Expression of MHC-I and II by Microglia and Lymphocytes in the Brain of Diet-Restricted Mice

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Summary Microglia are immunocompetent cells of the central nervous system whose function is to preserve nervous tissue homeostasis; however, under inflammatory conditions, they are associated with tissue damage. Dietary restriction is a nutritional intervention used to delay the onset of chronic disease and inflammation, in addition to improving the functions of the immune system. The aim of this study was to analyze the effect of dietary restriction on microglial expression of MHC molecules. Adult female and male BALB/c mice were fed ad libitum (controls) or kept under dietary restriction (30% reduction in food intake) for 4 wk. Purified brain mononuclear cells were analyzed by flow cytometry staining for CD45, CD11b, MHC-I and MHC-II. Our results show that female animals under dietary restriction had a significant increase in MHC-I expression in microglia (mean fluorescence intensity = 7.854 (control) vs. 10.628 (diet-restricted), arbitrary units; p=0.0108), along with increased frequencies of lymphocytes compared to controls (1.39% (control) vs. 7.85% (diet-restricted); p=0.0175), whereas male animals did not show significant differences between groups. Our data suggest a differential effect for dietary restriction on female and male animals, with this nutritional regimen predominantly affecting females. Increased expression of MHC-I by diet-restricted microglia may play a role in maintaining tolerance in the absence of antigenic stimulation.

Key Words microglia, lymphocytes, dietary restriction, MHC-I, MHC-II, sex

Microglia are considered resident immune system cells in the central nervous system (CNS), sharing many of the characteristics and functions of tissue resident macrophages. These cells are critical for the proper development and function of neurological synapses, as well as for the induction of innate and adaptive immune responses within the nervous tissue (1). At rest, microglia show a ramified structure and low expression of surface activation markers, which allow them to constantly sample their surrounding medium in search of pathogens or danger signals, while actively contributing to tissue homeostasis by the secretion of neurotrophic factors (2, 3). After stimulation, microglia undergo morphological changes to an ameboid structure, while upregulating their capacity to present antigens to adaptive immune system cells and activating an inflammatory response (4).

Classical microglial activation is characterized by increased expression of activation markers, such as major histocompatibility molecules (MHC) type I and II, as well as costimulatory molecules and the production of cytokines and other pro-inflammatory mediators (5, 6). Increased and/or chronic microglial activation has been related to neurotoxicity and development of a variety of neurological diseases, including Alzheimer’s, Parkinson’s and multiple sclerosis (7, 8). In comparison, expression of classical MHC molecules by lymphocytes has not been traditionally considered as highly relevant for the induction of neurological damage under different conditions, particularly since it is considered that microglia and dendritic cells are the principal antigen presenting cells within the CNS. Nonetheless, MHC-II expression on B cells has been demonstrated to contribute to the induction of CNS autoimmune responses in animal models of multiple sclerosis (9, 10).

Deregulated microglial activation is also seen as a normal consequence of aging, and is considered as a risk factor for the development of neurodegenerative disease (11, 12). Attenuation of abnormal microglial activation as a consequence of aging is considered an important goal for the prevention of neurodegenerative diseases.

Dietary restriction is a nutritional intervention that has been shown to decrease the detrimental effects of aging, extending lifespan and delaying the development of age-related degenerative diseases across a variety of organisms, including mammals (13, 14). In particular, dietary restriction, defined as controlled reductions in food intake ranging from 10–60%, on variable time frames, has been shown to reduce the activation of immune system cells, along with decreased production of pro-inflammatory mediators and reactive oxygen species (15–17). This restriction is suggested to have a beneficial effect on the prevention of age-related neurodegeneration by promoting pro-inflammatory and antioxidant mechanisms, as well as synaptic plasticity and neurogenesis in the CNS (18–20). Dietary restriction

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has also been shown to modulate microglial functions in murine models, where different dietary restriction regimes have been reported to decrease microglial activation after neonatal ischemia, traumatic brain injury and lipopolysaccharide administration in the brain (21–23), as well as having potential anti-inflammatory effects by reducing lymphocyte activation in vivo (24, 25).

So far, studies of microglial activation under dietary restriction regimens have focused on morphological changes from the ramified to the amoeboid configuration and expression of common microglial/macrophage markers such as ED-1 or Iba1 after antigenic stimulation (21–23), but no studies have determined the effects of dietary restriction on microglial expression of molecules related to their antigen presenting functions. The present study aims to contribute to this issue by exploring microglial expression of major histocompatibility molecules in mice under a 30% dietary restriction regimen, in order to better understand the effects of this kind of nutritional intervention on microglial functions.

**MATERIALS AND METHODS**

All experiments were performed following national guidelines for animal care in research and were approved by the Ethics in Research Committees from the authors' institution under protocol number 4,223/2016SF.

**Animals and experimental groups.** Eight-week-old BALB/c mice, male and female, were raised in the animal facilities at the Faculty of Medicine, Universidad Autónoma del Estado de México. Animals were kept in standard rodent breeding cages (3 mice per cage), with access to standard mouse chow (Rodent Laboratory Chow 5001, PMI Nutrition International) and purified drinking water, 12:12 h light cycle and 22°C ambient temperature. Chow composition is reported as follows: crude protein not less than 23%; crude fat not less than 4.5%; crude fiber no less than 6.0%; ash not more than 8.0% and: added minerals not more than 2.5%. This diet provides 28.507% calories from protein, 13.496% calories from fat and 57.996% calories from carbohydrates, according to the manufacturer. Breeding conditions and experimental procedures were performed following national guidelines for animal studies (NOM-062-ZOO-1999). Mice were separated by sex and randomly assigned to the following experimental groups: ad libitum (no dietary restriction) and dietary restriction (DR, fed 70% of the mean amount of food taken daily by the ad libitum group). Each group was comprised of 12 8-wk-old animals, 6 male and 6 female mice each. Dietary restriction was maintained 4 wk.

**Assessment of food intake and weight gain.** Food intake was measured daily. The initial amount of food provided for each cage was recorded and then measured again 24 h later, at a scheduled time (12:00 h) throughout the restriction period. The difference between each measurement was determined and consumption was established. As animals were kept 3 per cage, measured quantities were averaged. Individual animal weight was also recorded, weekly, in order to insure no severe variations in food and water intake were present in individual mice within each cage.

**Leukocyte purification from brain.** After 4 wk of treatment, individual animals were sacrificed by anesthetic overdose (sodium pentobarbital, 6.3 g/100 mL, 50 μL/g of weight) and perfused transcardially with 30 mL of ice-cold phosphate buffered saline solution (PBS, pH 7.4), to eliminate remaining cells in circulation. Brains were obtained and mechanically disaggregated using dissection scissors in petri dishes containing 3 mL of Dulbecco’s modified Eagle medium with glucose and l-glutamine (DMEM, Lonza Cat No. 12-604Q). The remaining tissue was filtered through a 70 μm nylon mesh (Fisherbrand Cat No. 22363548) using a sterile 3 mL syringe plunger, into a 15 mL centrifuge tube. The solution was centrifuged at 1,300 rpm, 10 min at room temperature (RT). Supernatant was removed and cells were resuspended in 3 mL of a 30% Percoll solution (GE Healthcare, Cat No. 17-0891-01) in PBS. Suspended cells were carefully layered onto 5 mL of a 70% Percoll solution in PBS, to form a gradient. Samples were centrifuged at 2,400 rpm, 30 min RT. After centrifugation, myelin and cell debris were removed from the top of the samples by aspiration with a glass pipette and purified cells were carefully recovered from the gradient interface and transferred to a 15 mL centrifuge tube containing 5 mL DMEM. Residual Percoll was removed by centrifugation at 1,200 rpm, 5 min RT. Supernatants were discarded and cells were resuspended in 1 mL DMEM for cell counting in a hemocytometer.

**Flow cytometry.** 1×10⁶ cells from each sample were suspended in 250 μL of DMEM and stained with the following fluorochrome-conjugated antibodies: anti-CD11b-FITC (BioLegend, clone M1/70); anti-CD45-APC (BioLegend, clone I3/2.3); anti-MHC-I-PE (H-2Kd-H-2Dd, eBioscience, clone 34-1-2S) and anti-MHC-II-PECy5 (I-A/I-E, BioLegend, clone M5/114.15.2). All antibodies were used at the manufacturer’s recommended concentrations. Antibodies were incubated for 60 min at 4°C in the dark. Unbound antibodies were removed by centrifugation at 1,200 rpm, 5 min RT and stained cells were resuspended in 250 μL of cold PBS. All samples were analyzed in an Accuri C6 flow cytometer (BD Biosciences) using C6 software (v. 1.0.264.21). Analysis gates were set to record 10,000 live cells. Unstained samples and isotype controls were used to establish dot plot quadrants and mean fluorescence intensity (MFI) parameters.

**Statistical analysis.** Results were compared using unpaired t tests with Welch’s corrections for comparisons between whole (i.e. female + male) groups and one-way ANOVA with Sidak’s multiple comparisons test for comparisons among individual male and female groups, using GraphPad Prism software (v.7.01). Values were considered significant at p<0.05.

**RESULTS**

**Feeding behavior and weight change in diet-restricted mice**

To determine the effect of the dietary restriction regimen on the experimental groups, we analyzed the
changes in feeding behavior, weight and weight gain throughout the study period. In male animals, dietary restriction showed a significant effect beginning after 1 wk of treatment, with diet-restricted mice showing decreased weight (20.417±0.458 g, \( p<0.0001 \)), as well as decreased weight gain (2.157±0.133 g, \( p<0.0001 \)) at the end of the study period, compared to the control group (22.083±0.279 g, weight and 3.233±0.197 g, weight gain) (Figs. 1A, B). Similarly, weight gain was also lower in diet-restricted female animals compared to the control group (2.033±0.151 vs. 2.650±0.138 g, respectively, \( p<0.0001 \)), showing a significant difference in this parameter beginning at week 2 (Fig. 1B); nonetheless, female diet-restricted mice did not show significant differences in body weight compared to controls at the end of the study period (22.700±0.460 vs. 23.417±0.458 g, respectively) (Fig. 1A). There were no significant differences in weight or weight gain within each experimental group. These data suggest that dietary restriction affects weight gain in all mice, although it has a more pronounced effect on body weight in male animals.

As expected from the experimental setup in this study, food intake was significantly lower in the diet-restricted groups throughout the study period, in both male (6.100±0.327 vs. 8.943±0.270 g, \( p<0.0001 \), dietary restriction and control, respectively) and female animals (4.971±0.706 vs. 5.986±0.485 g, \( p=0.0035 \), dietary restriction and control, respectively) (Fig. 1C). There were no significant differences in this parameter within experimental groups. These results suggest that animals in the same experimental group, housed in the same cage, consumed similar amounts of food throughout the study period.

**Leukocyte frequency in brain-derived mononuclear cells**

To determine changes in leukocyte populations within the brain of mice under dietary restriction, we determined the frequency of microglia (CD11b+ CD45lo),
lymphocytes (CD45+CD11blo) and macrophages (CD45+CD11bhi) in brain-derived mononuclear cells after the restriction regimen (Fig. 2A). Group comparisons did not reveal significant differences between control and diet-restricted animals, as they had similar frequencies for microglia (79.60±7.05 vs. 79.23±4.24%), lymphocytes (3.48±3.69 vs. 7.06±4.43%) and macrophages (0.54±0.26 vs. 0.89±0.98%, respectively), although there was an apparent increase in the frequency of lymphocytes in the dietary restriction group (Fig. 2B).

However, when comparing both groups while discriminating by sex, we found significant differences in the frequency of microglia between females and males from the control group (83.99±3.82 vs. 74.34±6.52%; $p=0.021$), as well as in the frequency of lymphocytes...
between females from the control and dietary restriction groups (1.39 ± 1.06 vs. 7.85 ± 4.61%; p = 0.0175) (Fig. 2C). No significant differences were found between males. These data provide an explanation for the apparent increase in lymphocyte frequencies observed in the whole group comparison, suggesting that dietary restriction may be having an effect on lymphocyte accumulation within the brains of diet-restricted female mice.

Microglial expression of MHC-I and II in diet-restricted animals

In order to explore the expression of major histocompatibility molecules on microglia under dietary restriction, we assessed the frequency and mean fluorescence intensity (MFI) for both MHC-I and MHC-II molecules on brain-derived CD11b+ CD45lo cells. Our results indicated no difference in the frequency of MHC-I+ or
MHC-II+ microglia between control and dietary restriction groups, even when compared by sex, with virtually all cells expressing MHC-I (group means: 99.66±0.49 vs. 99.70±0.40%, respectively) and having no detectable expression of MHC-II (Figs. 3A, B).

Nevertheless, MFI analysis for MHC-I expression identified a significant increase in MFI in the diet-restricted group as a whole (8,231±732 vs. 9,667±1,995, arbitrary values; p=0.0436) (Figs. 3B, C), with the disparity being clearly dependent on a significant difference in MHC-I MFI between females from the control and dietary restriction groups (7,854±443.3 vs. 10,628±2,278; p=0.0108), while male animals showed no significant difference (8,683±790.3 vs. 8,513±642.4), although both male groups showed a higher MHC-I MFI than females from the control group (Figs. 3B, D). Hence, our data suggest that dietary restriction promotes an increase in MHC-I expression on microglia in female mice, in the absence of antigenic stimulation.

Lymphocyte expression of MHC-I and -II in diet-restricted animals

Finally, since lymphocytes were present in significant proportions of the brain, MHC-I and -II expression were also examined in isolated brain-derived lymphocytes from control and dietary restriction groups. MHC-I expression was detected in both females and males from control and dietary restriction groups, with a trend towards higher expression in females from the dietary restriction group (Figs. 4A, B). Similarly, MHC-II expression was detected in both females and males, with no significant difference between groups (Figs. 4A, C).
frequencies in samples from brain-derived mononuclear cells, we determined the frequency and MFI for expression of both MHC-I and MHC-II on CD45\(^+\) CD11\(\text{bl}o\) cells. Macrophages were not considered for these analyses, as their frequencies were consistently very low in all samples. Results from these analyses showed no significant differences in the frequencies of either MHC-I or MHC-II between control and dietary restriction groups (MHC-I: 71.56±5.64 vs. 72.32±7.53%; MHC-II: 28.44±5.63 vs. 27.65±7.55%, respectively) (Figs. 4A, B). Similarly, group comparisons by sex did not indicate significant differences in the frequencies of either MHC-I\(+\) or MHC-I\(+/\)MHC-II\(+\) lymphocytes among groups, although male controls appeared to have an increased frequency of MHC-I single positive cells compared to control females (75.58±2.41 vs. 68.21±5.41%, respectively) (Fig. 4C). In contrast, the same male animals had a lower frequency of MHC-I\(+/\)MHC-II\(+\) cells than females from the control group (24.42±2.41 vs. 31.79±5.4%), although this difference was also not significant (Fig. 4C). No differences were observed among dietary restriction groups.

Fig. 5. Fluorescence intensity for MHC-I and II expression in lymphocytes from diet-restricted mice. (A) Histograms for mean fluorescence intensity (MFI) for MHC-I and II staining in gated CD45\(+\) CD11\(\text{bl}o\) brain-purified cells in control and diet-restricted animals. Isotype control is also shown. (B) and (C) MFI for MHC-I and II expression in brain-derived lymphocytes by group and comparing females and males from each group, respectively. Data are presented as mean±SD. n=6 females and 5 males per group. *\(p<0.05\) between female Ct and both male groups. **\(p<0.05\) between CR female and Ct male groups.
Regarding the analysis for MHC-I and MHC-II MFI in lymphocytes, we detected no significant differences in the expression of either molecule between control and dietary restriction groups (MHC-I: 75.112 ± 6.525 vs. 73.437 ± 9.814, arbitrary values, respectively) (Figs. 5A, B). In comparisons by sex, our results did not reveal significant differences for MHC-I MFI among groups, although there was an interesting trend showing decreased MHC-I MFI for diet-restricted females compared with their respective controls (71.715 ± 9.375 vs. 78.630 ± 23.226, arbitrary values), whereas male animals showed the contrary trend, with the dietary restriction groups showing increased MHC-I MFI compared to their respective controls (75.504 ± 11.007 vs. 70.890 ± 7.669) (Fig. 5C). Importantly, MHC-I MFI for lymphocytes was consistently higher than MHC-I MFI for microglia in all groups, demonstrating an increased level of MHC-I expression in brain-derived lymphocytes compared to microglia under both control and dietary restriction conditions in the absence of antigenic stimulation.

In contrast, analysis of MHC-II MFI showed significant differences between females from the control group and both male control (175.432 ± 30.785 vs. 227.993 ± 27.083; p = 0.0281) and dietary restriction groups (175.432 ± 30.785 vs. 224.691 ± 10.257; p = 0.0435), as well as between females from the dietary restriction group and control males (177.045 ± 31.565 vs. 225.993 ± 27.083; p = 0.0348) (Fig. 5C). There appeared to be no difference between control and dietary restriction groups for MHC-II MFI when comparing animals of the same sex, although it was clear that MHC-II expression was consistently higher in male animals (Fig. 5C). Thus, our results suggest that dietary restriction does not affect expression of either MHC-I or MHC-II on lymphocytes in the absence of stimulation.

DISCUSSION

Dietary restriction is a nutritional regimen that has been considered as a therapeutic alternative to prevent cellular changes occurring as a consequence of ageing in a variety of experimental models. It has been proposed as a useful alternative for the modulation of the immune system and the management of chronic degenerative diseases, as experimental evidence suggests that this particular kind of nutritional intervention provides anti-inflammatory, anti-oxidant and neuroprotective effects (13–20).

Dietary restriction has been proposed to inhibit CNS microglial activation under pathological conditions or after antigenic stimulation in vitro by demonstrating decreased morphological switch from the ramified to the amoeboid morphology, as well as decreased staining with the microglial markers ED-1 and Iba-1 (21–23); however, there is no further evidence on the functional effects of this nutritional regimen on immune system cells in the CNS. Furthermore, the possibility of sex-dependent differences in the effects of dietary restriction regimens on the immune and nervous systems has not been properly addressed yet.

The present study demonstrates that dietary restriction causes sex-dependent alterations in the distribution of leukocytes within the brain of mice, as well as in the expression of MHC molecules in CNS leukocytes, in the absence of antigenic stimulation. Importantly, data obtained from body weight and weight gain demonstrate that the 30% dietary restriction regimen used in our experimental setup has a profound effect on these parameters, particularly in male animals, in which it promoted an approximate 2 g decrease in body weight at the end of the study period. Although there were no significant differences in body weight between diet-restricted and control female mice at the end of the study, weight gain was also significantly lower in diet-restricted animals. These results were expected, as dietary restriction has previously been reported to promote reductions in weight gain both in the short and long terms (16).

Furthermore, since our experimental setup required animals to be housed 3 per cage, it was important to determine there were no significant differences in food intake within experimental groups and indeed, our results show that animals in the same experimental group consumed similar amounts of doos throughout the study period, with significant differences observed only between diet-restricted and control groups.

Although no specific tests were used to analyze the fine biochemical effects (such as vitamin and aminoacid concentrations in blood) of the 30% restriction regimen on the experimental animals, this kind of dietary restriction has been shown to be safely sustainable for relatively short time periods (up to 3.5 mo), preventing malnutrition while maintaining beneficial effects on biochemical parameters such as insulin sensitivity and glucose tolerance, as well as oxidative metabolism and inflammation (17). In addition, none of the experimental animals used in the study showed any signs of pathology or changes in normal behavior, suggesting that the restriction regimen did not have adverse effects on the health of these animals, although it would be necessary to address this point further by analyzing biochemical parameters such as liver and pancreatic function in our experimental model.

Our results suggest that a 30% restriction in daily food intake promotes CD45 + CD11blo lymphocyte accumulation in the brain of female, but not male, healthy adult BALB/c mice. Increased frequencies of CNS lymphocytes have usually been associated with antigen recognition within the CNS, as under steady state conditions lymphocyte traffic within the CNS is a highly regulated event where lymphocytes that gain entry into the brain through the blood-brain barrier (BBB) cannot remain within the CNS unless they recognize their cognate antigen being presented by microglia or dendritic cells, leading to activation of antigen-specific immune responses (24). However, it is now known that lymphocytes penetrate the CNS as part of physiological immunosurveillance mechanisms and that the presence of lymphocytes within the CNS parenchyma contributes to tissue homeostasis and immune tolerance in healthy animals (25–27). Therefore, the increased lymphocyte frequen-
cies observed in female animals under dietary restriction do not necessarily point to a pathological mechanism of leukocyte accumulation within the CNS and may rather be related to enhanced immunological surveillance of the nervous tissue under normal conditions.

Similarly, our data indicate a significant increase in MHC-I expression in microglia in the brain of female mice, which is not observed in male animals. Although there was no difference in the frequencies of MHC-I+ microglia among groups, increased MHC-I mean fluorescence intensity demonstrates an increased density of MHC-I molecules on the cell surface. Lack of MHC-II expression by microglia could readily be interpreted as a lack of activation, as proper activation of antigen presenting cells, including microglia, is known to be characterized by increased expression of both MHC-I and MHC-II molecules (5, 6). Along with a virtual lack of MHC-II expression on these same cells, enhanced MHC-I expression by resting microglia could be interpreted as a physiological mechanism for maintaining immune tolerance by presentation of self-antigens in the absence of antigenic stimuli. In addition, our results would be in line with previous reports of the effects of dietary restriction on the activation of microglia, wherein this nutritional regimen prevents microglial activation and their switch from a branched, resting morphology, to an amoeboid, activated morphology (21–23).

Likewise, the observed increase in microglial expression of MHC-I molecules in female mice under dietary restriction would also correspond with our results showing increased lymphocyte frequencies in the brains of the same animals, as enhanced antigen presentation could promote the permanence of lymphocytes within nervous tissue for more extended periods of time. Importantly, the fluorescence intensity for MHC-I expression in microglia was consistently lower than that observed in lymphocytes in all groups, showing that resting microglia constitutively express low levels of MHC-I on their surface. In contrast, we did not find any significant alterations in the expression of either MHC-I or MHC-II molecules on lymphocytes among groups, which would be consistent with a lack of lymphocyte activation within the brains of our experimental animals in the absence of other antigenic stimuli. As such, enhanced microglial expression of MHC-I would not trigger an immune response by presentation of self-antigens in the absence of co-stimulation. Indeed, we did not observe any adverse effects of the dietary restriction treatment on either male or female animals during the course of the study. Importantly, alterations in microglial MHC-I expression and increased lymphocyte frequencies in the brain were not observed in male animals, although lymphocytes in male mice from the control and dietary restriction groups had increased expression of MHC-II, compared to both female groups, an increase which may be assumed to be dependent on MHC-II expression by B cells. Enhanced MHC-II expression on CNS lymphocytes under steady state conditions has not been described previously, so the relevance of these data is unknown.

Hence, our data collectively suggest that a 30% dietary restriction regimen induces sex-dependent alterations to lymphocyte accumulation and microglial MHC-I expression in the brains of mice. Enhanced expression of MHC-I by microglia may promote MHC-I-dependent immune tolerance in the brains of female mice by enhancing self-antigen presentation to lymphocytes in the absence of antigenic stimulation or co-stimulatory signals. Nonetheless, further analyses, such as Western-blot analysis for MHC expression in purified microglia from diet-restricted animals and MHC expression in diet-restricted microglia after antigenic challenge, are required to corroborate the tolerogenic capacity of microglia under dietary restriction conditions, as well as to determine the causes for the sex-dependent effects observed herein.

Author contributions
José Estrada was the author of the study and manuscript, supervised experimental procedures and performed data analysis; Uriel Quijano-Juárez performed experimental procedures and data acquisition; Irazú Contreras contributed to manuscript writing, supervision of experimental procedures and data analysis. The present study did not receive funding.

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