B-CELL RESPONSE AND ANTIBODY PRODUCTION IN AN INFLAMMATORY MODEL OF ALZHEIMER'S DISEASE

By

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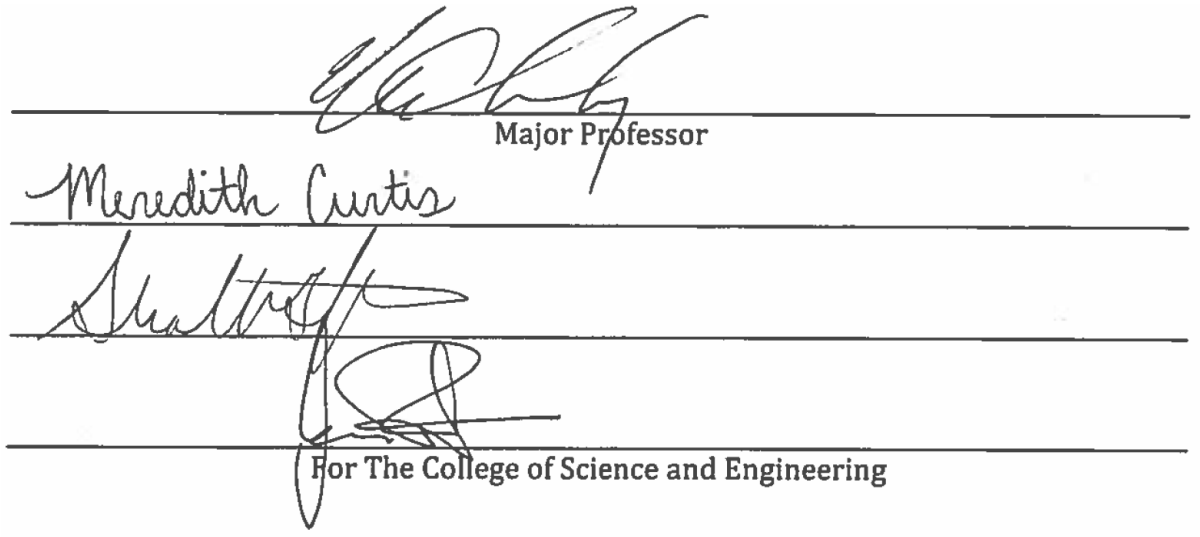

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1. Introduction

1.1 Characteristics of Alzheimer's disease

Alzheimer's disease (AD) is the most common form of dementia and the sixth leading cause of death in the United States (Tarawneh and Holtzman, 2012). It is estimated that more than 24 million people worldwide suffer from the disease. Furthermore, the rate of prevalence for AD rises exponentially with age. With advancements in modern medicine significantly increasing the life expectancy for humans, the number of adults with AD is expected to double in twenty years (Mayeux and Stern, 2012). As the disease progresses, mental function begins to deteriorate until eventually patients are completely unable to take care of themselves and require fulltime care. As a result, most AD patients reside in long-term care facilities for the last few years of their lives, until the disease progresses so far that their bodies can no longer function at all. The substantial amount of care that AD patients require, and the sheer number of patients we could be facing in the near future, will create an incredibly costly public health burden. It is estimated that AD healthcare will cost the nation $259 billion in 2017, and these costs are predicted to rise to as much as $1.1 trillion by 2050 (Alzheimer's Association, 2017).

Alzheimer's disease is usually diagnosed in a clinical setting, and begins with mild short term memory deficits. Patients experience difficulty remembering recent events and conversations, and this progresses to an inability to remember close friends and family members (Heneka and O'Banion, 2007). As the disease advances, severe dementia develops and patients exhibit multiple cognitive and behavioral deficits, with most eventually becoming bedridden. Basic motor functions such as swallowing and

walking become impaired and death typically occurs five to ten years after diagnosis (Tarawneh and Holtzman, 2012).

1.2 Amyloid beta

One devastating issue facing patients is that AD is difficult for health practitioners to diagnose. The disease can only conclusively be diagnosed through autopsy, when pathologists can examine a patient's brain and determine the presence of AD pathology (Murphy and LeVine, 2010). In living patients, physicians must apply an approximate diagnosis, and there is currently no single definitive test. Instead doctors use a variety of approaches for a clinical diagnosis, including cognitive and psychiatric tests, examination of family history, and brain imaging. Tragically, diagnosis typically comes much too late. While patients do not seek medical help until they start experiencing memory loss, actual onset of the disease typically begins as many as 15 years prior to clinical symptoms (Tarawneh and Holtzman, 2012). Many non-demented individuals exhibit AD pathology at autopsy, and this condition of early histopathology without detectable cognitive impairment is categorized as preclinical AD (Goldman et al., 2001).

One of the main pathologies associated with AD are amyloid beta (Aβ) plaques, which aggregate in the brains of patients. Aβ is a small peptide that is a product of the cleavage of amyloid precursor protein (APP). APP is a transmembrane protein found in high levels in the brain, and while its specific physiological function is as yet undetermined, it has been suggested to play a role in various cellular processes including cell adhesion and neuronal protein transport (Zhang et al., 2011). APP is processed rapidly in neurons, and it undergoes proteolysis during this processing. It

can be cleaved through multiple different enzymatic pathways, and some of these pathways lead to Aβ production while others do not. For example, cleavage by the proteases BACE1 and γ-secretase results in the production of Aβ, but cleavage by α-secretase followed by γ-secretase does not (O'Brien and Wong 2011). Soluble Aβ is secreted by neurons and other cell types, and it can be detected at low levels circulating in the blood and cerebrospinal fluid of non-demented individuals (Bates et al., 2009; Bateman et al., 2006). Though the precise function of the protein is unknown, at low levels Aβ has been found to be important to learning and memory (Hardy and Selkoe, 2002). In healthy individuals $A\beta_{1-40}$ is the predominant form that is produced, but mutations in one of three different genes (*APP, PSEN1,* and *PSEN2*) causes overproduction of Aβ and accumulation of the longer, more toxic $A\beta_{1-42}$ form of the protein (Bateman et al., 2006). These mutations cause early-onset Alzheimer's disease, usually developing in individuals before the age of 65. However, these instances of AD with a strong genetic link are incredibly rare, accounting for less than 1% of cases of the disease (Alzheimer's Association, 2017; van der Flier et al., 2011). Most cases of AD are spontaneous, and the underlying reasons for this sudden overproduction of amyloid beta are not fully understood. For the vast majority of patients living with Alzheimer's, the cause of the disease remains undetermined.

1.3 Inflammation

Though the causes of AD are not fully understood, certain lifestyle factors and conditions are known to increase a person's risk of developing the disease. Furthermore, many of these risk factors have an underlying inflammatory component. Several risk factors for AD such as obesity, sedentary lifestyle, and systemic infection

increase levels of pro-inflammatory cytokines in the blood over a person's lifetime. The elevated inflammatory state can also affect the brain, and this neuroinflammation accelerates cognitive decline and brain atrophy (Heneka et al., 2014). The link between AD and inflammation has been studied extensively. Just as inflammation has been shown to increase the risk of AD, Alzheimer's pathology is also well understood to cause inflammation. The presence of AD pathology in the brain initiates multiple different inflammatory mechanisms, including the recruitment of microglia which localize around plaques (McGeer et al., 1987). These microglia then enter an activated state in order to phagocytose the plaques, causing an upregulation of inflammatory cytokines and chemokines (McGeer and McGeer 2003; Solito and Sastre, 2012). Furthermore, this elevated inflammatory state in the brain has been shown to aggravate AD pathology and accelerate neurodegeneration (Craft et al., 2006). Treatment with non-steroidal anti-inflammatory drugs (NSAIDS) helps to alleviate some of this neuroinflammation and has even been found to delay the onset of the disease, lending further support to the fundamental link between inflammation and AD (Breitner et al., 1995).

In rodent studies, lipopolysaccharide (LPS) is frequently used to induce an inflammatory response. LPS is a bacterial endotoxin found on the outer membrane of Gram-negative bacteria. It is recognized by Toll-like receptor 4 (TLR4) and initiates systemic inflammation in the exposed host (Juskewitch et al., 2012; Qureshi et al., 1999). LPS induces production of pro-inflammatory cytokines including TNF-α, IL-6, and IL-1β (Li et al., 2015). In AD mouse models, LPS administered directly into the brain causes a neuroinflammatory state sufficient to aggravate AD pathology. Transgenic mice carrying mutations in APP exhibited increased Aβ deposition in brain

tissue after chronic intracerebroventricular (i.c.v.) administration of LPS over a period of two weeks (Qiao et al., 2001). This exacerbated Aβ pathology has been seen with intraperitoneal (i.p). administration of LPS in a similar transgenic model (Sheng et al., 2003). The effects of LPS have also been studied in the absence of Aβ, in a transgenic mouse model exhibiting a different form of AD pathology. These mice develop intracellular pathology known as tau tangles, and LPS similarly aggravates pathology in this mouse model (Lee et al., 2010; Kitazawa et al., 2005). Previous studies, including recent work in our lab, have shown that i.p. injection with LPS causes significant memory and behavior deficits, as well as increases in hippocampal Aβ, in non-transgenic mice (Lee et al., 2008 and Kahn et al., 2012). These studies support the notion that inflammation can exacerbate, and possibly even initiate, the progression of AD.

Though inflammation undoubtedly has an important role in the progression of Alzheimer's disease, there is also evidence of a beneficial role of inflammation in AD. Microglia enter an activated, pro-inflammatory state in AD that increases phagocytic activity and reduces pathology (Wyss-Coray, 2006). A single intrahippocampal injection with LPS has been shown to reduce the Aβ load in AD transgenic mice, and microglia have been implicated in this Aβ clearance as well (DiCarlo et al., 2001; Herber at al., 2007). It may be that the effect LPS has on AD pathology depends on the duration of exposure. Acute inflammation, as seen with a single exposure to LPS, may initiate an immune response that helps to clear AD pathology, while exposure to LPS over a longer period of time appears to create a chronic state of inflammation that worsens pathology.

Our lab further investigated the effects of chronic LPS-induced inflammation. In 2015 a previous graduate student, Amy Hardy, conducted a study to investigate the length of time required for Aβ levels to return to baseline in non-transgenic mice administered a week of LPS injections. She found that Aβ remained elevated after 16 days, but was no longer significantly different from controls after 23 days. A second study was then conducted to determine if a second 7-day course of LPS injections, beginning 15 days after the first round of injections, would induce even higher levels of Aβ. Interestingly, soluble Aβ in these mice returned to levels as low as that of control mice who had never been exposed to an LPS stimulus (Hardy 2015). The results of the second study indicated that an initial exposure to LPS protected the mice from the effects of a second course of injections.

1.4 B cells and anti-amyloid beta therapy

One potential explanation for the results of Hardy's work is that the immune system, after initial priming with LPS, mounts an adaptive immune response in which B cells generate antibodies capable of quickly eliminating the foreign antigen upon a second exposure. Antibodies bind to a specific antigen, allowing for neutralization or destruction of that foreign antigen upon antibody binding. We hypothesize that the immune system may be producing antibodies against either LPS or the Aβ that is produced as a result of LPS stimulation. Immune system cells residing in the peritoneal cavity are of particular interest because this is the site where we administered LPS injections. The peritoneal cavity is host to a wide variety of immune cells including macrophages, T cells, and B cells. There is a unique subset of antibody-producing B cells, called B1 cells, that are present in relatively high concentration in the peritoneal

cavity, and these cells will be the focus for the majority of this study. While B1 cells are only a minor subset of B cells, they are responsible for the production of more than 80% of circulating IgM (Moon et al., 2012). IgM, secreted as a pentamer, is the initial isotype to appear during immune challenge (Ehrenstein and Notley, 2010). Antigen-specific B cells secrete low-affinity pentameric IgM at a natural, baseline level prior to antigen exposure, providing a line of defense against pathogen invasion. When a B cell encounters its antigen during an immune challenge, it increases secretion of IgM that is specific for that antigen.

Self-reactive IgM and IgG autoantibodies circulate at higher levels compared to other antibody isotypes, and these autoantibodies may play a role in debris clearance of endogenous proteins (Nagele et al., 2013). Because of this important property, some naturally circulating antibodies have the ability to recognize endogenously produced proteins such as amyloid beta. In fact, previous studies have demonstrated that even people who do not have AD naturally produce IgM and IgG autoantibodies that are specific for amyloid beta (Szabo et al., 2008; Lindhagen-Persson et al., 2010). AD patients have a significantly higher amount of these anti-Aβ autoantibodies than non-demented individuals (Nath et al., 2003). IgG can also be found concentrated around Aβ plaques in the brains of AD patients, helping to alleviate the plaque burden (Kellner et al., 2009).

Anti-amyloid beta immunotherapy has been investigated as a way of treating AD early on, before pathology and cognitive deficits become too advanced. Immunotherapy involves the manipulation of the natural immune system to either induce or repress a specific immune response. With respect to AD, immunotherapy

research encompasses two different types of vaccination. One approach is active immunization, where patients are injected with a form of Aβ peptide. Immunization with Aβ in transgenic mice overexpressing APP prevented plaque formation and the development of other AD pathology (Schenk et al., 1999). A clinical trial with Aβ in humans showed promise, but the trial was halted when a small percentage of patients developed meningoencephalitis (Gilman et al., 2005). Future studies with active immunization hope to achieve similar clearing of Aβ pathology without causing autoimmune inflammation (Lambracht-Washington and Rosenberg, 2013).

A second approach to AD immunotherapy is passive immunization in which manufactured anti-Aβ antibodies, rather than the Aβ protein itself, are injected into the patient. Transgenic mice passively immunized against Aβ exhibit cognitive improvements, as well as microglial changes and reduced Aβ deposits in the brain (Wilcock et al, 2004). Several clinical trials of anti-Aβ immunotherapy have yielded success in reducing Aβ burden in AD patients, but many of these drugs do not significantly improve cognitive function (Lambracht-Washington and Rosenberg 2013; Salloway et al., 2014; Doody et al., 2014).

The problem may be that these immunotherapy drugs are not being administered to patients early enough. By the time AD is clinically diagnosed, the pathology caused by the disease has usually been wreaking havoc on a patient's brain for decades. Furthermore, it is crucial to differentiate between the multiple different forms Aβ can take, especially in the context of immunotherapy. It is well understood that the aggregation of Aβ causes neurotoxicity and cognitive impairment, but there has been a lack of evidence of similar neurotoxicity as a result of Aβ monomers (Cleary et

al., 2005; Benilova et al., 2012). In fact, it has been suggested that monomeric Aβ has an important role in normal neuronal function and memory (Lindhagen-Persson et al., 2010; Giuffrida et al., 2009). In order to effectively improve the cognitive deficits seen in AD without disrupting the natural function of amyloid beta, immunotherapy must target oligomeric Aβ and maintain Aβ in its monomeric form (Lindhagen-Persson et al., 2010; Lambracht-Washington and Rosenberg 2013).

1.6 Objectives

Lindhagen-Persson and colleagues in 2010 found that naturally secreted anti-Aβ IgM has a stronger affinity for oligomeric rather than monomeric Aβ. Since peritoneal B1 cells secrete such high levels of IgM, this makes them an ideal target for study of anti-amyloid beta therapy. Furthermore, since the B cell-rich peritoneum is the site where LPS injections were administered in Hardy's thesis work, we will investigate B1 cells more thoroughly in order to fully understand the protective mechanism implicated in these studies.

There are two main objectives of the present study: to investigate the activation of mouse B1 cells in response to LPS stimulation, and to study antibody production in the 5xFAD transgenic mouse model. We hypothesize that B cells traffic to the brain in Alzheimer's disease, localizing around Aβ plaques and producing antibodies on site. These studies may provide evidence for a way to exploit naturally produced anti-Aβ antibodies, particularly IgM, and use it for a natural, more effective form of immunotherapy in AD.

2. Materials and Methods

2.1 Mice

C57BL/6J mice were used, as used previously by Kahn et al. and by Hardy (Kahn et al. 2012, Hardy 2015). For the second part of the study, the 5xFAD mouse model was used. This is a transgenic mouse strain commonly used to model Alzheimer's disease, and is especially considered a useful Alzheimer's model because of the rapid development of amyloid pathology in the mouse (Oakley et al., 2006; Lee and Han, 2013). All mice used in these studies were bred in the TCU vivarium and 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 animal care protocols approved by the Institutional Animal Care and Use Committee (IACUC) of Texas Christian University. Food and water were made available *ad libitum* and mice were kept on a 12-hour light/dark schedule.

2.2 Cell isolation

After mice were euthanized via CO_2 asphyxiation, peritoneal cells were isolated using a protocol modified from Ray and Dittel, 2010. Briefly, cells were washed out of the peritoneal cavity with ice cold phosphate-buffered saline (PBS), and the cells were isolated from the collected cell suspension by centrifugation. After spinning, the supernatant was discarded and the cell pellet was resuspended in cell culture media. To isolate non-adherent B cells from the adherent peritoneal macrophage population, the total peritoneal cell collection was plated overnight on a 10-cm culture dish. The next day, the plate was gently washed with media, leaving adherent macrophages on

the bottom of the culture dish and aspirating the non-adherent B cells off of the plate. The non-adherent cells were again centrifuged and resuspended in culture media.

Spleen was also removed, and splenic cells isolated from tissue. Spleen tissue was ground and rinsed in Hanks' Balanced Salt Solution (HBSS, Sigma-Aldrich, St. Louis, MO), followed by filtration through a cotton filter pipet. Cells were then centrifuged at 300 rcf for 10 minutes. ACK solution (0.15M NH_4Cl, 1 mM $KHCO_3$, EDTA 0.1 mM) was applied to cells for 1 minute for lysis of red blood cells. The remaining cell suspension was rinsed twice more before cells were ready for antibody staining for flow cytometry.

2.3 LPS in vitro administration

Peritoneal B cells were collected, as described above, from C57BL/6J mice. After collection, cells were plated in a 96-well culture dish with 50,000 cells per well. Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Sigma-Aldrich, St. Louis, MO) with 1% Pen/Strep, 1% L-glutamine, and 10% FBS. LPS was administered to the cells at concentrations of 100 ng/ml, 500 ng/ml, 1 μg/ml, 5 μg/ml, and 10 μg/ml. Time points of 12, 24, and 36 hours of LPS exposure at each concentration were tested. LPS concentrations and time points were chosen based on previous LPS *in vitro* studies (Xu et al., 2008 and Enghard et al., 2010).

2.4 Cell viability assay

To determine B cell proliferation in culture, CellTiter-Glo Luminescent Cell Viability Assay (Promega, Madison, WI) was used in accordance with manufacturer instructions. Briefly, after LPS administration, 100 μl of assay buffer was added per well to a 96-well plate of B cells. The plate was mixed on an orbital shaker for 2 minutes to induce cell lysis and then incubated in the dark at room temperature for 10

minutes. Luminescent signal was recorded using a plate reader (BMG LabTech FLUOstar Omega, Cary, NC).

2.5 LPS in vivo administration

5xFAD or C57BL/6J mice were randomly assigned to treatment groups for each experiment. 250 μg/kg of LPS (*Escherichia coli* serotype: 055:B5, Sigma–Aldrich, St. Louis, MO) or saline were administered via intraperitoneal (i.p.) injection. This LPS dosage was chosen because it is commonly used in inflammation studies and has previously been used in our lab (Kahn et al., 2012 and Lee et al., 2008).

2.6 CellTrace CFSE

For tracking of B cell proliferation *in vivo*, CellTrace CFSE Cell Proliferation Kit was used (Thermo Fisher Scientific, Waltham, MA). The CFSE stock solution was prepared according to manufacturer instructions. For this experiment, C57BL/6J mice were administered 5μL of CFSE solution via i.p. injection with either saline or LPS, according to randomly assigned treatment group. Peritoneal and spleen cells were collected and centrifuged at 300 rcf and resuspended in flow staining buffer (PBS with 2% FBS and 0.1% Sodium Azide). Cells were counted using a hemocytometer. 100μL per well of the appropriate antibodies were plated on a 96-well round bottom dish, and 10μL of cells were added to each well for a total concentration of 200,000 – 300,000 cells per well. Antibodies used were anti-CD19 APC and anti-CD5 PE (eBioScience, San Diego, CA). The plate was incubated on ice for 45 minutes, then centrifuged and washed with flow staining buffer 3-4 times before cells were resuspended in buffer and transferred to polystyrene flow cytometry tubes.

2.7 Immunohistochemistry

All mice were euthanized via transcardial perfusion with PBS, and brains were removed for fluorescent immunohistochemical analysis. A series of three equally spaced sagittal brain sections (10 μm) were randomly selected from each animal. Sections were rinsed three times in phosphate buffered saline + 0.05% Tween-20 (PBST), then placed in blocking solution overnight (PBST + 2% donkey serum). Primary antibodies used were anti-6e10 (1:1000, Covance, Princeton, NJ), anti-IgM (1:1000, Jackson ImmunoResearch Laboratories, West Grove, PA), anti-IgG (1:2000, Jackson ImmunoResearch Laboratories, West Grove, PA), and anti-CD19 (1:500, BioLegend, San Diego, CA). Tissue sections were incubated overnight in primary antibody at 4˚C. Appropriate cyanine-conjugated secondary antibodies (for 6e10: donkey anti-mouse Cy2, 1:500; for IgM and IgG: donkey anti-goat Cy3, 1:500, eBioscience, San Diego, CA) were applied for 4 hours at room temperature. Sections were then mounted on slides and coverslipped using Aqua Polymount (Polysciences, Warrington, PA).

2.8 qRT PCR

Total RNA was isolated from hippocampal tissue using Maxwell 16 LEV simplyRNA Tissue Kit (Promega, Madison, WI). After analyzation 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 SYBR green reaction mix (ThermoFisher Scientific, Waltham, MA). All kits were used following manufacturer protocols. Two targets were chosen, purchased from Integrated DNA Technologies

(Coralvile, IA): CD19 (Primer 1: 5'-CCACCAGAGAAACCATACAGAA-3'; Primer 2: 5'-CACGTGAAGGTCATTGCAAG-3') and HPRT (Primer 1: 5'-AACAAAGTCTGGCCTGTATCC-3'; Primer 2: 5'-CCCCAAAATGGTTAAGGTTGC-3'). HPRT was chosen to act as the housekeeping gene. qPCR mixtures contained 0.36 µL of forward and reverse primers, 5 µL of SYBR green, 3.64 µL nuclease-free water, and 1 µL of cDNA. Samples were run in triplicate with a negative control, which contained 1 µL of nuclease-free water in place of cDNA. In accordance with the $\Delta\Delta C_T$ method, an optimization step was performed to determine optimal annealing temperatures for each primer. PCR efficiency was then determined by performing qPCR reactions on five serial dilutions of a solution containing cDNA from each sample. A modification of a method previously described by Pfaffl was used to account for differences in PCR efficiencies between the target gene and the house-keeping gene (Pfaffl, 2001). The relative quantification for each group was calculated using the following equation provided by Pfaffl:

$$\text{Ratio} = \frac{(\text{E}_{\text{target}})^{\Delta\text{CT}_{\text{target}}(\text{control}-\text{sample})}}{(\text{E}_{\text{ref}})^{\Delta\text{CT}_{\text{ref}}(\text{control}-\text{sample})}}$$

2.9 Detection of immunoglobulins

Following transcardial perfusion with PBS, hippocampal tissue was removed and lysed with protein extraction solution (PRO-PREP, Boca Scientific, Boca Raton, FL). Protein levels were determined using DC Protein Assay (Bio-Rad Laboratories, Hercules, CA). Arterial blood was also collected and centrifuged at 2000g for 10 minutes. Plasma was isolated from blood for analysis. IgM and IgG levels were assessed both in plasma and in hippocampal tissue using Affymetrix mouse total ELISA

kit (Thermo Fisher Scientific, Waltham, MA). Kits were used according to manufacturer instructions.

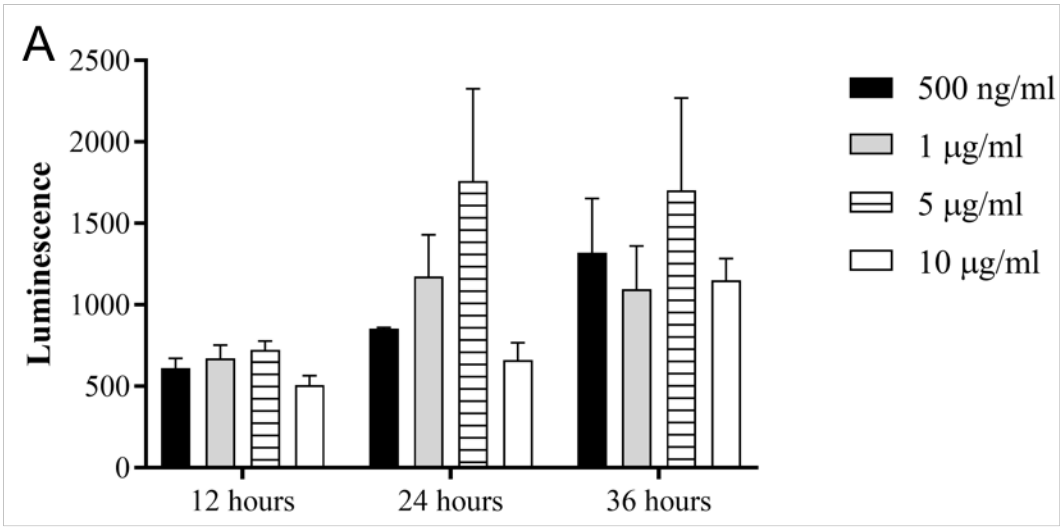
2.10 Statistical Analysis

Statistical analyses were performed using one-way analysis of variance (ANOVA) following each ELISA, cell proliferation assay, and qRT-PCR. A general linear model was used for analysis of IgG co-localization, with mean intensity of IgG determined for each group and 6E10 used as a covariate. Alpha level was set at 0.05 for all statistical analyses.

3. Results

3.1 B Cell Proliferation in culture

Peritoneal B cells were treated with increasing doses of LPS, from 500 ng/ml to 10 μg/ml, in culture to measure proliferative response. A dose-dependent response was not measured, as there were no significant differences in proliferation between any of the treatments (Figure 1A). Multiple time points were tested, and proliferation depended on duration of exposure to LPS. Regardless of dose, cells that were exposed to LPS for a longer amount of time exhibited increased proliferation, with a significant difference between amount of cells present after 12 hours of exposure and after 36 hours (Figure 1B).



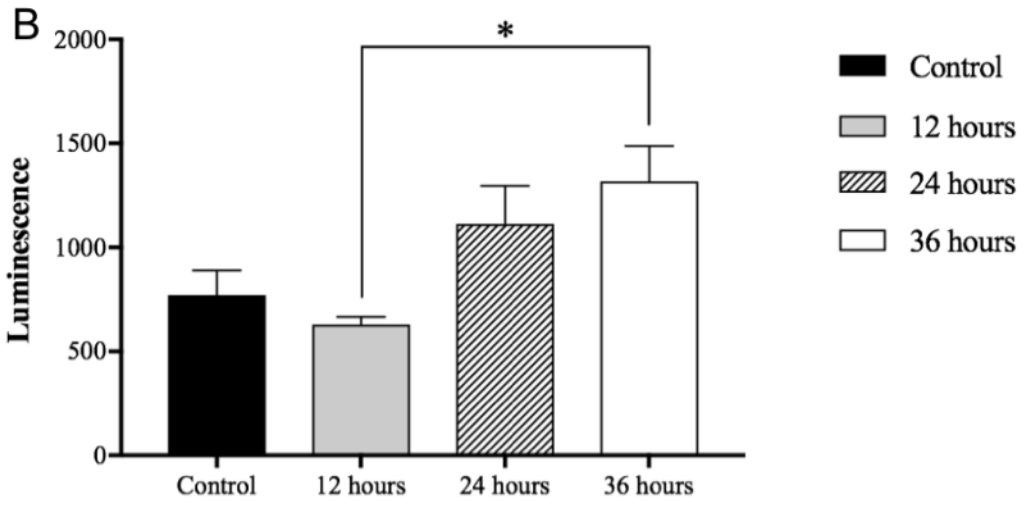


Figure 1. Proliferative response of peritoneal B cells after LPS exposure. Numbers shown are luminescence readings from Fluostar Omega plate reader after Cell Glo assay, corresponding to amount of proliferation *in vitro*. One-way ANOVA revealed no effect of dose on proliferation, with no significant differences between any of the doses at a given time point (A). Results indicate a significant difference in luminescence between 36 hours and 12 hours of LPS exposure (B). Values are expressed as mean ± SEM. *p=0.008.

3.2 Cell migration after LPS exposure

The CellTrace CFSE kit is optimized for *in vitro* use. Therefore, a protocol for *in vivo* use was established to determine if the kit was viable for use in a B cell migration study. C57BL/6J mice were administered i.p. injections of 250 μg/kg of LPS or saline, in addition to CFSE. 24 hours after injection with LPS and CFSE, there was a distinct population of CFSE-labeled B cells in the peritoneum, but not in the spleen (Figure 2A, 2B). After 48 hours, there is still a high percentage of CFSE-positive B cells in the peritoneum, though the population is not as distinct (Figure 2C). Additionally, there are a small amount of CFSE-positive cells starting to appear in the spleen at this time point (Figure 2D). Interestingly, when mice were administered CFSE with saline instead of LPS, CFSE signal was never detected by flow cytometry, both at the 24-hour time point and at 48 hours (Figure 2E, 2F).

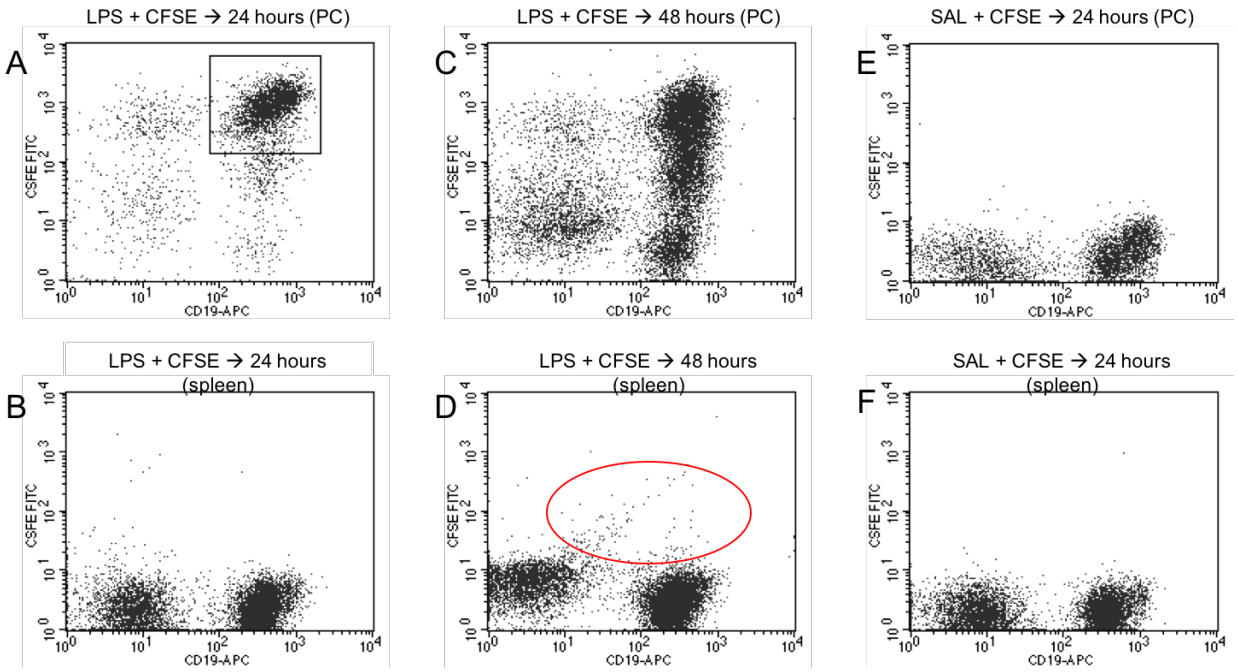


Figure 2. CFSE detection in B cells. 24 hours after injection with LPS and CFSE, there is a distinct population of CFSE+/CD19+ cells in the peritoneum (A), but this is population is not seen in the spleen (B). After 48 hours there are still CFSE+/CD19+ cells in the peritoneum and appears to be a small, less distinct population of CFSE+ cells in the spleen (C,D). Injection with saline and CFSE did not yield any CFSE+ B cells at 24 hours (E,F) or at 48 hours (data not shown).

Cells from these mice were also labeled with anti-CD5 antibody, to distinguish B-1 cells from conventional B cells. 24 hours after exposure to LPS, the B-1 cell population in the peritoneum was present at a much higher percentage than it typically is under natural conditions (73.59% of total lymphocytes, compared to 10-15% in saline and untreated animals, Figure 3A). Measurements were also taken 72 hours after exposure to LPS in order to detect LPS-induced changes in the B-1 cell population. After 72 hours, this population decreased substantially in the peritoneum (Figure 3B). This suggested an exodus of B-1 cells from the peritoneal cavity, and the spleen was investigated as a potential migration target for these cells. However, no changes in the B-1 cell population in the spleen were observed at either time point (Figure 3C, 3D). Furthermore, this splenic B-1 cell population remained unchanged from the percentage typically seen in saline and untreated animals, where B-1 cells are typically 1-2% of total lymphocytes (data not shown).

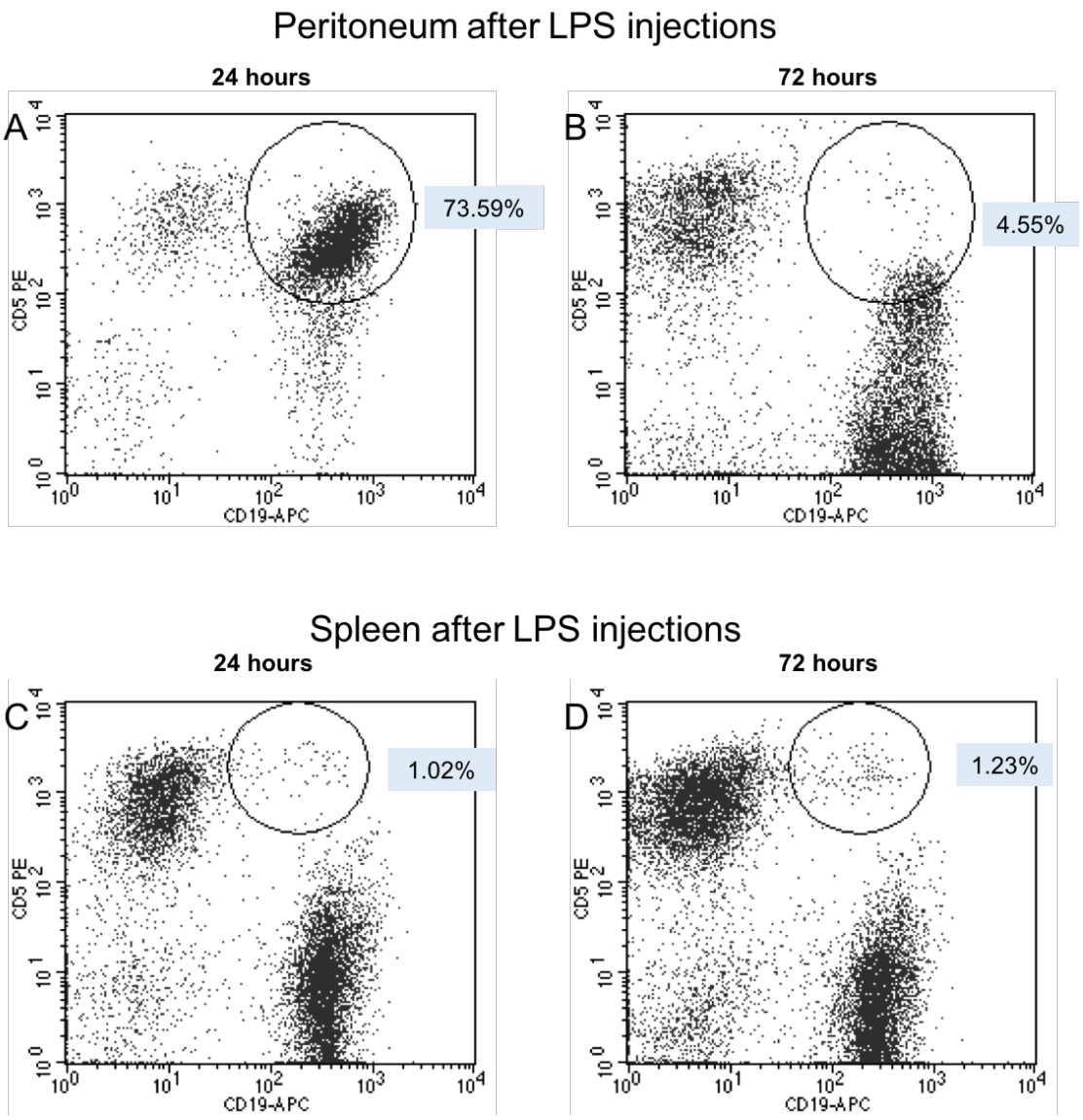


Figure 3. Changes in B-1 cell population after exposure to LPS. 24 hours after LPS injection, the B-1 cell population in the peritoneum is in relatively high concentration, with 73.59% of total peritoneal lymphocytes identified as CD5+/CD19+ B-1 cells (A) compared to saline controls with 10% of lymphocytes as B-1 cells (data not shown). After 72 hours this population has decreased (B). No changes in the spleen B-1 cell population were observed at these time points (C,D).

3.3 Antibody co-localization around amyloid beta plaques

Confocal microscopy showed IgG closely associated with amyloid beta plaques in the hippocampus of 5xFAD transgenic mice, both with and without exposure to LPS (Figure 4). We verified that our secondary antibody for IgG was not nonspecifically sticking to plaques with a control stain (Figure 5). In order to quantify this observed co-localization, mean intensity of IgG at each plaque was measured and determined for each treatment group. A significant effect of treatment was seen overall for amyloid beta, but not for IgG. There were also no significant differences in IgG intensity between treatment groups (Figure 6). Plasma and hippocampal IgG levels were further quantified by ELISA, and no significant differences were observed between groups (Figure 7).

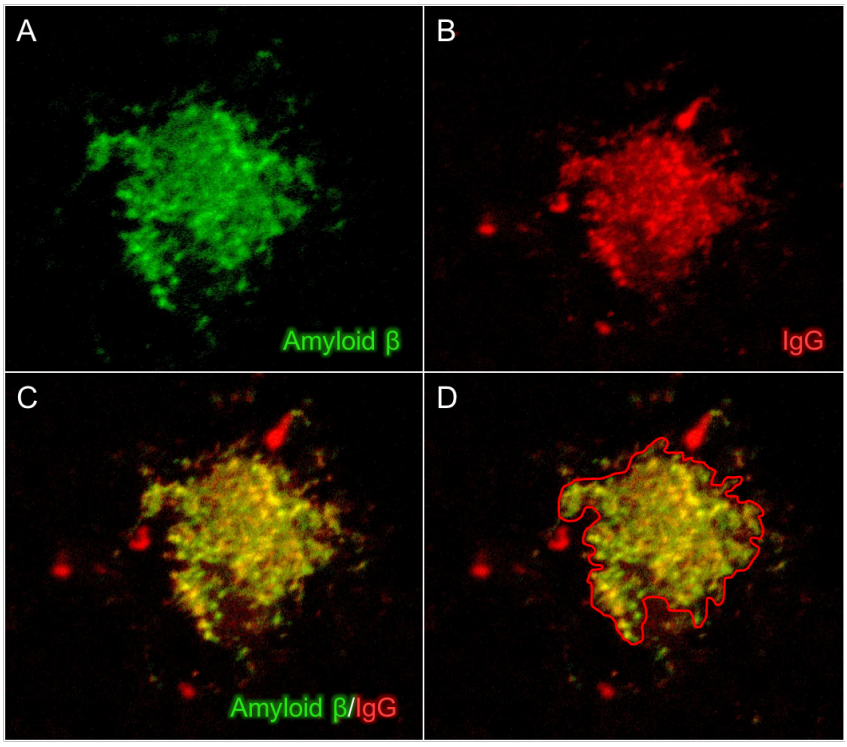


Figure 4. IgG co-localization around amyloid beta plaques in 5xFAD mouse hippocampus. Amyloid beta plaque is shown in green in panel A (6E10), and panel B shows the same plaque area with IgG stain in red. Panel C shows both images overlaid, with panel D demonstrating the outlining of the plaque area on confocal software, excluding IgG that is not directly on the plaque.

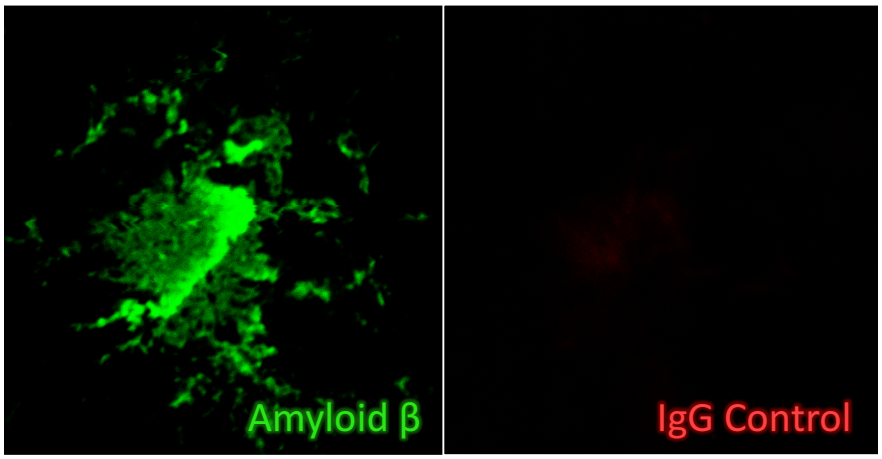


Figure 5. IgG control. Sections were stained for amyloid beta with secondary antibody, and for secondary antibody for IgG without the primary. No fluorescence was detected with the IgG secondary alone.

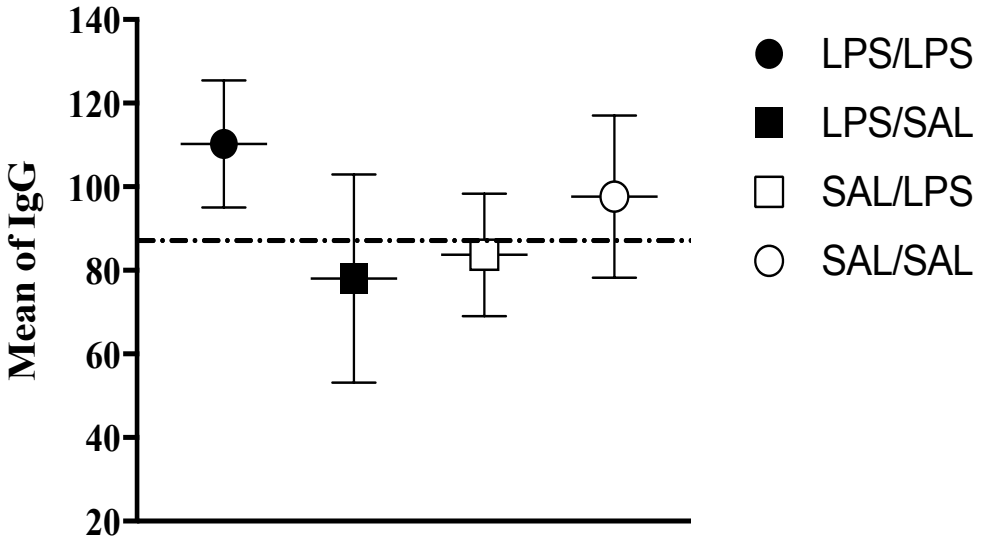
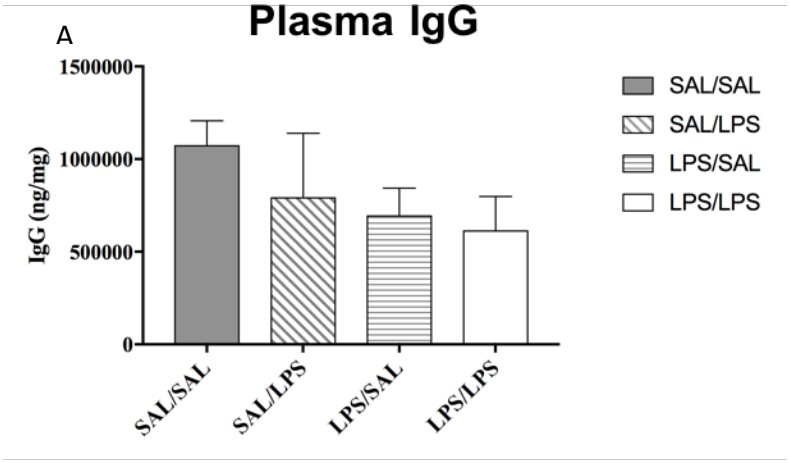


Figure 6. Factorial plot of mean IgG intensity. Figure shows mean intensity for each treatment group, and the central dashed line represents the grand mean. Using a general linear model, mean intensity of IgG was determined for each group, using 6E10 as a covariate. With IgG as the response variable, a significant effect was observed for 6E10 ($p<0.001$) but there were no significant differences in IgG between treatment groups.



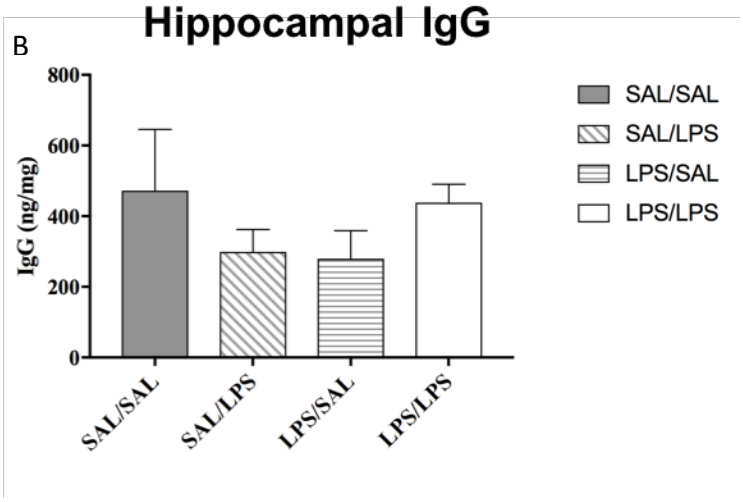


Figure 7. Plasma and hippocampal IgG levels. One-way ANOVA reveals no significant differences in plasma IgG or hippocampal IgG between treatment groups (p=0.468 and p=0.474, respectively).

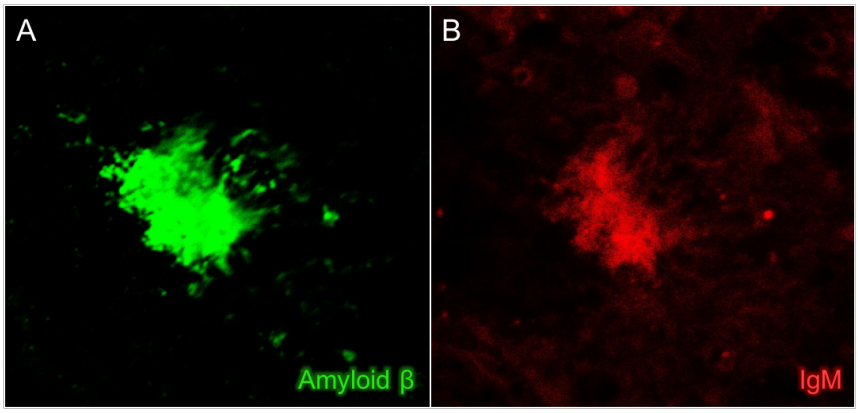


Figure 8. IgM co-localization on amyloid beta plaques in 5xFAD hippocampus. Staining demonstrates the presence of IgM antibody co-localization, similar to that of IgG. Panel A is staining for amyloid beta plaque alone, and panel B shows the same plaque area with IgM staining alone. Image shown is of a plaque from a mouse of the LPS/LPS treatment group, but IgM was observed in all treatments.

Immunohistochemistry was used to detect IgM presence in the hippocampus, following the same method previously described to measure IgG co-localization. Confocal images show IgM in close association with Aβ plaques regardless of treatment group, similar to what was seen with IgG but at a lower intensity (Figure 8).

3.4 CD19 detection in the brain

5xFAD mice were administered LPS or saline for one week, and sagittal sections of the brain were stained for CD19, a marker that is specific for B cells. Transcardial perfusion was performed on these mice prior to tissue collection, to ensure that CD19 fluorescence in the brain was due to CD19 presence in the tissue and not in the vasculature. Confocal microscopy images demonstrated CD19 expression in the brain tissue of 5xFAD mice, regardless of treatment (Figure 9A). Images suggested that this CD19 expression was present throughout the tissue and not localized around amyloid plaques (Figure 9B). Furthermore, closer inspection suggested the presence of CD19-

labeled cells (B cells) that aggregated in clusters (Figure 9C). A control stain confirmed that our CD19 antibody was not staining nonspecifically, as this effect was only visible when tissue was stained with anti-CD19 and not antibody against total-IgG (Figure 9D). In order to further investigate and quantify CD19 levels, qRT-PCR was conducted on hippocampal tissue of 5xFAD mice, as well as 5xFAD-negative mice. Mice were given i.p. injections of either LPS or saline for one week, after which hippocampus was collected for analysis. CD19 was found at detectable levels in all mice, with no statistical difference between treatment groups or genotype (Figure 10).

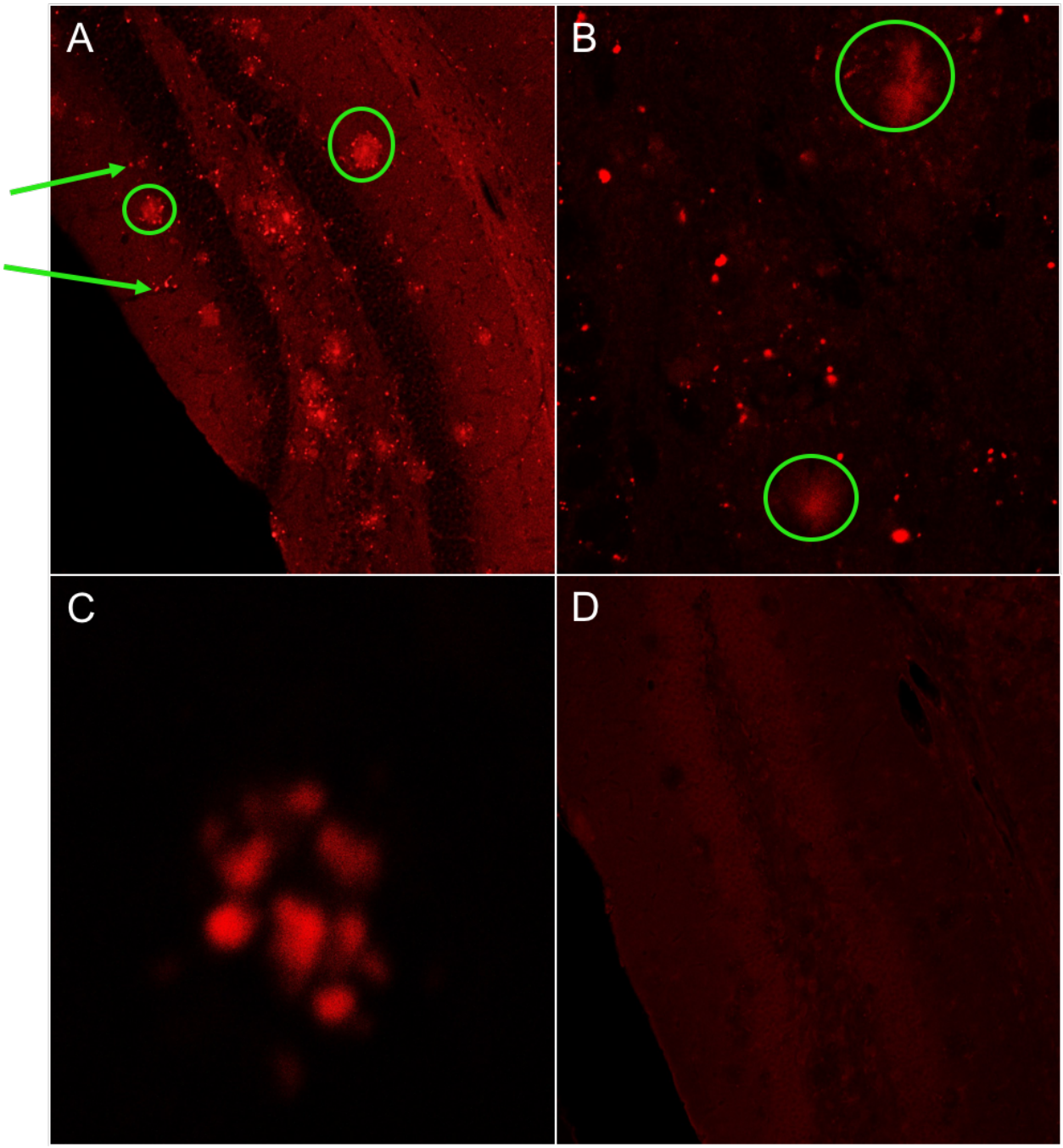


Figure 9. CD19 expression in 5xFAD hippocampus. Sagittal sections of 5xFAD mouse brain were stained with anti-CD19 antibody. Panel A shows CD19 expression in the hippocampus. Circles indicate non-specifically labeled amyloid plaques, and arrows indicate CD19 expression throughout the tissue. Panel B shows a similar image at higher magnification, suggesting CD19 expression is not centered around amyloid beta plaques. Images taken at the highest magnification suggest that each CD19 spot in tissue is actually a cluster of multiple CD19-positive cells (C). Panel D is a control stain with total IgG to rule out nonspecific staining.

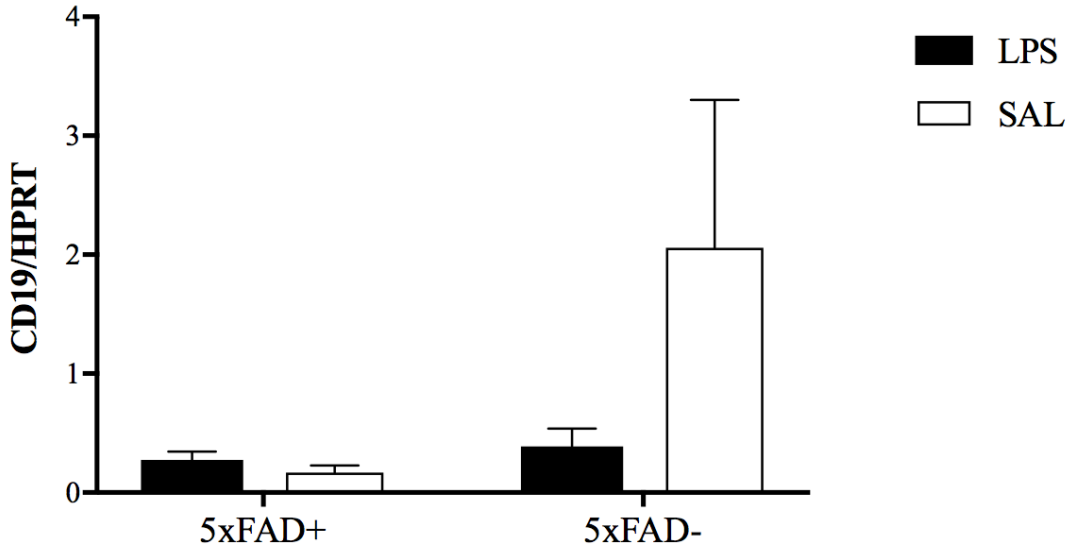


Figure 10. Quantification of CD19 in hippocampal tissue. qRT-PCR was use to analyze levels of CD19 in hippocampus. Values shown are CD19 expression divided by HPRT expression (used as a control). One-way ANOVA reveals no significant differences between LPS and saline-treated animals, or between 5xFAD-positive and negative mice.

4. Discussion

The first part of this study was designed to determine whether peritoneal B cells in our mouse model were binding and responding to LPS. Previous studies have established that LPS binds to TLR4 receptor on B cells to induce an inflammatory response, accompanied by cell proliferation and migration out of the peritoneal cavity (Ha et al., 2006; Barrio et al., 2013). Therefore, we expected to see this proliferative response when peritoneal B cells were isolated and administered LPS *in vitro*. Contrary to results reported in a previous study, a dose-dependent response to LPS was not observed (Xu et al., 2008). Rather, a time-dependent response was detected, with higher amounts of proliferation the longer cells were exposed to LPS. One explanation for the lack of dose-dependency seen in these results may be that the aforementioned study used splenic rather than peritoneal B cells. Moreover, the proliferative response of peritoneal B cells is known to be less robust than that of conventional B cells, which may make it difficult to measure substantial differences in cell counts (Sindhava and Bondada, 2012). Nevertheless, the time-dependent proliferative response observed confirmed that LPS activated peritoneal B-1 cells.

The next set of experiments studied the B cell response to LPS *in vivo* using CFSE Cell Trace dye. It has previously been established that i.p. administration of LPS induces B-1 cells to migrate out of the peritoneal cavity (Moon et al., 2012). The spleen has been implicated as one of the targets of this migration, so we investigated cell populations in the spleen as well as in the peritoneum (Pfau et al., 2013). 24 and 48 hours after injection with LPS and CFSE, there was a distinct B cell population labeled with CFSE in the peritoneum. This population was not observed in the spleen, but after

48 hours there was a small population of CFSE-labeled cells appearing in the spleen. Interestingly, CFSE was never detectable when it was administered with saline, both in the spleen and in the peritoneum at all time points measured. It is possible that the CFSE was actually binding to LPS. When LPS bound to its TLR4 receptor on B cells, it may have brought the CFSE with it, making it appear that our CFSE successfully labeled B cells. If CFSE had actually labeled B cells, the signal would have been detected for our saline animals, too. If CFSE is to be successfully used in the future for *in vivo* migration studies, other concentrations and methods of administration would need to be attempted.

Flow cytometry was also used to distinguish the B-1 cell population from conventional B cells, and to study the response of these cells to LPS *in vivo* without the use of CFSE. B-1 cells are native to the peritoneal cavity, and they are distinguishable from conventional B cells by their expression of CD5 (Baumgarth 2011). Therefore, these cells were labeled with anti-CD5 antibodies in addition to anti-CD19, the traditional B cell marker. 24 hours after injection with LPS, we observed a marked increase in the B-1 cell population in the peritoneum compared to saline controls. After 72 hours, this population had decreased substantially, from 73.59% of total lymphocytes to 4.55%. This suggests that i.p. injection with LPS induced B-1 cells to migrate out of the peritoneal cavity. However, the B-1 cell population in the spleen did not change. These cells remained about 1% of total lymphocytes for the duration of the experiment, and were in fact at the same percentage as saline controls.

The CFSE Cell Trace dye used for these experiments is optimized for *in vitro* use, and there were complications with using the dye *in vivo* that need to be addressed if

these experiments are to be repeated. Additionally, the data for these flow cytometry experiments were collected on separate days. Ideally, these experiments would be redone so that all of the collection events ended on the same day. This would help control for instrument settings and compensation settings, which can potentially vary each time the flow cytometer is used.

The second half of this study investigated antibody production and B cell migration within the 5xFAD transgenic mouse. It has previously been established that IgG aggregates around amyloid beta plaques in AD patients (Kellner et al., 2009). These experiments sought to determine if an LPS-induced inflammatory response would increase the amount of IgG decoration of Aβ plaques. Unfortunately, due to genotyping issues, many of the original 5xFAD-positive animals in this experiment turned out to be negative and did not exhibit any amyloid plaques after immunohistochemistry. This brought the sizes of some treatment groups down substantially, and we were unable to observe any significant differences in IgG co-localization between any of our groups. Interestingly, we compared our data to that of untreated 5xFAD-positive control animals and did see significantly more IgG at each plaque in our LPS/LPS treatment group compared to these controls. It may be that the stress of the injection itself is enough to significantly increase the IgG response for these mice, or that our saline was not sufficiently sterilized before injection and contained a low amount of endotoxin. These experiments are currently being repeated with much larger treatment groups to verify if our SAL/SAL control group will have lower IgG co-localization, similar to the untreated animals, than the LPS/LPS group.

Antibody trafficking into the brain is restricted by the blood brain barrier (BBB), consisting of tightly assembled endothelial cells and membranes that separate systemically circulating blood from the central nervous system. In fact, it is often assumed that larger antibodies such as IgM are unable to cross the BBB at all (Banks et al., 2002; Elahy et al., 2015). Most studies of antibody interaction with the BBB focus on IgG, and find that while IgG is capable of diffusing across the BBB at a relatively slow rate, it is not typically able to freely do so and must instead utilize certain extracellular pathways (Banks et al., 2007; Banks 2004). IgM is a much larger molecule than IgG, and the pentameric form of naturally circulating IgM further adds to the challenge of getting it into the brain. However, as recent studies have demonstrated, it is possible for certain forms of peripherally administered IgM to cross the BBB (Banks et al., 2007). Our observation of IgM in the hippocampus was novel in that this IgM was endogenously produced rather than manufactured and injected, and its mechanism of transport across the BBB needs to be investigated further. Additionally, the specificity of both IgG and IgM that was observed in the hippocampus needs to be determined. It was assumed for our purposes that because these antibodies appeared to directly bind to the amyloid plaques, they were specific for Aβ. In the future, this specificity needs to be confirmed with an ELISA.

B cell presence in the brain was investigated as a potential explanation for the hippocampal presence of IgM. B-1 cells have been shown to preferentially migrate to sites of inflammation (Geherin et al., 2016). As stated previously, the brain is in a heightened state of inflammation in AD (McGeer et al., 2003; Solito and Sastre, 2012). This makes the brain a site of inflammation and a potential migration target for B-1

cells. Previous studies have demonstrated that T cells, which are a type of lymphocyte similar to B cells, infiltrate the brain during AD (Ferretti et al, 2016). However, less is known about B cell infiltration. In order to investigate whether B cells were migrating into the brain in our 5xFAD mice, we administered LPS or saline to 5xFAD mice for one week, then collected and stained brain tissue with anti-CD19, a marker that is only expressed on B cells. CD19 was observed in the brains of all 5xFAD mice, regardless of treatment. Since CD19 is unique to B cells, this suggested the presence of B cells in the brain. Confocal microscopy suggested that these B cells were present throughout the brain, and were not localizing to a specific area or to the Aβ plaques. In order to further quantify this observation, qRT-PCR was conducted on hippocampal tissue from 5xFAD-positive mice, as well as 5xFAD-negative mice, that received similar injections. CD19 was detected in all treatment groups, with no significant differences between groups. However, these qRT-PCR results are likely confounded by the presence of B cells in the hippocampus. There are multiple arteries which supply blood to the hippocampus, and this blood is home to a number of naturally circulating B cells. Transcardial perfusion was performed on the tissue that was used for immunohistochemistry, ensuring that the CD19 we observed with confocal microscopy was being expressed in the tissue and not the vasculature. Future studies need to repeat this CD19 qRT-PCR using animals that have also undergone transcardial perfusion with RNAse-free saline. Furthermore, CD19 expression was observed throughout the brain tissue after immunohistochemistry, but qRT-PCR was only conducted on hippocampal tissue. Areas other than the hippocampus need to be investigated to determine where the majority of these B cells are present in the brain.

Flow cytometry results suggested that B cells migrated out of the peritoneal cavity when mice were injected with LPS. These B cells were suspected to migrate to the spleen, but no significant increase in CD5+ B cells, which are of peritoneal origin, was observed in spleen. Therefore, other potential targets for B cell migration in our inflammation model need to be further investigated. A recent study demonstrated that a subcutaneous inflammatory stimulus causes peritoneal B cells to preferentially migrate to skin (Geherin et al., 2016). Intraperitoneal injection with LPS likely causes inflammation of many organs within the abdominal cavity. Future studies should investigate targets such as mesenteric lymph nodes and small intestines as potential sites for LPS-induced B cell migration. These i.p. LPS injections also cause elevation of pro-inflammatory cytokines, as well as increased Aβ, in the hippocampus (Kahn et al., 2012). It may be that B cells are stimulated in the peritoneal cavity upon contact with LPS, and then migrate from there to the site of inflammation in the brain. Future studies will need to investigate the origin of the B cells seen in the hippocampus in these experiments, and to further investigate anti-Aβ IgG and IgM presence in the brain. A link between peripheral inflammation and trafficking of Aβ-specific antibodies to the brain would help scientists understand how the immune system of AD patients works to combat the disease. These studies may have a significant impact on the development of immunotherapy drugs, and even vaccinations, for AD.

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ABSTRACT

B-CELL RESPONSE AND ANTIBODY PRODUCTION IN AN INFLAMMATORY MODEL OF ALZHEIMER'S DISEASE

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Alzheimer's disease (AD) is characterized by the over-production of amyloid beta (Aβ), which aggregates into plaques in the brain. Our lab has previously demonstrated that intraperitoneal (i.p.) injection of lipopolysaccharide (LPS) for 7 consecutive days leads to a significant elevation of hippocampal Aβ in mice. However, mice given a two-week break after the first injections and administered a second 7-day course of LPS injections had reduced levels of Aβ, suggesting a protective effect of the immune system following the first exposure to LPS. Antibody-producing B cells were investigated as a potential mechanism for this protection. Peritoneal B-1 cells were activated by LPS, and data suggests that these cells migrate out of the peritoneal cavity after activation. The brain was investigated as a potential target of this migration and CD19 expression was detected in brain tissue. Antibody presence in the brain was analyzed and evidence of IgM co-localization at Aβ plaques was found in the hippocampus of 5xFAD transgenic mice. Together, these data suggest that B-1 cells may migrate to the brain in AD in response to neuroinflammation, and these cells may produce Aβ-specific antibodies that target amyloid plaques.