THE ROLE OF EXERCISE IN THE ANTI-INFLAMMATORY RESPONSE OF MICROGLIAL CELLS IN THE HIPPOCAMPUS

by

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ABSTRACT

Alzheimer's disease is currently the sixth leading cause of death in the United States, affecting an estimated 5.1 million Americans. This disease is characterized by amyloid-beta plaques and neurofibrillary tangles, which both lead to impaired neuron communication contributing to memory loss, disorientation, and eventually difficulty speaking and swallowing. Exact mechanisms leading to the development or treatment of AD are not well understood, but certain factors, such as chronic inflammation, are known to expedite onset and progression. That said, previous work from our laboratory showed that following an immune challenge, exercise led to decreased levels of amyloid-beta in mice. From this knowledge, this project was designed to determine specifically why exercise contributed to improved AD pathology in the hilus and subiculum, regions of the brain particularly susceptible to damage that are critical for learning and memory. We hypothesized that one week of exercise in mice would lead to a switch in phenotype of microglial cells, the resident immune cells of the brain, from pro-inflammatory to anti-inflammatory. If this were the case, there would be increased expression of Arg-1, a marker for the anti-inflammatory phenotype of microglial cells. However, results did not show a significant difference in Arg-1 expression across treatment groups. Future studies could increase the amount of inflammation induced, use a wider variety of cell markers to better account for the diverse phenotypes microglial cells can possess, or explore another mechanism by which exercise reduces inflammation.

Key words: Alzheimer's disease, neuro-inflammation, exercise, microglial cells, Arginase-1

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INTRODUCTION

Alzheimer's Disease (AD) is a neurodegenerative disease characterized by amyloid-beta (Aβ) plaques and neurofibrillary tangles. Aβ is a cleaved form of amyloid precursor protein (APP) that forms deposits amongst neurons leading to impaired communication and eventually cell death. Neurofibrillary tangles are a hyper-phosphorylated form of a microtubule-associated protein, tau, which can build up within cells, also causing neuronal death. Secondary to impaired neuron communication, those suffering form Alzheimer's disease exhibit memory loss, disorientation, social changes, and eventually difficulty speaking, swallowing, and even walking [1]. An inability to learn new information is typically the first sign of AD; this is due to the fact that the hippocampus, a region of the brain strongly correlated with the process of learning and memory, is incredibly sensitive to degeneration [2]. According to the Alzheimer's Association, AD is the sixth leading cause of death in the United States. It is estimated that 5.1 million Americans may suffer from AD, costing our nation up to $236 billion dollars in a single year [1]. While AD usually effects the older population, it is not simply a normal process of aging [3]. In fact, about 200,000 people today are living with early-onset AD, defined as onset prior to the age of 65. Only recently has technology improved enough to even diagnose AD while the patient is still alive [4].

At this point, exact mechanisms for the development, prevention, and cure for both early and late onset Alzheimer's disease are unknown. What is evident, though, is that there are both genetic components (i.e., gene mutations, genetic variants) and environmental components (i.e., diet, exercise, toxins, inflammation) contributing to the pathogenesis of AD [5]. Thus, uncovering the mechanism by which each component

contributes to neurodegeneration is critical to discovering the underlying factors involved in AD treatment and prevention. More recently, scientists have begun to study how chronic inflammation, which occurs when there are repeated immune challenges or a malfunction of the immune system, affects the development and progression of the disease. Particularly for late-onset AD, which includes about 90% of all AD cases, chronic systemic inflammation has become a major underlying factor for the progression of this disease as well as multiple other neurodegenerative diseases [6].

Within the realm of neuroinflammation, microglial cells are of particular interest as they are the resident immune cells of the brain. These cells, when functioning appropriately, are responsible for clearing pathogens and cellular debris and maintaining a stable neuronal environment [7]. However, these cells exhibit both pro-inflammatory and anti-inflammatory phenotypes, thereby acting as a double-edged sword [8]. Briefly, microglial cells in one state release pro-inflammatory cytokines and neurotoxic proteins, thus enhancing the immune response. In the other state, they release anti-inflammatory cytokines that lead to reduced inflammation and increased repair and regeneration. That being said, the state of each microglial cell can be altered by changes in the neuronal environment. In fact, when activated by LPS, they have been shown to produce pro-inflammatory cytokines that up-regulate the immune response for relatively prolonged periods of time [9]. This would suggest potential for the onset of an ongoing cycle of inflammation and cellular damage, which would result in an increased rate of progression of AD. As for determining which state particular microglial cells are in, there are a variety of markers that can be measured. The aim of this study was specifically to determine what promotes the anti-inflammatory response of microglial cells. Therefore,

we used an anti-inflammatory marker of microglial cells, Arginase-1, to determine the phenotype. While there are numerous indicators available, Arg-1 is one that has been used in numerous studies and is well established [10, 11].

Previous published work from our laboratory has shown that 7-days of peripheral injections with LPS, a cell wall component of Gram-negative bacteria that promotes an inflammatory response, led to increased levels of Aβ and associated cognitive declines in C57/BL/6J mice. It has been further demonstrated that voluntary exercise results in the reduction of AD pathology from the brain in genetic models of AD [12]. It has already been well established that exercise improves cognition [13], so one of the many explanations for this improved cognition could be the result of cleared Aβ. Following this information, we aimed to determine the cause of this Aβ clearance. We hypothesize in this study that this clearance is secondary to activation of the anti-inflammatory phenotype of microglial cells. Our primary region of focus was within the hippocampus, as it is vital to learning and memory. As it is well known that the hippocampus is one of the most vulnerable regions of AD pathology, we focused on two particular subdivisions: the hilus and the subiculum. Some studies have indicated that the subiculum is critical for Aβ to make its way to the hippocampus [14]. Similarly, the hilus has been shown to be one of the first regions damaged in instances of traumatic injury [15] or ischemic injury [16], suggesting this region is one of the most sensitive regions to degradation. Therefore, if microglial cells in either the subiculum or hilus are clearing Aβ, then the onset of learning and memory deficits due to degeneration of the hippocampus may be significantly delayed.

In sum, this project has been a part of an ongoing study to determine the mechanisms by which exercise reduces inflammation in the brain, thereby potentially delaying or preventing the onset of Alzheimer's disease. Our hypothesis is that one week of exercise induces the anti-inflammatory phenotype of microglial cells in the subiculum and hilus, thereby contributing to the clearance of Aβ and a potential delay in the onset of AD.

METHODS

Subjects

4-6 month old male C57BL/6 mice originating from a breeding stock from The Jackson Laboratory (Bar Harbor, ME) were used for all experiments. Mice from this stock were bred in the TCU vivarium and cared for under the protocol of the Guide for the Care and Use of Laboratory Animals (National Research Council, 2010) as well as the Institutional Animal Care and Use Committee (IACUC) of TCU.

Treatment conditions

Mice were given i.p. injections of either sterile saline (200 μL) or LPS (*Escherichia coli*, serotype 055:B5 Sigma-Aldrich, St. Louis, Missouri) once daily for 7 days. To ensure a dose concentration of 250 μg/kg, LPS was administered in a weight-dependent manner. Following seven days of injections, mice were separated into either exercise or sedentary conditions. Four treatment groups were established as follows: saline-sedentary, saline-exercise, LPS-sedentary, and LPS-exercise.

Housing

All mice were housed in standard polycarbonate cages (12.5cm x 15cm x 25 cm). Animals were individually housed in during the dark phase (1800h-0800h), with or without a running wheel according to their assigned condition, and then were group housed without a running wheel during the light phase (0800h-1800h) for a total of seven days. By moving the mice to individual cages only at night, we were able to ensure that mice of the exercise condition were running equivalent amounts while also minimizing potential isolation stress. Wheel running was monitored (Med Associates, Inc., Albans, VT) to ensure all animals ran equivalent distances.

Tissue collection

48 hours after the last night of exercise/sedentary condition, animals were anesthetized using ketamine (100 mg/kg) and xylazine (5 mg/kg). After proper sedation, mice were perfused transcardially with 1X phosphate buffered saline (PBS; pH 7.4) for 5 minutes followed by 4% paraformaldehyde (PFA; pH 7.4) solution for 7 minutes for fixation of the brain. Tissue was stored in 4% PFA at 2-8 degrees Celsius until they were sectioned into 40 μm sections using a vibratome (Leica Biosystems).

Immunohistochemistry

Tissue sections were stored in 1% PFA with .03% sodium azide in 48-well plates. To remove sodium azide and PFA, sections were washed three times with deionized water for a total of 30 minutes. Sections were then incubated with 1 mL of borohydride for 30 minutes then washed with PBS 3 times for a total of 30 minutes. Sections were blocked

in 1.5% donkey serum in PBST at 4°C overnight. The following day, 500 uL of PBST mixed with primary antibodies, rabbit anti-Iba-1 (1:4000, Abcam, Cambridge, UK) and goat anti-Arg-1 (1:250, Santa Cruz Biotechnology, Santa Cruz, CA), was added to each well. After 24 hours of incubation at 4 °C, the sections were washed in PBST three times for a total of 30 minutes. Sections were then incubated for 24 hours at 4 °C in 500 uL of peroxidase- conjugated AffiniPure anti-rabbit and Donkey anti-goat secondary antibodies (1:1000 in PBS), purchased from purchased from Jackson ImmunoResearch Laboratories, Inc.,West Grove, PA. Following incubation, samples underwent multiple washes in PBS for 45 minutes and then were mounted on slides.

Analysis of cells

Three sections per mouse were mounted on each slide, and four cells per hemisphere of each section were analyzed (for a total of 24 cells per animal). Iba-1 and Arg-1 positive cells were analyzed using a Zeiss LSM 710 confocal microscope (Jena, Germany) at 40x optic with a zoom of 8.2. Individual averages of fluorescence of Iba-1 and Arg-1 were taken for the hilus and the subiculum of each animal (Figure 1). Pixels, area, and background staining were also averaged and taken into account in analysis.

Statistics

Data was analyzed using IBM SPSS statistics software (Armonk, NY). A 2x2 ANOVA was utilized to determine any significant interactions or main effects ($p < .05$). Data in figures are shown as mean ± SEM.

RESULTS

One-week of exercise does not increase the number of anti-inflammatory microglial cells in the hippocampus

Contrary to our hypothesis, there were no significant main effects for treatment or condition (LPS or saline) on arginase-1 expression in the hilus (treatment $F(1,25) = .154$ and condition $F(1,25) = .014$, $p = NS$) or subiculum (treatment $F(1,25) = .235$ and condition $F(1,25) = .872$, $p = NS$), and no significant treatment x condition (exercise or sedentary) interaction for either the hilus ($F(1,25) = .002$, $p = NS$) or the subiculum ($F(1,25) = .010$, $p = NS$). This suggests that one week of exercise did not significantly up-regulate the presence of the anti-inflammatory phenotype of microglial cells in either the hilus or the subiculum as we had expected (Figures 2 and 3).

One-week of exercise does not alter the activation pattern of microglial cells in the hippocampus

No significance was found for Iba-1 in either the hilus or the subiculum (treatment $F(1,25) = .000$ and condition $F(1,25) = .716$, $p = NS$; treatment $F(1,25) = .117$, condition $F(1,25) =. 943$; $p = NS$; treatment x condition $F(1,25) = .222$, $p = NS$), suggesting no significant differences in the microglial cells themselves across groups (Figures 4 and 5).

There were no differences in the number of pixels or the total area of the confocal pictures taken for analysis

As expected, there were no significant effects of treatment or condition on pixels or area of the hilus (treatment pixels $F(1,26) = .348$, condition $F(1,26) = .001$, treatment x

condition F(1,26) = 1.365, p = NS; treatment area F(1,26) = .950, condition F(1,26) = .011, treatment x condition F(1,26) = 1.171, p=NS) and no significant effects of treatment or condition on pixels or area of the subiculum (treatment pixels F(1,26) = 1.194, condition F(1,26) = .436, treatment x condition F(1,26) = .309, p = NS; treatment area F(1,26) = .069, condition F(1,26) = .2.163, treatment x condition F(1,26) = .171, p = NS). By ensuring the pixels and area are not significantly different between animals, any results obtained cannot be attributed to differing settings of the microscope.

FIGURES

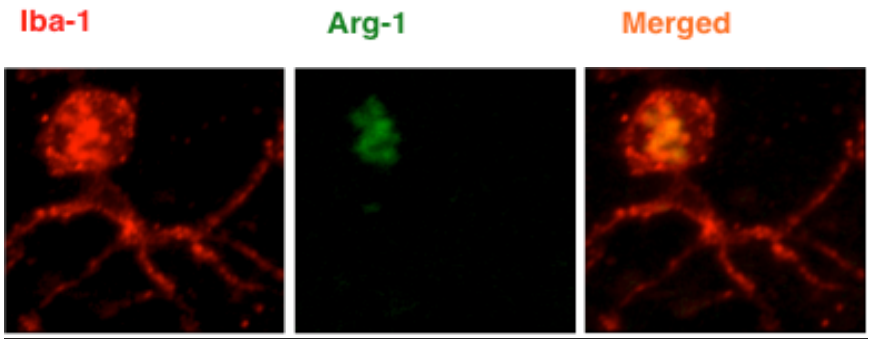

Figure 1. Images of microglial cells under the confocal microscope. Iba-1, a calcium binding protein, was used to identify activated microglial cells. Arg-1 was used to identify the anti-inflammatory phenotype of microglial cells.

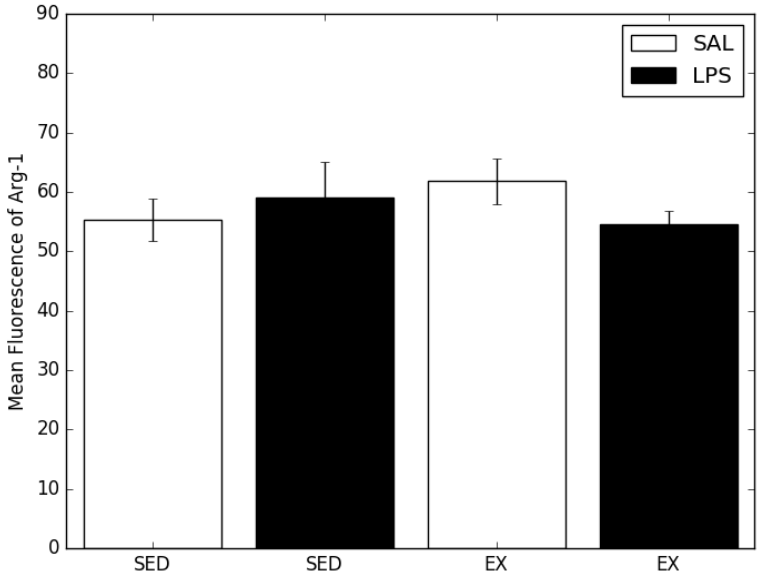

Figure 2. Mean fluorescence of Arginase-1 in the hilus. No significant differences found across treatment groups at $p < .05$.

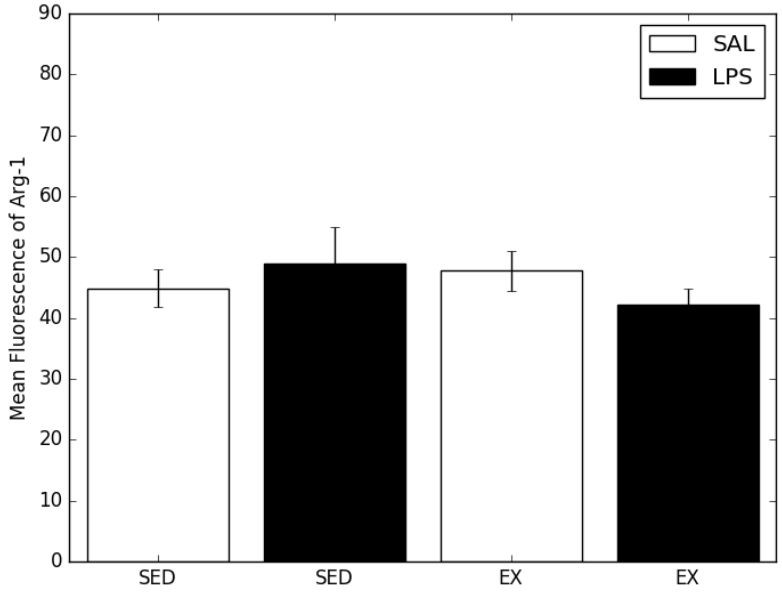

Figure 3. Mean fluorescence of Arginase-1 in the subiculum. No significant differences found across treatment groups at $p < .05$.

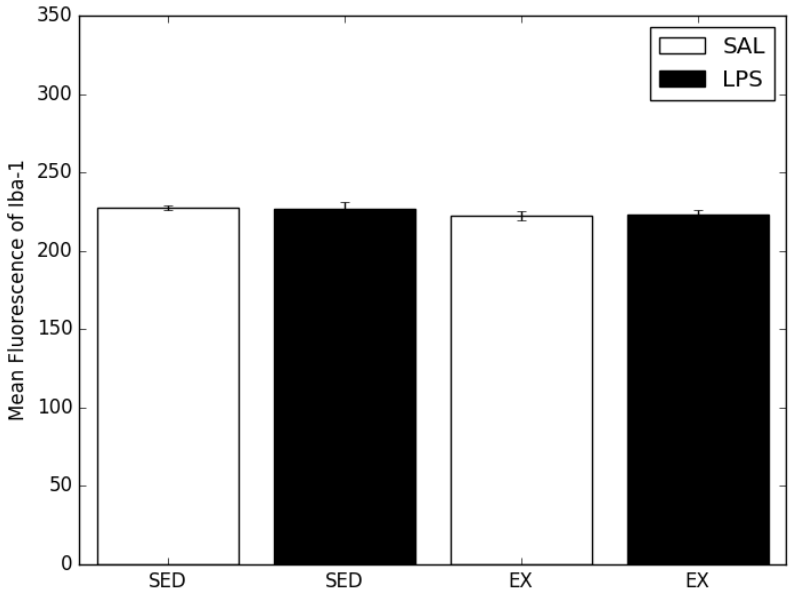

Figure 4. Mean fluorescence of Iba-1 in the hilus. No significant differences found across treatment groups at $p < .05$.

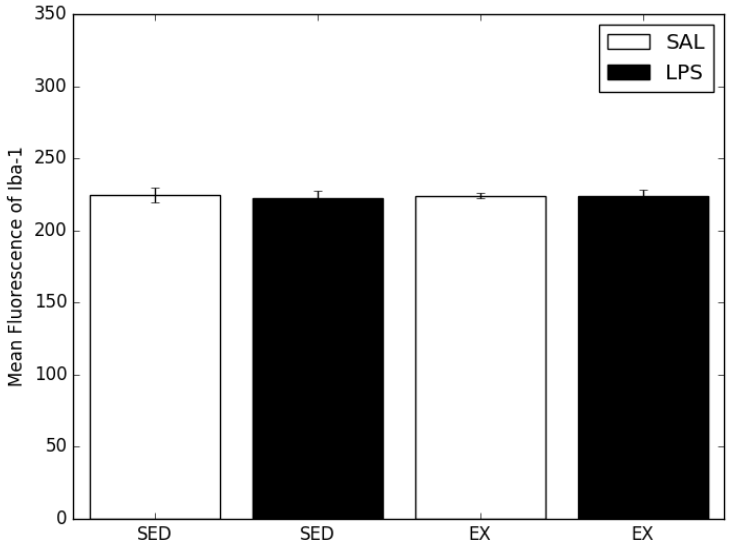

Figure 5. Mean fluorescence of Iba-1 in the subiculum. No significant differences found across treatment groups at $p < .05$.

DISCUSSION

The hypothesis of this study was that one week of exercise would up-regulate the expression of Arginase-1, a maker of the anti-inflammatory phenotype of microglial cells, in the hilus and the subiculum. However, we did not find this to be true. There are numerous possible reasons as to why our hypothesis could not be confirmed. First, one week of exercise may not be the correct amount of time. We currently have preliminary data from two-weeks of voluntary exercise that shows reduction in Aβ; however, we saw that both exercised and sedentary mice had elevated levels of Arg-1. This suggests that both treatment groups had an up-regulation of anti-inflammatory microglial cells after two weeks of recovery, whether they were exercised or not. We speculated that this could be because the sedentary mice eventually "caught up," that is, both end up in the anti-inflammatory state. If exercise sped up the process we were not seeing this in the data because the sedentary mice had long enough to recover. Based off this finding, we decided to look at one week, in case we were missing a critical window of time where microglial cells were switching to the anti-inflammatory phenotype more quickly in exercised mice than sedentary mice. All that said, after one-week of exercise we measured Aβ and found that one week of exercise, unlike two weeks of exercise, did not lead to significant reduction in Aβ [17]. Thus, if anti-inflammatory microglial cells are in fact responsible for the reduction in Aβ, then we would expect elevated levels of Arg-1 after two weeks, but not after one. Our data does show trends in support of this hypothesis. If this is so, one week of exercise is not enough to alleviate AD pathology, so in a "big picture" sense, the activation of anti-inflammatory microglial cells for one week would not make a significant impact on AD.

Another possible theory is that microglial cells are activated differently under different amounts of inflammation. Perhaps altering the dosage of LPS given could result in a different type of immune response, leading to a different prominent form of microglial cell expressed. One study, though not working specifically with neuro-inflammation or microglial cells, provided evidence of this hypothesis. In their study, they found dosage-dependent changes in immune responses, but not always in a linear way [18]. For example, a single injection of LPS significantly suppressed footpad swelling, while two injections produced a less obvious response. Again, while this does not directly correlate with inflammation in the brain, it does show that inducing an inflammatory response may not be as simple as we would like to think.

Further, it is possible that the saline mice could be under stress that is promoting an inflammatory response, resulting in insignificant differences between the control and treatment groups. While we aim to maintain low stress environments, it is impossible to keep animals stress free. Injections themselves, even if the injections are with saline, could induce a mild stress response. Moving the animals to isolated cages at night could also promote stress. Again, we minimize isolation stress by pairing the mice during the day, but they must be separated at night to encourage them to exercise on the running wheels. If this is promoting a stress response, it could be skewing the results of the control.

Lastly, it is possible that Arg-1 may not be the ideal marker for the anti-inflammatory phenotype of microglial cells. Other well-known markers for anti-inflammatory microglial cells include Ym1, a lectin that prevents degradation of the extracellular matrix, or FIZZ1, which mediates interactions between sensory nerves and inflammatory

cells [19]. Using only one marker could be ignoring the diverse and complicated means of microglial cells and it is possible that some could be more sensitive to up-regulation than others. Additionally, it could have been beneficial to also measure a marker of the pro-inflammatory phenotype. Such markers could include pro-inflammatory cytokines released, such as TNF-α, IFN-γ, IL-1β, or a commonly used marker, responsible for production of nitric oxide, iNOS [19, 20]. Forthcoming research should address these concerns by exploring other markers that could ensure that the results are more accurate and that microglial cells truly are not switching to the anti-inflammatory phenotype after one week of exercise. Another reason to measure the pro-inflammatory marker is that it is possible that one week of exercise down regulates pro-inflammatory microglial cells without necessarily up regulating anti-inflammatory microglial cells. In other words, exercise may be decreasing inflammation by suppressing pro-inflammatory microglial cells without actually promoting a switch in phenotype. If this were the case, Arg-1 would not be up-regulated, but inflammation would still be decreased.

Future studies may include alternative amounts of time spent exercising, greater periods of time inducing inflammation to account for mild stress responses from control mice, using numerous different markers to gain a broader understanding of how microglial cells are being activated, or exploring another mechanism by which exercise reduces AD pathology.

CONCLUSION

In sum, while exercise does correlate with decreased AD pathology, we were unable to elucidate the exact mechanism by which this occurs. Our hypothesis that exercise up-regulated the anti-inflammatory phenotype of microglial cells in the

subiculum and the hilus was not confirmed. While this could be attributed to a number of things, such as the amount of time spent exercising, the varying levels of inflammation, or the marker measured, further testing must be done to determine any one reason why our hypothesis was incorrect.

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