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Research report

Maternal separation induces retinal and peripheral blood mononuclear cell alterations across the lifespan of female rats

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HIGHLIGHTS

- MatSep induces morphological changes in the female rat retina across the lifespan.
- Aged MatSep rats show increased basal GFAP expression in the retina.
- Adolescent MatSep rats show higher retinal DJ-1 expression.
- MatSep disrupts the cell cycle and chronically increases mitochondrial activity in PBMCs.
- MatSep leads to long-term alterations in both the nervous system and the immune response.

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ABSTRACT

Early life stress alters the function and feedback regulation of the hypothalamic-pituitaryadrenal (HPA) axis, and can contribute to neuroinflammation and neurodegeneration by modifying peripheral blood mononuclear cell (PBMC) activity. The retina, as part of the nervous system, is sensitive to immune changes induced by stress. However, the consequences of stress experienced at an early age on retinal development have not yet been elucidated. Here we aimed to evaluate the impact of maternal separation (MatSep) across three stages of the lifespan (adolescent, adult, and aged) on the retina, as well as on progression through the cell cycle and mitochondrial activity in PBMCs from female Wistar rats. Newborn pups were separated from their mother from postnatal day (PND) 2 until PND 14 for 3 h/day. Retinal analysis from the MatSep groups showed architectural alterations such as a diminished thickness of retinal layers, as well as increased expression of proinflammatory markers DJ-1, Iba-1, and CD45 and the gliotic marker GFAP. Additionally, MatSep disrupted the cell cycle and caused long-term increases in mitochondrial activity in PBMCs from adolescent and adult rats. Changes in the cell cycle profile of the PBMCs from aged MatSep rats were undetected. However, these PBMCs exhibited increased sensitivity to H_2O_2-induced oxidative stress *in vitro*. Therefore, these results suggest that early life stress can have long-term effects on retinal structure and function, possibly elicited by neonatal immune preconditioning.

1. Introduction

Early life stress represents a risk factor for short- and long-term alterations in brain homeostasis (Fagundes and Way, 2014; Ganguly and Brenhouse, 2015). Maternal care is an essential requirement for newborns; its deprivation or absence in early life affects the behavior

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and development of the future adult, predisposing the offspring to chronic diseases. In mammals, the first postnatal days are characterized by high brain plasticity and neuroendocrine activity, and any alteration stands to potentially compromise brain function (Ganguly and Brenhouse, 2015; Kuhn and Schanberg, 1998). Neonatal maternal separation (MatSep) causes multiple short- and long-term effects on neuronal function and behavior, as well as on neuroendocrine and neuroimmune programming (Brenhouse et al., 2018; Huot et al., 2002; Zhang et al., 2013). Furthermore, the effects of this stressor are dependent on sex, age, time exposure, and frequency (Bereshchenko et al., 2018; de Melo et al., 2018; Gracia-Rubio et al., 2016).

MatSep induces proinflammatory activation, and microglial and glial reactivity in the brain (Roque et al., 2016, 2014). These events occur immediately with exposure to the stress and are maintained across the lifespan, inducing epigenetic modifications (Kundakovic and Champagne, 2015), among other outcomes. MatSep leads to oxidative damage and increases brain energy metabolism by promoting mitochondrial biogenesis, and higher neuronal sensitivity to future oxidative insults (Hoffmann and Spengler, 2018). On the other hand, recent studies show that acute or chronic psychological distress alters the number and activity of peripheral blood mononuclear cells (PBMCs) in humans (Boecka et al., 2016; Danese et al., 2007; Maydych et al., 2017). Moreover, chronic stress causes inflammation in the brain and generalized oxidative damage in PBMCs in rats (Grigoruţă et al., 2019, 2018; Mejia-Carmona et al., 2014; Vargas-Caraveo et al., 2015), linking the neurological and immune consequences of stress.

The immune and nervous systems are interconnected by hormones, neurotransmitters, and specific receptors to maintain healthy synaptic networks. Thus, deleterious events such as cell cycle alterations, mitochondrial dysfunction, inflammation, and increased oxidative stress affect both systems (Irwin and Cole, 2011). Consequently, it is possible to correlate brain alterations induced by psychological stress with changes in PBMCs activity.

The retina is part of the central nervous system (CNS) and is comprised of neurons, microglia, and glial cells. The latter respond to neuronal stress or injury through the processes of microglial activation and reactive gliosis, respectively (Burda and Sofroniew, 2014). In turn, reactive gliosis generates physiological and morphological changes, increasing proinflammatory cytokines and glial fibrillary acidic protein (GFAP) expression (Middeldorp and Hol, 2011). Microglial activation is characterized by increased inflammatory activity, as evidenced by Iba-1 and CD-45 expression (Rashid et al., 2019; Xu et al., 2007). Gliotic and proinflammatory activity in the retina can be stimulated by signals from circulating immune cells, which cause blood-retinal barrier (BRB) impairment and promote an inflammatory environment, elevated oxidative stress, and mitochondrial dysfunction. Consequently, neurons, glial cells, and immune system activity are dysregulated (Kauppinen et al., 2016). Moreover, antioxidant proteins, like DJ-1, modulate the stress response and control cellular homeostasis by activating pathways that protect cells against oxidative stress (Oh and Mouradian, 2018). Relatedly, DJ-1 deletion has been associated with retinal alterations and increased ROS production (Bonilha et al., 2017, 2015).

Excessive cytokines and chemokines, like interleukin 8 (IL-8) or vascular endothelial growth factor (VEGF), liberated by PBMCs disrupt the BRB. Such alteration elicits chronic inflammation, which has detrimental effects on retinal homeostasis, including age-related macular degeneration (Lechner et al., 2017) or glaucoma (Vohra et al., 2013). Also, similarly to the brain, the presence of glucocorticoid receptors (GR) in the retina makes it vulnerable to acute or chronic stress stimuli (O'Steen et al., 1987). Indeed, prolonged psychological distress is strongly associated with vision loss due to the dysregulation of the sympathetic nervous and vascular systems (Sabel et al., 2018). However, the effects of early life stress on the retina of adult and aged individuals have not yet been explored. Therefore, we evaluated retinal morphology and neuroinflammation across the lifespan of female rats exposed to MatSep as neonates (Fig. 1), along with alterations in mitochondrial function and the cell cycle in PBMCs. Significant changes in these measures were seen as a result of early life stress exposure.

2. Results

2.1. Morphological analysis of retina

Retinal thickness decreased in aged rats compared to adolescents ($p \leq 0.0001$), even in the absence of stress (Fig. 2A), demonstrating that reduced retinal integrity occurs naturally as an age-related physiological process (Nieves-Moreno et al., 2018). MatSep caused a significant decrease in the thickness of the outer nuclear layer (ONL) of the retina in adolescent and adult rats, and a further reduction in aged rats ($p \leq 0.0001$) compared to age-matched controls (Fig. 2A and B). Moreover, total DAPI fluorescence intensity (Fig. 2C) decreased in stressed adult and aged rats ($p \leq 0.05$) but not in the adolescent group ($p = 0.11$).

2.2. Immunofluorescence analysis

CD-45 expression increased in aged rats compared with adolescents from control groups ($p \leq 0.05$), but no changes were found in Iba-1 expression across the lifespan (Fig. 3), but MatSep induced changes in the expression of both microglial markers. Analysis of total fluorescence showed that CD-45 protein was increased in adult ($p \leq 0.01$) and aged ($p \leq 0.01$) MatSep groups compared to controls (Fig. 3A), while MatSep caused a significant increase in Iba-1 only in the adult group ($p \leq 0.01$) (Fig. 3B). The antioxidant marker DJ-1 had increased expression in control adult and aged retinas compared to adolescents ($p \leq 0.0001$) and was significantly increased by MatSep in adolescent rats only ($p \leq 0.01$) (Fig. 4).

The expression of GFAP, a gliotic marker (Fig. 5A), and NFkB, an inflammatory marker (Fig. 5B), was studied in retinal sections from aged rats. GFAP was significantly increased in the aged MatSep group ($p \leq 0.001$), whereas NFkB protein expression was not changed in these animals ($p = 0.45$).

2.3. Cell cycle analysis of PBMCs

An age-related increase ($p \leq 0.05$) in DNA fragmentation was observed in the unstressed PBMC population from aged animals compared to adolescent and adult groups, as demonstrated by an increased number of events in the sub-G0/G1 phase that represents the dead cell population, exhibiting apoptosis-induced DNA fragmentation (Fig. 6A-C). MatSep adolescents showed a nearly-significant reduction in the level of DNA fragmentation in PBMCs compared to their age-matched control group (diminished sub-G0/G1 subpopulation, $p = 0.07$), while also suggestively demonstrating a higher capacity to proliferate (increased G2/M, $p = 0.06$, Fig. 6A). However, the dead cell population increased in stressed adults ($p \leq 0.05$, Fig. 6B).

The PBMC cell cycle progression in stressed aged rats (Fig. 6C) did not seem affected by MatSep. However, under the influence of an additional stressor, those cells showed an impaired response (Fig. 6D). Oxidative stress-induced *in vitro* in PBMCs from aged MatSep rats by exposure to H_2O_2 resulted in a significant increase in the population of dead cells ($p \leq 0.0001$) and in cells in phase G0/G1 ($p \leq 0.01$), and a diminished cell population in the S phase ($p \leq 0.05$) and the G2/M phase ($p \leq 0.01$); similar responses were observed for both concentrations of peroxide (0.2 mM and 1 mM) tested. PBMCs from control aged rats were less affected by the peroxide than aged rats previously exposed to the MatSep paradigm, showing increased damage at the 1 mM H_2O_2 dose, seen as an increase in sub-G0/G1 ($p \leq 0.01$) and a decrease in G2/M ($p \leq 0.05$).

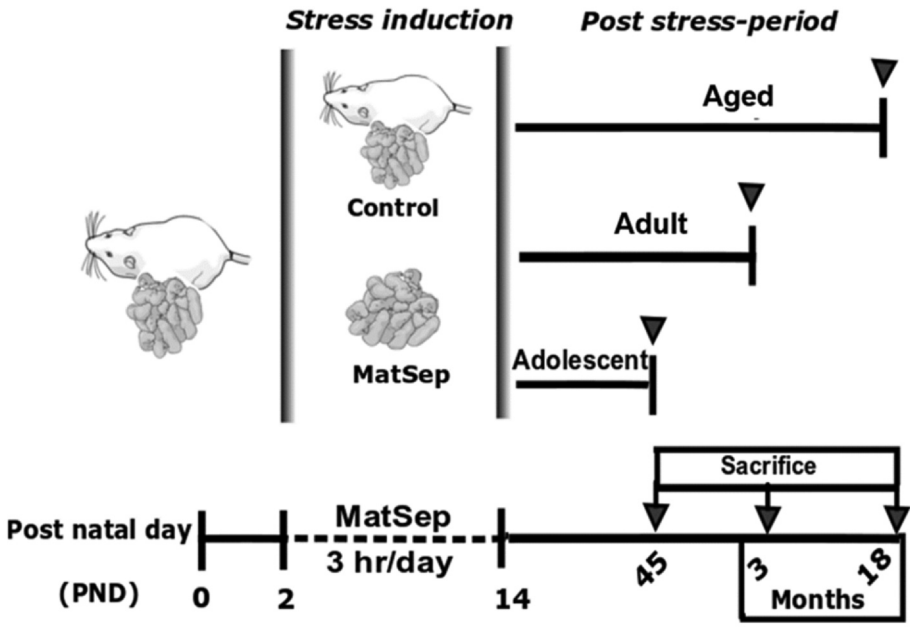

Fig. 1. Induction of early life stress by maternal separation. Female Wistar newborn rat pups were randomly divided into control or maternal separation (MatSep) groups by litter. Pups in the MatSep group were separated from their dam for 3 h daily, for 13 consecutive postnatal days (PND 2–14). Control pups were handled intermittently but not separated, and both groups remained with their dam uninterrupted from PND 15 until weaning on PND 21. Post-weaning, animals were allowed to grow without further manipulation until they reached an adolescent, adult, or aged life stage, and were sacrificed (gray triangle) for tissue harvest.

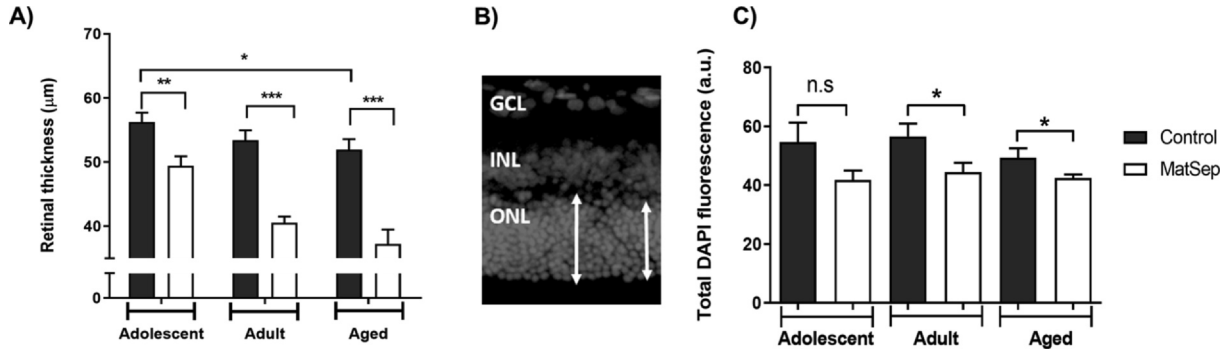

Fig. 2. Maternal separation causes retinal layer thickness. Retinal sections (12 μm) were cut on a cryostat from control and maternally separated (MatSep) groups at the adolescent, adult, or aged timepoints. Sections were fixed and stained with 4′,6-diamidino-2-phenylindole (DAPI). A) Retinal thickness measurement for control (dark bars) and MatSep (light bars) was calculated by the average of five measurements of the outer nuclear layer (ONL) per animal. B) Image of a DAPI-stained retina, indicating example sites (arrows) of ONL thickness measurement. C) Quantification of the total number of cells in the retina, as determined by the average of the gray mean of DAPI fluorescence in 20–30 slices from each treatment and age group (n ≥ 5 rats in each analysis). Graphs represent the mean ± SEM by group and age range. An unpaired *t*-test was used to compare control versus MatSep data, and a one-way ANOVA was used for comparison across age groups. Asterisks represent * p ≤ 0.05, ** p ≤ 0.01, *** p ≤ 0.001, **** p ≤ 0.0001. Abbreviations: GCL, ganglion cell layer; INL, inner nuclear layer; a.u., arbitrary units; n.s., not significant.

2.4. PBMC energized mitochondria analysis

Early life stress-induced a chronic increase in mitochondrial metabolism in PBMCs, shown by an increased cell population with MitoTracker high fluorescence (Fig. 7A), which is considered to be a cell population with highly energized mitochondria (Pendergrass et al., 2004). This occurrence was seen in adolescent ($p \leq 0.01$) and adult ($p \leq 0.05$) rats, while no changes were found in aged rats (Fig. 7B). However, the aged rats had diminished mitochondrial activity in their PBMCs under control conditions, as compared to the younger groups ($p \leq 0.01$), indicating an age-related physiological change. No significant changes were found when the cells from old rats were stressed *in vitro* (data not shown).

3. Discussion

The nervous and immune systems are both implicated in the complex regulation of stress responses and, therefore, both systems are susceptible to the deleterious effects of psychological stressors or oxidative injury (Grigoruţă et al., 2020, 2019, 2018; Vargas-Caraveo et al., 2015, 2014). Prolonged early life stress leads to dysregulation of the HPA axis, the sympathetic nervous system, and the immune response, in part through the modified expression of several genes that are functionally linked to stress reactivity (Fogelman and Canli, 2019). Chronic release of catecholamines and corticosteroids is highly correlated with increase inflammatory markers, oxidative stress, neuronal and vascular dysregulation. As the retina is part of the CNS, it can be affected by emotional stress due to the overstimulation of the corticosteroids and adrenergic receptors from the inner blood-retinal barrier. Thus, it can lead to inflammation, endothelial dysfunction, and blood flow alterations that can increase intraocular pressure, ischemia, optical nerve damage, and retinal disease (Malan et al., 2020; Sabel et al., 2018).

Altered corticosteroid synthesis and specific corticosteroid receptor levels increase vulnerability to future stress situations and has been associated with pathologies such as depression-like behavior and systemic inflammation (Fogelman and Canli, 2019). Systemic inflammation stimulates the migration of circulating monocytes to the CNS,

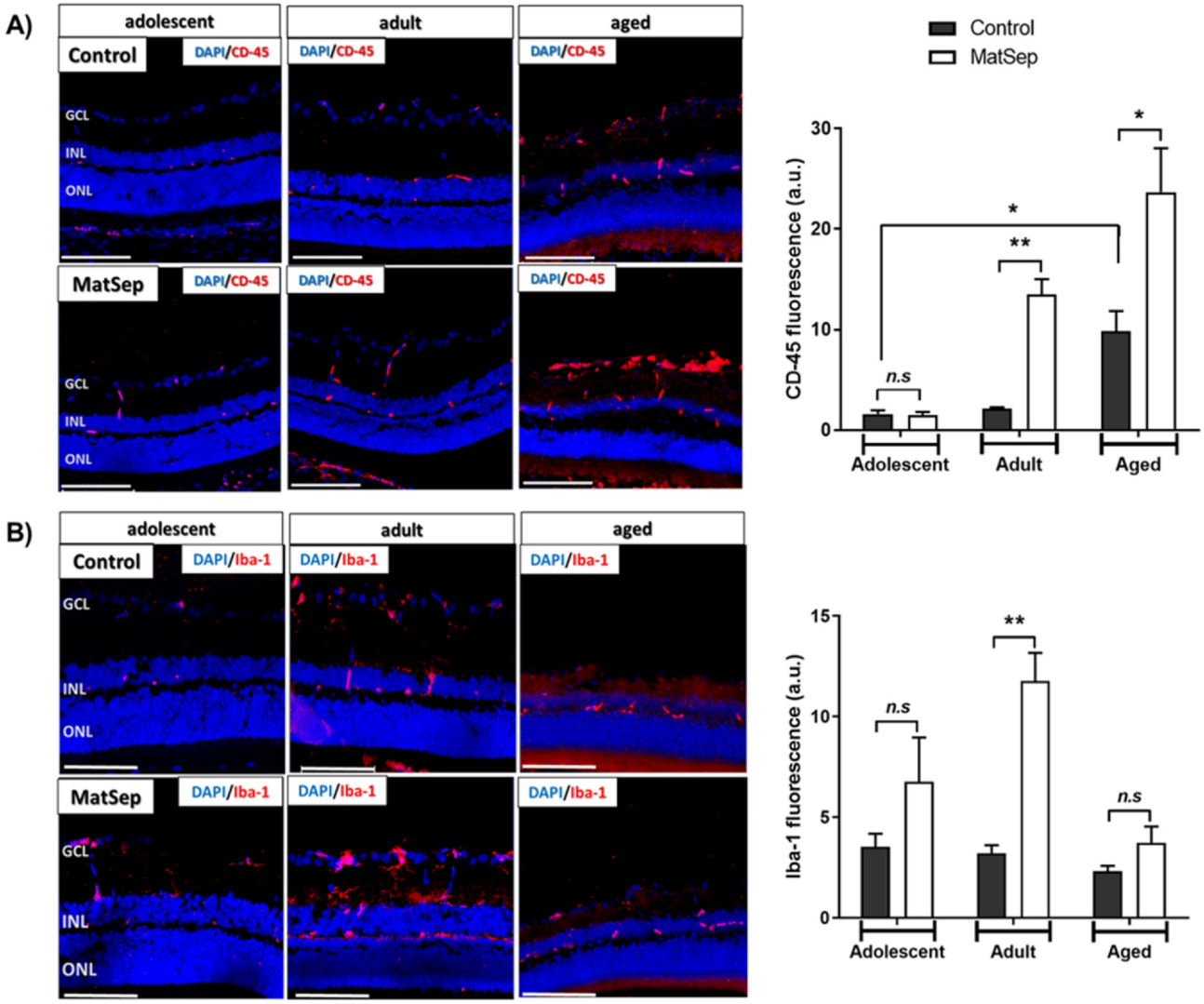

Fig. 3. Maternal separation rats exhibit an increase in microglial markers. Retinal sections (12 μm) from control (upper panel) and MatSep (lower panel) rats were used for the analysis of A) CD45 expression (red color; upper panel) and B) Iba-1 (red color; lower panel) at three different ages: adolescent, adult, and aged. Nuclei of retinal layers were stained with 4′,6-diamidino-2-phenylindole (DAPI; blue color). Images were obtained by confocal microscopy and the fluorescence quantification corresponds to at least eight sections from every of five animals per age and treatment group. Graphs represent the mean ± SEM of fluorescence by treatment group and age range. Statistical analyses were done with unpaired t-tests. Asterisks represent * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$. Abbreviations: GCL, ganglion cell layer; INL, inner nuclear layer; a.u., arbitrary units; n.s., not significant. Scale bar, 100 μm.

including the retina, where they can differentiate into microglia and induce neuroinflammation (Rangasamy et al., 2014). Chronic activation of retinal microglia facilitates the release of proinflammatory molecules that promote oxidative damage and has detrimental effects on the development of retinal neuronal and vascular systems in newborns. Vascular aberrations can lead to progressive neuronal damage and retinopathy, and can have long-term effects on visual function (Tremblay et al., 2013). Moreover, the chronic activation of retinal microglia by systemic inflammation accelerates retinal degenerative diseases (Noailles et al., 2018). In the present study, we investigated the effects of MatSep stress (3 h/day; PND 2–14) on retinal morphology and physiology, as well as on PBMCs response to oxidative stress, across different stages of life.

At young ages, the neural circuitry and immune system are still immature. Stress experienced in early life can impact brain development and cause long-lasting negative effects (Hensch, 2005). Similarly, acute and chronic MatSep at an early age is associated with morphological and physiological alterations in various mammalian brain structures (Andersen and Teicher, 2004; Fabricius et al., 2008; Huot et al., 2002; Mirescu et al., 2004). Stressed females maintain stable their corticosterone levels (Lundberg et al., 2017) and showed less behavior changes (Dimatelis et al., 2016) compared to males. Although various authors showed that females are less vulnerable to early stress, the mechanism and mediators of the alterations on the neuroinmune system across life span remain unclear. Females carry high inflammatory load (Bereshchenko et al., 2018) which has been strongly associated with declining retinal function (Kauppinen et al., 2016). Reduced levels of circulating gonadal steroids impact retinal degeneration, as demonstrated in aging rats and post-menopausal women. The loss of estrogen and progesterone, the latter mediating anti-inflammatory effects in the retina (Benlloch-Navarro et al., 2019), promotes macular degeneration. Relatedly, estrogen production or delivery via hormone replacement therapy protects against the development of this pathology (Snow et al., 2002). On the other hand, it has been shown that a normal estrus cycle in rats promotes retinal function and maintenance (Chaychi et al., 2015). These findings suggest that age and the concomitant decrease in steroid hormone levels in females would be relatively more critical to retinal architecture than the normal hormonal variations seen throughout the estrus cycle in younger rats. The fact that we observe changes in the aged MatSep female retina

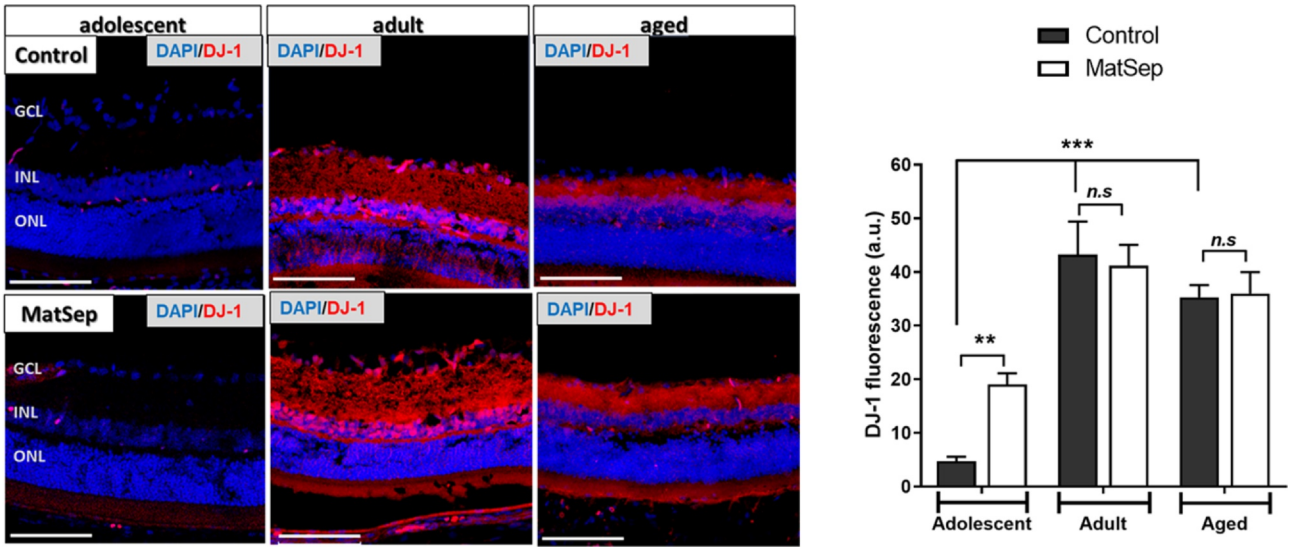

Fig. 4. Maternal separation increases the expression of the antioxidant protein DJ-1 in adolescents but not adult or aged animals. Retinal sections (12 μm) from control (upper panel) and MatSep (lower panel) rats were analyzed for immunofluorescent staining of DJ-1 (red color) at three different ages: adolescent, adult, and aged. Nuclei of retinal layers were stained with 4′,6-diamidino-2-phenylindole (DAPI; blue color). Images were obtained by confocal microscopy and the quantification of total DJ-1 fluorescence was made from at least eight sections from every of three animals per age and treatment group. Graphs represent the mean ± SEM by group and age range. Statistical analyses consisted of unpaired t-tests. Asterisks represent * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$. Abbreviations: GCL, ganglion cell layer; INL, inner nuclear layer; a.u., arbitrary units; n.s., not significant. Scale bar, 100 μm.

compared to the Control group supports our interpretation that the early life stress exposure has consequences that add to or occur in concert with age-related changes in sex steroids. This does not preclude the fact that hormone levels at different estrus cycle stages may also influence the retina.

We reported that MatSep rats have a diminished total retinal thickness, mainly in the retinal ONL. Such changes were similar to architectural alterations induced by maternal deprivation that have been reported in the brain. Indeed, deprived rats exhibited hypoplasia of mossy fiber density, leading to alterations in the hippocampal cytoarchitecture in adults (Huot et al., 2002), as well as long-lasting synaptic changes (Andersen and Teicher, 2004). Also, MatSep animals showed diminished total neuronal number (Fabricius et al., 2008) with decreased immature forms but unchanged mature neurons number in hippocampus (Mirescu et al., 2004), indicating that maternal deprivation leads to decreased neuronal proliferation and neurogenesis in adulthood.

Both the hippocampus and the amygdala are rich in glucocorticoid receptor (GR), which makes them highly vulnerable to stress (McEwen et al., 2016). The retina is also rich in GR, expressed exclusively in Müller glial cells (Gallina et al., 2014). The MatSep model has been established as a stress model that induces early increases in corticosteroids, specifically glucocorticoids, that bind these receptors (Huot et al., 2002). The aging brain naturally decreases in its number, function, and sensitivity of GR (Djordjevic–Markovic et al., 1999), revising stress reactivity over time.

O'Steen et al. (1987) demonstrated that an escapable stress stimulus, administered to young, mid-age, and aged rats, diminished total retinal thickness, mainly in the ONL (O'Steen et al., 1987). In agreement with this observation, our results showed that MatSep causes alterations to retinal architecture, mostly in the ONL, with decreased overall retinal layer thickness. This effect was exacerbated in aged retinas. On the other hand, several studies have demonstrated that retinal thickness changes are associated with inflammatory and age-related diseases (Wood et al., 2011). Noteworthy is the knowledge that increased CD-45 and Iba-1 expression is considered a hallmark of microglial activation and retinal aging (Chen et al., 2002; Xu et al., 2009). Thus, to evaluate whether an inflammatory state was present in MatSep retinas, the expression of both CD-45 and Iba-1 was studied. We found significant increases in both proteins in MatSep retinas across all of our age groups. Our results suggest that MatSep may predispose the retina to microglial activation and consequently to a more advanced aging process. Therefore, to assess gliotic activity in the aged retina, GFAP expression was analyzed. Our results showed that GFAP was increased in the aged MatSep group alone. Our data are supported by several studies showing that physical damage, chemical stressors, aging, mutations, and inflammatory diseases could lead to an increase in GFAP expression in the retina (Chavez-Solano et al., 2016; Kalesnykas et al., 2008; Lewis and Fisher, 2003; Mansour et al., 2008). GFAP is the major protein of intermediate filaments in glial cells and is commonly used as a gliotic marker in the damaged brain and retinal tissues. Our results showed that GFAP expression increased by more than 70% in the stressed aged group. The normal retina has poor and restricted GFAP expression in the GCL. However, GFAP expression increases across all retinal layers during disease (Lewis and Fisher, 2003; Sarthy et al., 1991). Likewise, it has been reported that GFAP-positive cells increase by over 30% in the hippocampus of chronically stressed rats (Lambert et al., 2000).

NFκB is a cytoplasmic protein expressed in both neuronal and glial cells (O'Neill and Kaltschmidt, 1997) that translocates to the nucleus during early inflammation as a response to harmful stimuli (Shih et al., 2015). Therefore, we also evaluated NFκB expression in aged retinas and no significant differences were observed. The unchanged levels of NFκB in the aged MatSep group suggest that the retinal inflammation we observed was likely due to an increase in immune cell infiltration (high CD-45 expression) and GFAP expression that were induced by early life stress instead of a recent event.

Additionally, DJ-1, a neuroprotective protein implicated in retinal homeostasis (Bonilha et al., 2017, 2015), showed significantly increased expression only in the stressed adolescent group. This expression is likely an early response to the intense oxidative stress that occurs during the postnatal period. Together with the chronically increased expression of CD-45, Iba-1, and GFAP in MatSep groups, DJ-1 can initiate the activation of antioxidant pathways and inflammatory-like responses. Moreover, DJ-1 expression increased over time in a sustained way until advanced age, suggesting a possible modulation of basal DJ-1 expression with age in the retina.

Previous studies have shown that early-life stress has long-term consequences on the development and function of the HPA axis

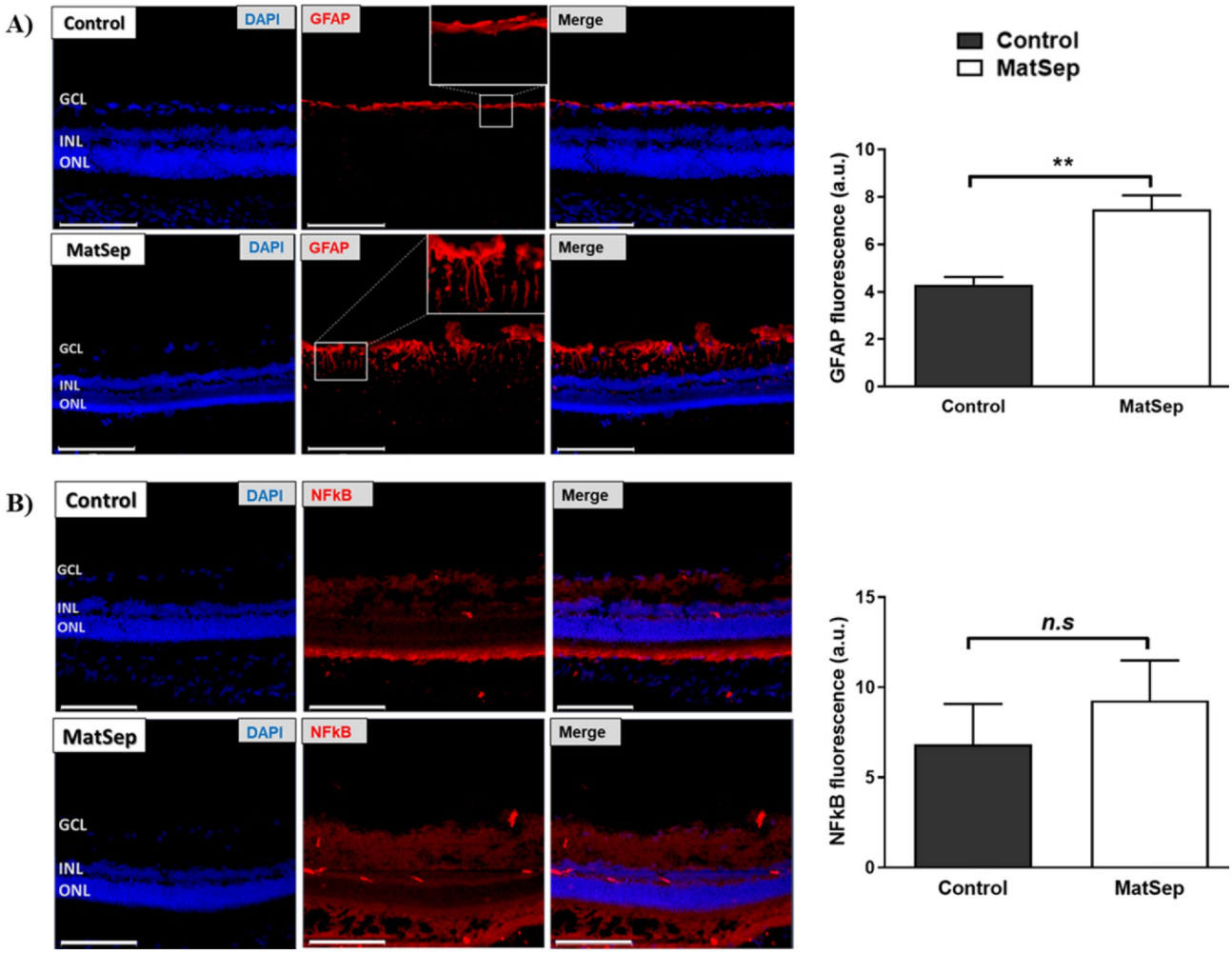

Fig. 5. Maternal separation increases GFAP expression but not NFkB in the aged retina. A) GFAP expression (red color; upper panel) and B) NFkB (red color; lower panel) in retinal sections (12 μm) from aged rats under control (upper panels) and MatSep (lower panels) conditions. Nuclei of retinal layers were stained with 4′, 6-diamidino-2-phenylindole (DAPI; blue color). Images were obtained by confocal microscopy and are representative of the quantitative data gathered from at least eight sections from every of five different animals per age and treatment group. Graphs represent the mean ± SEM by group and age range. Unpaired t-tests were used for statistical analysis. Asterisks represent * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$. Abbreviations: GCL, ganglion cell layer; INL, inner nuclear layer; a.u., arbitrary units; n.s., not significant. Scale bar, 100 μm.

(Schmidt, 2010) and induces chronic neuroinflammation (Gracia-Rubio et al., 2016). Moreover, early life stress induces a chronic increase in inflammatory markers in serum (Boecka et al., 2016; Danese et al., 2007) with permanent changes to neuroimmune signaling and an altered capacity to adapt to new experiences (Brenhouse et al., 2018). We recently demonstrated that chronic stress induces neuroinflammation and oxidative stress in PBMCs (Grigoruţă et al., 2018; Vargas-Caraveo et al., 2015, 2014). Our current data suggest that the morphological and physiological changes observed in the retina that were induced by MatSep could be caused by a dysregulation of the PBMC stress response. Indeed, MatSep animals showed PBMC cell cycle dysregulation in adolescence and adulthood. Also, the aged MatSep group was more sensitive to H_2O_2 compared to controls and followed the all-or-none law, giving a maximal response to stress starting from the lowest tested H_2O_2 concentration. This increased sensitivity to H_2O_2 suggests that MatSep alters PBMC activity, potentially through impaired feedback of the HPA axis and the epigenetic changes induced by early life stress, as previously described in the brain (Darnaudéry and Maccari, 2008; Kundakovic and Champagne, 2015). Perhaps the exposure to another stressful event later in life could induce a new state of immune reactivity; such a hypothesis deserves further exploration.

In line with the notion of a new and higher immune responsivity due to early life stress exposure, we investigated the possible involvement of mitochondria in the adaptive response mechanism. This mechanism was tested by identifying energized mitochondria using a cationic fluorescent dye sensitive to mitochondrial membrane potential (Pendergrass et al., 2004). Adolescent and adult MatSep rats showed an increase in the high fluorescent events cell population that is associated with an increase in the number of cells with high mitochondrial activity. These data suggest that early life stress induces chronic activation of PBMCs, a result that corroborates the changes found in the cell cycle profile in adolescent and adult rats. Our results expand on previous reports of increased mitochondrial allostatic load and energy demand in PBMCs, increased number of PBMCs, increased inflammatory biomarkers, and ROS production in serum from adults exposed to maltreatment during childhood (Boecka et al., 2016; Danese et al., 2007).

To the best of our knowledge, this is the first study that shows long-term alterations in the retinal structure and function that are caused by early life stress and correlated with changes in PBMCs and the immune response.

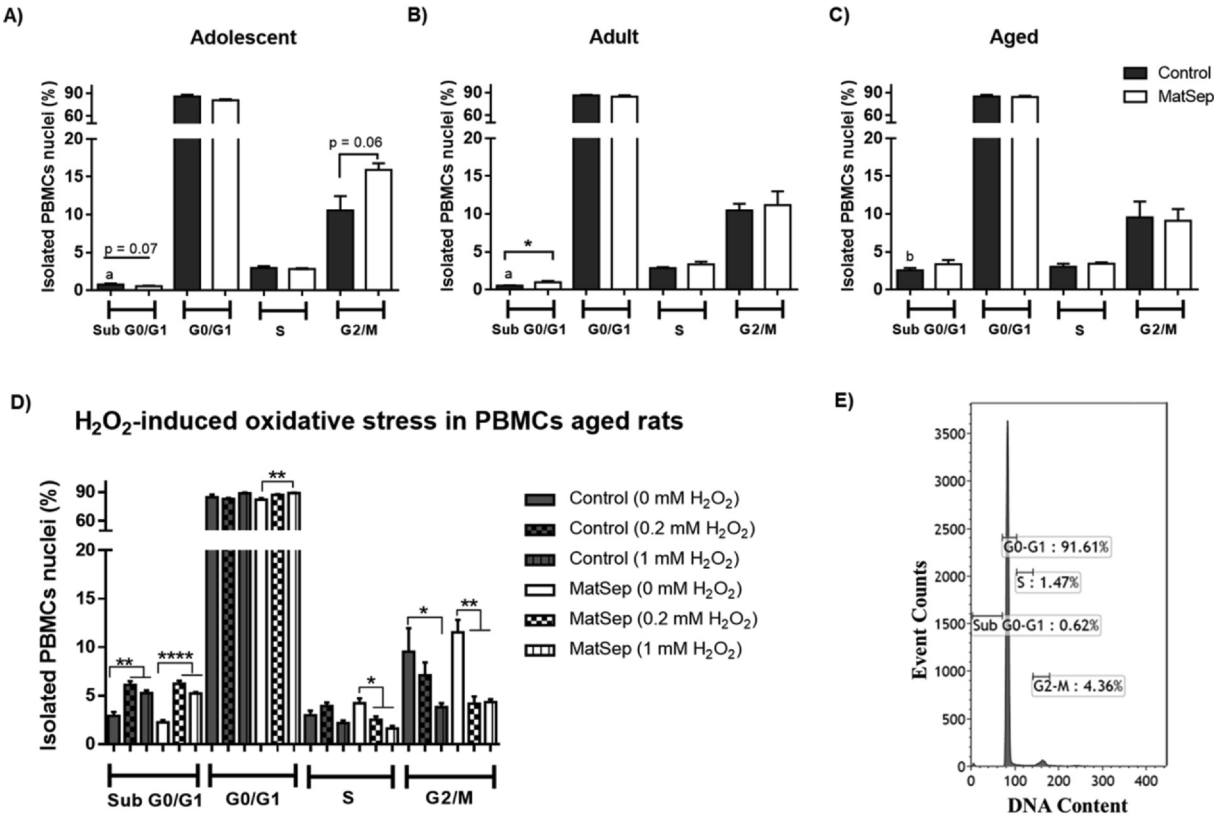

Fig. 6. Maternal separation has negative long-term effects on the response of PBMCs to subsequent cellular stress. The percentage of isolated PBMC nuclei in each cell cycle phase is shown from A) adolescent, B) adult, and C) aged groups. D) In isolated PBMC nuclei from aged rats, MatSep alters the response to H_2O_2 exposure in terms of cell cycle phase compared to control rats. E) A representative single parameter histogram is shown, where the four gates represent the percentage of cells in each phase of the cell cycle evaluated by DNA content. Sub G0/G1 represents the hypodiploid cell population associated with an apoptotic subpopulation. G0/G1 represents diploid cells, while the S phase includes hyperdiploid cells. In the G2/M phase are the tetraploid cell population. Graphs represent the mean percentage ± SEM, n = 3–5 per group. Data were analyzed by unpaired *t*-test. Asterisks represent * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$. Letters indicate significant differences between cell population in subG0/G1 phase across age groups, $p \leq 0.05$.

4. Experimental procedure

4.1. Animals

Wistar rats were housed in standard cages with *ad libitum* access to food and water and maintained on a 12–12 h dark/light cycle in a temperature- and humidity-controlled room. All experiments were approved by the Institutional Animal Care and Use Committee from the University of Texas at El Paso, USA. Litters were randomly assigned in two groups: control or maternal separation (MatSep). After the

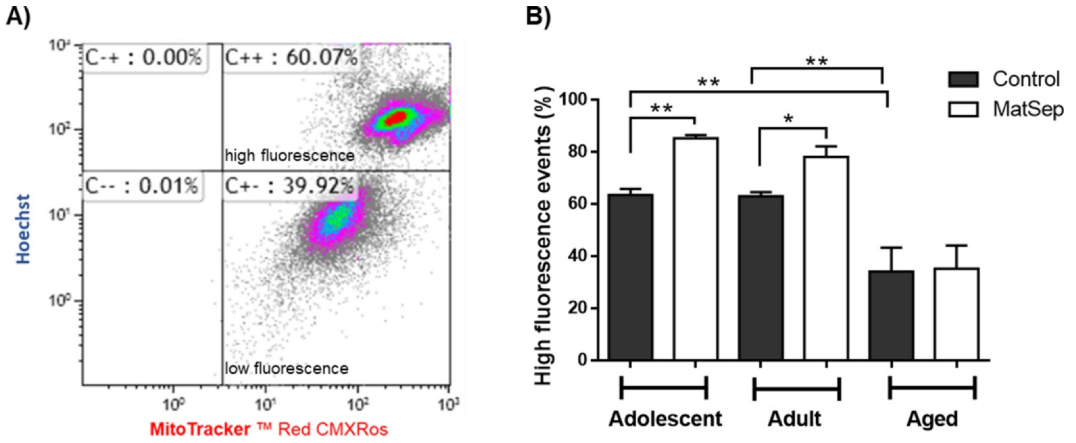

Fig. 7. Maternal separation has long-term effects on mitochondrial activity. A) A representative dual parameter flow cytometry histogram is shown of PBMCs with energized mitochondria identified by using MitoTracker™ Red CMXRos (100 nM), a cationic fluorescent dye that accumulates in active mitochondria, and Hoechst (1:1000), which provides an estimated number of cells. The cells were distributed in two clusters: low and high fluorescence of MitoTracker which was associated with cells with low or high energized mitochondria. B) Percentages of PBMCs with high fluorescence of MitoTracker differ in the aged compared to the adolescent and adult groups for both control and MatSep rats. Graphs represent the mean percentage ± SEM, n = 3–5 per group. Data were analyzed by unpaired *t*-test. Asterisks represent * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.

experimental procedure, both control and MatSep groups were consistently maintained under similar conditions until sacrifice.

4.2. Maternal separation (MatSep) stress model

The day of birth was established as postnatal day (PND) 0. Stress induction by MatSep started on PND 2 and finished on PND 14. MatSep consisted of removal of the litters from their dam for a period of 3 h/day (from 8 AM to 11 AM) for 13 days. All littermates were carefully separated from their dam and placed in a new cage with surgical bedding that was placed over a circulating water heating pad to avoid hypothermia-induced stress. Control litters were handled intermittently during the same timeframe, but not separated from their dams. From PND 15–21, the dams and litters were undisturbed; at PND 21, all litters were weaned, separated by sex, and pair-housed until sacrifice at the following times: adolescent (45–50 days old), adult (75–80 days old), and aged (18 months old).

The animals were anesthetized using sodium pentobarbital (100 mg/kg, i.p.) and sacrificed by decapitation after blood collection by cardiac puncture. A schematic of this experimental design is provided in Fig. 1 (created using the freely available Mind the Graph software).

4.3. Immunofluorescence

Eyes from control or MatSep groups were enucleated and immersion fixed overnight in 4% (w/v) paraformaldehyde in the dark, at room temperature. The next day, eyes were washed with 1X PBS (137 mM NaCl, 8.2 mM Na_2HPO_4, 1.5 mM KH_2PO_4 and 3.2 mM KCl, pH 7.4) and transferred to 30% (w/v) sucrose solution overnight at 4 °C. Then, eyes were embedded in Tissue Tek (Sakura, cat 4583) and cut with a cryostat (Leica, CM 1510S). Retinal sections (12 μm) at 120–500 μm from the optic nerve were obtained and blocked in a solution containing 3% (w/v) BSA, 0.1% Triton X100 in 1X PBS for 2 h at room temperature. Briefly, sections were incubated overnight with Iba-1 (Wako, cat 019–19741), CD45 (Abcam, cat AB33923), NFκB (Abcam, cat AB16502), GFAP (Abcam, cat AB7260), or DJ-1 (Abcam, cat AB76008) antibodies at 4 °C. After washing with 1X PBS, retinal sections were treated with fluorochrome-conjugated secondary antibodies (Alexa Fluor 594, Abcam; cat AB150080 and AB150116) for 2 h at room temperature with protection from the light. Finally, cellular nuclei were stained with 1.5 μM of 4′, 6-diamidino-2-phenylindole (DAPI; Invitrogen cat D1306) diluted in glycerol. Digital photomicrographs of the stained retina were captured using a confocal microscope (LSM 700, Zeiss), equipped with 20x/0.5 EC Plan-Neofluar objective lens, and using 1 Airy-Unity (1 AU) pinhole setting consistently; the 405 nm, and 555 nm lasers were used. The intensity and total fluorescence were calculated with Fiji-ImageJ software. Values represent a mean of threshold gray intensity ± SEM for each group.

4.4. Isolation of peripheral blood mononuclear cells (PBMCs)

Blood was obtained by cardiac puncture and collected in vacutainer tubes containing 7.2 mg of K_2EDTA. Whole blood was diluted with 1X PBS 1:1. This mixture was carefully placed onto a bed made from a mix of 40% 1X PBS and 60% Percoll™ (Sigma-Aldrich, cat GE17-0891–01). The preparation was centrifuged at 1000 × g for 30 min, at 30 °C, and the white layer containing the PBMCs was harvested by careful pipetting. Next, cells were washed with 1X PBS and centrifuged three times at 800 × g for 5 min at room temperature. Finally, the cell pellet was resuspended and separated into two fresh tubes for cell cycle and mitochondrial membrane potential (MMP) analysis.

4.5. PBMC cycle analysis by flow cytometry

Cell pellets from PBMCs were resuspended in 100 μl of 1X PBS and 200 μl of nuclear isolation medium (NIM)-DAPI solution (NPE Systems, Inc. Pembroke Pines, FL). The cell suspension was incubated in the dark for 3 min at room temperature. Next, the cellular DNA content was measured via a Gallios flow cytometer (Beckman Coulter, Miami, FL), with the aid of a 405 nm laser and an FL9 detector. Approximately 50,000 events per animal were collected and analyzed using Kaluza software (Beckman Coulter).

4.6. Analysis of PBMC energized mitochondria by flow cytometry using MitoTracker™ red CMXRos

PBMC cell pellets were resuspended in a solution containing 1:1000 Hoechst 33,342 (Thermo Scientific™, cat 62249) diluted in 1X PBS and supplemented with 25 mM glucose and 0.1% (w/v) BSA. After 30 min of incubation at 37 °C, MitoTracker™ Red CMXRos (Invitrogen™, cat M7512) was added, mixed with Hoechst 33,342 (1:1000) and diluted in 1X PBS with 25 mM glucose and 0.1% (w/v) BSA. The final concentration of MitoTracker was 100 nM. After 45 min of incubation at 37 °C and protection from light, the PBMCs (50,000 events per animal) were analyzed at 579 nm using a Gallios flow cytometer (Beckman Coulter, Miami, FL). Data were analyzed using Kaluza flow cytometry software (Beckman Coulter).

4.7. PBMC stress induction in vitro using H_2O_2

Cells were incubated for 1 h at 37 °C with 0.2 mM or 1 mM of H_2O_2 (Sigma-Aldrich, cat H1009) diluted in 1X PBS supplemented with 25 mM glucose, 0.1% (w/v) BSA. Subsequently, the cells were washed once with 1X PBS, centrifuged, and gently resuspended. Next, the cell cycle and energized mitochondria identification assays were performed as described.

4.8. Statistical analyses

All immunofluorescence data were expressed as mean ± SEM of intensity by treatment group and age group. The flow cytometry assay data were expressed as mean percentage ± SEM. The statistical analyses were made in GraphPad Prism software (version 6.0); unpaired *t*-test was used to compare control versus MatSep data per age, and one-way ANOVA was used to compare across age groups. The *p* values of less than 0.05 were considered statistically significant.

CRediT authorship contribution statement

Mariana Grigoruta: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data curation. **Marbella Chavez-Solano:** Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data curation, Visualization, Project administration, Supervision. **Armando Varela-Ramirez:** Conceptualization, Methodology, Resources. **Jorge A. Sierra-Fonseca:** Investigation. **Ernesto Orozco-Lucero:** Formal analysis. **Jameel N. Hamdan:** Investigation. **Kristin L. Gosselink:** Conceptualization, Methodology, Resources, Supervision, Funding acquisition. **Alejandro Martinez-Martinez:** Conceptualization, Methodology, Resources, Supervision, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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