HOW EARLY LIFE INFLAMMATION AFFECTS BRAIN AMYLOID-BETA ACCUMULATION FOLLOWING SUBSEQUENT INFLAMMATION IN AGED MICE

by

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ABSTRACT

Alzheimer's disease (AD) is characterized by short term memory loss which progresses into dementia due to the buildup of amyloid beta (Aβ) plaques as well as neurofibrillary tangles. These pathologies in the brain disrupt neuronal signaling and lead to brain atrophy and cognitive deficits. While age is the greatest risk factor in developing this malady, inflammation has been implicated in initiating the production of Aβ in the brain. This study was designed to assess the effects of the bacterial endotoxin lipopolysaccharide (LPS) on inflammation and subsequent production of Aβ in the hippocampi of 22-25 month-old mice that had either received saline or LPS injections when at a young age (4-month-old). Aged C57BL/6J mice were given intraperitoneal injections of saline, and 4 hours later blood was drawn via cheek bleed in order to measure base levels of pro-inflammatory cytokines. The following day the same mice were given intraperitoneal injections of 125 μg/kg of LPS, and 4 hours later blood was drawn via cheek bleed in order to measure acute reaction levels of pro-inflammatory cytokines. These mice were injected with LPS for 2 more consecutive days before their hippocampi were removed and analyzed for levels of Aβ. A control group of aged mice only received saline injections during all 4 days of injections. We were unable to acquire significant cytokine data, but we showed that aged mice who received LPS when they were young had less hippocampal Aβ compared to aged mice that had only received saline when they were young.

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INTRODUCTION

Alzheimer's Disease (AD) is a malady that, unless some major cure or preventative measure is discovered, is projected to affect up to 40% more people in the United States than it already does by 2025. 5.2 million Americans currently suffer from this disease, and this number is expected to increase to at least 7.1 million by 2025 (32). AD typically begins by negatively affecting short term memory and, over the course of the disease, progresses to severe dementia with death occurring within a decade after initial clinical diagnosis, on average (1).

In 1907, Dr. Alois Alzheimer's original paper on the disease discussed his discovery of "miliary foci which are caused by the deposition of a special substance" as well as "striking changes of the neurofibrils" in the brain of a woman exhibiting memory loss and cognitive deficits (2). These "miliary foci" that Alzheimer noticed are now known as senile or amyloid beta (Aβ) plaques and the "striking changes of the neurofibrils" are now known as neurofibrillary tangles (NFTs). The plaques result from the cleavage of amyloid precursor protein (APP) by β- and then γ-secretase enzymes in neurons in the brain (3). Aβ has been determined a major hallmark of AD, and it has been shown to compromise neuronal synapse structure and/or function in the brain, thereby negatively affecting memory and learned behavior (4). Mouse lines which are transgenic for human (h) APP have been used as models to study Aβ and its effect on AD clinical symptoms and, in these mice, it has been shown that inhibiting or reversing synapse deficits also inhibits or reverses cognitive impairment. These findings add further support to the hypothesis that synapse disruption via Aβ is at least one important component of cognitive deficits seen in AD [(5,6,7) reviewed by Mucke and Selkoe (8)].

While Aβ has been generally accepted as a likely source of the memory and cognitive deficits in AD, it has been questioned what actually causes Aβ to present itself in the brain. However, over the past several decades, research has shown that peripheral and central inflammation could act as a potential mechanism in the instigation and continuation of AD, as well as other neurodegenerative diseases such as Parkinson's Disease (9). When a biological insult presents itself in the body an immune reaction and inflammatory response is initiated for defense. The cells which are associated with these inflammatory responses are themselves stimulated by what are known as inflammatory cytokines, which are upregulated in an inflammatory state. Interleukin-1α (IL-1α), IL-1β, IL-6, and tumor necrosis factor- α (TNF-α) are the most potent inflammatory cytokines, and they have been shown to be upregulated in AD brains [(11, 12, 13, 14) reviewed by McGeer (10)]. This neuroinflammation is not just present as a byproduct of Aβ or other pathologies of AD, however: evidence over the past several decades has shown that it is, in fact, a trigger for both neurodegeneration as well as even more neuroinflammation, creating a self-perpetuating cycle (10, 15-16).

How does this cycle of neuroinflammation and neurodegeneration begin? One hypothesis that is gaining support is the notion that peripheral or systemic inflammation can lead to an increase in neuroinflammation (15-16). For example, Cunningham et al. (15) demonstrated that levels of pro-inflammatory cytokines are expressed at low levels in the brain with chronic neurodegeneration but increase significantly when subjects are introduced to lipopolysaccharide (LPS), a bacterial mimetic. This LPS elicits an immune inflammatory response which, in turn, upregulates neuroinflammation (including IL-1β and TNF-α) and results in a decline in cognitive ability. Another study by Kahn et al. (17)

also used LPS to assess the effects of peripheral inflammation on Aβ and cognitive ability. It was found that a single peritoneal injection of LPS resulted in a significant upregulation of IL-1β and IL-6 both in and out of the central nervous system (CNS), that 7 consecutive days of LPS injections resulted in a significant increase in $A\beta_{1-42}$ (a neurotoxic peptide component of Aβ plaques) in the brain, and that cognitive deficits could be attributed to this increase in Aβ due to inflammation.

The greatest risk factor for random onset AD is age. Why is this the case? First, age seems to have an effect on endotoxin tolerance. This is a phenomenon in which prior exposure to a microbial insult programs the defending cell to react less powerfully upon a subsequent recognition of that same microbial insult. This effect is characterized by a diminished release of pro-inflammatory cytokines in order to avoid excessive self-harm due to the inflammatory response (18). As has already been discussed, inflammation has been heavily implicated in the development of neurodegeneration and Aβ, so it seems logical to assume that less regulation of endotoxin tolerance with age would lead to more inflammation in response to infections as one ages, thereby making one more susceptible to AD. Ying Sun et al. isolated peritoneal macrophages from both young (2-month-old) and middle-aged (12-month-old) mice and introduced them to LPS which showed that macrophages from the younger animals produced a significantly higher amount of all cytokines measured, including TNF-α, when compared to the older mice's macrophages. More importantly, however, when the aforementioned macrophages were reintroduced to LPS the younger macrophages produced significantly less amounts of TNF-α, indicating that the ability to develop endotoxin tolerance was more excellent in the younger animals (18). Though this study is not in the CNS, as previously mentioned, there has been a

correlation between increased production of peripheral inflammatory cytokines and production of pro-inflammatory cytokines in the brain (17).

Age has also been shown to have an effect specifically on neuroinflammation via potentiation of pro-inflammatory cytokine response to LPS. Microglia are critical in mediating neuroinflammation within the CNS due to their ability to secrete pro-inflammatory cytokines into neuronal tissue as a response to infection or injury. Frank et al. showed that hippocampal microglia from aged (24-month-old) mice exhibited a potentiated IL-1β and IL-6 cytokine response to LPS administration ex vivo when compared to young (4-month-old) mice. It was also found that expression of several microglial activation markers were also significantly increased in the aged mice (19). These data suggest that increased age plays a significant role in sensitizing one's ability to produce an inflammatory response. Additionally, age has been implicated in an overall increase in the baseline levels of circulating pro-inflammatory cytokines in human centenarians, namely TNF-α, when compared to younger humans. A statistically significant correlation was also found between increased levels of TNF-α and the degree of cognitive impairment/dementia in these individuals regardless of confounding factors such as other diseases (inflammatory or cancerous) or anti-inflammatory medication intake (20).

Of the 5.2 million individuals suffering from AD, five million are age 65 or older, which has begged the particular question: why and how does AD overwhelmingly target those in old age? A novel hypothesis has been recently developed in this lab, questioning if susceptibility to inflammation-induced Alzheimer's disease later on in life could be affected by previous bouts of inflammation earlier in life. The elderly who live in nursing

homes have a high rate of infections, most commonly respiratory, urinary, skin and soft tissue, and gastrointestinal. They also have a high rate of dementia. Our lab wants to investigate if perhaps early life infections, which result in peripheral inflammation, increase the inflammatory response later in life and lead to the onset of dementia- or AD-like symptoms. Our hypothesis is that animals which have already experienced a bout of inflammation via LPS when they were young will have a hypersensitivity to LPS at an old age, leading to higher levels of Aβ in the brain compared to old animals being injected with LPS for the first time.

MATERIALS & METHODS

Experimental Subjects

Experimentally naïve 4–6 month-old male C57BL/6J mice, bred in the Texas Christian University vivarium from a breeding stock obtained from Jackson Laboratory (Bar Harbor, ME), were utilized in all experiments. All subjects were housed in groups of three or four in standard polycarbonate mouse cages. All subjects were on the same 12-h light/dark schedule, and both food and water were available ad libitum. All animals were housed and cared for in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council, 2010), and in accordance with protocols approved by the Institutional Animal Care and Use Committee (IACUC) of Texas Christian University.

Treatment Conditions

Animals were randomly assigned to their original treatment conditions for each of the biological experiments. To determine whether a later immunological insult could alter the level of Aβ (taking into account the previous immunological insult) in the C57BL/6J

mouse, intraperitoneal (i.p.) injections of 250 μg/kg LPS (Escherichia coli serotype: 055:B5; Sigma–Aldrich, St. Louis, MO) or saline were administered for both the first and second injection series. This protocol produced 5 treatment groups depending on the injections received at 4-months-old and then at 22-25 months-old, respectively: LPS-LPS (LL), Saline-LPS (SL), Saline-Saline (SS). Two groups, LPS-None (LN) and Saline-None (SN), were added as control groups in which mice only received injections at 4-months-old before their hippocampal tissue was analyzed. We used the 250 μg/kg dose of LPS on young mice because prior studies had shown that this dose reliably induces sickness behavior and learning deficits in the C57BL/6J mouse, and is a dose that is commonly used for studies in which behavioral measures are collected after injection (21-26). However, we used a 125 μg/kg dose of LPS on old mice in order to avoid a reaction which would result in death as observed in prior experiments.

Tissue Preparation

At the appropriate times after completion of LPS or saline treatment, mice were euthanized and hippocampal tissue samples were extracted and prepared for protein assay and $A\beta_{x-42}$ ELISA procedure. For the ELISA procedure, the tissues were homogenized with protein extraction solution (PRO-PREP, Boca Scientific, Boca Raton, FL) containing protease inhibitors, and were allowed to further lyse for 30 min on ice. The lysate was centrifuged at 16,000 × g for 30 min and the clear lysate removed for DC Protein Assay (Bio-Rad Laboratories, Hercules, CA), and subsequent $A\beta_{x-42}$ ELISA (Covance Research Products, Dedham, MA).

Amyloid-β ELISA Procedure

The Chemiluminescent BetaMark $A\beta_{x-42}$ ELISA (Covance Research Products, Dedham, MA) was performed in accordance with manufacturer instructions. Briefly, the aged mice samples were diluted 20:1 and the young mice samples were diluted 8:1 with working incubation buffer, which includes the HRP-labeled detection antibody, loaded into duplicate wells, and the plate and was incubated over night at 2–8°C. On the following day, the wells were washed and the chemiluminescent substrates were added to each well. The plate was then immediately shaken within the luminometer at room temperature for 15 seconds prior to reading (BMG LabTech FLUOstar Omega, Cary, NC).

Blood Assays

Blood was collected via cheek bleeding of mice 4 hours after a saline injection for a baseline control of cytokine levels, and then blood was collected via cheek bleeding the next day 4 hours after a single injection of LPS. Serum was isolated and used to measure peripheral pro-inflammatory cytokines TNF-α by Mouse TNF-α ELISA MAX Deluxe Set (BioLegend, San Diego, CA), in accordance with kit instructions.

RESULTS

Base Weight Data

At the start of the second phase of this experiment, where on day 1 animals received either a second injection of LPS or saline (randomly assigned), baseline weights were collected. Animal weights, analyzed using one-way analysis of variance, revealed no significant main effect $F(2,26)= 2.205$, $p = 0.13$ (NS). This tells us that there were no

significant differences between groups prior to second phase of injections (data not shown).

Repeated Measures Analysis of Variance (ANOVA) of Weight

During the 3 consecutive days of either saline or LPS injections, weight data was collected prior to each daily injection. There were significant main effects of treatment ($F(2,21) = 3.638$, $p < 0.05$), day ($F(2,42) = 62.24$, $p < 0.001$), and interaction ($F(2,42) = 5.60$, $p < 0.001$). As hypothesized, animals injected with LPS lost significantly more weight than those that had been injected with saline, and that weight loss continued over the three day period. Also, as hypothesized, further post-hoc analysis revealed no significant differences in weight on days 2 and 3 between animals in groups SL and LL, while on both days 2 and 3 groups SL and SS, as well as LL and SS, continue to be significantly different from each other ($p < 0.05$). These data suggest that LPS is having the desired biological effect on the animals consistent with prior studies (17).

Amyloid-β Data

To understand how an early immune insult can later affect inflammatory responses to a similar insult, injections of either LPS or saline were administered later in life (22-25 months of age) to the same mice who received either LPS or saline injections at 4 months of age. This produced a main effect of treatment = $F(4,33) = 33.794$, $p < 0.0001$ (See Figure 1). Fisher's PLSD post-hoc comparisons revealed numerous significant differences between groups (data not shown). Although contrary to our original hypothesis, these data revealed that early immunological insult may in fact lead to reduced reactivity to a later life immunological insult.

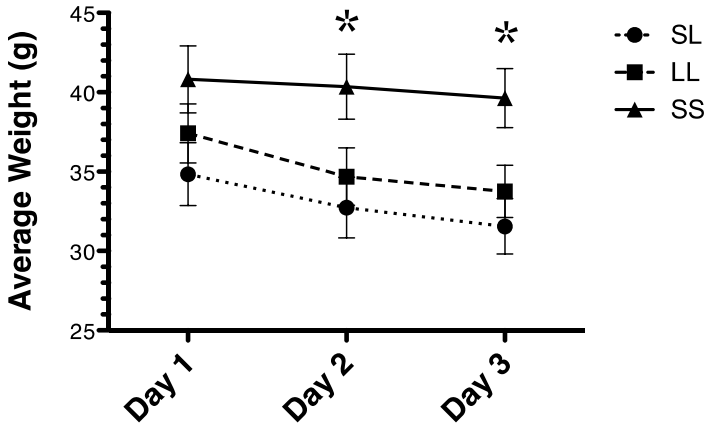

Figure 1. Repeated measures ANOVA for mean weight of animals in all three aged groups following injections of LPS or saline for three consecutive days. Average weights for the mice in groups LL and SL decreased over the three day course of injections and, across all three days, were not significantly different from one another. On days 2 and 3, average weights of mice in the SS group were significantly different from both SL and LL, indicating that LPS had the desired biological effect on the mice regardless of early-life treatment. (*Abbreviations*: LPS: lipopolysaccharide; SL: Saline-Lipopolysaccharide; LL: Lipopolysaccharide-Lipopolysaccharide; SS: Saline-Saline.)

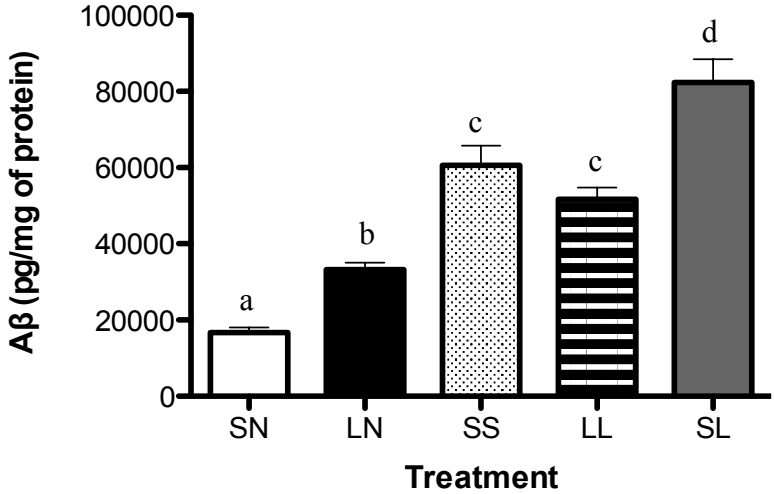

Figure 2. Amount of hippocampal Aβ measured following single intraperitoneal injection of LPS. Groups LL and SL are comprised of animals that encountered LPS during old age, and they are both significantly different from groups SN and LN comprised of animals who received either saline or LPS at a young age ($p < 0.05$). The SS group is comprised of old animals who received saline when young and old and was not significantly different from the LL group, whereas the SL and LL groups are significantly different from each other. Means with different letters (a,b,c,d) are significantly different ($p < 0.05$) from each other. Bars represent mean ± SEM. (*Abbreviations*: Aβ: amyloid beta; SN: Saline-None; LN: Lipopolysaccharide-None; SS: Saline-Saline; LL: Lipopolysaccharide-Lipopolysaccharide; SL: Saline-Lipopolysaccharide.)

DISCUSSION

The purpose of this study was to determine whether the production of hippocampal Aβ elicited by peripherally-injected LPS is affected by a similar inflammatory response, or lack thereof, earlier in life. Inflammation has been implicated in causing acute upregulation of Aβ in the brain (17), and age has been implicated in increased baseline levels inflammation as well as potentiation of neuroinflammatory responses (19-20), so it makes intuitive sense that aged animals would have higher levels of baseline Aβ in their hippocampi when compared to young animals. Indeed, results seen in *Figure 2* reveal that our data supports the aforementioned idea: the SS group of aged mice has significantly more hippocampal Aβ than the LN and SN groups, both of which consist of young mice. In other experiments from this lab it had been noticed that aged mice that had received LPS at a young age would die upon repeated injections of the same dose of LPS at old age. Because of these observations we opted to decrease the LPS dose for old animals in our experiment. However, due to increased susceptibility of aged animals to inflammation, their decreased ability to develop endotoxin tolerance (18), and the link between inflammation and Aβ production it was hypothesized that the LL group of mice would have significantly more hippocampal Aβ when compared to the other groups. As our data reveal, however, that is not the case (see Figure 2). Contrary to our hypothesis not only does the SL group have significantly more Aβ than the LL group, but also the LL and SS group are not statistically different from each other. These results seem to contradict intuitive reasoning, which means some other factor(s) must be involved.

As shown by the weight data on both Day 2 and Day 3 (see Figure 1) there is no statistically significant difference in weight between SL and LL, but both SL and LL weight losses are statistically different from SS (which had no significant weight loss). This shows that the immediate injections of LPS had the desired biological effect, as mentioned in the prior section. Also, due to having serum from a small number of aged mice in the study it was determined that cytokine analysis should be performed in order to measure the inflammatory response and correlate the results with the Aβ data. To that effect we attempted an ELISA for TNF-α. However, due to the limited amount of serum per subject as well as too small of a sample number of subjects, no significant data could be gained (data not shown). Measurement of cytokines should be a priority going forward in performing a follow up of this study in order to determine if our treatment conditions alter the innate inflammatory response. However, we do not believe that the inflammatory response is attenuated in these old mice for a couple reasons: a) literature concludes that inflammation typically worsens with age and b) our observations that old mice injected with LPS (which had encountered injections early in life) tended to decline rapidly and die during prior experiments in the lab. These lethal inflammatory responses in old mice are, in fact, what led to the genesis of this project and the source of our original hypothesis that this would correlate with also-heightened levels of Aβ. The remainder of this discussion will focus on possible explanations for our observed Aβ results.

First, one needs an explanation of the mechanism by which LPS is processed by the body in order to determine if age affects this process. Specifically, *Escherichia coli-derived* LPS acts as an agonist against toll-like receptor 4 (TLR4) which is an cell surface

receptor utilized by the various cells of the immune system including macrophages, neutrophils, and dendritic cells (DCs) (18, 27-28). At the site of infection/LPS injection macrophages and neutrophils will encounter LPS, which in turn binds to TLR4 and initiates a signaling cascade to ultimately produce pro-inflammatory cytokines. Upon subsequent encounters with LPS, however, endotoxin tolerance develops within these macrophages and neutrophils in order to prevent sepsis. This is usually accomplished by a reduction in expression of TLR4 and many of the signaling components inside the cell (18, 28). Therefore, downstream proinflammatory cytokine production is reduced, as has been shown by Kahn et al. after seven consecutive days of LPS injections (17). However, being part of the innate immune system (though dendritic cells do play a role in adaptive immunity), these cells tend to be short lived – the original, tolerant cells which interacted with LPS injected in a mouse while it was young would not be present in the same mouse should it encounter LPS at an old age up to 20 months later. Therefore, any kind of memory response would not be likely from these cells.

A unique type of cell in the peritoneal cavity, however, could potentially provide some sort of long-lived response or form of memory. B-1 cells are an important cell population in the peritoneal cavity of mice with unique characteristics including the ability to self-replicate and participate in immunological memory. B-1 cells also interact with other cells of the innate immune response which cause B-1 cell proliferation and differentiation into antibody-secreting plasma cells (29-30). It has even been shown that LPS (and other TLR agonists) play an unexpected role in eliciting a B-1 cell response to infection in the peritoneal cavity by regulating these cells' surface proteins and their

ability to migrate throughout the body (31). Theoretically, these cells could explain why Aβ levels were lower in LL animals who received (and survived) LPS reintroduction.

When encountering LPS for the first time as young animals a normal immune response would have mounted and caused typical inflammation and sickness behavior. However, another normal response would be that the B-1 cell population expanded within the peritoneal cavity. Due to self-replication and indefinite existence in the peritoneal cavity this expanded population of cells would continue to constitute a larger percentage of the overall cell population in this area as other, short-lived cells (i.e. macrophages and neutrophils) died during a normal aging process. With a higher ratio of B-1 cells per amount of LPS injected at old age, along with their possible memory function, these cells could phagocytose/neutralize a larger amount of LPS and, therefore, decrease the production of peripheral Aβ. Inflammation would still be present or increased despite decreased numbers of other innate immune responders, however, due to the previously-mentioned discovery that old-age cells (macrophages and microglia) exist in an inflammation-producing sensitized state. Another possibility is that B-1 cells could have APP- or Aβ-specific antigen receptors that recognized APP or Aβ produced following the initial injection of LPS and subsequently proliferated to increase their number. Then, upon reinjection of LPS at old age, this expanded pool of specific B-1 cells recognized the APP or Aβ proteins and endocytosed them through their antigen receptors in the periphery. This would have eliminated Aβ from the peripheral pool and effectively reduced it from reaching the CNS. Such a possibility would provide an intriguing piece of evidence in support of the theory of vaccination against AD. More studies need to be performed in order to analyze B-1 cell response in young and old mice

following LPS injections, as well as in-depth cytokine analysis to determine the level of inflammatory response elicited in these animals.

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