Stimulating Innate Immunity via TLR9 agonist CpG ODN in a Non-Human Primate Model

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Abstract

Alzheimer's disease (AD) is the most common form of dementia characterized by hallmarks such as neurofibrillary tangles, amyloid plaques, and cerebral amyloid angiopathy (CAA). Additionally, inflammation and glial function have been recognized to play an important role in neurodegeneration. There is currently no effective treatment for AD. Previous research indicates immunomodulation has been successful in reducing AD pathology in mice. The current study aims to determine if squirrel monkeys are an appropriate model to use for AD research, if innate immune stimulation via TLR9 agonist class C CpG ODN is safe to use in squirrel monkeys, and if class C CpG ODN is an effective treatment for AD. To decide if the monkey model was acceptable for AD research, the current study compared young and old monkeys for the presence of astrocytes, T-cells, and glial cells via histological staining. Results indicated that old monkeys had a greater presence of T-cells and glial cells present, suggesting that aging had taken place in older monkeys, like that of humans. Through histological staining and semi-quantitative analysis, it was determined that CpG ODN did not cause any adverse effects in squirrel monkeys when compared to the monkeys receiving a saline control, suggesting its safety. Additionally, CpG ODN injections increased microglial activation, reduced T-cells in the squirrel monkey model, and increased cytokine presence in plasma associated with inflammatory, and anti-inflammatory response.

Review of Literature

Alzheimer's disease (AD), the most common cause of dementia, is a neurodegenerative disorder that can be characterized by, neurofibrillary tangles, and deposition of amyloid-beta (Aβ) in the brain causing massive neuronal cell death leading to cognitive decline (Serrano-Pozo, 2011; Bennett, 2017). Additionally, cerebral amyloid angiopathy (CAA), or the deposition of amyloid plaques in arterioles has been associated with cerebral microhemorrhages, and inflammation in the brain (Kinney, 2018; McCaulley, 2015). While previous research has determined that inflammation in the brain is part of the normal aging process, extensive inflammation can promote a neurodegenerative cascade (Cribbs, 2012). Alzheimer's disease deals directly with the immune system attacking the nervous system and causing dysfunction in many proteins and signaling cells (Cao, 2018).

Innate immune responses are greatly impaired in subjects with AD (Sochocka, 2019). Microglial cells play a key role in the immune system (Lenz, 2018). They are responsible for maintaining neuronal homeostasis and initiating the inflammatory response (Mandrekar-Colucci, 2010). Microglial activation is a major aspect of the immune system and can enhance neuroprotection (Subramaniam, 2017). Additionally, microglial activation in Alzheimer's has been shown to be beneficial, and aid in the uptake and clearance of amyloid-beta (Lai, 2012). As a person ages, glial activity deteriorates and becomes less efficient in preventing immune diseases and regulating immune responses (Ojo,2015). While microglial activation can be beneficial in AD patients, chronic microglial activation results in neuron damage and is neurotoxic (Leyns, 2017). Additionally, long term glial activation can result in an abundant release of pro-inflammatory cytokines, promoting nerve tangles, and increasing neuronal death (Li, 2014). This phenomenon is known as gliosis and is emerging as a common hallmark of neurodegenerative diseases (Kim, 2019). Astrocytes a type of glial cell also plays an important role in cytokine release (González-Reyes, 2017). In a brain affected by Alzheimer’s, the astrocytes are compromised and contribute to the inflammatory response (Myles, 2016) While there are many pro-inflammatory cytokines, there are also anti-inflammatory cytokines which play a role in neutralizing the inflammation, attempting to return the body to a homeostatic state (Su, 2016). T-cells are another major aspect of the immune system which are responsible for mediating a fast cell response during pathogen invasion (Nathan, 2013). In AD patients, high frequencies of reactive T-cells have been found in the patient's bloodstream when compared to a

healthy person's bloodstream suggesting that they play a role in neurodegeneration. (Ciccocioppo, 2019).

Squirrel Monkeys, a new world monkey model, naturally develop CAA (Heuer, 2017). CAA is a common hallmark of AD found in humans pathology making squirrel monkeys very useful for Alzheimer's research (Toledano, 2014; Walker, 1990). Additionally, the pattern of Aβ deposition in squirrel monkeys is very similar to that of humans, allowing for a more accurate comparison between pathology in humans and pathology in squirrel monkeys (Heuer, 2019). Previous research has also determined that like humans, squirrel monkeys develop senile plaques, and exhibit similar cognitive deficits as humans with AD (Walker, 1987; Rosen, 2016). Importantly, Alzheimer's can also manifest in new world monkeys such as squirrel monkeys through the same gene as in humans: APOE gene (Morelli, 1996). As a result of these essential traits, squirrel monkeys are becoming an important animal model used for AD experimentation (Bading, 2002)

Immunotherapy has become the favored treatment for AD (Bittar, 2018). Previous research has demonstrated that innate immunity stimulation helps to reduce AD pathology (Wang, 2018). CpG ODN is a synthetic oligodeoxynucleotide that contains unmethylated deoxycytosine-deoxyguanosine which has been used to stimulate innate immunity via a toll-like receptor. CpG ODNs have been used in clinical trials for various diseases such as cancer and HIV to test for their immune-stimulating response (Scheiermann, 2014; Jahrsdörfer, 2008). Several clinical trials have reported success from the use of CpG ODNs showing potential for its use in other diseases (Shirota, 2017). A toll-like receptor is responsible for recognizing invading microbial pathogens and activating the appropriate signaling pathways in response to a threat (Lim, 2013). There are three toll-like receptor agonists: A-Class, B-Class, and C-Class (Jurk, 2004). Class B CpG ODN has been used to stimulate innate immunity in AD transgenic mice models and has been shown to ameliorate AD pathology (Scholtzova, 2017; Scholtzova, 2014;Scholtzova, 2009) There are still, however, concerns over the safety of using CpG ODNs such to possible toxicity, increased inflammation (Wiśniewski, 2010; Heneka, 2015). Current studies have been using class C CpG ODN, which is a combination of Class A, and Class B (Martinson, 2007). In the current study, the aim was to determine the safety of class C CpG ODN in squirrel monkeys *(Saimiri boliviensis)*, determine the efficiency of class C CpG ODN in stimulating immune response, and complete a neuropathological evaluation in young and old

squirrel monkeys.

Materials and Methods

Squirrel monkey brain tissue and plasma was acquired from the Squirrel monkey Breeding Research Resources (SMBRR) located at the University of Texas MD Anderson Cancer Center Michale E. Keeling Center for Comparative Medicine and Research. At this center a cohort of aged female squirrel monkeys (19-20 years old) , and a cohort of young female squirrel monkeys (4 year old) were subcutaneously injected with CpG ODN or with saline (vehicle) control.

Histological Studies

All histological stainings were performed on 8-micron thick paraffin brain sections. All sections were deparaffinized going from xylene to ethanol prior to staining.

PERL Prussian Blue Staining: A PERL staining was performed to stain for microhemorrhages. 5% Potassium ferrocyanide with 5% HCL was used as the reactant. The slides were heated in the microwave in a slide jar submerged with a volume of 250ml of Potassium ferrocyanide for 75 seconds. Once heated, potassium ferrocyanide is toxic; therefore, for safety, a mask, gloves, and a lab coat were worn. The slides were then left to sit in the solution under the fume hood for ten minutes and were then washed under tap water for eight minutes. Nuclear fast red was then applied for one minute, followed by dehydrating, and cover slipping.

T-Cell Staining: A t-cell staining was performed in order to detect t-cells in the brain tissue. Using the TRIS-EDTA buffer, antigen retrieval was performed in the microwave for 20 minutes. The slides were then washed with PBS followed a 30-minute H_2O_2 incubation. Following incubation, the slides were washed in PBS and subjected to a one-hour goat blocking. Following three rounds of five- minute PBS washes, an anti-rabbit primary was then applied with a 1:1000 dilution and left overnight. The next morning, the primary was taken off, and the slides were washed with PBS-T to ensure for a more thorough washing of the section. An anti-rabbit secondary was then applied for one and a half hours made in a 1:800 dilution. Following the secondary, there were three five-minute PBS-T washes followed by an incubation in A+B for one and a half hours. The DAB reactant was made using nickel sulfate, and DAB powder. The

reaction was then done starting with a concentration of 100ul of H202 per 10ml of DAB. Sodium acetate was used to stop the DAB reaction, and the slides were then washed with PBS, and dehydrated and cover slipped.

6E10 Staining: A 6E10 staining was performed in order to detect amyloid burden. Using formic acid, antigen retrieval was performed under the fume hood for 15 minutes. The slides were then washed with PBS followed by a one-hour MOM blocking. Following three rounds of five-minute PBS washes, an anti-mouse primary was then applied with a 1:1000 dilution and left overnight. The next morning, the primary was taken off, and the slides were washed with PBS-T to ensure for a more thorough washing of the section. An anti-mouse secondary was then applied for one and a half hours made in a 1:800 dilution. Following the secondary, there were three five-minute PBS-T washes followed by an incubation in A+B for one and a half hours. The DAB reactant was made using nickel sulfate, and DAB powder. The reaction was then done starting with a concentration of 100ul of H202 per 10ml of DAB. Sodium acetate was used to stop the DAB reaction, and the slides were then washed with PBS, and dehydrated and cover slipped.

GFAP Staining: A GFAP staining was done to determine the presence of astrocyte pathology. Using the TRIS-EDTA buffer, antigen retrieval was performed in a water bath for 25 minutes at 95°C. The slides were then washed with PBS followed by a one-hour goat blocking. Following three rounds of five- minute PBS washes, an anti-rabbit primary was then applied with a 1:1000 dilution and left overnight. The next morning, the primary was taken off, and the slides were washed with PBS-T to ensure for a more thorough washing of the section. An anti-rabbit secondary was then applied for 1 hour made in a 1:800 dilution. Following the secondary, there were three five-minute PBS-T washes followed by an incubation in A+B for 1 hour. The DAB reactant was made using nickel sulfate, and DAB powder. The reaction was then done starting with a concentration of 50ul of H202 per 10ml of DAB. Sodium acetate was used to stop the DAB reaction, and the slides were then washed with PBS, and dehydrated and cover slipped.

Iba-1 Staining: An Iba-1 staining was performed in order to determine microglial pathology. Using the TRIS-EDTA buffer, antigen retrieval was performed in a water bath for 25 minutes at

95°C. A goat blocking was then applied, and the slides were then washed with PBS. An anti-rabbit primary was then applied with a 1:1000 dilution and left overnight. The next morning, the primary was taken off, and the slides were washed with PBS-T to ensure for less precipitate on the slide. An anti-rabbit secondary was then applied for 1 hour made in a 1:800 dilution. Following the secondary, there were three five-minute PBS-T washes followed by an incubation in A+B for 1 hour. The DAB reactant was made using nickel sulfate, and DAB powder. The reaction was then performed beginning with a concentration of 50ul of H202 per 10ml of DAB. In order to stop the DAB reaction, the slides were rinsed in sodium acetate followed by PBS. The slides were then dehydrated, and cover slipped.

Cytokine Assays

Cytokines IL4, IL6, IL12p70, MCP1, IL1β, TNFα, IP10, IL10, and IFNγ where quantified using a custom nine-plex detection kit. The assays were performed as per the manufacturer's protocol. Plasma samples taken from a separate cohort of aged female squirrel monkeys were incubated with a mixture of antibodies conjugated with fluorescent beads and left overnight. The Luminex 200 analyzer (New York University Langone Medical Center Immune Monitoring Core) was used to measure the levels of each cytokine. Using ExPONENT software Median fluorescent intensity was analyzed. Concentrations were calculated from standard curves and are expressed in picograms per milliliter.

Semi-Quantitative Analysis

Analysis of GFAP, Iba1, and T-cell immuno-stained sections was based on a semiquantitative analysis. Immuno-stained sections were evaluated on scales ranging from 0 to 5 based on pathological severity. Approximately nine cortical sections were analyzed per animal.

Results

Neuropathological Evaluation in Aged and Young Squirrel Monkeys

Squirrel monkeys were assessed as an acceptable model for Alzheimer's disease through histological staining and analyzed via semi-quantitative analysis. Old monkeys were compared to young monkeys (4 years old). A 6e10 staining was performed to determine the amyloid burden in an old monkey compared to a young monkey (Figure 1). According to the histological

staining, greater amyloid pathology is seen in the older, aged monkeys than in the young monkey. An Iba-1 staining for glial pathology showed no significant difference between glial presence in old and young monkeys (Figure 2). Squirrel monkey tissue also underwent T-cell analysis through immunohistochemical staining. Results showed significantly more T-cells present in older monkeys than in younger monkeys (two-tailed t test**p=.0011, Figure 3). Further histological analysis measuring astrocyte presence reported significantly more astrocytes present in older aged monkeys than in younger monkeys (two-tailed t test*p=.0338, Figure 4).

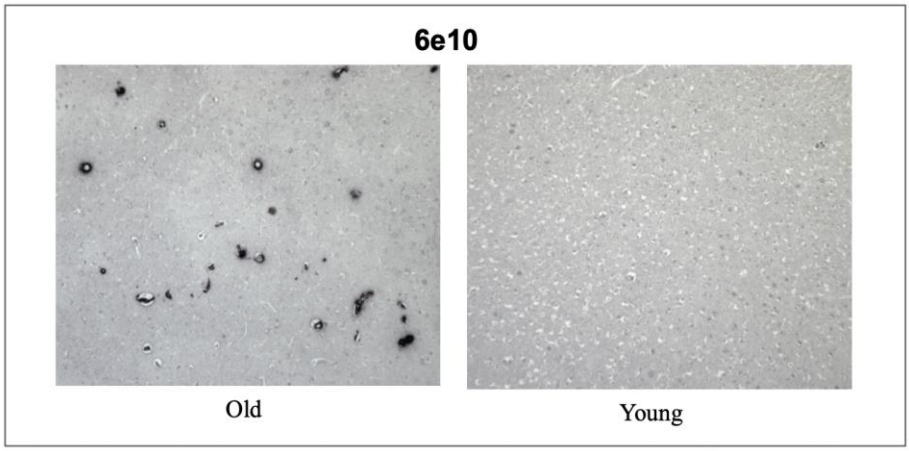

Figure 1. Amyloid burden in aged monkeys compared to young monkeys. Greater amyloid burden is seen throughout the brain of the old monkey, while the young monkey region is clear.

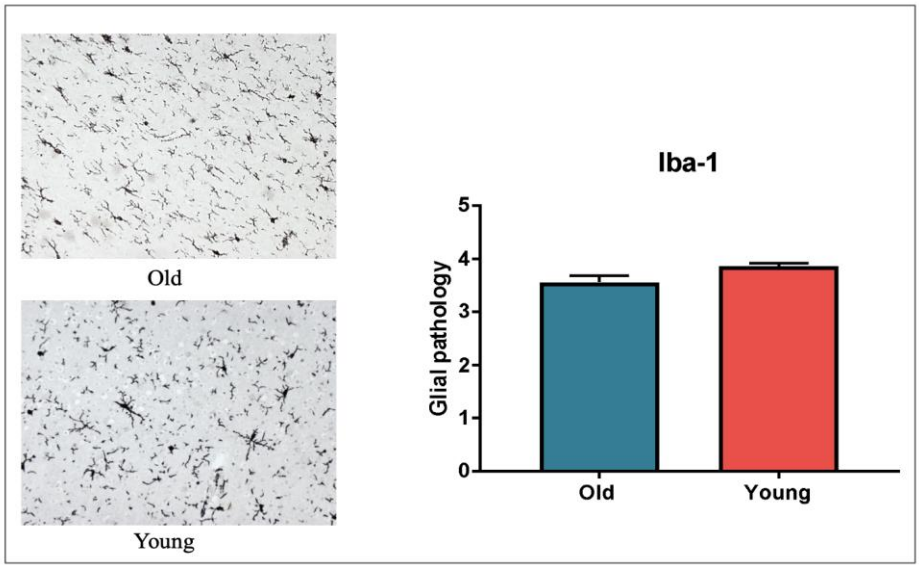

Figure 2. Glial presence in aged monkeys compared to young monkeys. According to semi-quantitative analysis and histological staining, there is no significant difference in glial pathology between old and young monkeys.

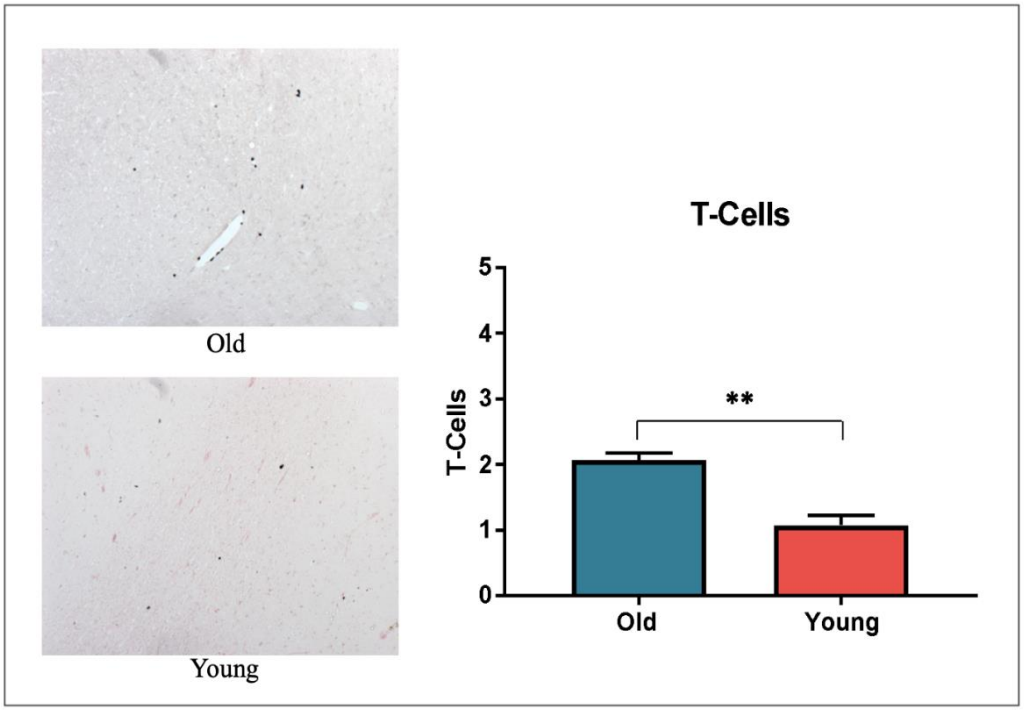

Figure 3. T-cell presence in aged monkeys compared to young monkeys. Semi-quantitative analysis comparing old and young monkeys indicated a significantly higher amount T-Cells present in old monkeys than in young monkeys (**p=.0011)

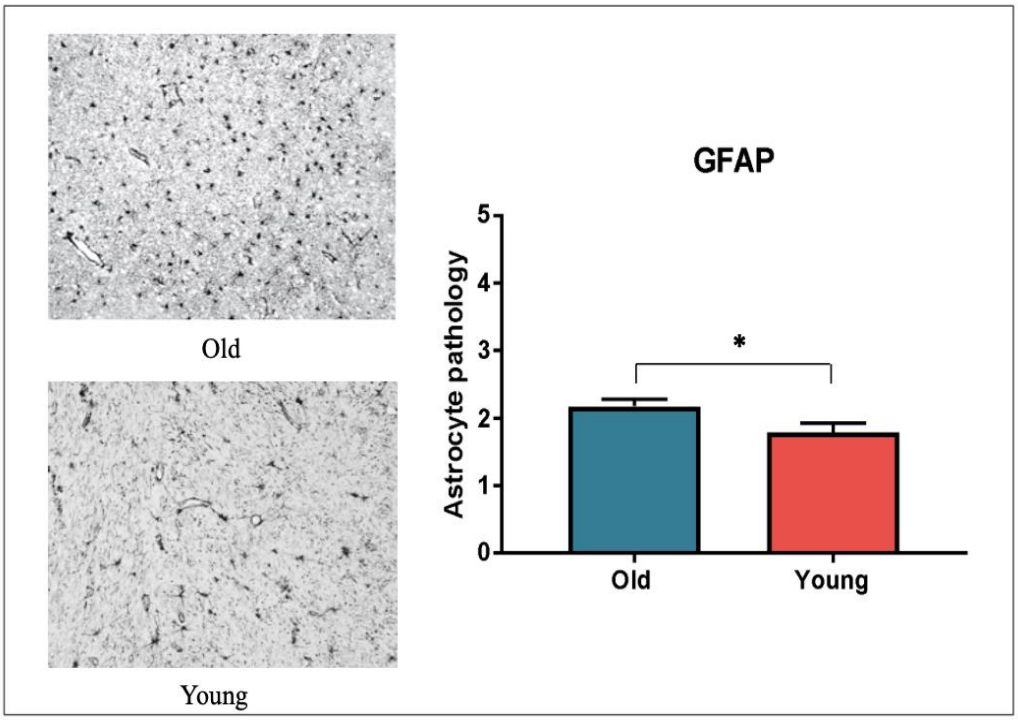

Figure 4. Astrocyte presence in aged monkeys compared to young monkeys. Semi-quantitative analysis indicates a significant increase in astrocyte presence in old monkeys compared to young monkeys (*p=.0338).

CpG ODN Safety in Squirrel Monkeys

Histological and semi-quantitative analysis was performed on squirrel monkey tissue to determine the safety of class C CpG ODN. A PERL Prussian blue staining was performed to determine the presence of microhemorrhages in the brain. Histological staining shows that older monkeys had more microhemorrhages than monkeys injected with CpG ODN (Figure 5). An Iba-1 staining for glial presence was performed showing a significant increase in monkeys administered with CpG ODN compared to monkeys only receiving saline (two-tailed t test****p = < .0001; Figure 6). Additional analysis revealed a significant decrease in T-Cells in monkeys administered with CpG ODN when compared to control monkeys (two-tailed t test****p = < .0001; Figure 7). In order to determine astrocyte presence, a GFAP staining was performed. Results showed no significant difference between monkeys administered with CpG ODN and monkeys that were given saline (Figure 8).

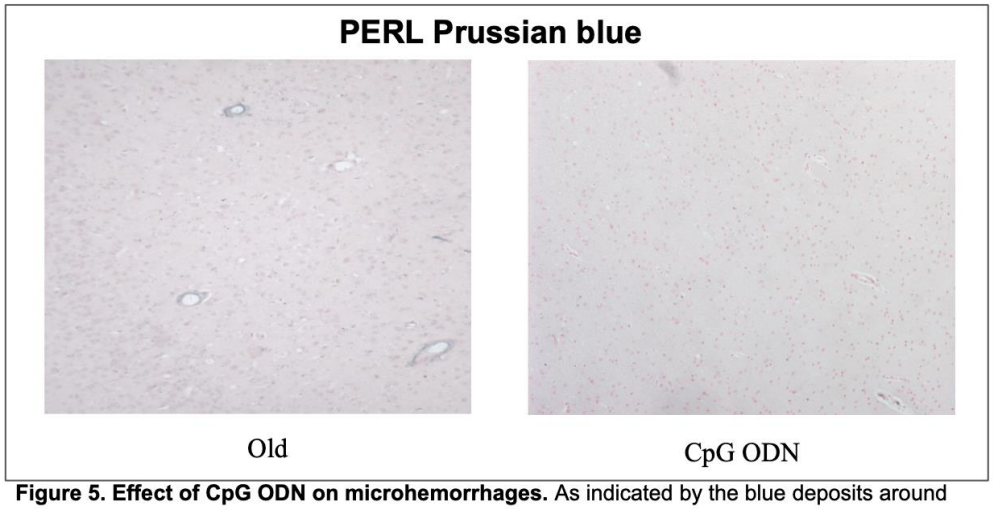

Figure 5. Effect of CpG ODN on microhemorrhages. As indicated by the blue deposits around the vessels, there are more microhemorrhages present in the old monkey compared to monkeys administered with CpG ODN.

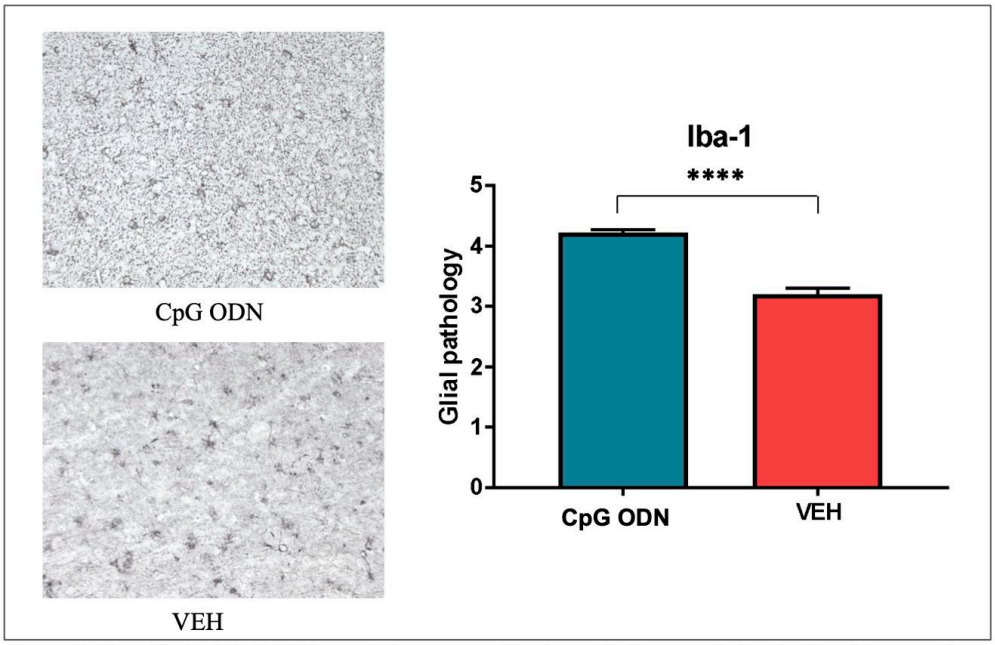

Figure 6. The effect of CpG ODN on glial presence. Semi-quantitative revealed a significant increase in glial pathology in monkeys receiving CpG ODN compared to monkeys receiving VEH (****p = < .0001)

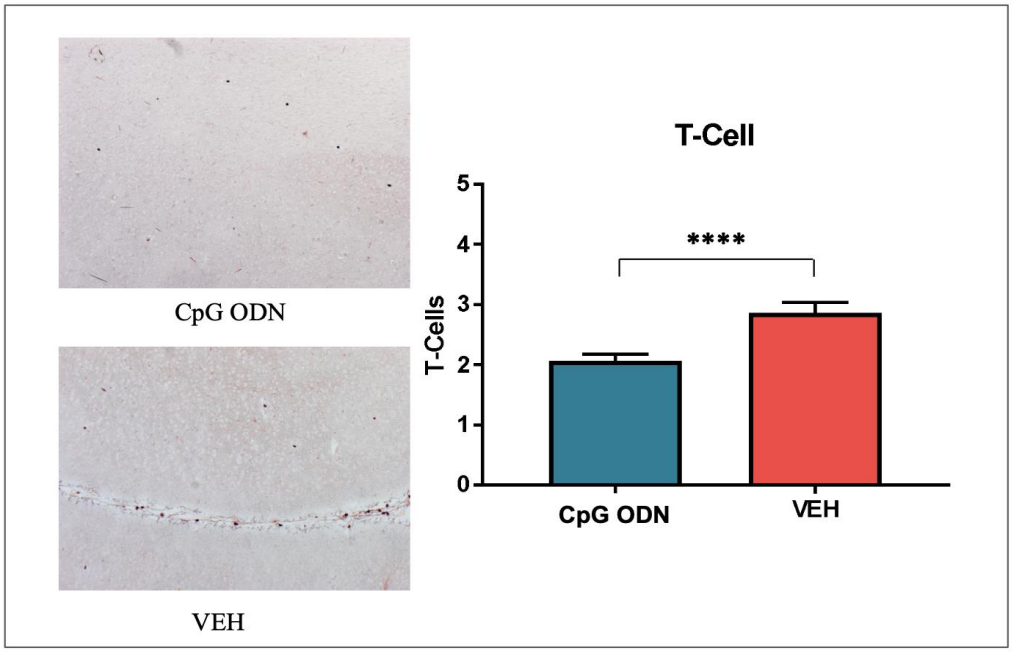

Figure 7. The effect of CpG ODN on T-cell presence. Semi-quantitative analysis showed a significant reduction in T-Cells (****p = < .0001) in monkeys administered with CpG compared to monkeys given VEH.

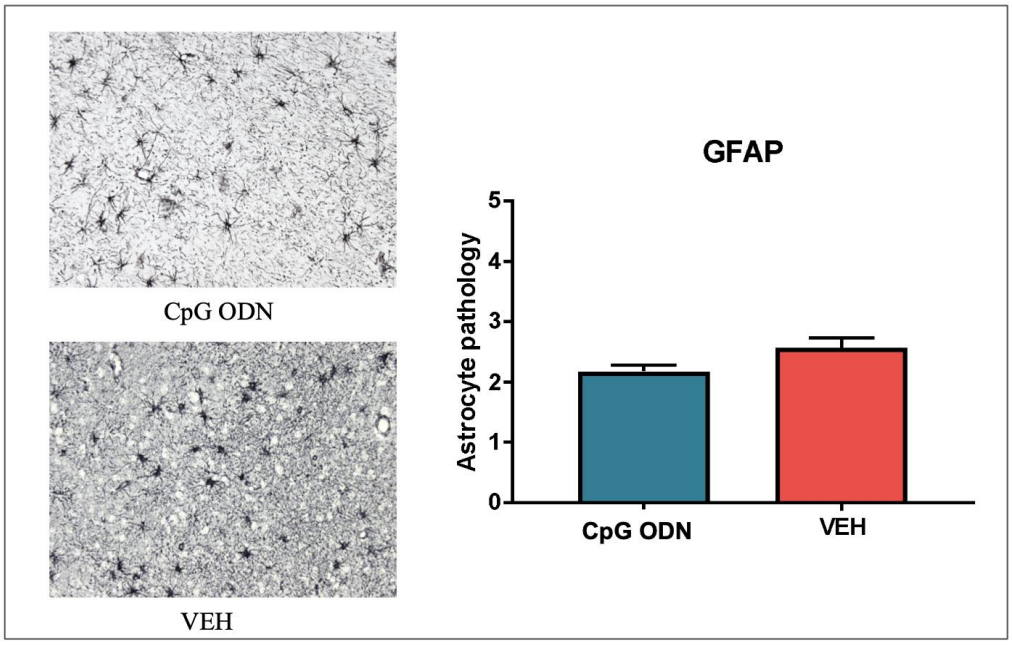

Figure 8. The effect of CpG ODN on astrocyte presence. Semi-quantitative analysis was performed following histological staining, but no significant difference was detected between the two groups.

CpG ODN immunomodulatory response

Cytokine data was retrieved from the plasma of a current chronic ongoing treatment study of a separate cohort of aged female squirrel monkeys. Results indicate that 10 hours after CpG ODN injection, there was a significant increase in cytokine levels in the CpG ODN group. No change was detected in cytokine levels of the saline group. (TNFα: *p=.0276, MCP1: *p=.0248, IL12p40: **p=.0051, IL4: *p=.0496, IFNγ: *p=.0277, IL1RA: *p=.0312, IL10: **p=.0022, Figure 9).

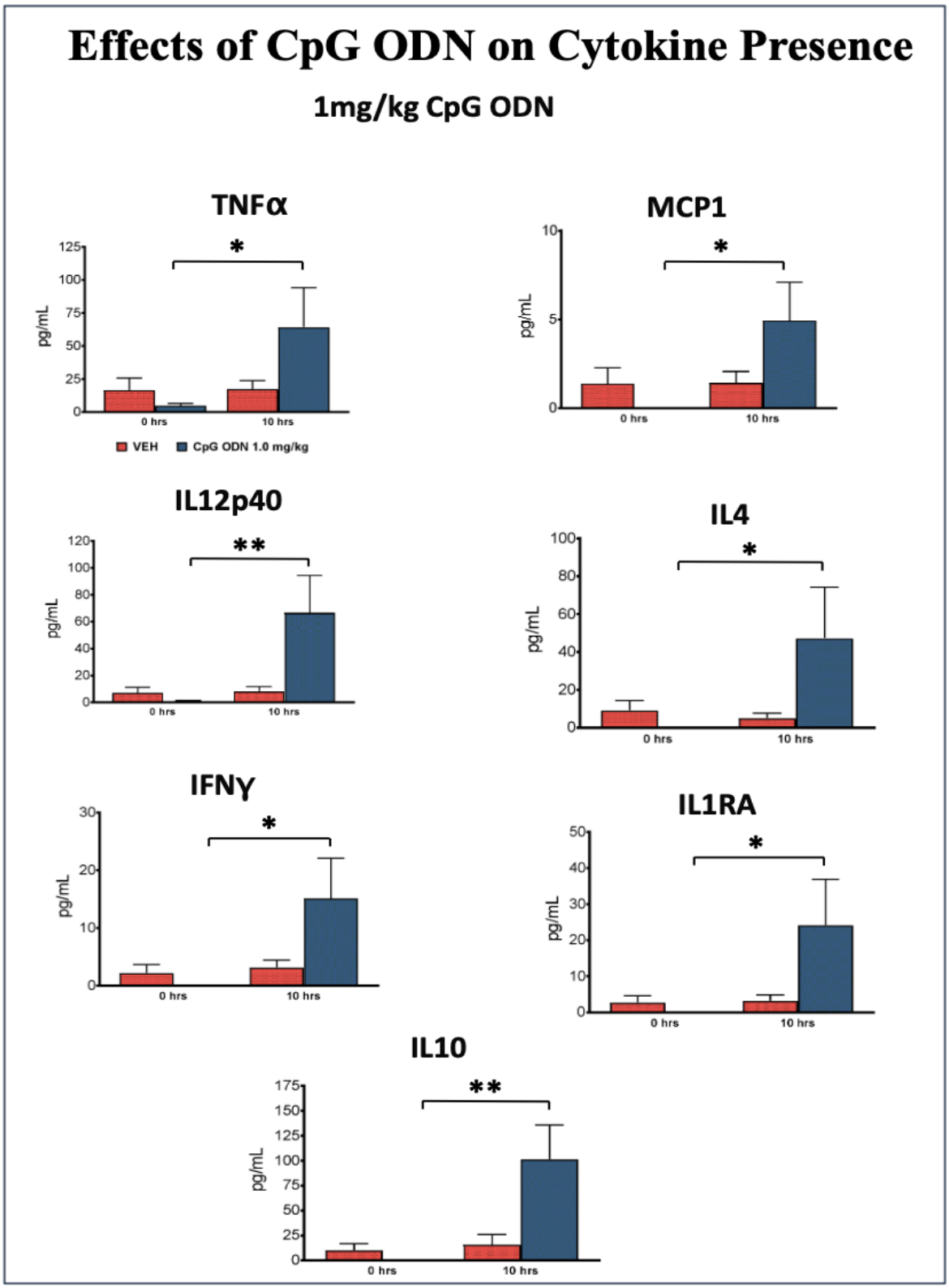

Figure 9. Effects of CpG ODN on Cytokine Presence. Cytokine analysis indicated a significant increase in cytokines. (TNFα: *p=.0276, MCP1: *p=.0248, IL12p40: **p=.0051, IL4: *p=.0496, IFNγ: *p=.0277, IL1RA: *p=.0312, IL10: **p=.0022.)

Discussion

Multiple immunotherapies have been developed to treat Alzheimer's; however, many have been unsuccessful and come with many safety concerns. Such toxicity leads to greater amyloid burden and the development of CAA which can give way to hemorrhages and inflammation in the brain (Kumar-Singh, 2008; Brenowitz, 2015). The current study aims to investigate the safety of TLR9 agonist class C CpG ODN in squirrel monkeys, characterize neuropathology in aged squirrel monkeys, and to asses CpG ODN efficiency in stimulating innate immunity.

This study confirms neuropathology in squirrel monkeys indicating they are an appropriate model of CAA and are, therefore, acceptable for AD experimentation. Immunohistochemical analysis revealed that when comparing younger monkeys to older aging monkeys for T-cells and astrocyte presence, older monkeys exhibited significantly higher levels of these pathologies. This implies that squirrel monkeys display similar aging pathology to humans, suggesting that they are an effective model for Alzheimer's research. In addition, histological data revealed amyloid beta to be more prominent in older monkeys than young monkeys providing further evidence for the use of squirrel monkeys in Alzheimer's research. Due to a small sample size, results showed the same number of glial cells in old and young monkeys. Additional comprehensive analysis of microglia and microphages would need to be performed in order to further characterize the differences between young and old monkeys.

Histological data and semi-quantitative suggests the safety of class C CpG ODN in squirrel monkeys. Previous research confirms microhemorrhages are a common hallmark in individuals with Alzheimer's disease and one of the main aspects of CAA (Yates, 2011). When comparing aging control monkeys to aging monkeys injected with CpG ODN, there was a greater presence of microhemorrhages in control monkeys. This evidence suggests that the use of class C CpG ODN is safe in squirrel monkeys and that it did not result in any negative implications. When testing astrocyte presence in monkeys administered with CpG ODN compared to monkeys injected with saline, both groups showed the same presence of astrocytes suggesting that CpG ODN caused no toxicity or adverse effects in monkeys. Additionally, monkeys receiving a CpG ODN injection exhibited a significant increase in the presence of glial cells– a housekeeping cell–compared to monkeys receiving saline indicating CpG ODN caused no negative consequences. The presence of T-cells in the brain suggests neuronal inflammation

or toxicity (Scholtzova, 2014). Monkeys injected with CpG ODN showed significantly fewer T-cells present compared to monkeys receiving saline. This implies that CpG ODN did not cause T-cell infiltration indicating a lack of evidence for toxicity in the CpG ODN treated monkeys. Due to cross-reactivity of the reagents during histological staining, there is limited knowledge on what types of T-cells (CD3, CD4) were present in the monkeys', so it is unclear whether cytotoxic or helper T-cells were present on the brain tissue.

TLR9 agonist CpG ODN has immune-stimulating qualities (Chen, 2016). Luminex analysis revealed significantly higher cytokine/chemokine levels in monkeys injected with CpG ODN compared to monkeys injected with the saline control. This data suggests that CpG ODN has immune-stimulating qualities and can successfully activate the immune system in the aged monkey population without toxicity. Final behavioral evaluations and assessment of brain pathology will be performed on squirrel monkeys at the completion of the chronic ongoing CpG ODN study.

Conclusion

The present study indicates that the squirrel monkey model is an appropriate Alzheimer's model for experimentation and that it exhibits the same pathologies as humans. In addition, safety for TLR9 agonist class C CpG ODN was confirmed in squirrel monkeys. This suggests that there are no adverse effects such as inflammation, or neural toxicity caused by the CpG ODN injection. Further data also suggests that class C CpG ODN is an effective method of stimulating innate immunity as demonstrated through cytokine/chemokine analysis. Success with CpG ODN as an immunomodulator in squirrel monkeys can suggest future studies in human clinical trials.

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