UNIVERSITY OF CALIFORNIA, SAN DIEGO

The effects of altered DNA methylation on regulation of inflammatory gene expression during the progression of Alzheimer's disease.

A Thesis submitted in partial satisfaction of the requirements for the degree Master of Science

in

Biological Science

by

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The Thesis of Roxana Wiswell is approved and it is acceptable in quality and form for publication on microfilm and electronically:

Co-Chair

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University of California, San Diego

2016

DEDICATION

This thesis is dedicated to my parents, Lydia, Joe, and Jennifer, in gratitude for their unconditional love and support.

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ABSTRACT OF THE THESIS

The effects of altered DNA methylation on regulation of inflammatory gene expression during the progression of Alzheimer's disease.

by

Roxana Wiswell

Master of Science in Biology

University of California, San Diego, 2016

Professor Paula Desplats, Chair
Professor Nicholas Spitzer, Co-Chair

Alzheimer's disease (AD) causes devastating decreases in cognitive ability and quality of life for more than 5 million Americans. Genetic AD risk factors have been well researched, but emerging evidence calls attention to epigenetic changes. In order to screen for changes in DNA methylation during AD, we analyzed genome-wide methylation data from postmortem human frontal cortex samples from AD (n=26), mildly cognitively impaired (n=24), and healthy subjects (n=22). We identified significant changes in methylation in genes related to inflammation, including *CXCL17,*

IKBKAP, IKBKG, IL3, IL13, IL17RB, TNFRSF8, A2M, BDKRB1, and *TNF* in association with AD pathology.

The relationship of epigenetics and inflammatory pathways was further investigated with an *in vitro* model. It was hypothesized that the aberrant DNA methylation observed in AD brains causes dysregulation of inflammatory pathways in glial cells, contributing to the neuro-inflammation that is characteristic of AD. Global DNA methylation was modulated *in vitro* using S-adenosyl methionine to increase or Decitabine to decrease methylation in cultured human microglia, which resulted in activation of inflammation-related genes. Moreover, we identified similar transcriptional changes between cells treated with Decitabine and those activated by amyloid-β (Aβ), including deregulation of *TNF, ICAM1, ITGB1,* and *TNFRSF1B*. Importantly, Decitabine treatment of microglia also caused an increase in *APP* expression, further suggesting a link between changes in DNA methylation and elements of AD pathology.

INTRODUCTION

Alzheimer's disease (AD) is a devastating neurodegenerative disorder associated with a wide range of cognitive deficits. Over time, the symptoms of AD deprive affected individuals of their memories, communication skills, and ability to live independently [1].

During the process of AD neurodegeneration, neuronal death occurs mainly in the hippocampus, leading to memory loss, and the frontal cortex, leading to decline in cognition and decision making capabilities [2]. This is accompanied by deposition of amyloid-β (Aβ) protein into masses called plaques outside brain cells, as well as abnormal formation of tau protein into neurofibrillary tangles inside neurons [3]. Amyloid plaques result from an accumulation of misfolded Aβ peptide aggregated into insoluble fibrils; soluble oligomers of Aβ are often present as well [2, 4]. Tau is a protein normally associated with microtubules in neuronal axons that becomes prone to aggregation when it is misfolded due to hyperphosphorylation [2].

Another important pathological hallmark of AD is inflammation in the brain [3]. It remains open to debate whether neuroinflammation is a cause or a consequence of the aggregation of misfolded proteins in AD – it may well be both [5]. However, inflammation in the brain undoubtedly contributes to neuronal aging and death [6,7]

AD in the United States

There are an estimated 5.2 million Americans currently living with AD, making it the most common cause of dementia [8,1]. As the American population ages over the coming decades, prevalence will continue to rise [8]. According to the National Center

for Health Statistics, AD was the fifth leading cause of death for women in the US in 2013, accounting for 4.6% of deaths; it was the sixth leading cause of death overall [9]. While AD is the leading single cause of dementia, it should be noted that mixed dementias arising from more than one disease are thought to predominate in the elderly, highlighting the complexity inherent to neuropathological conditions [10].

At this time, there is no effective strategy for treatment nor prevention of AD. The management of this chronic disease requires an annual expenditure of more than $150 billion [11]. Taxpayers cover around 7% of this cost through Medicaid, while the rest is paid by private insurance and individual cost [11]. Caregivers for those with AD, often family members, dedicated approximately 18.1 billion hours of unpaid labor to this task in 2015 [1]. In order to combat this growing health and financial burden, the National Alzheimer's Project Act (NAPA) took effect in 2011 and calls for a National Plan to Address Alzheimer's Disease, with a goal of identifying effective prevention and treatment for AD by 2025 [12]. While several drugs are now in Phase III clinical trials for AD intervention, many questions remain unanswered regarding the causes and natural history of the disease.

Progression of AD

AD progresses over decades from a preclinical phase (with pathological changes occurring in the brain, but no detectable impact on cognition), to mild cognitive impairment, and finally to AD dementia [13] **(Figure 1).** Slow decrease in cognitive ability manifests many years before AD diagnosis, and progresses more rapidly during the AD phase [14].

Mild cognitive impairment (MCI) is a condition diagnosed in the elderly based on reduced cognitive function that does not yet meet the criteria for dementia [15]. The difficulty of collecting longitudinal data on the human brain, an inaccessible organ, creates a need to use patients diagnosed with MCI as a proxy for the early stages of AD. Such patients have been shown to progresss to AD at a rate of 12% per year – a significant risk considering that after 6 years, 80% of elderly amnestic MCI patients develop the full symptoms of AD [16]. Further research shows that the amount of accumulated Aβ in the brain of MCI patients correlates well with risk of progression to AD, supporting the view of MCI as early AD because of the known link between Aβ and AD pathology [17].

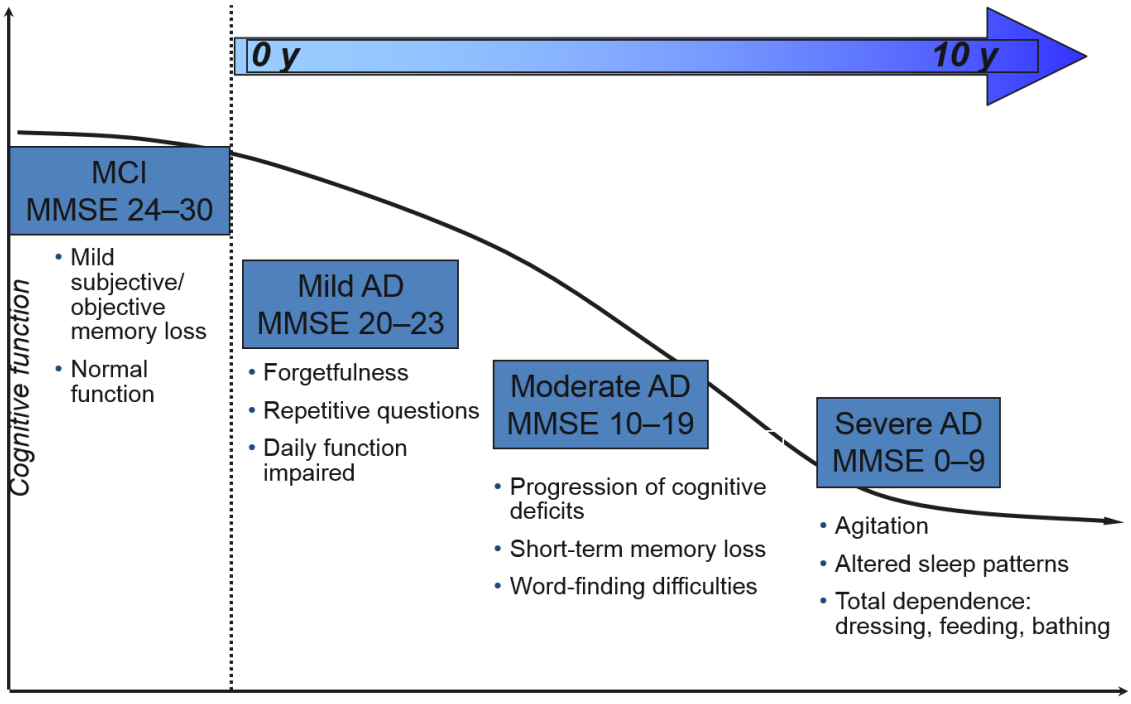

Figure 1. Clinical progression of AD represented in terms of cognitive function (indicated by Multiple Mini State Examination score) over time.

The amyloid hypothesis

Although AD has been the subject of much research, the exact cause remains unknown. The most widespread hypothesis among researchers posits that a buildup of the Aβ protein causes aggregation into plaques, triggering the pathological cascade and ultimately resulting in cognitive decline and death [3]. The idea that Aβ plaques are inherently capable of causing disease has been questioned in recent years, as several Aβ-targting antibodies have failed to ameliorate AD symptoms in clinical trials [18]. However, it remains widely accepted that the Aβ peptide is a key player in the disease. Antimicrobial properties of Aβ have been recently discovered, leading to the novel idea that Aβ plaques can result from the immune response to invading pathogens from the brain [19].

The amyloid-precursor protein (APP) must undergo several cleavages to yield the 42-amino acid fragment of Aβ that has been identified in AD [4] **(Figure 2)**. Mutations in the *APP* gene, as well as the *Presenilin 1* and *Presenilin 2* genes (*PSEN1* and *PSEN2*) that regulate the cleavage of APP, can lead to early-onset AD that occurs with a Mendelian dominant inheritance pattern [20]. Mutations in the *APP* gene that are protective against AD have been identified as well [21]. This provides strong evidence that Aβ dysregulation is sufficient to cause AD; however, only a small percentage of AD cases can be linked to a genetic predisposition to Aβ accumulation. This reinforces the view of AD as a multifactorial disease, combining elements of Aβ and tau pathology with neuroinflammation.

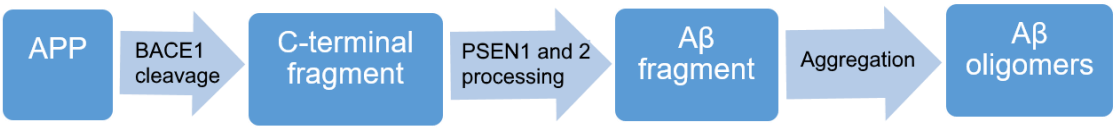

Figure 2. APP post translational processing involves an initial cleavage by the membrane-bound β-site APP cleaving enzyme 1, or β-secretase (BACE1). Out of the two resulting fragments, the larger is released outside of the cells and the smaller C-terminal fragment remains in the membrane for further processing by the γ-secretase complex, composed of PSEN1 and PSEN2. These enzymes cleave the C-terminal fragment into an Aβ peptide fragment between 37 and 42 amino acids long, which can then accumulate into oligomers [6].

Neuroinflammation

Inflammation in the central nervous system (CNS) is executed by pathways of the innate immune system [6]. The source of cytokine signaling in the CNS is glial cells, which can release signals increase or decrease inflammation, such as interleukins (IL) and tumor necrosis factor (TNF) [6]. Key cytokine functions include activating the complement system and inducing expression of nitric oxide (NO) through activation of glial nitric oxide synthases (NOS) [6]. Increased levels of NO are neurotoxic, and also causes nitration of peptides including Aβ – this makes Aβ more prone to aggregation [6].

The resident immune cells of the brain (glia) have two major subsets: astrocytes and microglia [22]. Microglia are derived from neural stem cells, unlike most immune cells [23]. The blood-brain barrier prevents the passage of systemic immune cells and other potentially dangerous molecules from the circulatory system into the CNS tissue – although the recent discovery of lymphatic vessels in the brain shows that the CNS is not as isolated as previously believed [23, 24]. It has also been shown that the blood-brain barrier decreases in efficacy with age, leading to minor perfusion of signaling molecules

into the brain and consequent inflammation, although leukocytes still incapable of entering the brain [25].

Microglia are similar to macrophages but not of hematopoietic origin [26]. During brain development, microglia play a key role in directing the formation of neural synapse networks [27]. In the healthy adult brain, they continue to aid in the formation and repair of synapses [6]. Microglia are also responsible for responding to danger-associated molecular patterns (DAMPs) and pathogen-associated molecular patterns (PAMPs) by producing pro-inflammatory cytokines [6]. One such DAMP is Aβ [28]. When brain cells undergo apoptosis, glial cells clear the debris so that it will not interfere with the functioning of neurons [29]. If microglial cells fail to clear the routine accumulation of cell debris in the brain, then synapse regeneration is unable to proceed [30]. When it comes to Aβ, microglia can help remove it from the brain by two mechanisms: phagocytosis of insoluble fibrils, or protease cleavage of soluble oligomers and fragments [6].

Microglial activation

Microglia can exist in distinct states of activation, denoted M1 and M2 [6]. The M1 activated phenotype is characterized by increased antigen presenting capacity and decreased phagocytic ability, along with increased pro-inflammatory cytokine release [6, 31]. In this state, microglia can damage neurons by secreting cytotoxic factors **(Figure 3),** and also fail to aid with the clearance of Aβ due to reduced phagocytosis [31].

Microglia also can display an anti-inflammatory activation state called M2. This promotes clearance of undesirable biomolecules from the brain, helping to protect

neighboring neurons [6, 31]. During M2 activation, expression of *TREM2* is upregulated [7]. This receptor's activation promotes phagocytosis without inflammatory cytokine production, and is thought to help with clearance of Aβ as well as neuronal debris [7]. *TREM2* loss of function mutations are a known risk factor for AD [7]. While microglia have been well studied in the context of AD, there is still little consensus as to the exact role of these cells in the disease.

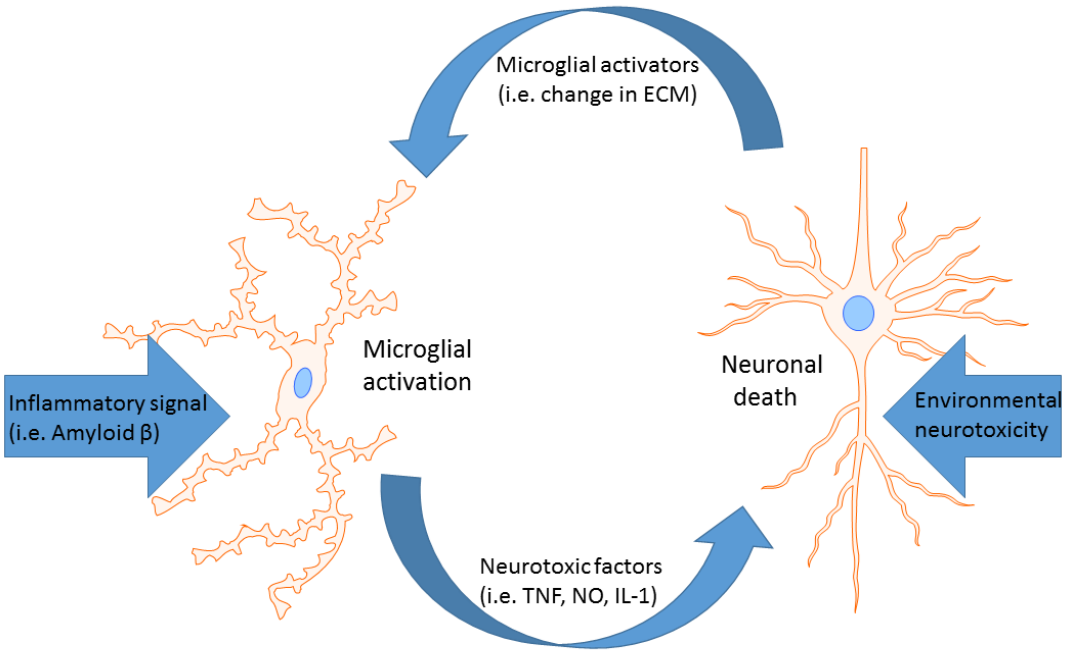

Figure 3. The relationship between microglial activation and neuronal cell death. Activated microglia secrete pro-inflammatory gene products such as interleukins, tumor necrosis factor, and reactive nitrogen species (produced by nitric oxide synthase), which can have negative consequences for neighboring neurons. Changes in neuronal cells can have a positive feedback effect on microglia, reinforcing activation.

Genetic and environmental factors in late-onset AD

The vast majority of AD cases are sporadic and not from familial origin [20]. This means they do not arise from mutations in APP or PSEN genes [20]. Instead, the majority

of cases have multifactorial genetic and environmental causes [20]. For late-onset, non-familial AD, many genetic risk factors have been identified that are neither necessary nor sufficient for the development of the disease, but confer additional risk. The most well-known such gene is *APOE* ε4 allele [20]. Normal ApoE is involved in cholesterol homeostasis, and is hypothesized to help clear Aβ oligomers out of the brain [32]. *APOE* variant ε4 is the single most influential risk factor for the development of sporadic AD, and carriers of this allele have been shown to have greater Aβ accumulation in the brain [33].

Interestingly, a link has been established between the function of the *APOE* gene and the status of microglial cells in the brain [7]. *APOE* expression in the brain is predominantly in astrocytes and microglia, and the ApoE protein is responsible for decreasing inflammation by downregulating TLR signaling, TNF, and nitric oxide production, which are a result of M1 activated microglia [7, 31]. The presence of the deleterious *APOE* ε4 allele increases pro-inflammatory nitric oxide production by microglia *in vitro* [34].

Epigenetic factors in sporadic AD

Research has historically focused on the genetic basis of AD, but emerging studies point to the involvement of epigenetics as well. Histone acetylation and DNA methylation patterns are known to be altered in the progression of AD [35, 36]. These epigenetic mechanisms help modulate transcriptional activity of genes [36]. DNA methylation, the addition of a methyl group to cytosine bases in DNA, is one form of epigenetic modification that can modulate gene expression [37]. Enzymes called DNA

methyl transferases (DNMTs) add methyl groups to DNA at cytosine-phosphate-guanine (CpG) sites [38]. The DNMT3a and DNMT3b enzymes are primarily responsible for adding new methylation marks to previously unmethylated CpG sites, while DNMT1 maintains the methylation state through DNA replication by methylating the daughter strand to match the parental strand [39]. All three DNMTs use S-adenosylmethionine (SAM) as a methyl donor in order to transfer a methyl group to the DNA [38]. After surrendering its methyl group, SAM is converted to S-adenosylhomocysteine and subsequently to homocysteine; folate and Vitamin B12 are necessary for the conversion of homocysteine back to SAM [40].

The effect of methylation on gene expression varies – methylation in the promoter tends to decrease transcription of a gene, while methylation in the 5' untranslated region tends to increase transcription [38]. It has been shown that some genes become over or under-methylated in patients' brains early in AD [41]. De Jager and colleagues have shown differential methylation in several genes including *RHBDF2*, involved in the TNF inflammatory pathway, in AD brains versus healthy controls [41].

The role of DNA methylation in modulating neuroinflammation is not well understood, but transcriptional programs for microglial activation are known to be under epigenetic control [42]. Previous studies have shown alterations of DNA methylation in inflammation-related genes for AD patients' brain tissue [41, 43]. This raises the question of whether there is a direct link between the differential gene methylation and neuroinflammation in AD. The role of epigenetic dysregulation in microglia has not been fully explored, and I therefore hypothesized that changing the global methylation level of the DNA in microglial cells will alter the expression of inflammation-related genes. I also

investigated a potential connection between Aβ peptide exposure and methylation changes in microglia.

METHODOLOGY

In silico analysis of global DNA methylation in human postmortem brain samples

Postmortem brain samples were previously used in the Desplats lab to profile genome-wide methylation in the frontal cortex. Differential methylation analysis was performed with Genome-Studio software applying the Illumina custom error model, after background subtraction and computing for false discovery rate. Methylation levels were expressed as β scores, which represent the average intensity of signals measured for the methylated and unmethylated status of each particular CpG site. Differential methylation was considered significant at a threshold of $\Delta\beta > 0.17$ (in each direction) at $p < 0.01$.

Methylation values at specific CpG sites mapping to the inflammatory-related genes profiled by real-time PCR was extracted using GenomeStudio software (Illumina). Prism software (GraphPad) was used to test for differences by group in methylation at specific CpG sites using Partek analysis. One-way ANOVA with the Bonferroni post hoc test or Tukey's test was used to test for correlations between site-specific methylation and MMSE scores at $p < 0.05$. CpG sites for analysis were chosen by known relation to pro-inflammatory genes.

Cell culture

Human microglia derived from adult primary microglia and immortalized with SV40 were purchased from Applied Biological Materials. Cells were grown in PriGrow III media (Applied Biological Materials) containing 10% fetal bovine serum (FBS) and 1% Penicillin-Streptomycin, at 37ºC in a 5% CO_2 enriched environment. Cultureware

was coated with Extracellular Matrix (ECM, Applied Biological Materials) diluted 1:2 in PBS. The cells were collected using TrypLe Express detachment reagent (Gibco). The viability of the cells was determined using the Countess automated cell counter and Trypan blue stain.

Immunocytochemistry

Microglia were grown on acid-washed coverslips coated with diluted ECM and stained with antibodies against GFAP (1:300 dilution, Cell Signaling Technology), NeuN (1:500 dilution, Abcam), and Iba-1 (1:2000 dilution, Wako). FITC secondary antibody and an Olympus phase contrast microscope at 400x magnification were used for detection.

Treatment to increase DNA Methylation *in vitro*

Human microglia were treated with different concentrations of SAM (Cayman Chemical) as a methyl donor to induce increased methylation of DNA. SAM (dissolved in PBS, diluted in media) was applied to cells at concentrations of 50 µM, 500 µM, 750 µM, 1000 µM, and PBS (diluted 1:10 in media) was used as a vehicle control. After 24 hours of treatment, the cells were collected and viability was determined using Trypan Blue exclusion measured by a Countess cell counter (Thermo Fisher). DNA was isolated from approximately 1 $x10^6$ cells using the DNeasy Blood and Tissue Mini Kit (Qiagen). The DNA was concentrated using the Wizard SV Gel and PCR Clean-Up System (Promega).

Treatment to decrease DNA Methylation *in vitro*

Human microglia were grown as described above. Cells were treated with Decitabine (5-Aza-2' deoxycytidine, Cayman Chemical), which inhibits DNMT activity. Cultured microglial cells were treated with Decitabine (dissolved in DMSO, diluted in media) at 1 µM, 10 µM, 20 µM, and 50 µM, with DMSO (1:1,200 dilution in media) used as a vehicle control. The treatments were replenished every 24 hours. After a 48 hour treatment, approximately 1 $x10^6$ cells were collected and DNA was prepared as described previously.

Treatment with Amyloid-β Peptide *in vitro*

Human microglial cells were grown as described above and treated with Aβ 1-42 peptide fragments (rPeptide). Cultured microglial cells were treated with Aβ (dissolved in DMSO, diluted in media) at 0.5 µM, 1 µM, 2.5 µM, or DMSO (diluted 1:100) as a vehicle control. After a 24 hour treatment, approximately 1 $x10^6$ cells were collected and DNA was prepared as described above.

Enzyme-linked Immunosorbent Assay

The proportion of 5-methyl cytosine (5-mC) residues in DNA was measured using the Global DNA Methylation LINE-1 Kit (Active Motif). Long Interspersed Nucleotide Element 1 (LINE-1) sequences methylation levels can be used as a representation of methylation in the overall genome [44]. DNA was quantified using a Nanodrop spectrophotometer at 260 nm. 600 ng of DNA were digested with MseI, and 100 ng of the resulting DNA fragments were then hybridized to a biotinylated LINE-1 probe in

duplicate. The probes, along with the DNA of interest, were then bound to a streptavidin-coated plate. Methylated DNA was detected with an antibody for 5-mC and a secondary antibody conjugated to horseradish peroxidase. Results were quantified using spectrophotometry with the DTX 880 Multimode Detector (Beckman Coulter) at 450 nm. Data was analyzed using Prism 6 software (GraphPad) and expressed either as percentage of 5-mC content in comparison to methylated DNA standards, or as a proportion of 5-mC compared to the vehicle.

Assessment of Cytotoxicity

The degree of cell death triggered by the different experimental treatments was assessed using the CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega). Human microglia were cultured in a 96-well plate and cell lysis in response to treatment conditions was measured by quantifying levels of lactate dehydrogenase (LDH). Absorption at 490 nm was quantified with the DTX 880 Multimode Detector (Beckman Coulter). The level of cytotoxicity elicited by a treatment was expressed as a percentage, considering the LDH released by cells lysed in control reactions as 100%.

Transcriptional analysis of inflammation-related genes

Human microglial cells were grown as described above and treated with 500 µM SAM, 10 µM Decitabine, 2 µM Aβ, or the corresponding vehicle. Approximately 2 $x10^6$ cells were used for RNA extraction, while 1 $x10^6$ cells were used for DNA isolation for ELISA to verify that treatments were effective in modulating methylation as previously described. RNA was extracted using the RNeasy Mini Kit (Qiagen). The RNA was

quantified with a nanodrop spectrophotometer at 260 nm and 2 µg of total RNA were reverse transcribed using the Superscript VILO cDNA Synthesis Kit (Life Technologies).

Levels of gene expression in the treated microglia were quantified by real-time PCR using the Taqman Human Inflammation Panel (Life Technologies) and the StepOne Plus Real-Time PCR System (Applied Biosystems). This panel contains primers for 84 human inflammation-related genes and 4 control housekeeping genes. Data was analyzed using the DataAssist software (Life Technologies). Transcript abundance was determined using the comparative Delta Ct method, normalized to the geometric mean of *HPRT1* and *GUSB* as reference genes. The Gene Network Central Pro tool (SA Biosciences) was used to investigate the interactions between differentially expressed genes.

Transcriptional analysis of selected genes

RNA was prepared and reverse transcribed as described previously. Real-time PCR was performed using Taqman Fast reagents and the StepOne Plus Real-Time PCR System with primers for *DNMT1, DNMT3a, DNMT3b,* or *APP*, multiplexed with primers for *β-actin*. Transcript abundance was determined using the comparative Delta Ct method normalized to *β-actin* as a reference gene.

Statistical analysis

Statistical analysis was performed on Prism software (GraphPad). Non-parametric t-test (Mann-Whitney) analysis was used to compare the effect of treatment versus vehicle on methylation and gene expression. One-way ANOVA with Tukey's post-hoc

test was used to compare methylation and gene expression between AD, MCI, and control groups.

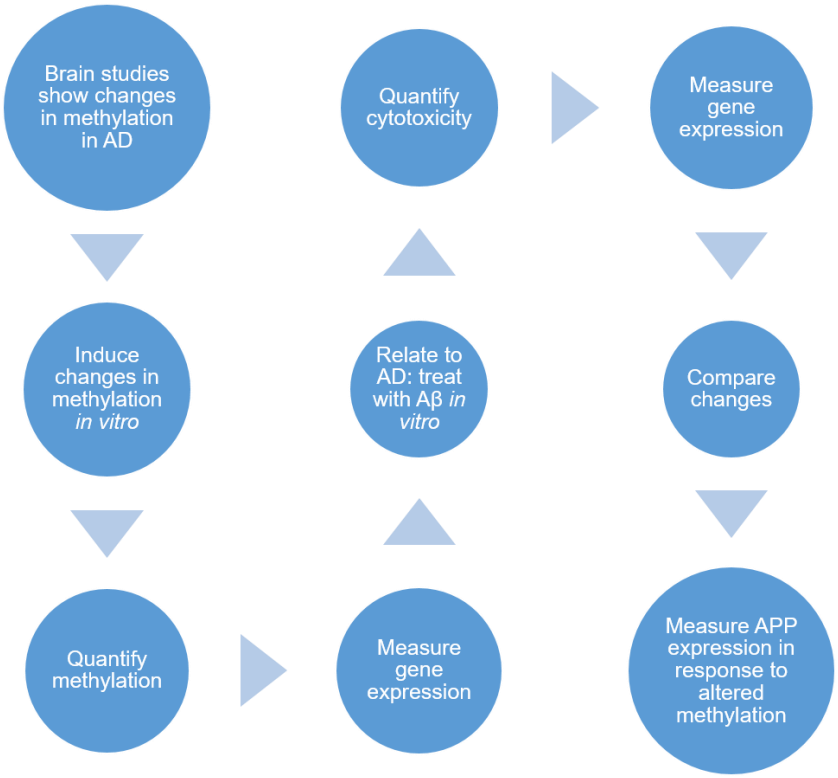

Figure 4. Workflow of DNA modulation in human microglial cells. Based on findings *in silico*, cultured cells were treated with compounds to increase or decrease DNA methylation. Global DNA methylation was quantified by ELISA. Using RNA from the same pool of cells, the expression of inflammation-related genes was determined with real-time PCR. Cultured cells were also treated with Aβ, and cytotoxicity and gene expression responses were quantified.

RESULTS

In silico analysis of genome-wide methylation data from human brains

In order to understand the role of DNA methylation on neuroinflammation in AD, we started by performing *in silico* analysis of genome-wide methylation data already generated by the Desplats lab. This comprehensive database includes intensity signals from more than 450,000 probes interrogated using the Illumina Human Methyl 450K bead array, which enables wide coverage of promoters, 5'UTR, 3'UTR and gene body regions across the genome. For this parental study, DNA was extracted from postmortem midfrontal cortex samples (Brodmann's area 9) obtained from the UCSD Shiley-Marcos Alzheimer's Disease Research Center. The cohort included 72 cases that were stratified according to pre-mortem cognitive evaluation and postmortem neuropathology (summarized in **Table 1**) as mild cognitively impaired individuals, MCI (n=24), which presented Mini Mental State Examination (MMSE) scores < 27 and >18, with AD Braak stage for neurofibrillary pathology 3-4; AD cases (n=26), with MMSE <18 and AD Braak stage 5-6; and a control group (CT; n=22) including age and sex matched non-demented individuals with MMSE ≥ 27 and AD Braak stage 0-2.

Table 1. Clinical and demographic characterization of cases used for genome-wide profiling of DNA methylation. Disease duration calculated as time elapsed from clinical diagnosis to death.

Group	Age	Gender	Disease duration	MMSE	Braak	Plaques (MF)
Control	83.9 ± 5.9	13/9	N/A	27.3 ± 6.4	0 - 1	10.7 ± 14.7
Mild Cognitive Impairment	87.3 ± 4.5	13/11	5.0 ± 5.4	22.6 ± 5.4	1.2 - 3	30.2 ± 19.7
Alzheimer's Disease	80.5 ± 6.0	13/13	9.5 ± 5.5	9.2 ± 6.6	6.2	48.79 ± 3.1

The methylation status per specific CpG site was expressed as a β value, calculated as the ratio of the fluorescence intensity signals of the methylated (M) and unmethylated (U) alleles, $\beta = \text{Max}(M,0)/[\text{Max}(M,0) + \text{Max}(U,0) + 100]$. β values range between 0 (non-methylated) and 1 (completely methylated). The Illumina GenomeStudio Software (version 2011.1) was used to assess quality and extract the DNA methylation signals from scanned arrays.

In order to incorporate biological and clinical factors into the analysis, normalized β values were imported into PARTEK Genomic Suite software [45]. Mixed-model multi-way analysis of variance was used to compare the methylation at individual CpG loci across different groups. We applied multivariable ANOVA using gender (female or male); age (coded as decades) and disease group (CT, MCI or AD) as

categorical variables with fixed effects, since they represent conditions of interest and may influence methylation levels.

Differential methylation analysis by multiway ANOVA yielded 1180 individual CpGs with significant differences in methylation when computing the interaction between disease, age, and gender; with 938 probes associated with known genes. Importantly, gene ontology (GO) analysis interrogating for biological processes using Panther (www.panther.org) showed that many of these genes were related to the immune system; including both pro- and anti-inflammatory genes (**Table 2**).

Table 2. Specific CpG sites with significant changes in DNA methylation state between control, MCI, and AD postmortem brain tissue, as compared to all CpG sites profiled. P-values were calculated by multiway ANOVA of disease, age, and gender of subjects.

Gene	CpG probe ID	p-value
IL13	cg06584121	0.009968
IL17RB	cg16448309	0.006607
IL3	cg16545743	0.005593
IKBKAP	cg07281640	0.005204
TNFRSF8	cg03422094	0.003802
CXCL17	cg15937958	0.003459
IKBKG	cg00813156	0.003176

Focused *in silico* analysis of human brain data

In silico analysis was continued using the data from human brain tissue to detect changes in methylation at specific CpG sites between control, MCI, and AD groups using one-way ANOVA. Genes for analysis were selected based on broad associations with the inflammatory response in AD.

At a CpG site within the *A2M* gene denoted as probe cg00146928, significant changes in DNA methylation were observed between control and MCI cases as well as

control and AD cases **(Figure 5).** Similar changes were identified at *BDKRB1* gene site cg10528989.

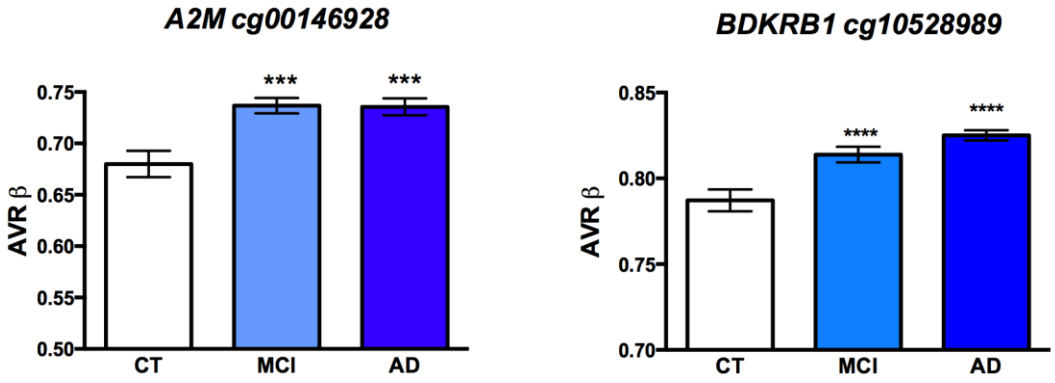

Figure 5. Comparison of average β methylation values between control, MCI, and AD postmortem brain tissue at individual CG sites in genes related to inflammation.

The *A2M* cg00146928 and *BDKRB1* cg10528989 sites are examples of significant differences in methylation by group. Additionally, the *TNF* gene shows statistically significant changes at 10 of the 27 CpG loci identified within the gene.

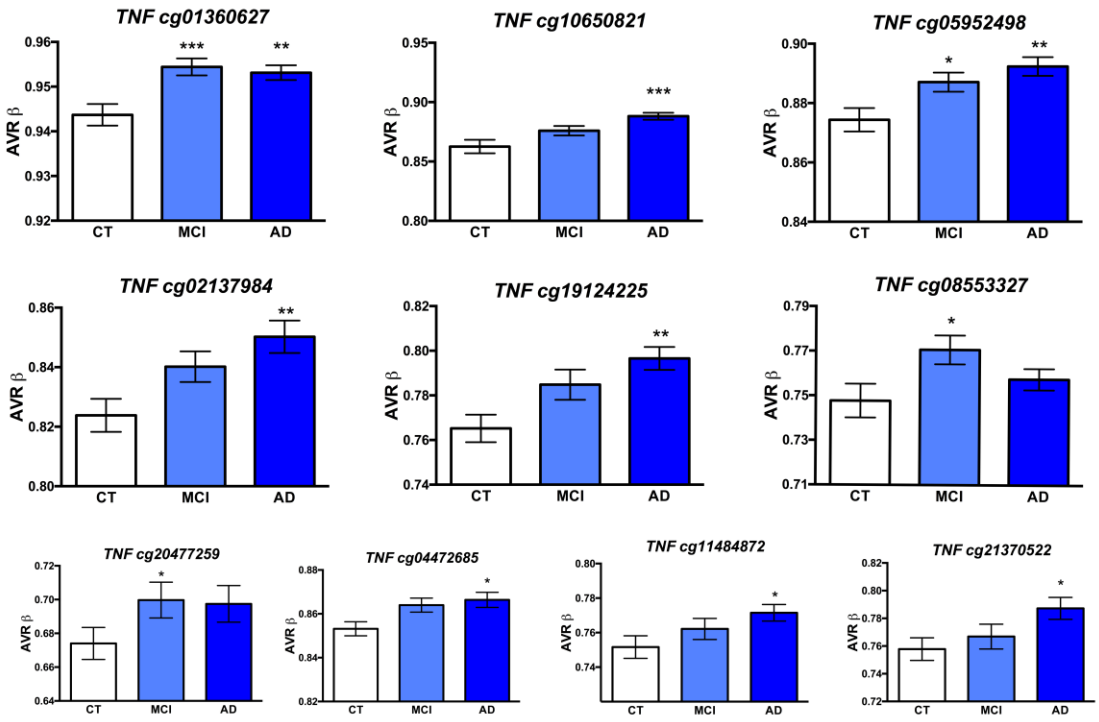

Figure 6. Comparison of average β methylation values between control, MCI, and AD postmortem brain tissue at individual CG sites in the *TNF* gene.

A major limitation inherent to studies in postmortem brain samples is the presence of multiple cell types, each with characteristic epigenotypes, which may confound the methylation signal quantified. This creates difficulty in conducting a focused study of inflammatory processes, which mainly involve microglial cells. Patterns of DNA methylation are known to differ across cell types in the brain, and the broad epigenetic differences between neurons and glia in the brain may confound the detection of methylation changes during AD progression [46]. To complement an in depth analysis of the role of DNA methylation in mediating neuroinflammation, my work utilized a cell culture model in order to isolate the effects on a specific cell type.

Characterization of the human primary microglia cell model.

Activation of microglial cells and the ensuing inflammatory cascade is one of the known mechanisms of neurotoxicity mediated by Aβ in AD [6]. A commercially available immortalized primary human microglial cell line was selected as an *in vitro* model. Cells were first characterized by immunocytochemistry, using the Iba1 protein as a marker of mature microglia **(Figure 7).**

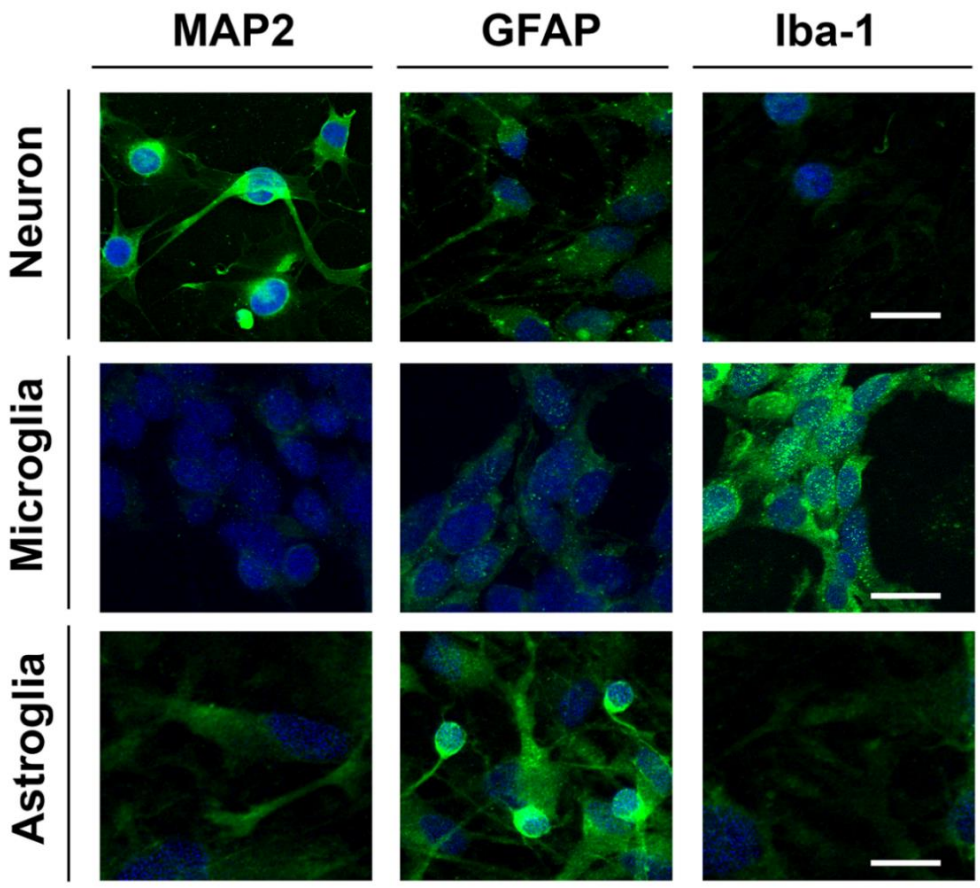

Figure 7. Immunocytochemistry of brain cell types, stained for MAP2 to indicate a neuronal phenotype in B103 rat neuroblastoma cells, Iba1 to indicate microglial markers in immortalized human microglial cells, and GFAP to identify primary human astrocytes.

The immortalized human microglial line used for *in vitro* experiments shows expression of markers for mature microglia, but not for markers of other brain cell types – namely neurons and astroglia **(Figure 7).** With this model established, we modulated the levels of global DNA methylation using pharmacological tools.

Increase in global DNA methylation of human microglial cells

SAM is the donor molecule of methyl groups used by DNMTs to modify cytosine bases in the DNA, and is known to increase global levels of methylation *in vitro* [47]. We first assessed the effect of various concentrations of SAM on cultured microglia using ELISA to quantify methylated cytosine residues in the DNA **(Figure 8).** We compared the total proportion of methylated DNA in each epxerimental group to the levels of methylation in the vehicle treated cells.

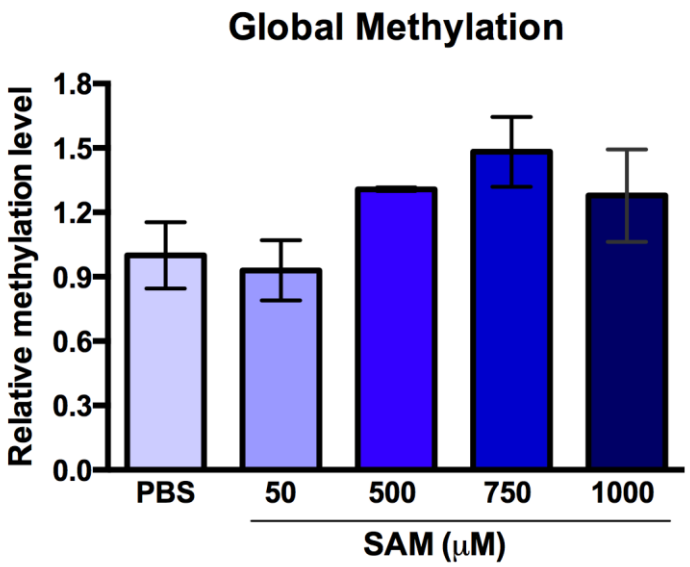

Figure 8. DNA methylation of human microglia treated with SAM as quantified by the Global DNA Methylation LINE-1 Kit. Relative global methylation shows the proportion of DNA methylated in each of the treatment groups in comparison to the vehicle condition (PBS) set to 1.

Cell viability was recorded as percentage of live cells using Trypan Blue exclusion, and changes in morphology were monitored. Viability was slightly decreased in cells treated with the higher concentrations of SAM (750-1000 μM), therefore we selected 500 μM SAM for further treatment to increase DNA methylation without compromising viability.

In order to evaluate whether changes in DNA methylation induced by SAM treatment in microglia directly modulate the expression of genes involved in inflammatory responses, we used a PCR focused array panel to measure expression of 84 genes known to regulate the human inflammatory response **(Table 3)**.

Table 3. Categorization of genes included in the Human Inflammation Taqman Array for real-time PCR from Applied Biosystems. The panel also included the genes *HPRT1*, *GUSB*, and *GAPDH* to function as endogenous controls for normalization.

Enzymes and Inhibitors	**Receptors**
Kinases: *MAPK1, MAPK3, MAPK8, MAPK14*	G-Protein Coupled Receptors: *BDKRB2, HRH3, PTGDR, PTGER2, PTGER3, PTGFR, PTGIR, TBXA2R*
Lipases: *PLA2G10, PLA2G1B, PLA2G2A, PLA2G2D, PLA2G4C, PLA2G5, PLA2G7, PLCB2, PLCB3, PLCB4, PLCD1, PLCE1, PLCG1, PLCG2*	Interleukin Receptor family: *IL1R1, IL1R2, IL1RAPL2, IL1RL1, IL2RA, IL2RB, IL2RG*
Prostaglandin Metabolism: *ALOX5, ALOX12, HPGD, KLK1, KLK2, KLK3, KLK14, KLK15, KLKB1, PTGIS, PTGS1, PTGS2, TBXAS1*	Adhesion molecules: *ICAM1, VCAM1, ITGAL, ITGAM, ITGB1, ITGB2*
Phosphodiesterases: *PDE4A, PDE4B, PDE4C, PDE4D*	Tumor Necrosis Factor receptor family: *TNFRSF1A, TNFRSF1B, CD40*
Other: *A2M, CASP1, CES1, LTA4H, LTC4S, NOS2*	Nuclear receptors: *NR3C1*
Factors	**Channels**
Tumor Necrosis Factor family: *TNF, TNFSF13B, CD40LG*	L-type calcium: *CACNA1C, CACNA1D, CACNA2D1, CACNB2, CACNB4*
Annexins: *ANXA1, ANXA3, ANXA5*	Ligand-gated receptor: *HTR3A, HTR3B*
Other: *IL13, NFKB1, KNG1*	

Expression of genes in **Table 3** was quantified in microglia after 24 hours of treatment with 500 µM SAM, in comparison to the corresponding vehicle, performed in duplicate. DNA and RNA were isolated from the collected cells, with DNA used to validate the effectiveness of SAM in causing increased methylation while RNA was used to profile the expression of the selected genes using the Human Inflammation Taqman Array.

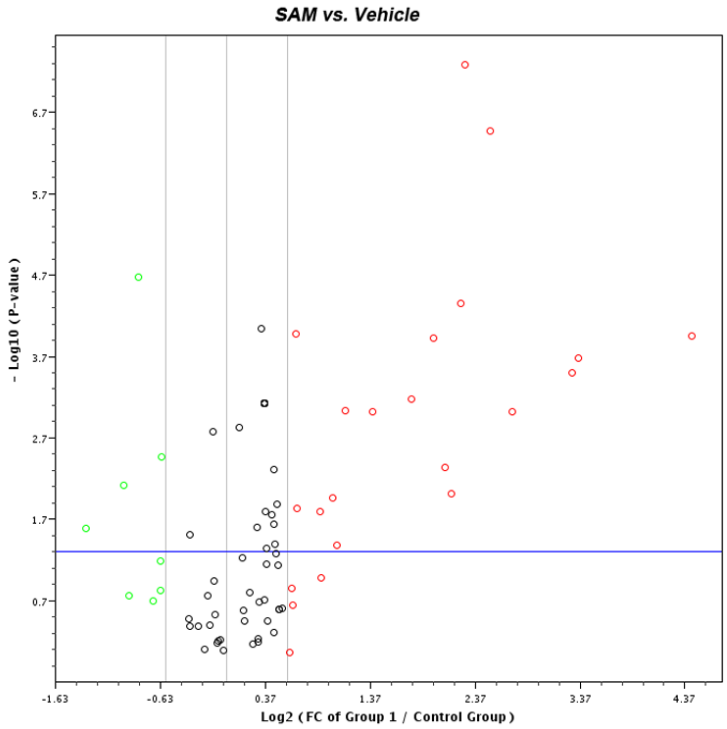

Figure 9. Volcano plot for SAM-treated cells, comparing fold change of expression and p-value of the significance of this change. Each point represents a gene assayed for changes in microglia with increased DNA methylation. Red indicates increased expression, while green indicates decreased expression. Data points above the blue line display statistically significant changes with a p-value < 0.01. Data points outside the vertical bars increase or decrease in expression by more than 1.5 fold.

Figure 9 shows that this treatment modulated the expression of many genes in the panel, with statistically significant changes at a magnitude of more than 1.5 fold. The top changes are reported in detail in **Table 4**.

Table 4. Expression of inflammation-related genes in microglia after SAM-induced increase of DNA methylation. Fold regulation indicates the fold change, (2^(- Delta Delta Ct)), for up-regulated genes and the negative inverse of the fold change for down-regulated genes. The reported p-values are calculated based on a Student's t-test of the 2^(- Delta Ct) values for two replicates.

Gene	Fold regulation	p-value
TNF	21.631	0.000111
IL1RL1	10.2372	0.000211
BDKRB2	9.8084	0.000323
IL1R2	6.6095	0.00095
PTGS2	5.6957	0
PDE4D	4.6992	0.000045
ITGAM	4.4142	0.009868
PTGS1	4.2405	0.004636
IL1R1	2.6207	0.000963
TNFRSF1B	2.0696	0.042169
TNFRSF1A	1.8479	0.016105
MAPK3	1.5864	0.014967
CACNB2	-1.5419	0.003427
HPGD	-1.7932	0.000021
PLCB4	-1.9684	0.00776
ANXA3	-2.5364	0.026062

Although by definition increased methylation is associated with gene repression, there is a position effect involved in this epigenetic mechanism [48]. The majority of the changes we observed correspond to gene activation, and may therefore be associated with changes in methylation at sites in the gene body instead of promoters. On the other hand, these genes may be indirectly affected by methylation changes; the observed increase in expression may be due to changes at the level of regulators upstream of the inflammatory signaling cascade. We then interrogated the functional relation of the up-regulated genes (**Figure 10)**.

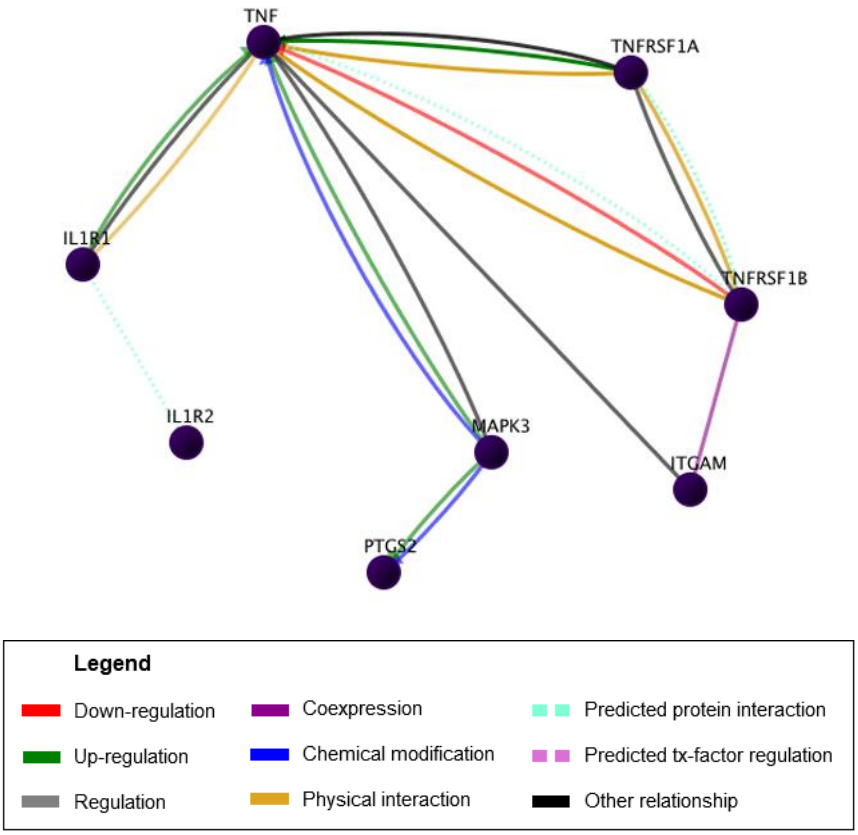

Figure 10. Interactions known to occur in human brain tissue for products of genes with significantly increased expression after treatment with SAM. These relationships were identified *in silico* using Gene Network Central Pro from SA Biosciences.

Of the genes that showed significant changes in expression after SAM treatment, eight are part of a known network. Most of the interactions reported in brain tissue converge on *TNF*. These effects may account for the 20-fold increase observed in the transcription of this gene **(Table 4)**, which may result from the additive effect of direct methylation changes in *TNF* and signaling from other pathway members.

Decrease in global DNA methylation in human microglial cells

To investigate the effects of overall decreased methylation on microglial cells *in vitro*, we used Decitabine to inhibit the activity of DNMTs. This drug has been previously reported to induce a global decrease in DNA methylation [49]. Similarly to the analysis we performed with SAM, the effects of different concentrations of Decitabine in cultured microglia were tested by ELISA to quantify methylated cytosine bases in the DNA **(Figure 11).**

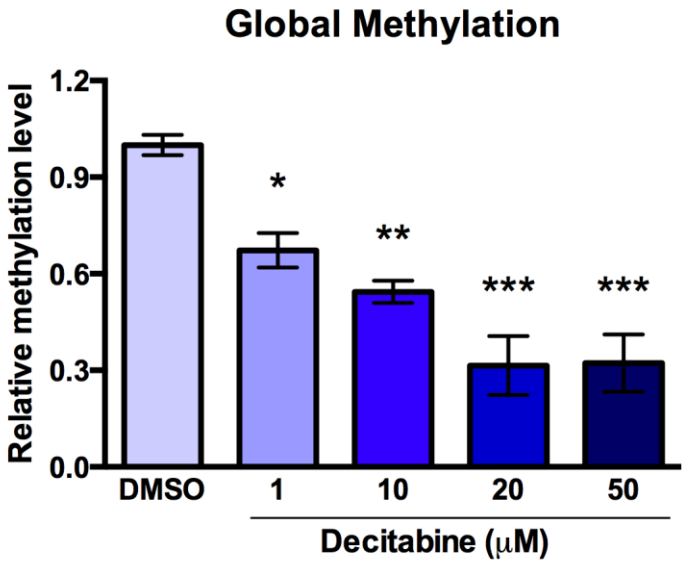

Figure 11. DNA methylation of human microglia treated with Decitabine as quantified by the Global DNA Methylation LINE-1 Kit. Relative global methylation shows the proportion of DNA methylated in each of the treatment groups in comparison to the vehicle condition (DMSO) set to 1.

Decitabine treatment elicited a dose-response decrease in DNA methylation which reached statistical significance at every concentration tested. Treatment with 20 μM Decitabine resulted in a 30% decrease in global methylation in comparison to the vehicle

condition. A higher dose of Decitabine (50 µM) enacts similar effects on methylation, and none of the treatments significantly altered cell viability **(Figure 11)**.

After defining the optimal condition for Decitabine treatment, cultured microglia were treated with 20 µM Decitabine for 48 hours. The effect of Decitabine treatment on methylation was validated in microglia used for real-time PCR with ELISA, showing the expected reduction of DNA methylation to about 35% of the vehicle. Results of the Human Inflammation real-time PCR panel are shown in terms of fold change and statistical significance in **Figure 12** and top changes are detailed in **Table 5.**

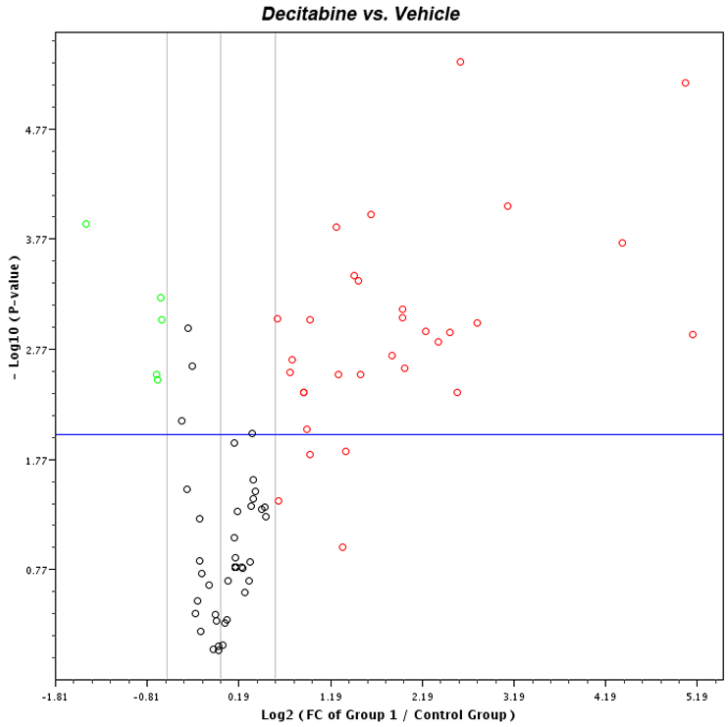

Figure 12. Volcano plot for Decitabine-treated cells, comparing fold change of expression and p-value of the significance of this change. Each point represents a gene assayed for changes in microglia with decreased DNA methylation. Red indicates increased expression, while green indicates decreased expression. Data points above the blue line display statistically significant changes with a p-value < 0.01. Data points outside the vertical bars increase or decrease in expression by more than 1.5 fold.

Table 5. Expression of inflammation-related genes in microglia with decreased DNA methylation. Fold regulation indicates the fold change, (2^(- Delta Delta Ct)), for up-regulated genes and the negative inverse of the fold change for down-regulated genes. The reported p-values are calculated based on a Student's t-test of the 2^(- Delta Ct) values for replicates.

Gene	Fold regulation	p-value
TNF	35.3658	0.001233
IL2RG	33.461	0.000006
PLCB2	20.7318	0.000184
IL2RB	6.9392	0.000966
IL1R2	6.0924	0.000004
ITGB2	5.96	0.004148
ITGAM	5.632	0.001189
PLA2G7	5.1667	0.001445
PLA2G10	4.7065	0.001154
PTGER3	4	0.002513
ITGAL	3.9451	0.000869
NOS2	3.933	0.000727
KLK1	3.6416	0.001931
ICAM1	2.8591	0.002815
PTGS1	2.8241	0.000398
HTR3B	2.7408	0.000363
IL2RA	2.5735	0.014058
CD40	2.4194	0.002843
PTGS2	1.9579	0.000916
PTGIS	1.951	0.015268
PTGIR	1.916	0.008927
IL1RL1	1.8711	0.004177
PLA2G4C	1.8711	0.004177
PLCB4	1.7132	0.002084
PTGFR	1.6882	0.002728
TNFRSF1B	1.5457	0.040045
PLCD1	1.5243	0.00089
PLCG2	-1.5692	0.000902
ITGB1	-1.5784	0.000568
CACNA2D1	-1.6204	0.003177
PTGER2	-1.624	0.002828
CACNA1C	-2.7794	0.000123

Decitabine treatment of induced the activation of a large number of inflammation-related genes **(Table 5, Figure 13)**. We detected 32 genes with differential expression,

many of which are related to the TNF superfamily of cytokines and receptors. Increased transcription was recorded for 84% of regulated genes.

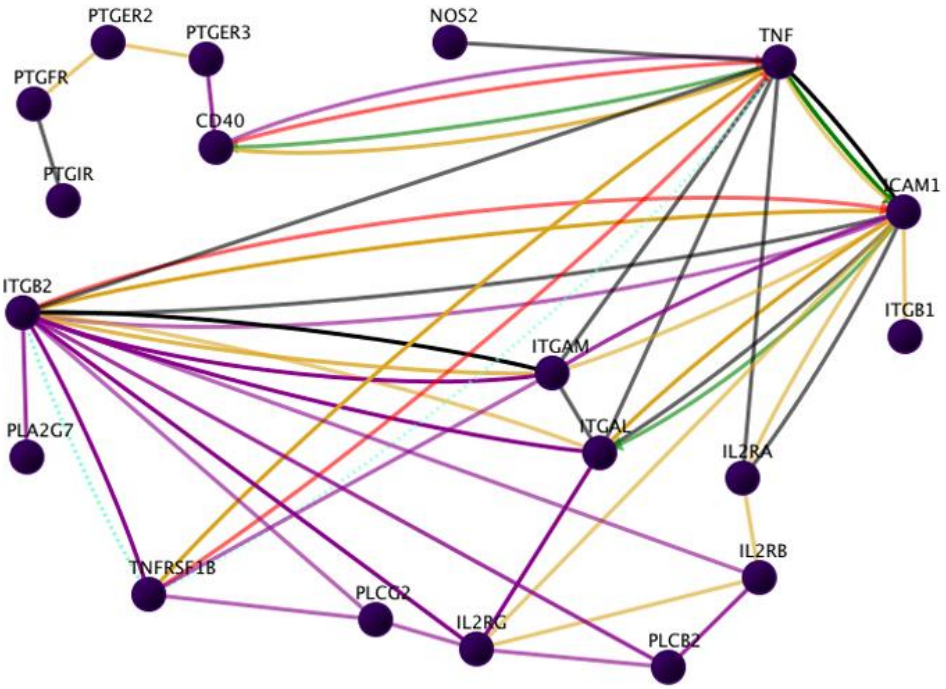

Figure 13. Interactions in human brain tissue for products of genes with significantly altered expression after treatment with Decitabine. These relationships were identified *in silico* using Gene Network Central Pro from SA Biosciences.

Analysis of the interactions between genes with transcription modified by Decitabine showed a more complex network, where Integrin Beta 2 (*ITGB2*); Intercellular Adhesion Molecule 1 (*ICAM1*) and TNF are the major interaction hubs.

Notably, these molecules play an important role in the inflammation cascade associated with AD pathology **(Figure 13).**

Effects of Aβ 1-42 peptide on human microglial cells

Taken together, the previous results show that DNA methylation is directly implicated in the regulation of gene expression in human microglia, thus contributing to neuroinflammation. We next evaluated these results in the context of AD pathology. The response of microglia to Aβ treatment *in vitro* has been well studied, in order to explore the behavior of microglia in AD conditions. Aβ peptide treatment is known to activate transcription of inflammation-related genes [50].

We further characterized our cell culture model of immortalized human microglia by treating cells with different concentrations of Aβ 1-42 peptide and testing cell viability **(Figure 14).**

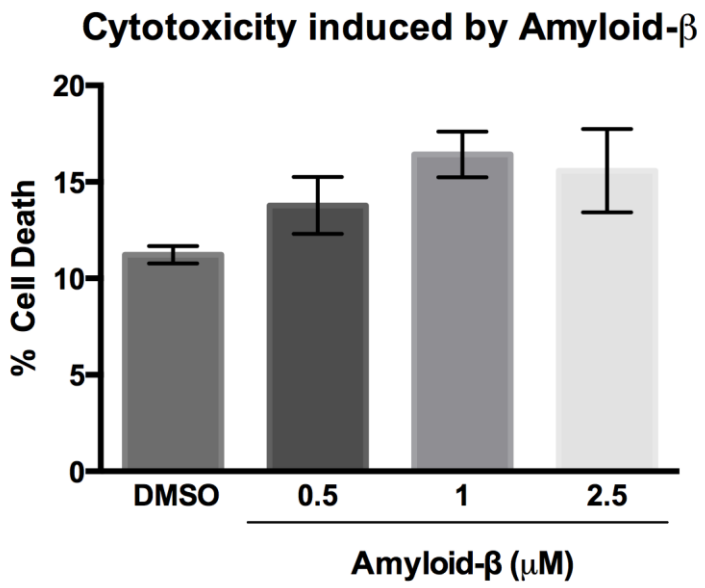

Figure 14. Cell death of human microglia treated with Aβ 1-42 as quantified by the CytoTox 96 Non-Radioactive Cytotoxicity Assay.

Treatment with Aβ peptide induced cell toxicity as previously reported. Based on previous studies and our viability assay, we selected a concentration of 2 μM for a preliminary analysis of the effects of Aβ on the expression of inflammation-related genes **(Table 3).** Since these studies are undergoing, only two replicates have been analyzed so far, with variable results that hindered the statistical significance of the changes. Results are therefore presented in terms of fold regulation alone. Importantly, we measured several changes consistent with the observations of Walker et al. in a similar *in vitro* system, providing an indication that treatment was effective [51].

Table 6. Expression of inflammation-related genes in microglia activated with Aβ treatment. Fold regulation indicates the fold change, (2^(- Delta Delta Ct)), for up-regulated genes and the negative inverse of the fold change for down-regulated genes. As this is preliminary data pending further validation, p-values are not given.

Gene	Fold Regulation	Gene	Fold regulation
ICAM1	4.228	ITGB1	-1.6607
PTGS2	2.8378	MAPK14	-1.6611
TNFSF13B	2.3494	PDE4A	-1.7474
TNFRSF1B	1.9674	PLCD1	-1.7657
BDKRB2	1.9156	MAPK3	-1.7747
HTR3A	1.8538	PLCB4	-1.8059
TNF	1.7813	CACNB2	-1.849
VCAM1	1.5616	PTGS1	-1.8696
PLCG2	-1.5106	PTGIS	-1.8806
ANXA5	-1.5342	CES1	-1.9106
CACNB4	-1.5359	CACNA1C	-1.9904
PTGFR	-1.5802	ANXA1	-2.0278
PLA2G1B	-1.6	PTGER2	-2.0559
TNFRSF1A	-1.6113	KLK3	-2.3245

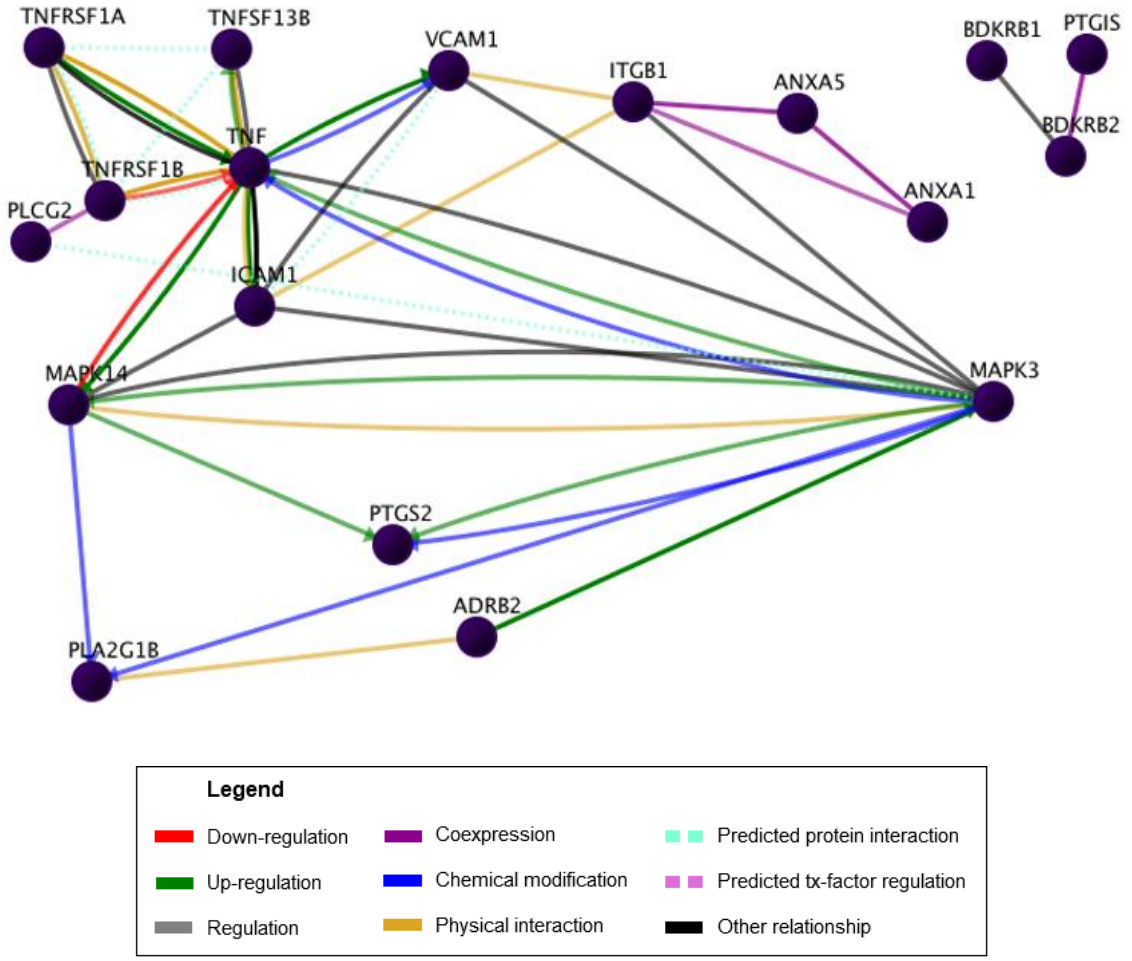

Figure 15. Interactions in human brain tissue for products of genes with altered expression after treatment with Aβ. These relationships were identified *in silico* using Gene Network Central Pro from SA Biosciences.

Similarly to the effects observed for Decitabine, most differentially transcribed genes interact and TNF appears as the converging point. Two novel hubs appear in this group of genes, centering on in Mitogen-Activated Protein Kinases (MAPK) 3 and 14 **(Figure 15).**

After identifying the transcriptional changes induced by increased or decreased methylation and Aβ, we compared treatment effects as a first step in revealing a potential mechanistic relation. Upregulation of *TNF, TNFRSF1B* and *PTGS2* was a common

change observed after all treatments, therefore we reasoned that these genes are likely regulated by the inflammatory signaling cascade and represent secondary targets. This hypothesis is supported for the position of these genes as receiver molecules in the interaction networks (**Figures 10, 13 and 15**). Overall, changes elicited by Aβ treatment overlap better with Decitabine-induced transcriptional alterations, suggesting that a decrease in methylation mediates inflammation in AD.

A major question in AD research is whether inflammation and DNA methylation represent a cause or consequence of disease. We tested two alternate hypotheses that may relate Aβ and methylation. First, accumulation of Aβ peptides during the course of AD may alter the expression of methylation enzymes, resulting in a global decrease in methylation that induces inflammation-related genes. The effects of Aβ on microglia were further tested using real-time PCR for the expression of DNMTs **(Figure 16)**.

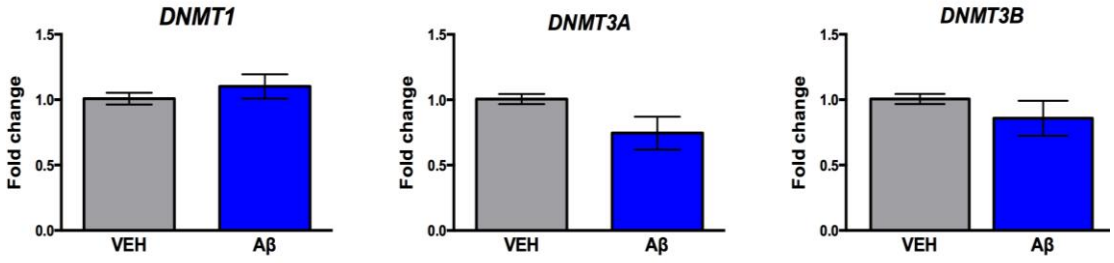

Figure 16. Quantification of levels of DNMTs in human microglia after treatment with 2 μM Aβ using real-time PCR.

The expression of DNMT family members did not change significantly in response to 2 μM of Aβ. These results suggest that Aβ fragments do not act upstream of

DNMTs. Still, other mechanisms including altered protein stability or subcellular localization may deregulate DNMTs in AD.

A second hypothesis was made following reports that regulation of BACE and PSEN1 by DNA methylation modify Aβ levels [40].We tested whether changes in methylation can directly modulate APP expression. Increased levels of the amyloid precursor protein are known to cause increased abundance of Aβ fragments, which in turn may trigger inflammation [4]. Quantification of APP expression in microglia showed that Decitabine treatment caused a significant increase in *APP* expression **(Figure 17)** supporting this second mechanism and placing demethylation upstream of the toxic amyloid cascade that includes inflammation.

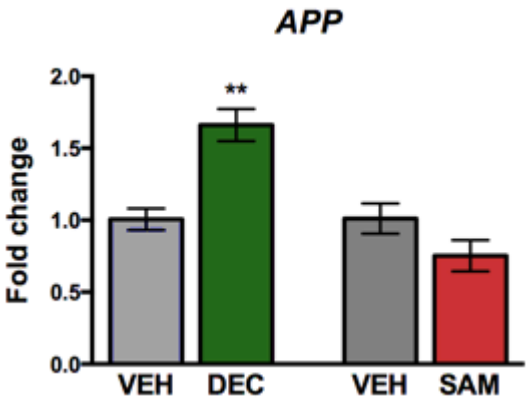

Figure 17. Expression of *APP* in human microglia treated with either Decitabine or SAM, compared to the corresponding vehicle. Decitabine-induced decrease and SAM-induced increase in global methylation were validated by ELISA.

Part of the results section of the thesis is currently being prepared for submission for publication of the material. Wiswell, Roxana; Cronin, Peter; Fields, Jerel and Desplats, Paula. “Alterations in DNA methylation contribute to neuroinflammation in

Alzheimer's disease". The thesis author was the primary investigator and author of this material.

DISCUSSION

In this study, DNA methylation was investigated for its role in modulating neuroinflammation in AD. This research provides evidence that alteration of DNA methylation causes broad changes in the expression of inflammation-related genes in human microglia.

Epigenetic alterations are recognized as a component of AD pathology, but the mechanisms remain unclear. Reductions in global DNA methylation, DNMTs, and DNMT recruitment factors have been identified in AD brains [35]. A recent development shows that the *APOE* gene, whose genotypic variation is the strongest risk factor for late-onset AD, is differentially methylated in the brain tissue of AD patients [32]. DNA methylation changes in AD patients have also been shown to correlate with age, suggesting a connection with disease progression [52]. AD-related reduction in DNA methylation and related cellular machinery has been previously studied in neurons, but less explored in microglia [35].

This study in AD brains shows dysregulation of methylation in genes directly implicated in the inflammatory response including *A2M*, *BDKRB1*, and *TNF*. A2M, or α-2-macroglobulin, is a serum protein capable of binding and inhibiting the action of proteases and cytokines [53, 54]. Elevated A2M has been reported as a blood biomarker in AD, as well as cardiovascular diseases, and *A2M* polymorphisms have been investigated for potential conferral of AD risk, though no consensus has been reached [55, 56, 57]. These results show that epigenetic changes occur in the *A2M* gene in AD **(Figure 5).** Interestingly, A2M binds and inhibits signaling of TNF, another factor

displaying alterations across multiple CpG sites in AD brains [54] **(Figure 6).** Aberrant methylation of the TNF promoter has been reported previously in AD [58]. We found significantly altered methylation of the *TNF* gene in MCI cases as well, suggesting that dysregulation may be an early step in pathology.

Another gene showing changes in methylation in AD is *BDKRB1* **(Figure 5).** Bradykinin signaling, which can occur through the BDKRB1 receptor, modulates expression of the beta-adrenergic receptor implicated in AD susceptibility and pathogenesis [59, 60, 61].

A major limitation of methylation studies in brain tissue is the mix of neurons and glia present, as such cell types are known to have different methylomic signatures [46]. Therefore, an *in vitro* model of an isolated cell type was warranted. Another limitation of studies of AD epigenetics is the inaccessibility of brains for experimental studies. This project makes a novel contribution by investigating the mechanisms of epigenetic changes in terms of a cell type known to be a key player in AD pathology, and yet understudied in terms of the epigenome.

Methylation of cultured human microglia was manipulated in order to focus on the inflammatory response. Changes were observed from both an increase and decrease in global DNA methylation, with more robust changes associated with the Decitabine treatment. During the normal aging process *in vivo*, alterations in DNA methylation are known to accumulate over time. This phenomenon, called “epigenetic drift”, consists of a decrease in global methylation, paired with an increase of methylation at specific promoter sites [62]. Considering that age is the largest risk factor for AD, and is

correlated with AD hallmarks such as accumulation of Aβ plaques, this link between epigenetic changes and aging may indicate a role of epigenetic drift in AD [1]. Age-associated epigenetic changes may directly contribute to inflammation, which is a known early contributor to neuronal death in AD [6]. Notably, treatment of microglia with Decitabine to decrease global DNA methylation caused an increase in expression of *APP*. Overexpression of *APP* can contribute to the formation of Aβ plaques [40]. This key finding places epigenetic alterations as a possible contributor to AD pathology.

In order to further relate these findings to molecular mechanisms in AD, we investigated the changes in inflammatory genes after treatment with exogenous soluble Aβ. Microglial responses to Aβ fibril administration have been previously characterized, showing significant induction of *TNF* and *ICAM1* transcription in addition to other inflammation-related genes [51]. This correlates well with the results of our study of Aβ administration, where *TNF* and *ICAM1* were shown to be up-regulated **(Table 6).**

Among the genes with altered transcription in response to Aβ, *TNF*, *MAPK3,* and *MAPK14* appear as key players – all genes that have been previously implicated in the Aβ response **(Figure 15).** TNF signaling is known to be significantly up-regulated in AD, with downstream effects culminating in neuroinflammation and, critically, an increase in BACE expression that may promote Aβ formation [63]. Aβ is known to modulate signaling of MAPK3, also known as ERK1 [64]. Inhibition of MAPK 14 (aka P38 MAPK) suppresses cytokine up-regulation and ameliorates synaptic and behavioral defects in transgenic mouse models of AD [65].

Treatment of microglia with Decitabine caused up-regulation of multiple genes, with *TNF, ICAM1,* and *ITGB2* emerging as important effectors **(Figure 13).** *TNF* was also upregulated in response to Aβ and SAM treatments, and is known to regulate *ICAM1* expression. Polymorphisms in the *ICAM1* gene are associated with AD risk, while *ITGB2* expression has been shown to be upregulated in AD [66, 67].

The inflammatory response to Decitabine treatment showed more overlap with gene expression changes induced by Aβ treatment, compared to SAM. Therefore, a decrease in global DNA methylation may more accurately reflect AD conditions than a global increase. Future studies will investigate whether the inflammatory effects of exogenous Aβ administration in microglia can be ameliorated by sustaining high levels of DNA methylation using SAM. This idea is supported by previous studies showing that reduced SAM levels *in vitro* caused by a lack of folate and vitamin B12 can increase levels of Aβ fragments [40]. This effect is mediated through increased expression of PSEN1 and BACE, enzymes that work together to modify APP into Aβ [40]. Addition of exogenous SAM to the system was sufficient to reverse the increase in Aβ, leading to the idea of SAM as a possible treatment for AD [40]. This pharmacological intervention has shown promise in mouse models of APP overexpression [68, 69].

Taken together, these results position DNA methylation high up in a pathological cascade that triggers neuronal death via increased APP production and altered processing, promoting aggregation of Aβ fragments and resulting in neuroinflammation.

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