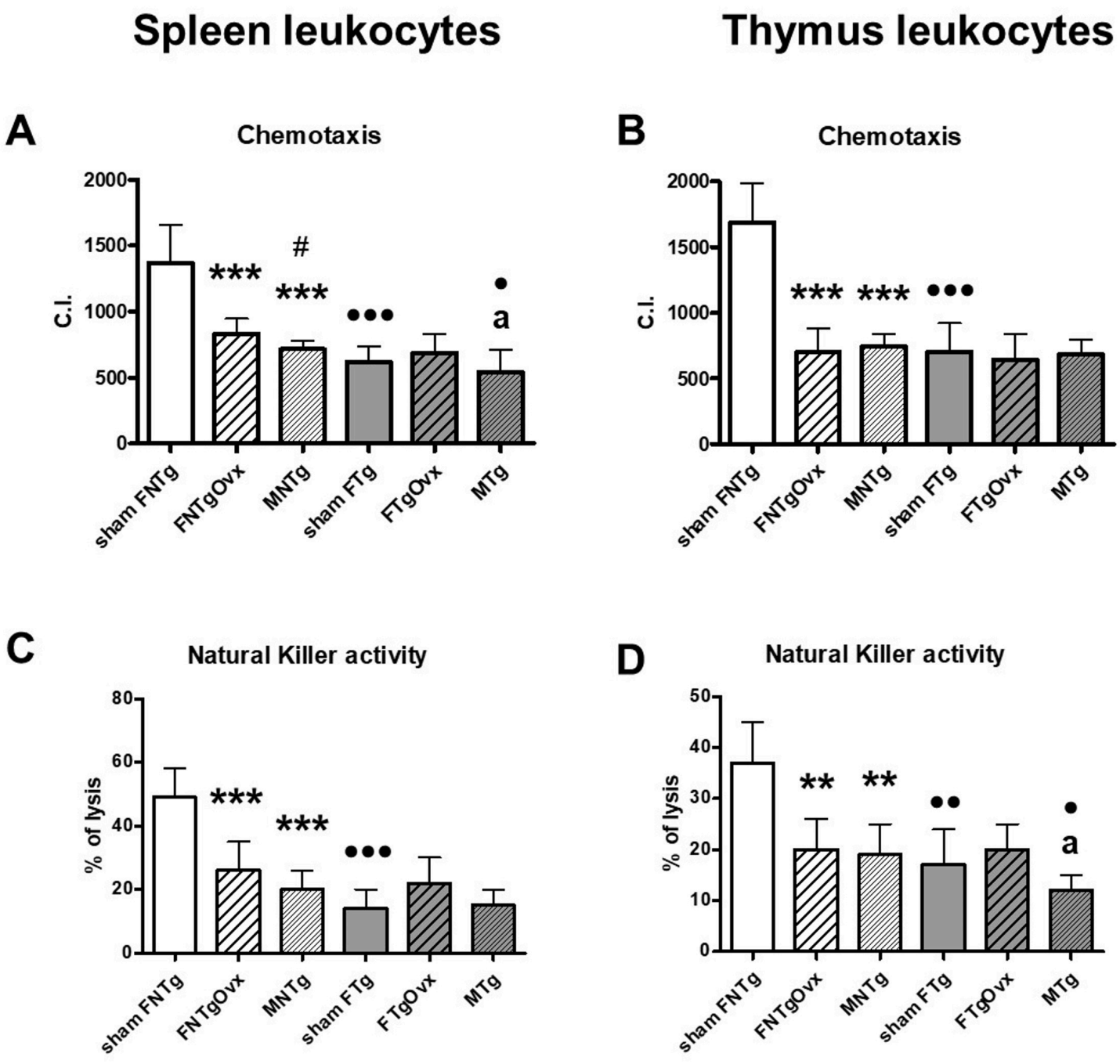


Figure 1

Effects of ovariectomy on innate spleen and thymus immune functions from 3xTg-AD and non-transgenic animals. Chemotaxis (Chemotaxis index: C.I) evaluated in spleen (**A**) and thymus (**B**) leukocytes. Natural Killer activity (% of lysis) analyzed in spleen (**C**) and thymus (**D**) leukocytes. ** $p<0.01$ and *** $p<0.001$ compared to the sham FNTg group; # $p<0.05$ compared to the FNTgOvx group; • $p<0.05$, •• $p<0.01$, and ••• $p<0.001$ compared to the same condition in non-transgenic animals; a $p<0.05$ compared to FTgOvx animals. Each bar represents the mean ± standard deviation of 8 values. FNTg: sham female non-transgenic mice; FNTgOvx: female non-transgenic ovariectomized mice; FTg: sham

female triple transgenic mice; FTgOvx: female triple transgenic ovariectomized mice; MNTg: male non-transgenic male; MTg: male triple transgenic mice.

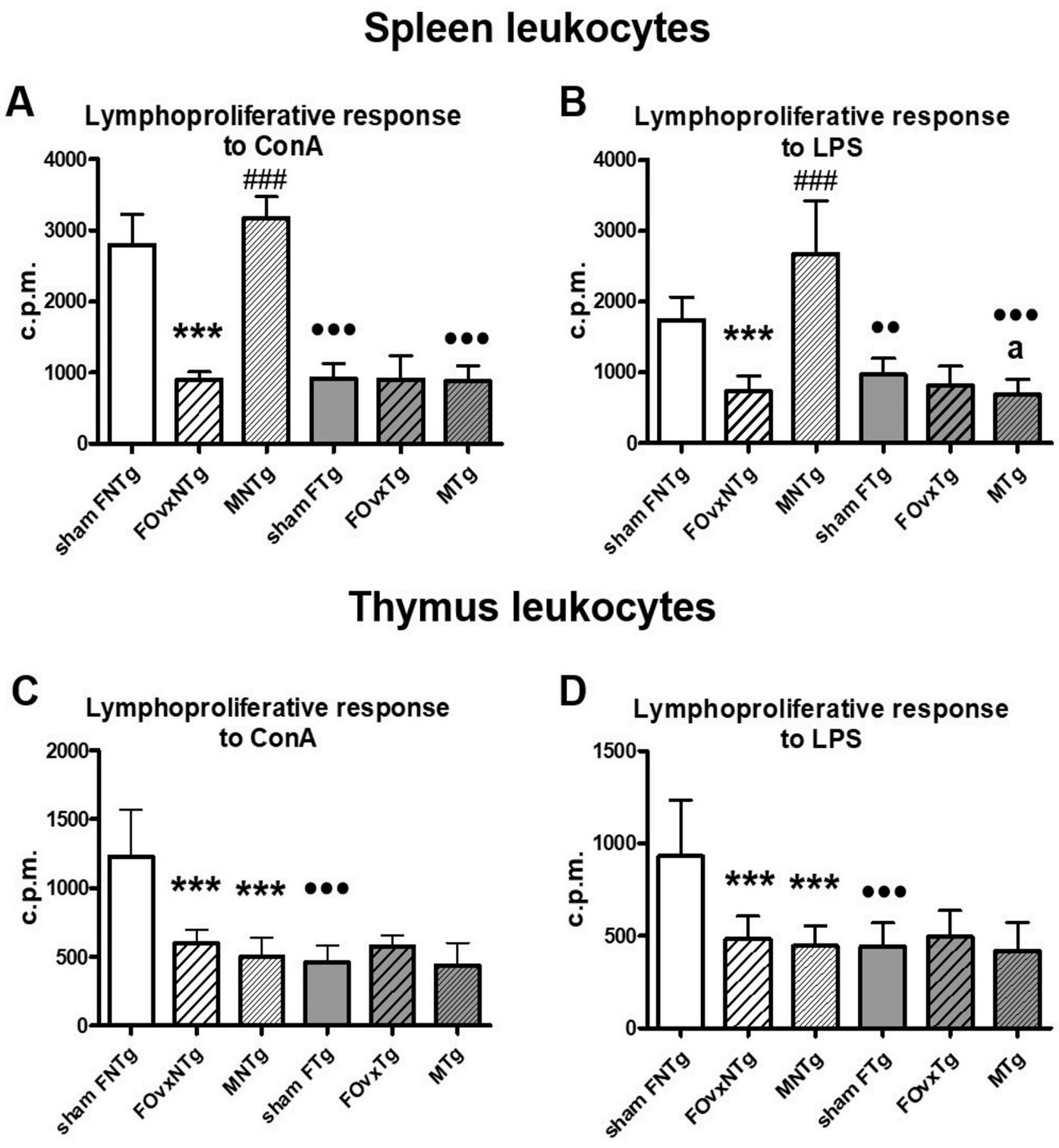


Figure 2

Effects of ovariectomy on acquired immunity in spleen and thymus leukocytes from 3xTg-AD and non-transgenic animals. Spleen lymphoproliferative response to ConA (**A**) and LPS (**B**). Thymus

lymphoproliferative response to ConA (**C**) and LPS (**D**). *** p<0.001 compared to the sham FNTg group; ### p<0.001 compared to the FNTgOvx group; •• p<0.01 and ••• p<0.001 compared to the same condition in non-transgenic animals; a p<0.05 compared to FTgOvx animals. Each bar represents the mean ± standard deviation of 8 values. FNTg: sham female non-transgenic mice; FNTgOvx: female non-transgenic ovariectomized mice; FTg: sham female triple transgenic mice; FTgOvx: female triple transgenic ovariectomized mice; MNTg: male non-transgenic male; MTg: male triple transgenic mice.

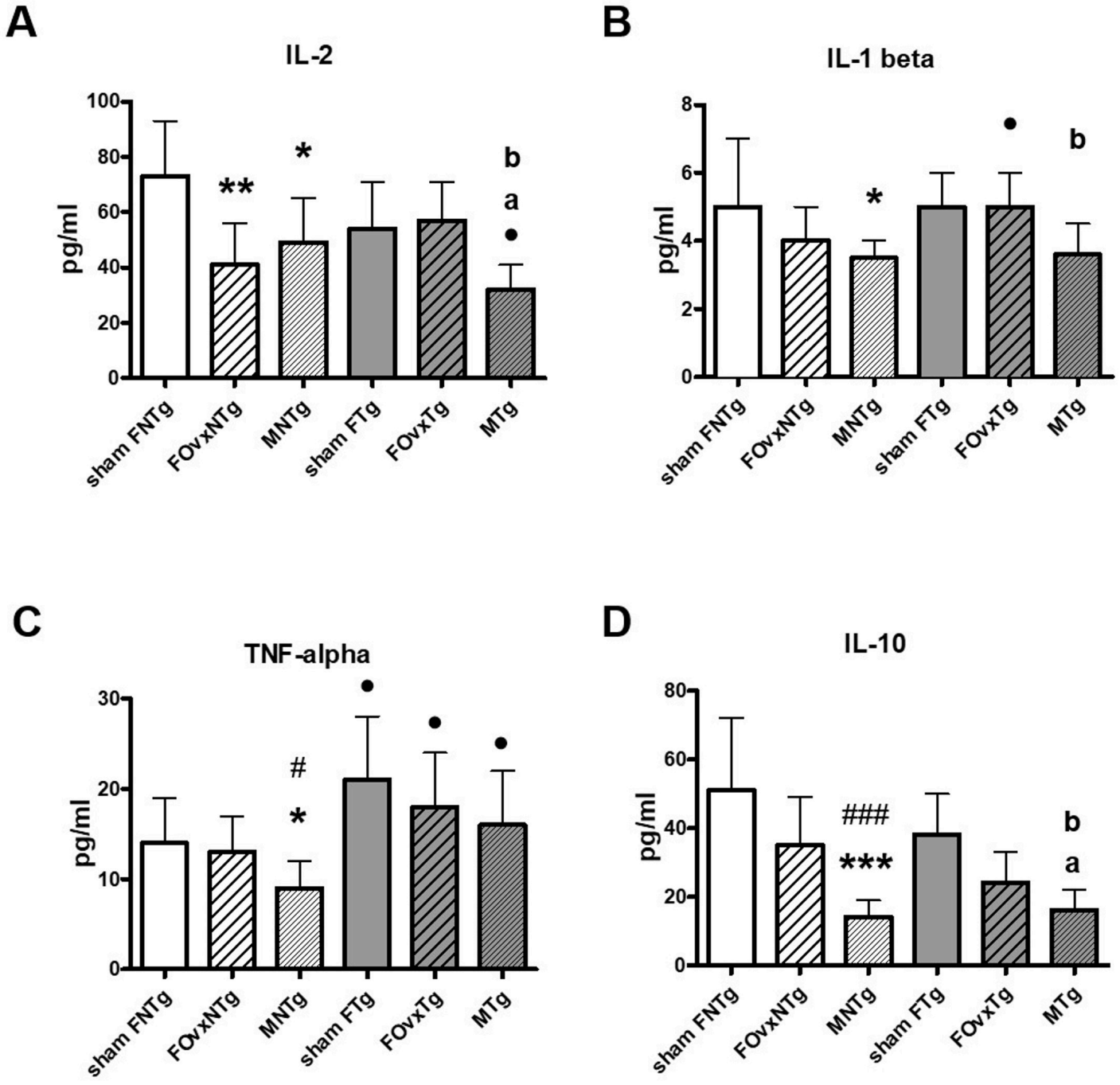


Figure 3

Effects of ovariectomy on several cytokines released in spleen leukocyte cultures from 3xTg-AD and non-transgenic animals after ConA stimulation. IL-2 (**A**), IL-1 beta (**B**), TNF-alpha (**C**) and IL-10 (D) concentrations (pg/ml). *** p<0.001 compared to the sham FNTg group; ### p<0.001 compared to the FNTOvx group; •• p<0.01 and ••• p<0.001 compared to the same condition in non-transgenic animals; a p<0.05 compared to FTgOvx animals; [b]p<0.05 compared to the sham FTg. Each bar represents the mean ± standard deviation of 8 values. FNTg: sham female non-transgenic mice; FNTgOvx: female non-transgenic ovariectomized mice; FTg: sham female triple transgenic mice; FTgOvx: female triple transgenic ovariectomized mice; MNTg: male non-transgenic male; MTg: male triple transgenic mice. ConA: Concanavalin A.

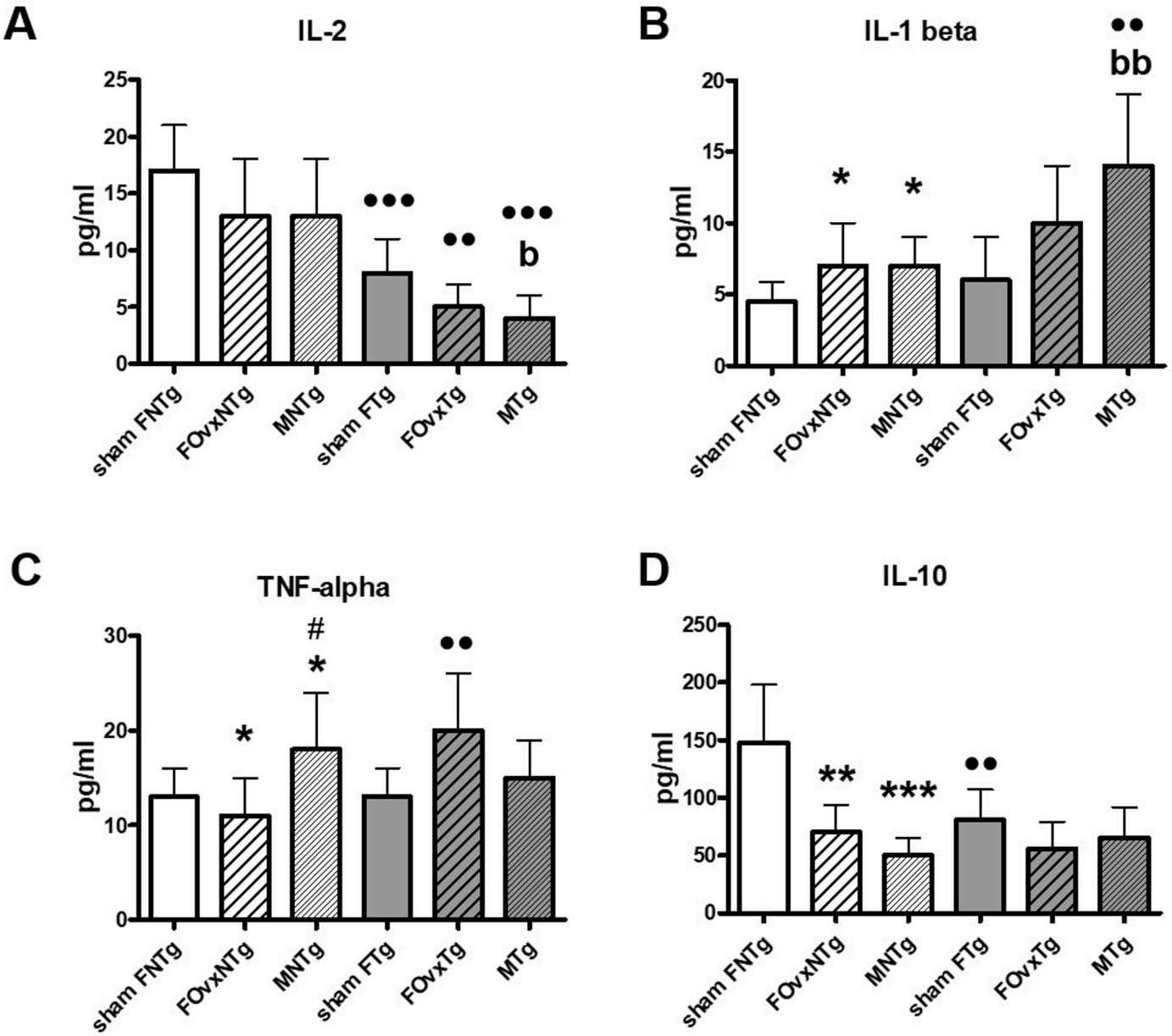


Figure 4

Effects of ovariectomy on several cytokines released in spleen leukocyte cultures from 3xTg-AD and non-trangenic animals after LPS stimulation. IL-2 (**A**), IL-1 beta (**B**), TNF-alpha (**C**) and IL-10 (**D**) concentrations (pg/ml). *** $p<0.001$ compared to the sham FNTg group; ### $p<0.001$ compared to the FNTOvx group; •• $p<0.01$ and ••• $p<0.001$ compared to the same condition in non-transgenic animals; a $p<0.05$ compared to FTgOvx animals; $^{b}p<0.05$ compared to the sham FTg group. Each bar represents the mean ± standard deviation of 8 values. FNTg: sham female non-transgenic mice; FNTgOvx: female non-transgenic ovariectomized mice; FTg: sham female triple transgenic mice; FTgOvx: female triple transgenic ovariectomized mice; MNTg: male non-transgenic male; MTg: male triple transgenic mice. LPS: Lipopolysaccharide

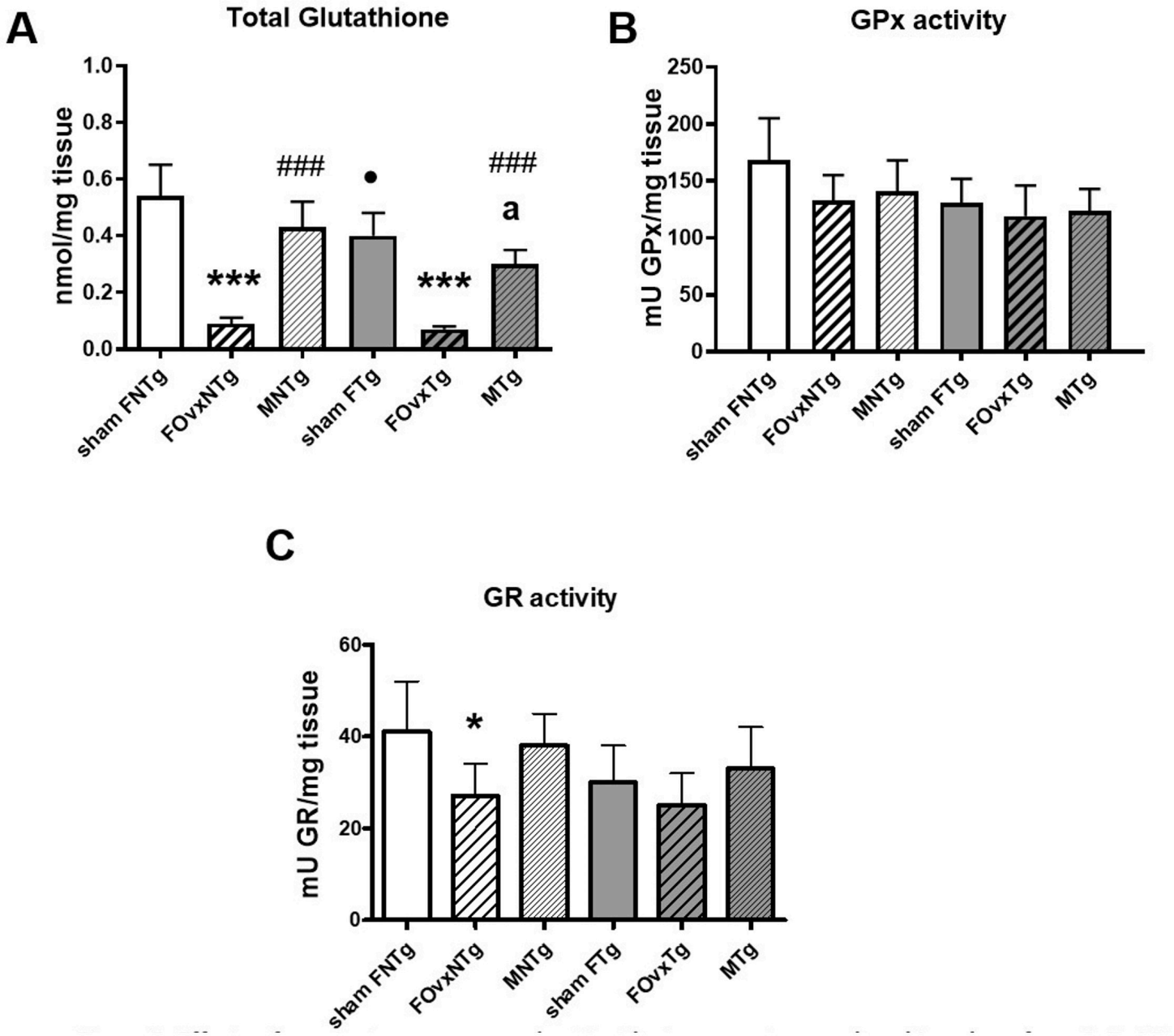


Figure 5

Effects of ovariectomy on several antioxidant parameters analyzed in spleen from 3xTg-AD and non-trangenic animals. Total glutathione (nmol/mg tissue) (**A**), glutathione peroxidase (GPx) activity (mU GPx/mg tissue) (**B**), and glutathione reductase (GR) activity (mU GR/mg tissue) (**C**). * $p<0.05$ and *** $p<0.001$ compared to the sham FNTg group; ### $p<0.001$ compared to the FNTOvx group; • $p<0.05$ compared to the same condition in non-transgenic animals; a $p<0.05$ compared to FTgOvx animals. Each bar represents the mean ± standard deviation of 8 values. FNTg: sham female non-transgenic mice; FNTgOvx: female non-transgenic ovariectomized mice; FTg: sham female triple transgenic mice; FTgOvx:

female triple transgenic ovariectomized mice; MNTg: male non-transgenic male; MTg: male triple transgenic mice.