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Thrilling Monotony: A Summer of Alzheimer's Research

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SENIOR THESIS APPROVAL

This Honors thesis entitled

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By Baronger Bieger

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Table of Contents:

Thrilling Monotony: An Erratic Narrative of my Establishment in Science

But let that man with better sence aduize,
That of the world least part to vs is red:
And dayly how through hardy enterprize,
Many great Regions are discouered,
Which to late age were neuer mentioned.
Who euer heard of th'Indian Peru?
Or who in venturous vessell measured
The Amazon huge riuer now found trew?
Or fruitfullest Virginia who did euer vew?

Yet all these were, when no man did them know;
Yet haue from wisest ages hidden beene:
And later times things more vnknowne shall show.
Why then should witlesse man so much misweene
That nothing is, but that which he hath seene?
What if within the Moones faire shining spheare?
What if in euery other starre vnseene
Of other worldes he happily should heare?
He wonder would much more: yet such to some appeare.

—Edmund Spenser, *The Faery Queene*, Book Two, Stanzas 2 & 3

NOTE TO THE READER: The above quotation is important for two reasons. Its primary significance derives from the fact that it was this quotation that adorned the outside of my cubical during my two months of summer research. Secondly, this quotation is drawn from Spenser's 1590 masterpiece *The Faerie Queene*, a work that is possibly equal parts great and confusing in its attempt to educate. Alas, the only real comparison I can make here is that my narrative might also seem confusing. It might seem like an erratic narrative told with arbitrary speed and detail, and that is probably because it is. It is simply a collection of some of the experiences from my summer research that stood out to *me*. Most were included in this narrative

because they served as pivotal points in my journey to feeling established and comfortable as a studier of science. Other experiences were included in this tale simply for their interesting or educational qualities. Any lessons that may come from these, however, I will leave for the reader to tease out. Above all else, I am simply trying to tell the facts, though some of the names have been changed. But, for fear the reader might get unduly high hopes for what is to come, I will say no more.

An awkward silence jarred the closing of the initiation. Sensing a *faux pas*, both the program directors and the budding scientists they were in charge of looked at me with a sort of curious pity. Was it really *that* unusual to suggest the members of my medical research program get to know one another by having a tea party? I was once again reminded that my exposure to science had not fully converted me into a "science person." My heart, intricate and interesting device though it may be, still pumped the blood of an English major. Though I loved this fact, it amplified my fear that I was in over my head.

Two years earlier I had added a B.S. to my degree in English and begun a vigorous frolic through the biology and chemistry courses OBU offered. Eventually, the time came for me to apply to research opportunities. Due to their highly sequential nature and my accelerated timetable, I had not had many classes that would have aided me in comprehending my research. Micro- and cellular biology, genetics, and biochemistry all lay ahead of me. The resulting conceptual deficiency made it difficult to decipher even the projects to which I was applying. Trying to be diligent, I researched countless topics before applying to the programs they concerned. Even though I felt compelled to state the gaps in my knowledge, I somehow got

accepted into a few programs, and I settled on joining Dr. Steven Barger's Alzheimer's research at the University of Arkansas for Medical Sciences.

Dr. Barger was studying the effects of different isoforms of apolipoprotein E on the uptake of amyloid beta plaques by rat microglia. Microglia! A word I recognized! Looking to get a head start, I requested Dr. Barger send me some primer reading to help me get oriented. He did so in the form of several journal articles. After spending two weeks trying to translate sixty pages of what seemed to be a highly obscure English dialect, I was perfectly oriented. I knew right where I stood—six feet under the waters of perplexity.

The start date rolled around, though, and I moved into the dorms at UAMS. A few days later, I was at the initiation, frantically trying to think of a way to tell science folk that nothing breaks ice better than hot-water infusions. To my great relief, the gal next to me, one Miss Distefano, piped up with the enthusiastic acceptance of the idea of a tea party. She never came to a single one, but I owe her much for this initial embrace. A little while later, I was picking my mentor out of a lineup across the table and trying to figure out how to initiate conversation.

I do not remember exactly how I did that, but soon we were walking through the labyrinthine buildings on the way to Dr. Barger's lab. Our conversation naturally turned to the literature he had sent me. I told him that the shorter articles held my attention like good page-turners, keeping me interested in seeing how they would turn out. Upon hearing this, he rightly seemed surprised and tickled. It was a bit harder to classify his response when I told him that the longer articles had remained absolutely impenetrable to me.

Less than three minutes after setting foot in his lab for the first time, I was ecstatically looking at living microglia under a microscope. Having only encountered microglia in text, I elatedly tried to fathom that scientists could extract microglia from the brain of a rat and keep

them alive and replicating in flasks. Sheepishly, I admitted that I knew nigh nothing of the electrophoresis, polymerase chain reaction, and the other laboratory procedures he had casually brought up. I simply had not had the classes yet. In response to my concern, he said seven words that would change the entire way I approached the summer: "I'm going to assume you know nothing."

Those seven words freed me from having to meet anyone else's expectations, allowing me to focus on my own. In retrospect, this might have increased my work load. I wanted to learn everything I could from this opportunity, so I asked infinite questions, investigated every instrument, and accosted everyone in the surrounding labs. When Dr. Barger began training a volunteer in the evenings after our work was finished, I would hover about, gleaning all I could from their projects. This pesky curiosity usually led to my work days being 12-14 hours long, but Dr. Barger's were always longer.

Though long, the days were not entirely filled with work. Especially early on, many hours were spent standing cross-armed outside my cubical, discussing the philosophical, ethical, and political implications of science; the physiological explanations of biological phenomena; and the joys of the outdoors and music. There were also frequent meetings with my fellow program members, and even more excitingly, I was allowed to attend the weekly interdisciplinary meetings that my lab participated in.

As I became more competent in the lab, I was eventually able to do much of the up-keep of the microglia. I could come in on the weekend and feed or collect the cells, while playing music unabashedly. I could plate harvested cells at a desired concentration and prepare treatments for them. I could even extract their DNA and assess its purity with various instruments. After I was taught different techniques and protocols, I would retreat to my cubical

to research the concepts behind them. I found myself perpetually awed by the simple creativity employed by scientists when they make the seemingly impossible possible.

The days were not devoid of their stresses, of course. I spent many sleep-deprived hours in my cubical, listening to the incessant whining of my tinnitus as I strained to figure out concepts which were well beyond my comprehension. To remedy the tedium, I would occasionally adjourn to one of the scenic outdoor benches around campus and continue my work while watching a storm roll in. During these times, I felt most sharply the loss of calluses, scars, and physical exertion that were associated with my adventures as an English major. As a biology major I learned how muscles worked, but I rarely got to use mine. It was extremely enjoyable to work in a lab that had been pulled straight out of the first scene of a zombie movie, but I found the lack of being outdoors taxing.

Other stressful situations arose when I did something clumsy, either physically or mentally. Whenever I'd forget to put the lid on the centrifuge, spill or contaminate a culture, or misdo a dilution, my fellow lab members would always put the incident into perspective by sharing tales of all the things they had messed up in the past.

Weeks of thrilling monotony passed, dusted throughout with papers, presentations, dinners, concerts, and roving adventures. Before I knew it, it was time to get my poster printed for my final presentation. I sat in my cubical for 14 hours straight, listening to Trampled by Turtles and trying to connect the different facets of our research into coherent findings. The poster was printed, and the next day I was setting it up. In spite of all I had learned, I was still pointedly aware of my vast ignorance regarding how my project fit into the grand scheme of things. The work that I had done was only a fraction of Dr. Barger's overall project, a fact that made seeing the grand scheme difficult.

Before I had a chance to review my poster, a small, balding man walked up and said, "I'm Dr. Smith. I went to Harvard." My head involuntarily cocked sideways and one eye narrowed as I tried to grasp the relevance of his statement. He asked several very precise questions, but before I had finished answering them, he moved on to critiquing the methods and patterns of some of the people I worked with. Then he trailed into talking about research he had done that was tangentially related to mine. Then he left, and I was disheartened. Was this how real scientists behaved? Did many of them have such inflated egos and such a competitive spirit?

I did not have long to ponder these questions, because soon after the old man left, a young, long-haired kid named Jarrett came up. I believe he was a sophomore at Hendrix. After eyeing my poster from a distance, he came into range. I pounced at him energetically, asking if he would like to hear my spiel. Through a timid smile, he told me that he would prefer to read over it himself first. For a moment he did. Then, speaking softly, he said that he had been interested in Alzheimer's for a while and had been doing some personal research into it. We relayed what we knew of the disease, each filling in the gaps in the other's knowledge. Into this review he incorporated a number of thought-provoking questions about my research, using his fresh perspective and different experience to make connections I hitherto had not.

This encouraging encounter set the tone for the rest of the poster session. Though I felt as though I didn't understand the grand scheme of my research when I started, as I repeatedly explained it to people, answered their questions, and heard their comments, the logic of it all fell into place. By the end of the session, I was pumped about my research, and sad to see it go. That night, instead of letting me finish off my written report, my lab took me out for a going-away party. I left for home the next day, amid invitations to return to visit labs and houses and even to make jelly.

As soon as I was out of the lab and back at school, the memories of my research rapidly began to degrade. This was unfortunate, as several months later, when I had had time to thoroughly forget the workings of my project, I had to present again. This time it was at the University of Arkansas. Though I had intended to review my poster before I went, I didn't get a chance to until the night before. When I began to look over it, I realized that I was once more entirely lost, and I regretted ever signing up to present again. In a small panic, I lamely stayed up late into the night nervously reviewing the PDF version of my poster instead of joining the rest of my program's members for a night on the town.

As I began to present the next morning, the anxiety produced by my perceived ignorance had not dispersed. Though I had spent half the night preparing, I still felt as though I were winging it with the butterflies in my stomach. As before, however, my sleep deprivation and stress gave way to a thrilled enthusiasm as people asked questions and provided insights. It would seem that this enthusiasm was contagious, because the judges ended up awarding me first place out of the 84 biology poster presentations. I still feel as though my enthusiasm gave me an unfair advantage, but I could not help it!

This recently happened again when I was able to go with a group from OBU to the National ACS Conference in Denver. There, in addition to being able to watch tons of enlightening lectures, I was able to present my research again. I ran through another cycle of panicking and getting an award.

That brings us just about up to the present, as far as my research is concerned. Cumulatively, this experience has encouraged me in my pursuit of science and led me from being an uncertain Englishy science person to one both humbled and inspired by the "many great Regions [to be] discouered." Though, according to my standards, I am a long way off from

general scientific competence, my experience with research has shown me that persistence and curiosity is a viable way to pursue this goal.

To effect an even more merry conclusion, I will resolve the stress with which I opened. During the course of the summer, I was able to conjure the scientists, young and old, to numerous tea parties. Some came hesitantly, picturing polite society and fancy teaware, but they soon learned that I do not do daint. Most of the tea parties were held in the lobby of my dorm. There, people from all over the world congregated with teas from their homelands and beyond. Sometimes we would talk for over six hours, sitting amid the chaotic collection of teas, pastries, and cheeses that was strewn over the couches and ping-pong table. Sipping out of mismatched mugs, we would discuss our research, theories, and interests. People would come and go as they pleased. Often strangers passing through the lobbies would stop for a while to see what was going on and tell us what they had seen that night while working in the emergency room.

We also enjoyed having more exclusive tea times in a conference room that overlooked the campus from the fifth floor of my lab building. On one such occasion, a friend and I talked late into the night with Dr. Barger as he sipped on tea I had collected from the woods of Arkadelphia.

You might condemn this ending as out-of-order or irrelevant to my research, but above all else, it was experiences like these that proved to me that the English and the scientific halves of my heart could work together. Science functions as the left half of my heart, driving me and invigorating my body and mind, while English functions as the right half, replenishing my life-force and reminding me to breathe. It is only in the collaboration of these two halves that I thrive, and it was this summer research experience that showed me just how well they mesh.

The Effects of Apolipoprotein E on M2 Microglia

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Abstract

The primary genetic risk determinant for late-onset Alzheimer's disease (LOAD) is the apolipoprotein E gene (APOE). Variations in this gene produce three different isoforms of the apolipoprotein E protein (ApoE): ApoE2, ApoE3, and ApoE4. ApoE3 is the most common isoform, so rates of LOAD among other genotypes are indexed to this variant. ApoE2 is rather rare, but its carriers are less likely to get LOAD; when they do, they get it later. The second most common variant is ApoE4, and its carriers are significantly more likely to get LOAD. They also tend to succumb earlier. Once developed, LOAD is characterized by inflammation-related (M1) changes in microglia, non-neuronal cells that mediate innate immunity in the central nervous system (CNS). Microglia are also capable of expressing gene products associated with an alternative, non-inflammatory (M2) form of activation in the periphery, but the degree to which microglia recapitulate those peripheral phenomena and the influence of LOAD etiology on the M1-M2 spectrum remain to be determined. Thus, identifying the effects of ApoE3 and ApoE4 on activated microglia could be helpful in two important ways: 1) by elucidating the differences in activity between ApoE3 and ApoE4 during LOAD and 2) by characterizing the M1-M2 spectrum of activation in the CNS. To explore these areas, we cultured primary microglia harvested from neonatal rats and treated them with ApoE3, ApoE4, an M2 activator—interleukin-4—or combinations thereof. We then measured expression of M2 genes, phagocytic

activity, and mitochondrial respiration. Preliminary results have shown substantial effects of ApoE and IL-4 on all these parameters, notably, expression of CD33 and TREM2, modulators of phagocytic activity, and other genetic determinants of LOAD risk. Ongoing experiments seek to further characterize these effects.

Introduction

Alzheimer's Disease (AD) is the most prevalent form of dementia (Ridge 2013). One of the two best-known features of AD is the accumulation of plaques in the brain composing of amyloid β (Aβ) peptide, a protein derivative with a natural tendency to self-aggregate into oligomers and fibrils (Masters 2012). Aβ is a normal proteolytic product derived from the processing of its larger amyloid precursor protein (APP), and while it can be over-produced in rare cases of inherited forms of AD, the accumulation of Aβ is typically a pathological result of age-related failures in the mechanisms of removal (Figure 1) (Mandrekar 2009).

Microglia are macrophage-like cells that reside in the central nervous system (CNS) and perform many of the same functions that macrophages do in the periphery, scavenging infectious agents and insoluble debris (Lynch 2014). Microglia contribute to the normal clearance of Aβ. Soluble Aβ is collected through macropinocytosis, but if the peptide has already formed insoluble plaques, microglia collect the Aβ via phagocytosis (Mandrekar 2009).

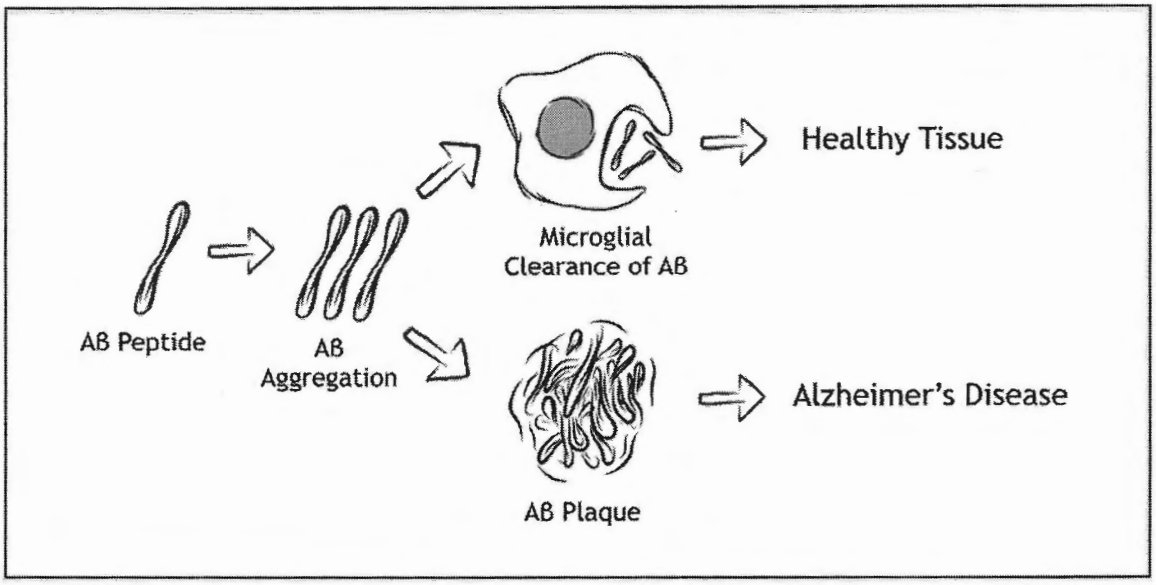

Figure 1: When the amyloid β regularly produced in neurons is not efficiently cleared by microglia, it aggregates, eventually forming the plaques characteristic of LOAD. This study is aimed at understanding the differences in microglial activity associated with different proteins known to be closely linked to LOAD. (Special thanks to Ashley Keathley for making this lovely illustration.)

As with many other types of neurological disorders, an immune response plays a large role in AD (Lynch 2014). Macrophages in the periphery are capable of a spectrum of immune activation states ranging from M1 to M2 (or "classical" to "alternative") (Lynch 2014). Generally, M1 is an inflammatory state that is known for causing collateral damage to healthy tissue in its effort to combat invaders. M2, however, is non-inflammatory and is associated with tissue repair. M1 is often induced by molecules produced by infectious pathogens and facilitates phagocytic activity, whereas M2 is typically induced by host-produced cytokines such as interleukin-4 (IL-4) and has been proposed to be counter-productive to the role microglia play in clearing Aβ from the brain (Sudduth 2013). Though the immune spectrum in the periphery has been well characterized, the extent to which the CNS's immune spectrum is analogous to the periphery's is still being determined (Wilcock 2011).

Late onset AD proved hard to predict genetically. In 1992, however, the apolipoprotein E gene (APOE) was discovered to have three variants that correlated strongly with varying degrees of risk for developing late onset AD. APOE3 is the most common allele, and so is associated with the baseline rates of AD. People with an APOE2 allele, the rarest form, are much less likely to get AD than those homozygous for APOE3, and if they do get it, it is generally later in life. Those with an APOE4 allele, on the other hand, have a higher probability of getting AD earlier than those with APOE3 (Roses 1997). It is important to note, though, that all the APOE alleles are producing useful apolipoprotein E proteins (ApoE). These proteins play a key role in metabolism and the distribution of cholesterol and other lipids to neurons, especially under conditions of injury or other stress (Horsburgh 2000).

To begin characterizing the influences of ApoE genotype on the M1-M2 spectrum in micoglia, we treated rat primary microglia with IL-4, ApoE3, and ApoE4, alone and in combination. The oxidative metabolism of microglia was assessed using a Seahorse Bioscience Extracellular Flux Analyzer. Quantitative real-time polymerase chain reaction (qRT-PCR) was performed to measure the expression of M2 markers such as arginase 1 (ARG1), chitinase 3-like 1 (Ch3L1), and mannose receptor C1 (MRC1) (Chhor 2013). We also measured expression of CD33 and the triggering receptor expressed on myeloid cells 2 gene (TREM2). An increase in CD33 is associated with suppressed phagocytic activity (Griciuc 2013), while increased levels of TREM2 have been found to promote the phagocytic breakdown of Aβ (Jiang 2014). Finally, we directly assessed the ability of microglia to internalize Aβ after pretreatment with IL-4, ApoE3, ApoE4, or their combination.

The purpose of this study is twofold. Its primary goal is to tease apart the differences in microglial response to ApoE3 and ApoE4, both by themselves and accompanied by an M2

inducer. Since these isoforms are so closely tethered to AD risk, any differences in effects could help elucidate the harmful aspects of ApoE4. A secondary benefit is that our study deals with the relatively understudied M1-M2 spectrum of the CNS. Consequently, our results could help develop a clearer image of immune activation in the CNS.

Experimental Procedures

Primary microglia

Cerebral cortices were removed from neonatal (P0-2) Sprague-Dawley rat pups and dissociated by trituration and digestion in trypsin (1 mg/ml). The dissociated cell suspension from each neonate was plated in 75-mm flasks in minimum essential medium (MEM) containing 10% fetal bovine serum (FBS) and gentamycin (10 μg/ml). Cultures were incubated at 37 °C in a humidified incubator with 5% CO_2 approximately 12 days. Microglia were then physically dislodged from the astrocyte monolayer by sharp blows to the sides of the flasks and collected by centrifugation at 800 rpm for 10 min. The supernatant was aspirated away, leaving ~4 mL per conical left. The cells were then resuspended in the existing medium and counted on a hemacytometer.

Microglia were plated in MEM/FBS at approximately 150,000 cells/well in 96-well Seahorse plates, 350,000 cells/well in 24-well plates (for Aβ uptake assays), or 10^6 cells/35-mm plate (for RNA isolation). After 24 h, the culture medium was washed to serum-free MEM/F12 (1:1), and microglia were incubated an additional 18-22 h with the following: 40 ng/ml IL-4, 30 nM ApoE3, 30 nM ApoE4, or combinations of the IL-4 treatment with each of the ApoE's.

Production of recombinant ApoE

The T98G human astroglioma cell line was stably transfected with plasmids encoding ApoE3 or ApoE4. Cultures were incubated 96 h in MEM/F12 supplemented with 0.5 mM L-glutamine, 10 nM Na selenite, and 50 μM ethanolamine. After clearing by centrifugation, conditioned medium was concentrated through two steps: first on Centricon Plus-70 and second on Amicon Ultra-4 filters (both with a 10-kDa cutoff), ultimately achieving ~2000-fold concentration. This concentrate was resolved on a Superdex 200 10/300 GL column (mobile phase: 20 mM $NaPO_4$, pH 7.5, 50 mM NaCl, 1 mM EDTA). Fractions were analyzed by western blot for ApoE and by Coomassie staining for total protein; fractions highest in ApoE and lacking other visible protein contaminants were pooled, sterile-filtered, and quantified by immunoblot comparison against standards comprising highly purified ApoE.

qRT-PCR

Microglia to be used in qRT-PCR were plated in 35-mm dishes (Falcon). After treatment, the RNA was purified using the QIAGEN RNeasy Mini Kit Plus according to manufacturer's directions. (It should be noted that the optional drying spin after the final column wash was performed and the RNA elution was performed with one volume of 30 μl.) The isolated RNA was then quantified either by spectrophotometry or use of a LabChip with Agilent Technologies's 2100 Bioanalyzer. Reverse transcription was performed on 200 or 250 ng of RNA using the ImProm II Reverse Transcription System (Promega) with random primers. Finally, qRT-PCR was performed with SYBR Green mix (Applied Biosystems) and primers for CD33, MRC1, Ch3L1, ARG1, and TREM2; 18S rRNA was also quantified as an internal reference. The cDNA was amplified on an Applied Biosystems 7900HT Fast thermocycler, and

quantitation was achieved by interpolation in a standard curve produced from a mixture of all samples.

Seahorse

The oxidative metabolism of microglia was assessed using a Seahorse Bioscience Extracellular Flux Analyzer (Figure 2). This instrument characterizes various aspects of cells' metabolism by monitoring their oxygen consumption and acid production while injecting drugs that isolate various aspects of mitochondrial respiration. The analysis was performed as three 3-min measurements of O_2 consumption under each of the following conditions: initial (no addition), oligomycin (1.5 μM), carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP; 0.7 μM), and antimycin A/rotenone (0.75/1.5 μM). The timepoint achieving greatest average magnitude of effect for each condition was selected. Respiration after antimycin A/rotenone was considered non-mitochondrial respiration. The difference between this and the initial reading was considered basal mitochondrial respiration. The difference between initial and oligomycin readings was considered ATP-linked. The difference between non-mitochondrial and FCCP readings was considered maximal mitochondrial respiration.

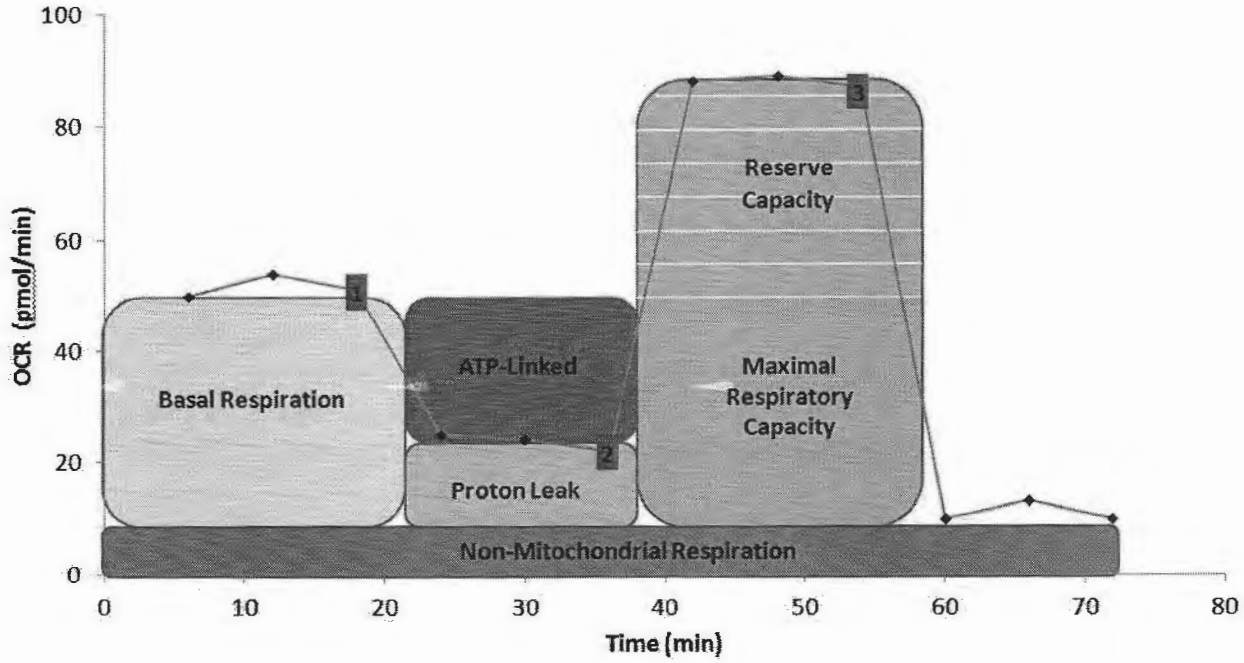

Figure 2: After monitoring their basal respiration, oligomycin is injected to inhibit respiration tied to ATP. Once this state is documented, carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP) is used to relax the inner mitochondrial membrane's proton gradient, permitting maximal respiration. Finally, antimycin A and rotenone are added to inhibit complexes III and I, respectively. These drugs disrupt the electron transport chain, providing a measure of oxygen consumption that is not related to the mitochondria (Rose 2014).

Aβ-uptake assays

Aβ (22 nmol) and Aβ-HiLyte488 (4.4 nmol) were dissolved in hexafluoroisopropanol, dessicated, resuspended at 2 mM in anhydrous dimethylsulfoxide, then incubated 30 h in phenol red-free F12 medium at 4 °C at 150 μM. (These conditions favor the formation of oligomers, soluble aggregates of 2-15 molecules of Aβ.) Primary microglia were plated at 350,000/well in 24-well plates and seated overnight in MEM/10% FBS. Microglia were washed to serum-free MEM (3X) and treated with ApoE and IL-4 for 18 h. Microglia were washed again (2X) and the Aβ mixture was applied. After 12 h, a 25-μl aliquot of medium was removed, diluted with 75 μl

PBS, and read on a FITC channel on a fluorescent plate reader. (Three pre-reads were made with PBS alone to establish a background reading for each well. All readings were made in the same black 96-well plate, with each fraction of the same original culture well in a consistent reading-plate well throughout.) Cells were washed briefly with PBS then exposed to 200 µl of an acid wash solution consisting of 0.2 M acetic acid and 0.5 M NaCl at 4 °C for 15 min. 100 µl of this solution was assessed for fluorescence. Cells were washed briefly with 50 µl PBS. 50 µl of radioimmunoprecipitation buffer (20 mM phosphate buffer, pH 7.4, 150 mM NaCl, 2 mM EDTA; 1% NP-40; 1% sodium deoxycholate) was then applied, and the plate was frozen. The plate was thawed and each well was scraped with a cell scraper cut down to fit in a 24-well. The lysate was centrifuged at 16,000 x*g* for 10 min, and the entire supernatant was diluted in 50 µl PBS and read in the plate reader. The pellets were dissolved in 100 µl 70% formic acid for 60 min at room temp.; the entire 100 µl was read in the plate reader. Readings were subtracted by the background, and each fraction was multiplied by its dilution factor in the fluorescence reading (1 for the formic acid fraction). These adjusted values were summed for each well, and the data are expressed as a percent of the total.

Results and Discussion

Seahorse

The first thing to note about the data shown in Figure 3 is that, across all treatments, the maximal respiration is less than the basal respiration. This seems unusual given Figure 2. We speculate, however, that the oligomycin actually killed many of the cells. This seems unusual, as the concentrations used are standard in such assays. If such results continue to be found with microglia, we might opt to do a dose-response curve with oligomycin, while monitoring cell

viability. This Seahorse dataset is still valuable, though, as it is readily seen that some treatments remained healthier throughout the injections.

For instance, the Seahorse assay highlights the increase in metabolism produced by ApoE, especially in conjunction with IL-4. Though IL-4 has been associated with trophic effects (Araujo 1993, Soria 2011), it did not show much effect on the mitotic respiration until combined with ApoE. Then the effects are substantial. Regardless of M2 activation, the addition of ApoE increased metabolism. This suggests increased cell health and robustness, though it is also possible that the elevated levels of respiration represent a response to the stress of activation. To wit, IL-4 also elevated the leak-associated respiration, a parameter associated with a loss of integrity of the mitochondrial membrane, resulting in O_2 consumption that is less efficient with regard to ATP production.

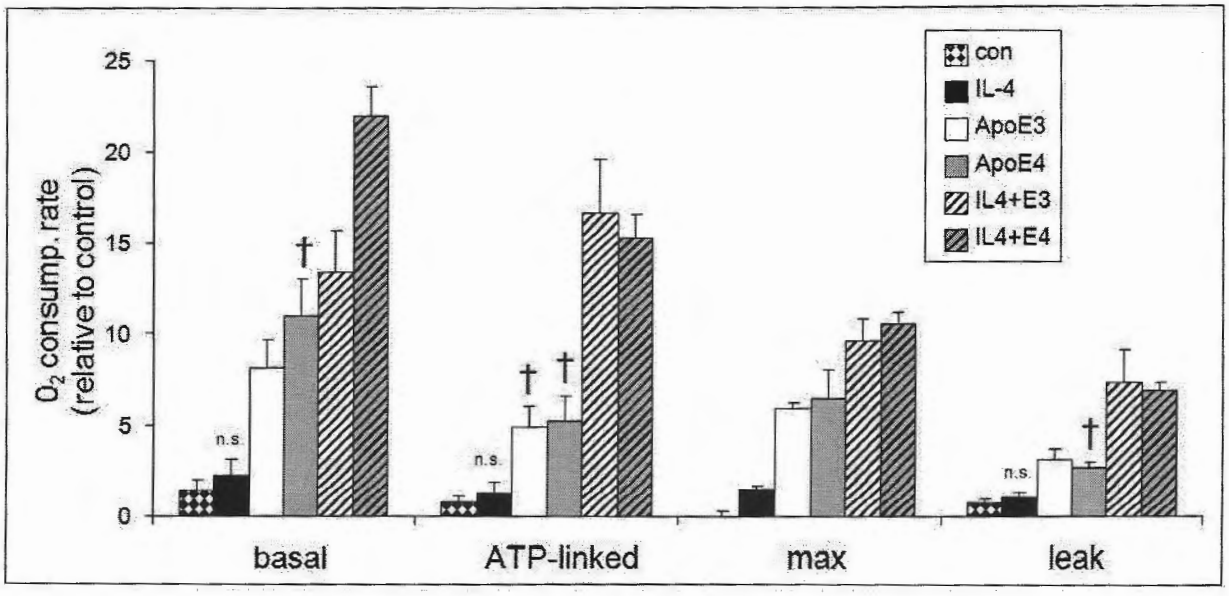

Figure 3: Mitochondrial respiration as impacted by ApoE. Microglia were treated as indicated and then subjected to assays of mitochondrial oxygen consumption by Seahorse technology (see Methods). Components of respiration are indicated below each grouping. All treatments were significantly different from control except IL-4 in the basal, ATP-linked, and leak components († = $p<0.05$ vs ApoE + IL-4, n.s. = not significant)

qRT-PCR

As expected, our preliminary data have shown an increase in gene expression of the M2 indicators ARG1, Ch3L1, and MRC1 in the IL-4 treatment (Figure 4). ApoE3 tended to suppress the M2 activation state, as indicated by its effects on these three genes. The fact that ApoE4 did not exhibit this suppression of ARG1 and Ch3L1 is potentially significant and could prove to be a clue to what ApoE4 is doing differently that elevates AD risk. For example, as an M2 state is hypothesized to suppress phagocytic activity and thereby allow Aβ buildup in the brain (Wilcock 2011), the lack of M2 suppression seen in ApoE4 might be part of the reason it is associated with greater Aβ deposition and greater risk of AD. However, in MRC1, the same mitigation of IL-4-associated M2 that is seen in ApoE3 is seen with ApoE4. Overall, the differing expression of these M2 markers demonstrates that the interactions between ApoE and the M2 state are complex and emphasize the importance of testing a wide range of markers to comprehensively elucidate the impact of ApoE.

The expression of CD33 was found to be increased in treatments with ApoE and decreased with those in an M2 state, effects that seem largely to cancel each other out when combined (Figure 5). CD33 expression levels appear to be inversely correlated with Aβ clearance by microglia (Griciuc 2013). However, the implication that IL-4 suppression of CD33 would increase phagocytosis is hard to reconcile with other evidence that an M2 state promotes the buildup of Aβ plaques (Wilcock 2011). This may indicate that the clearance of Aβ by microglia is not entirely dependent on classical phagocytic mechanisms (see below).

The situation is made even more intriguing by the fact that the relative expression of TREM2 across the treatments almost exactly mirrors that of CD33 (Figure 6). This is

counterintuitive because TREM2 appears to facilitate the phagocytosis of Aβ (Jiang 2014) while CD33 appears to inhibit the phagocytosis of Aβ. It is possible that one of these gene products dominates the other, however, which could be tested by measuring phagocytic activity directly. As the M2 state appears to be conducive to Aβ buildup, it is possible that the suppression of Aβ phagocytosis associated with CD33 is less important than the ability of TREM2 to enhance Aβ clearance.

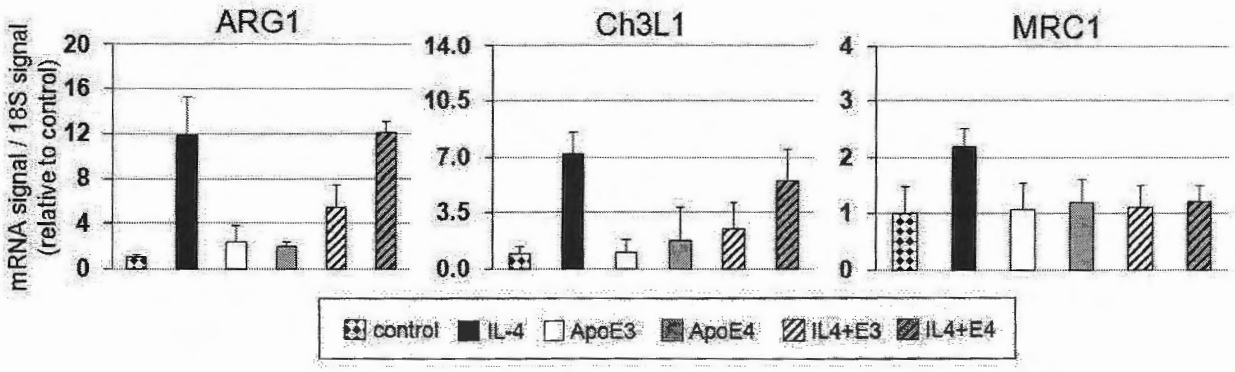

Figure 4: Influence of ApoE on M2-related gene expression. Microglia were treated as indicated and RNA was isolated and subjected to qRT-PCR for ARG1 (left), Ch3L1 (middle) or MRC1 (right). A limited number of replicates to date has obviated statistical analysis, but initial trends indicate that ApoE3 inhibits the effects of IL-4, and ApoE4 may do the same for MRC1.

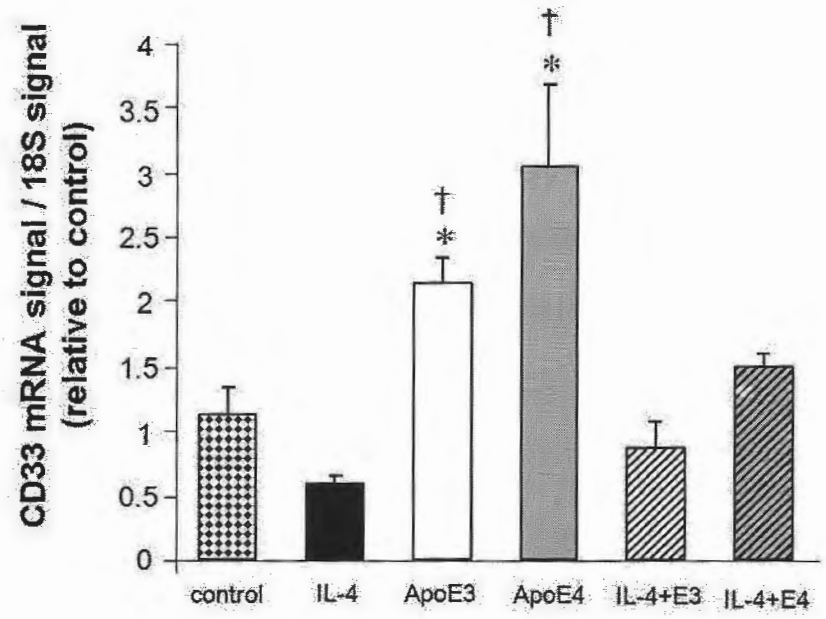

Figure 5: ApoE elevates expression of CD33. Microglia were treated as indicated and RNA was isolated for qRT-PCR of CD33. ApoE3 and ApoE4 elevated expression of this phagocytosis inhibitor, and IL-4 blocked the effects of both. († = $p<0.05$ vs ApoE + IL-4, * = $p<0.05$ vs control)

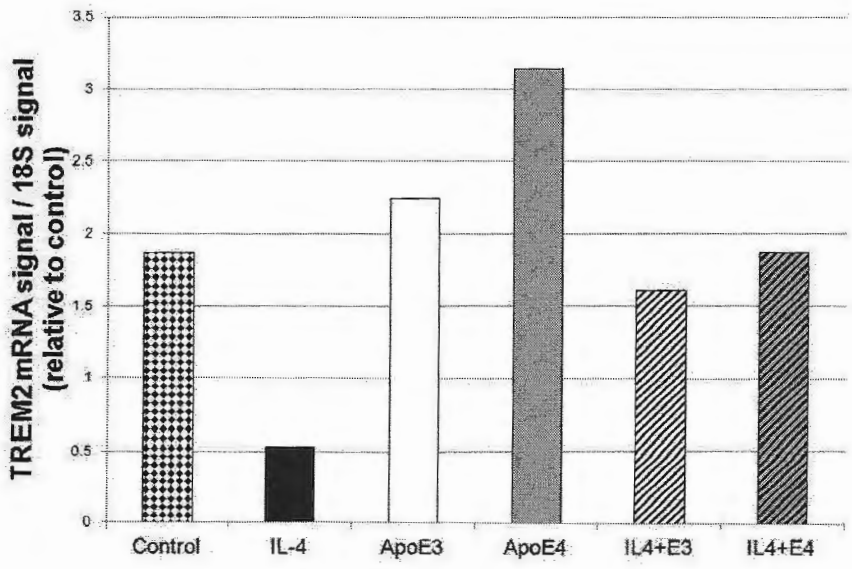

Figure 6: TREM2 expression follows the same pattern as CD33, increasing with ApoE and decreasing with IL-4.

Aβ uptake

ApoE is known to form complexes with Aβ that influence the peptide's transport across cellular membranes. To test the effects of ApoE on Aβ uptake mechanisms that would be independent of this role as a shuttle, we applied ApoE3 and ApoE4 to microglia as pretreatment and washed them away prior to conducting Aβ uptake assays. After a 12-h incubation with fluorescently tagged Aβ oligomers, microglia were subjected to an acid wash to remove surface-bound Aβ. Fractions of this wash showed no significant effect of the pretreatments (data not shown).

After the acid wash, cells were lysed in a mild detergent. This lysate was cleared by centrifugation; Aβ that was soluble in the supernatant, as well as the formic acid-extractable insoluble material in the pellet, was quantified. These measurements indicated that ApoE evoked stable changes in the microglia that favored Aβ uptake (Figure 7). Surprisingly, ApoE4 was significantly more effective than ApoE3 in this regard. IL-4 reversed the effect of both ApoE's, even though it had no impact on its own. As these findings reflect the uptake of Aβ, they seem at first to conflict with the phagocytic activity as determined by CD33. This may be explained by the fact that the Aβ in this test was prepared as oligomers rather than insoluble fibrils. As such, the microglia would be expected to take it up via macropinocytosis (Mandrekar 2009). This process is independent of the molecules and mechanisms that mediate phagocytosis and thus may also be independent of CD33. On the other hand, these influences on Aβ uptake generally mirror the effects of the ApoEs (and their reversal by IL-4) on TREM2 expression. Thus, it is possible that the TREM2 plays a role in regulating Aβ uptake.

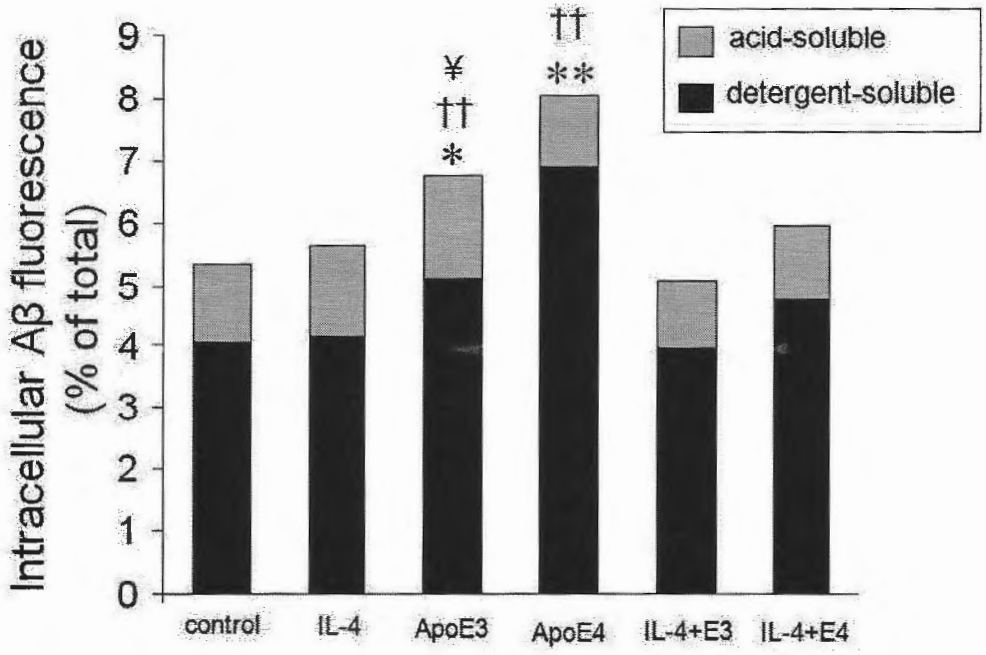

Figure 7: ApoE4 increased the uptake of Aβ. Microglia were treated with IL-4, ApoE, or combinations thereof, then assayed for internalization of Aβ as described (Methods). Two fractions are reported: a detergent-soluble fraction obtained from the supernatants of cell lysates and a fraction ("acid-soluble") liberated by formic acid hydrolysis of material that was insoluble in the initial lysates. ApoE3 and ApoE4 increased the uptake and IL-4 blocked the effects of both. (* = $p<0.05$ vs control, ** = $p<0.01$ vs control, †† = $p<0.01$ vs ApoE + IL-4, ¥ = $p<0.05$ vs ApoE4)

Conclusions

- ApoE stimulated microglial respiration, and this was generally enhanced by IL-4, even though IL-4 had no measurable effect on its own.

- ApoE4 seems to interact with M2 activation differently than does ApoE3, according to ARG1 and Ch3L1 expression. ApoE3 may counteract the tendency of microglia to enter the M2 state of activation. As the latter is associated with relatively poor phagocytic

activity, this may mean ApoE3 carriers have generally lower rates of AD because their microglia more efficiently remove Aβ aggregates.

- The relative expressions of CD33 and TREM2, genes producing opposite effects on Aβ phagocytosis, mirror each other across the treatments. This suggests a surprising similarity in their contributions to the M2 phenotype.

- ApoE3 is somewhat less effective than ApoE4 at inducing microglia to sequester soluble Aβ. While this seems counter-intuitive to the suppression of M2 by ApoE3, it is possible that this effect is limited to macropinocytosis of soluble Aβ and that the effects on insoluble aggregates require distinct processes of phagocytosis.

This is merely the tip of the iceberg, though. In the future, we are also interested in more directly characterizing the phagocytic activity of the microglia, perhaps by monitoring latex bead uptake. This would help us determine whether CD33 or TREM2 is dominating in this process. Also, though the Aβ-uptake test was designed to measure Aβ absorbed through macropinocytosis, we would like to confirm the trends seen by experimental manipulation of the macropinocytotic mechanism. This further testing would help us have a more solid understanding of the role of ApoE and M2 activation have on phagocytic and macropinocytotic clearance of Aβ.

As MRC1 seems to display a different trend from the other M2 activators we investigated, we could also test the expression of more genes and look into how ARG1 and Ch3L1 expression differs from that of MRC1. These tests would help us determine whether the

two genes gave us an accurate representation of the M2 activation in each treatment and help us uncover the difference in ApoE3 and ApoE4 interactions with the M2 state. We might try to determine if one gene is dominating the other through combined treatments with ApoE3 and ApoE4. Finally, we would, of course, like to do more repetitions of these experiments to further validate the data we have gathered thus far.

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References

Araujo DM, Cotman CW. Trophic effects of interleukin-4, -7 and -8 on hippocampal neuronal cultures: potential involvement of glial-derived factors *Brain Res.* **1993**, 600(1), 49-55.

Chhor V, Le Charpentier T, Lebon S, Ore M, et al. Characterization of phenotype markers and neuronotoxic potential of polarised primary microglia in vitro. *Brain, Behavior, and Immunity* **2013**, 32, 70-85.

Griciuc A, Serrano-Pozo A, Parrado AR, Lesinski AN, et al. Alzheimer's disease risk gene CD33 inhibits microglial uptake of amyloid beta. *Neuron.* **2013**, 78(4), 631-43.

Horsburgh, McCarron OM, White F, Nicoll JAR. The role of apolipoprotein E in Alzheimer's disease, acute brain injury and cerebrovascular disease: evidence of common mechanisms and utility of animal models. *Neurobio. of Aging.* **2000**, 245–255.

Jiang T, Tan L, Zhu XC, Zhang QQ, et al. Up-regulation of TREM2 Ameliorates Neuropathology and Rescues Spatial Cognitive Impairment in a Transgenic Mouse Model of Alzheimer's Disease. **2014**.

Lynch MA. The impact of neuroimmune changes on development of amyloid pathology; relevance to Alzheimer's disease. *Immunology.* **2014**, 141(3), 292-301.

Mandrekar S, Jiang Q, Lee CY, Koenigsknecht-Talboo J, Holtzman DM, et al. Microglia mediate the clearance of soluble Abeta through fluid phase macropinocytosis. *J Neurosci.* **2009**, 29(13), 4252-62.

Masters CL, Selkoe DJ. Biochemistry of Amyloid β-Protein and Amyloid Deposits in Alzheimer Disease. *Cold Spring Harb Perspect Med.* **2012**, 2(6).

Ridge PG, Ebbert MT, Kauwe JS. Genetics of Alzheimer's disease. *Biomed Res Int.* **2013**, 2013:254954.

Rose S, Frye RE, Slattery J, Wynne R, Tippett M, et al. Oxidative Stress Induces Mitochondrial Dysfunction in a Subset of Autism Lymphoblastoid Cell Lines in a Well-Matched Case Control Cohort. *PLoS ONE.* **2014**.

Roses, AD, Saunders AM. ApoE, Alzheimer's disease, and recovery from brain stress. *Ann N Y Acad Sci.* **1997**, 826:200-12.

Soria JA, Arroyo DS, Gaviglio EA, Rodriguez-Galan MC, Wang JM, Iribarren P. Interleukin 4 induces the apoptosis of mouse microglial cells by a caspase-dependent mechanism. *Neurobiol Dis.* **2011**, 43:616-2

Sudduth TL, Schmitt FA, Nelson PT, Wilcock DM. Neuroinflammatory phenotype in early Alzheimer's disease. *Neurobiol Aging.* **2013**, 34:1051-9

Wilcock DM, Zhao Q, Morgan D, Gordon MN, et al. Diverse inflammatory responses in transgenic mouse models of Alzheimer's disease and the effect of immunotherapy on these responses. *ASN NEURO*. **2011**, 3(5):art:e00069.