

THE ROLE OF B CELLS IN TARGETING ALZHEIMER'S PATHOLOGY

by

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ABSTRACT

Alzheimer's disease is the foremost type of dementia that causes deficits in memory, thinking, and behavior. Alzheimer's disease is the sixth leading cause of death in the United States, yet there is no known cure. Amyloid-beta ($A\beta$) is a peptide fragment that at normal levels of expression helps memory and learning by improving long-term potentiation (LTP). Too much $A\beta$ will diminish LTP because $A\beta$ will accumulate in synapses and block the signaling of neurotransmitters, thus leading to the deficits characteristic of Alzheimer's disease. Our lab has proven that acute inflammation leads to an increase in $A\beta$ in the hippocampus of mice. $A\beta$ levels in the hippocampus, however, do not increase or lead to more cognitive deficits if a second bout of LPS-induced inflammation is administered to mice while the $A\beta$ levels from the first LPS injections are still significantly high. The objective of this study was to determine how mice were conferring resistance to this second bout of LPS. We investigated the antibodies produced after the second course of LPS in 5xFAD mice. We observed co-localization of IgG and IgM around the amyloid beta in the brain, suggesting these antibodies might specifically target amyloid beta. Furthermore, we wanted to investigate the source of these antibodies in the brain. We stained for CD19, a specific marker for B cells, in the hippocampus of 5xFAD mice. We found what we believe are B cells diffuse throughout the brain tissue. Therefore, B cells are crossing the BBB and releasing their products, IgG and IgM, at the site of inflammation.

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TABLE OF CONTENTS

INTRODUCTION	1
METHODS.....	4
Mice.....	4
Injections.....	4
Immunohistochemistry.....	5
RT PCR.....	5
RESULTS.....	6
FIGURES.....	7
DISCUSSION.....	9
CONCLUSION.....	10
REFERENCES	11

INTRODUCTION

Alois Alzheimer first described the neurodegenerative disease known as Alzheimer's disease (AD) in 1901 [1]. A 49-year-old woman, Auguste, was brought to a mental hospital in Frankfurt, Germany where Alois Alzheimer, a physician specializing in neuropathology, interviewed her. Auguste showed signs of disorientation, forgetfulness, and problems with reading and writing. Auguste's condition worsened over the next several years while in the mental hospital. Eventually she experienced hallucinations and lost all cognitive function. When she died in 1906 at age 55, Alois performed an autopsy of her brain. Alois presented his findings of brain atrophy and two abnormalities in her brain—neurofibrillary tangles and amyloid plaques [1]. Today, the pathological diagnosis of AD is very similar to how Alois interviewed Auguste upon her arrival at the mental hospital. Little did Alois Alzheimer know, this form of dementia would be termed Alzheimer's disease and would become a leading cause of death in the world.

Alzheimer's disease currently affects more than 5.4 million Americans and is the 6th leading cause of death in the United States [2]. Alzheimer's disease is a degenerative brain disease and the most common form of dementia. One in three seniors dies with AD or another form of dementia [2]. Symptoms of Alzheimer's disease can present themselves differently in individuals and progress at different rates. Early symptoms include difficulty remembering new information, names, events, and disorientation. Later symptoms consist of impaired communication, confusion, behavior changes, and difficulty speaking and walking. In these later stages, a person living with AD will need help with their day-to-day activities, such as cooking their meals and showering. Therefore, Alzheimer's disease also profoundly affects the loved ones of people suffering from AD. In 2015, an estimated 15 million caregivers provided 18.1

billion hours of unpaid care [2]. AD cost the nation 236 billion dollars last year and is estimated to cost over one trillion dollars by 2050, as the prevalence of people over the age of 65 with AD nearly triples [2]. The damage done by Alzheimer's disease is irreversible.

Currently, the exact mechanisms for the prevention and cure of Alzheimer's disease are unknown. The medications currently available for AD do not slow or stop the progression of the disease. These medications merely aim to improve symptoms on a daily basis by increasing the amount of neurotransmitters in the brain [3]. One in nine people over the age of 65 have AD and one in three people age 85 and older have AD [2]. As medical advances increase the life expectancy of future generations, it is anticipated that the prevalence of Alzheimer's disease will grow substantially. The "baby boomer" generation has also begun to reach the age 65 and older and therefore many new cases of AD will be presented. Over time, an AD patient's cortex shrivels, the hippocampus shrinks, and ventricles grow. These changes in the brain associated with Alzheimer's may begin up to 20 years before symptoms appear [3]. A cure for AD is clearly at the forefront of our nation's concern as researchers race to beat time.

One of the main biological hallmarks of AD includes amyloid-beta ($A\beta$) plaques composed of $A\beta$ aggregates [4]. $A\beta$ is a 42 amino acid protein that is formed upon cleavage of amyloid precursor protein [4]. It is postulated that $A\beta$ plays a role in neurons ability to adapt over time. In a healthy brain, $A\beta$ can be cleared effectively. In AD, these protein fragments accumulate to form plaques. Enzymes, such as β -secretase and γ -secretase, cleave APP into $A\beta$ in response to a stimulus, such as inflammation [4,5]. Upon over production of $A\beta$, the $A\beta$ peptides are able to cross the blood-brain barrier (BBB), enter the brain, and aggregate into senile plaques [5]. These plaques will further exacerbate inflammation and are neuro-toxic. Ultimately, $A\beta$ plaques lead to neuronal cell death.

It is unknown what causes the overexpression of A β in Alzheimer's disease. Genetic mutations in APP, or the enzymes that cleave APP, leading to A β , only account for 5% of known AD cases [6]. However, there are many known risk factors that exacerbate the pathology of AD. There is much evidence that supports chronic inflammatory diseases, such as cardiovascular disease and hypertension, increase the risk of developing AD [7]. Studies in our lab have shown that neuroinflammation can be stimulated by repeated bouts of inflammation via LPS injections. Kahn et al. demonstrated that 7 consecutive days of peripheral LPS administration in nontransgenic mice leads to elevations in A β in the hippocampus [8]. These mice also demonstrated hippocampal hippocampus-dependent cognitive deficits.

Our lab has previously shown that an acute bout of inflammation induced by 7 days of LPS leads to significant increases in levels of A β in the hippocampus. These elevations in A β also led to cognitive dysfunction [8]. Our lab also determined that 15 days following a 7-day period of LPS injections A β were still significantly elevated over controls [8]. Not until 23 days post injections did A β levels return to non-significant levels and cognitive function was restored [8]. Our lab therefore hypothesized that a second bout of LPS induced inflammation could lead to even more significantly elevated A β levels compared to mice that received only one bout of LPS. In addition, it was hypothesized A β levels would also remain elevated for an additional 15 days after the second bout of inflammation. Hardy et al. unexpectedly found that there was no difference in CFC performance and A β levels following a second bout of LPS injections. Cognitive function remained unaffected 15 days post a second LPS injection [8].

Therefore the purpose of this study was to determine why mice did not produce A β following the second bout of LPS injections. We wanted to look into the mechanism of how mice are able to confer resistance to additional acute inflammatory stimuli. Specifically, we

looked at the role of B-1 cells. B-1 cells can be found mainly in the peritoneal and pleural cavities [9]. B-1 cells express surface immunoglobulin receptors that, upon activation, can lead to B-1 cell differentiation into plasma cells and secretion of antibodies [9]. It is known that B-1 cells have memory function and recreate immunoglobulins in response to LPS [9,10]. We hypothesized that LPS induced inflammation leads to an expansion of the B-1 cell population that is protective against inflammation and amyloid beta aggregation.

METHODS

Mice:

Experiments were performed using 5xFAD transgenic mice. This transgenic model presents with many phenotypic similarities to what is seen in AD and have a relatively early and aggressive presentation. Importantly, the 5xFAD transgenic mice rapidly develop amyloid pathology. 5xFAD mice were bred in the TCU vivarium under proper animal care and safety controls approved by the Institutional Animal Care and Use Committee (IACUC) of Texas Christian University. Mice were on the same light/dark schedule; lights were on from 0700-1900 every day.

Injections:

Mice were randomly assigned to either the experiment (LPS) or control group (saline). Mice were given intraperitoneal (IP) injections once daily of either sterile saline (200 μ L) or LPS

(*Escherichia coli*, serotype 055:B5 Sigma-Aldrich, St. Louis, Missouri) for 7 days per each bout. LPS was administered in a weight dependent manner to ensure correct dosage was administered.

Immunohistochemistry:

All mice were euthanized via transcardial perfusion with PBS, fixed in 4% paraformaldehyde, and brains were removed for fluorescent immunohistochemical analysis. Three sagittal brain sections (10 μ m) in series were randomly selected from each animal. Sections were washed three times for 10 minutes each in phosphate buffered saline + 0.05% Tween-20 (PBST). Afterwards, sections were placed in 500 μ l blocking solution overnight (PBST + 2% donkey serum). Primary antibodies used were 6e10 (1:1000), IgM (1:1000), and IgG (1:1000). Tissue sections were incubated overnight in primary antibody at 4°C. The next day three 10-minute washes with PBST were performed. Appropriate cyanine-conjugated secondary antibodies (for 6e10: donkey anti-mouse Cy2, 1:500; for IgM and IgG: donkey anti-goat Cy3, 1:500) were applied for 4 hours at room temperature while protected from the light. After 4 hours, another round of three 10-minute washes with PBST were performed. Sections were then mounted on slides and cover-slipped.

RT-PCR

After CO₂ euthanasia, the hippocampal tissue was removed under RNase-free conditions. Total RNA was isolated from hippocampal tissue using Maxwell 16 LEV simplyRNA Tissue Kit (Promega, Madison, WI). After analysis for purity with NanoDrop, RNA stock was standardized

so that equal amounts of each sample were used for cDNA synthesis using Invitrogen SuperScript III kit. Quantitative RT-PCR was performed on a thermal cycler using SsoAdvanced SYBER green reaction mix (ThermoFisher Scientific, Waltham, MA). All kits were used following manufacturer protocols. CD19 expression was assessed using the primers. Two targets were chosen, purchased from Integrated DNA Technologies (Coralville, IA): CD19 (Primer 1: 5'-CCACCAGAGAAACCATACAGAA-3'; Primer 2: 5'-CACGTGAAGGTCATTGCAAG-3') and HPRT (Primer 1: 5'-AACAAAGTCTGGCCTGTATCC - 3'; Primer 2: 5'-CCCCAAAATGGTTAAGGTTGC-3').

RESULTS

It is known that IgG is capable of crossing the blood brain barrier (BBB) [11]. Previous work in our lab determined IgG production increased with LPS-induced inflammation, crossed the BBB, and aggregated around A β plaques. Female 5xFAD⁺ mice were injected with LPS for one week, followed by 2-week recovery period and a second week of LPS injections. Brain tissues were removed 24 hours after their last injection. Hippocampal tissue and blood samples were collected and analyzed by ELISA to determine antibody concentration. Brain tissues were collected for immunohistochemistry and confocal microscopy imaging to determine antibody presence around A β in the hippocampus. Data revealed an increased amount of plasma IgG present in mice that were given two bouts of LPS injections versus saline controls. In addition, IgG co-localization with hippocampal A β plaques was observed. Furthermore, staining for IgM was conducted and it was observed IgM also aggregated around A β plaques (Figure 1).

Next we stained sagittal sections with anti-CD19 in the hippocampus of 5xFAD⁺ mice. B cells are the only cells in the human body that express CD19. We observed what we believe are B cells to be diffuse throughout the brain tissue (Figure 2). If you zoom in on the red fluorescence even further, it is visible that each aggregate is actually composed of several B cells clustered together.

After the detection of B cells in the brain, we used RT-PCR to detect mRNA expression for B cell production. RT-PCR allowed us to quantify the amount of expression for B cells in all four of our treatment groups. We observed no significant difference in the LPS or saline treated groups, and no difference between the 5xFAD⁺ and 5xFAD⁻ mice.

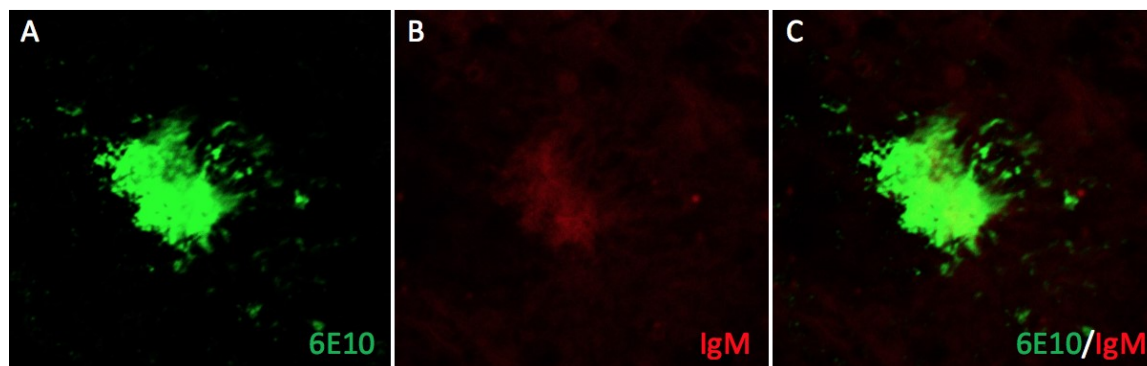


Figure 1. IgM co-localization on amyloid beta plaques in 5xFAD hippocampus. Staining demonstrates the presence of IgM antibody co-localization, similar to that of IgG. Panel A is staining for amyloid beta plaque alone, and panel B shows the same plaque area with IgM staining alone.

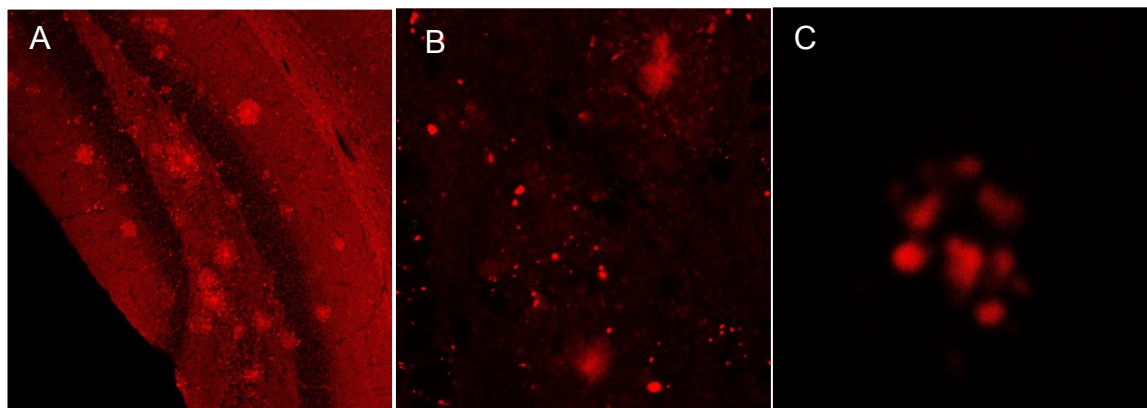


Figure 2. CD19 expression in 5xFAD hippocampus. Sagittal sections of 5xFAD mouse brain were stained with anti-CD19 antibody. Panel A is CD19 expression in 5xFAD hippocampus. Panel B shows CD19 expression in another region of the brain, suggesting B cells are not localized in the hippocampus. Panel C shows a similar image at higher magnification.

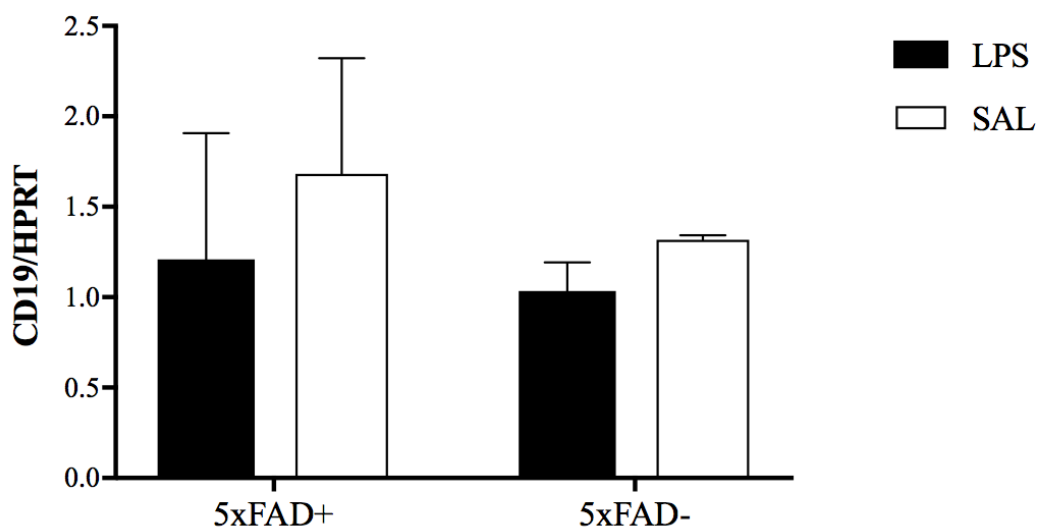


Figure 3. Quantification of CD19 in hippocampal tissue. qRT-PCR was used to analyze levels of CD19 in hippocampus. Values shown are CD19 expression divided by HPRT expression (used as a control). One-way ANOVA reveals no significant differences between LPS and saline-treated animals, or between 5xFAD-positive and negative mice.

DISCUSSION

Previous studies have not found significant BBB differences between 5xFAD and non-transgenic mice [12]. IgM is too large to freely cross the BBB and enter into the brain [13]. However, our data showing that IgM can be found in the hippocampus suggests the BBB is altered in our LPS/FAD mouse model (Figure 1). IgM may be entering the brain in one of two ways. IgM is either actively being transported into the brain or B cells cross the BBB and produce IgM at the site. Our results implicate the role of the adaptive immune system in targeting A β plaques in 5xFAD mice. Therefore, infiltration of IgM into the brain indicated a need to investigate B cell trafficking to the brain to determine the source of hippocampal antibodies. Our results showed IgM co-localized around A β . It can be proposed that IgG and IgM are anti-A β due to the co-localization of IgM with A β in the hippocampus.

Sections of the hippocampus stained with anti-CD19 antibody show that CD19 staining is diffuse throughout the brain tissue (Figure 2). Negative controls confirmed CD19 was a specific marker for B cells and did not stick nonspecifically to FC receptors. Therefore since CD19 is a specific marker for B cells, B cells must be entering the brain and producing antibodies that could potentially be anti-amyloid beta.

The results from the RT-PCR did not show statistically significant differences between the four treatment groups. There was no difference between the 5xFAD+ or 5xFAD- groups, and in addition, there was also no difference whether the mice received LPS or saline. Unfortunately, these results could potentially be confounded with the fact that we did not perfuse the mice brain tissues before running the assay. There is a lot of vasculature in the hippocampus, and typically B cells will be found there. Therefore, any B cell that could have been floating around in the

vasculature at the time of tissue removal would have been collected and quantified in our results. We cannot be sure if we solely measured B cells in the brain or B cells in the brain and the vasculature. Consequently, we need to repeat this assay and perfuse the brains to control for this factor in the future.

CONCLUSION

We have shown antibodies, IgG and IgM, are in fact crossing the blood brain barrier of 5xFAD mice administered two bouts of LPS. The mechanism of how these antibodies are crossing the blood brain barrier continues to be under investigation. We believe that upon LPS administration, the BBB of 5xFAD mice become more leaky, thus allowing B cells to cross. Once in the brain tissue, B cells release their products, IgG and IgM. However, B cells were discovered in the brain tissue of all four treatment groups of mice we used in this study. Future studies need to be done to examine whether this is a novel function of B cells or if B cells are crossing the BBB upon a change in permeability to the BBB after an inflammatory response. Furthermore, studies need to be conducted on whether these antibodies in the brain are anti-amyloid beta or anti-LPS. If in fact these antibodies are anti-amyloid beta, we could potentially be on the path to the development of a vaccine for Alzheimer's disease by preventing the build up of amyloid beta in the brain.

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