sTREM2 and ApoE isoforms differentially regulate cytokine expression in myeloid-derived cell models.

Ryan Arsenault

The thesis is submitted as a partial fulfillment of the requirements for the degree of Master of Science in Cellular and Molecular Medicine

Department of Cellular and Molecular Medicine

Faculty of Medicine

University of Ottawa

© Ryan Arsenault, Ottawa, Canada, 2023

ABSTRACT

TREM2 is an innate immune receptor expressed in microglia and macrophages. ApoE isoforms (E2/E3/E4) are ligands of TREM2. TREM2 variants and ApoE4 are top risk factors of Alzheimer's disease. TREM2 can be cleaved from cell membrane as soluble TREM2 (sTREM2), the level of which fluctuates during Alzheimer's progression. However, the mechanisms that sTREM2 and the interactions between TREM2 and ApoE may contribute to Alzheimer's neuroinflammation are largely uncharacterized. The project objectives were to investigate whether sTREM2 and ApoE isoforms can affect cytokine expression profiles in myeloid-derived cell models. My results show that sTREM2 can stimulate inflammatory cytokine expression at early time-point but anti-inflammatory cytokine expression at later time-point mainly via MAPK-JNK signaling pathway. sTREM2 has differential effects on cytokine expression in M0, M1, and M2 macrophages. ApoE isoforms also differentially induce cytokine expression and regulate TREM2 expression in M0, M1, and M2 macrophages. My study reveals a complex interplay of sTREM2, TREM2 and ApoE isoforms and differential effects of those in the models.

ACKNOWLEDGMENTS

I would like to first extend my sincerest gratitude towards my thesis supervisor, Dr. Wandong Zhang for the opportunity to work with the Human Health Therapeutics Research Center at the National Research Council of Canada and to work on this project. His wealth of knowledge as well as his guidance throughout this project was strongly encouraging and essential to the completion of this work.

I would also like to thank my thesis co-supervisor Dr. Qiao Li for allowing me to complete this work in her laboratory at the University of Ottawa. Her input and help throughout was valuable and supportive.

My thesis advisory committee¸ Dr. Jagdeep Sandhu and Dr. Maxime Rousseaux, also deserves recognition and thanks for their inputs, collaboration and supports, Dr. Jagdeep Sandhu and Dr. Maxime Rousseaux.

Lastly, I would like to thank my mother Terri, my father Michael and step-mother Susan for their distanced love, support, encouragement and motivation as this work was inconceivable without it.

Table of Contents

ABSTRACT ii

ACKNOWLEDGMENTS iii

ABBREVIATIONS vi

1.0 INTRODUCTION 1

1.1 Hypothesis and Aims 21

2.0 Materials and methods 22

2.1. Cell Cultures 22

2.2 Treating THP-1 cells with sTREM2 23

2.3. Differentiation and Polarization of THP-1 Monocytes into Macrophages 24

2.4 Reverse Transcription-polymerase chain reaction (RT-PCR) 26

2.5. Enzyme linked-immunosorbent assay (ELISA) 27

2.6. Western blot analysis 28

2.7. Live cell imaging 29

2.8. Statistical analysis 30

3.0 RESULTS 30

Part A: sTREM2 induces cytokine expression in THP-1 monocytes through MAPK-JNK Signaling 30

i. sTREM2 Induces Expression of Cytokine in THP-1 Monocyte Model. 30

ii. TREM2 mAb Partially Alleviated sTREM2-Induced Expression of Cytokines 34

ii. sTREM2 induced expression of cytokines in THP-1 differentiated macrophages 43

iii. Polarized macrophages differentially shed sTREM2 50

Part B: ApoE isoforms differentially affect cytokine and TREM2 expression in THP-1 derived macrophages. 53

i. ApoE isoforms differentially induced the expression of inflammatory cytokines in polarized macrophages. 53

ii. ApoE isoforms differentially regulated TREM2 expression in polarized macrophages. 61

4.0 Discussion 63

4.1. sTREM2 induced cytokine expression in THP-1 monocytes 63

4.2. The effects of TREM2 antibody on sTREM2-induced cytokine expression 66

4.3. The signaling pathways involved in sTREM2-induced cytokine expression. 68

4.4. The effects of sTREM2 on cytokine expression in polarized macrophages 71

4.5 Shedding of sTREM2 by M0, M1 and M2 macrophages 73

4.6. The effects of ApoE isoforms on cytokine expression in macrophages 74

4.7. The effects of ApoE isoforms on TREM2 expression in macrophages 77

5.0 Conclusions 78
References 81
Contributions of Collaborators 93
Appendices 94

ABBREVIATIONS

Aβ: Amyloid-β peptide

Ab: Antibody

AD: Alzheimer’s Disease

ADAD: Autosomal Dominant AD

ADAM: A disintegrin metalloproteinase

ALDOA: Aldolase A

ALS: Amyotrophic lateral sclerosis

ApoE: Apolipoprotein E

APOER: ApoE receptor

APP: Amyloid precursor protein

Arg: Arginine

BSA: Bovine serum albumin

CNS: Central nervous system

CRP: C-reactive protein

CSF: Cerebrospinal fluid

Cys: Cysteine

DAM: Damage associated microglia

DAMP: Damage associated molecular patterns

DAP-12: DNAX activation protein-12

dNTP: Deoxy nucleotide triphosphates

DTT: Dithiothreitol

EDTA: Ethylenediaminetetraacetic acid

EOAD: Early onset Alzheimer’s disease

FABPH: Fatty acid binding protein

FAD: Familial AD

FBS: Fetal bovine serum

FTD: Frontotemporal dementia

GM-CSF: Granulocyte macrophages colony stimulating factor

HI: Heat inactivated

HIF-1: hypoxia-inducible factor 1

IFNγ: Interferon γ

Ig: Immunoglobulin

iMGL: immortalized microglia-like cells

IL-1α: Interleukin-1alpha

IL-1β: Interleukin-1beta

IL-4: Interleukin-4

IL-6: Interleukin-6

IL-8: Interleukin-8

IL-10 : Interleukin-10

IL-13: Interleukin 13

ITAMs: Immunoreceptor-tyrosine activation motifs

JAK: Janus Kinase

JNK: c-Jun N-terminal kinases

LDL: Low density lipoprotein

LDLR: Low density lipoprotein receptor

LOAD: Late onset Alzheimer's disease

LPS: Lipopolysaccharide

MAPT: microtubule associated protein tau

MCP-1: Monocyte chemoattractant protein 1

MCAF: Monocyte chemotactic activating factor

MS: Multiple Sclerosis

NaCl: Sodium chloride

NDD: Neurodegenerative diseases

NFκB: Nuclear factor kappa B

NHD: Nasu-Hakola disease

PD: Parkinson's disease

PI3K: Phosphatidylinositol 3-kinase

PLCγ: Phospholipase Cγ

PLOSL: Polycystic lipomembranous osteodysplasia with sclerosing leukoencephalopathy

PMA: Phorbol 12-myristate 13 acetate

PMSF: Phenylmethylsulphonyl fluoride

SCARA1: Scavenger receptor A1

SNP: Single nucleotide polymorphism

STAT: signal transducer and activator of transcription

sTREM2: soluble TREM2

Syk: Spleen tyrosine kinase

TARC/CCL17: Thymus-and activation regulated chemokine

TGF-β: Transforming growth factor β

TNF-α: Tumor necrosis factor-alpha

TREM2: Triggering receptor expressed on myeloid cells -2

YKL40: Chitinase 3 like protein 1

WCE: Whole cell extract

1.0 INTRODUCTION

Alzheimer's Disease (AD) is a progressive neurodegenerative disease (NDD) and the most common form of dementia worldwide accounting for between 60-80% of dementia cases (Alz.org). Clinically, symptoms manifest as dementia including cognitive decline faster than usual senescence, changes in behavior and personality, increased memory loss and generally presents itself in people older than 65; however, cases of earlier diagnosis are not still uncommon. Suspected AD may be difficult to confirm at first and requires a diverse array of neurological and medical tests to assess the patient's memory, thinking, mental and emotional state, as well as structural brain imaging to identify any possible abnormalities of the brain or central nervous system (CNS) and even then, the complexity of the disease makes it difficult to definitively tell from an early stage. Researchers today are still searching for validated biomarkers of AD through neuroimaging, cerebrospinal fluid (CSF), blood, urine tests as well as genetic profiling but none have yet proved reliably conclusive in large scale clinical trials (McQuade and Blurton Jones, 2019). Although recently, the United States Food and Drug Administration (FDA) has released the first in vitro diagnostic test for early detection of beta-amyloid associated with AD (FDA.gov). AD currently has no cure with only limited treatment options that are designed to help patients cope with disease complications rather than targeted therapies aimed at stopping, reducing, or reversing disease progression.

AD was first described over a century ago in 1906 by Dr. Alois Alzheimer and his work with a 51-year-old patient named Auguste Deter who experienced memory loss, paranoia, and severe

psychological changes. Dr. Alzheimer's pathological examination detected both beta-amyloid plaques and neurofibrillary tangles which would later be coined defining features of the disease (Alzheimer et al., 1995). Pathologically AD is characterized by two main characteristics, including extracellular accumulation of amyloid-beta (Aβ) peptides found in plaques and intracellular accumulation of hyperphosphorylated tau proteins found in neurofibrillary tangles (Brion et al., 1985, Pollock et al., 1986). These progressively lead to synaptic dysfunction and loss, chronic neuroinflammation, neuronal death, severe cognitive decline and ultimately death (Alzheimer's Association, 2019). Although AD patients do not normally develop clinical symptoms until later in life, key disease-initiating events such as Aβ seeding and neurofibrillary tangle development likely begins decades before deficits in cognitive function are observed (Jack et al., 2013). Thus, the key to treating AD patients may lay in preventive treatment and primary identification of those at risk rather than trying to mitigate to treat patients after diagnosis as most times, and cognitive function appears non-repairable. To do so, we need to understand how these factors and pathologies interact with each other and their surrounding environment throughout the development of these key pathogenic features to drive neurodegeneration.

AD diagnosed in people over the age of 65 is known as late-onset AD (LOAD) which is mostly sporadic, accounting for ~ 95% cases; however, patients presenting AD symptoms prior to the age of 65 are said to have early onset AD (EOAD). EOAD comprises approximately 5% of all diagnosed AD cases (Tellechea 2018). Some of the EOAD is heritable in a rare autosomal dominant fashion known as autosomal dominant AD (ADAD) representing less than 1% of all cases (Bateman et al., 2011). EOAD may also be familial sharing a genetic component but not all EOAD cases are heritable and may be sporadic due to causative gene variants and plausible

environmental factors. EOAD is not just LOAD occurring at an arbitrarily younger age cut off; EOAD differs from LOAD in many respects such as greater neurofibrillary tangle load per stage of dementia and per gray matter atrophy (Mendez, 2017). EOAD often involves a significantly greater extent of evaluation required for diagnosis (Eriksson et al., 2014), an increased impact of dementia risk factors such as cardiovascular and cognitive fitness (Nyberg et al., 2014). There are also many psychosocial problems specific to early-onset dementia which may include sudden loss of independence (Clemerson et al., 2013).

The exact pathogenic reasons for developing LOAD are not currently known, but likely multi-factorial; however, the science community has joined a consensus that the long-standing theory known as the Aβ theory may be the best and most logical explanation for the development of AD. The theory is that mutations accumulate in gene expression throughout life which alters the production and subsequent proteolytic cleavage of amyloid precursor protein (APP). This results in excess Aβ peptides composed of either 40 or 42 amino acids (Zheng and Koo, 2011) in the extracellular environment and due to some genetic mutations/variations. Additionally, the clearance of extracellular Aβ is impaired. This then causes Aβ to aggregate into oligomers, then fibrils which then form toxic insoluble Aβ plaques. The accumulation of these oligomers and plaques throughout life then drives neuronal dysfunction and synapse damage as well as chronic neuroinflammation, causing neurodegeneration, dementia and ultimately death (Hardy and Selkoe, 2007). It was also shown that the toxicity of Aβ seems to depend on the presence of microtubule-associated protein tau (Roberson et al., 2007), the hyperphosphorylated forms of which aggregate and deposit in AD brains as neurofibrillary tangles (Liu et al., 2013). While the reasons for developing LOAD remain elusive, since EOAD generally has a heritable component,

researchers have been able to identify a few key genes that greatly increase the predisposition for EOAD and possibly LOAD that also coincides with the previous described Aβ theory.

EOAD and ADAD have been identified to occur as a result, or largely because of mutations that are involved in the synthesis and cleavage of Aβ peptide including APP (Goate et al., 1991), presenilin-1 (PSEN1) (Sherrington et al., 1995) and presenilin-2 (PSEN2) (Levy-Lehad et al., 1995), which are proteins involved in gamma secretases that cleave APP from the membrane to generate $A\beta_{40}$ and $A\beta_{42}$. These genes alone were shown to account for approximately 71% of all EOAD (<61 years old) in a large population-based study in the city of Rouen which was comprised of 426,710 residents (Campion et al., 1999). The discoveries that mutations in APP, PSEN1 and PSEN2 were all significant factors in EOAD and may play roles in the development LOAD strongly supported the generally well accepted Aβ theory that, improper Aβ processing and cleavage leads to accumulation of Aβ oligomers and plaques thus driving disease progression. Together these gene interactions are thought to initiate disease progression at an earlier stage of life through improper Aβ processing which could lead to earlier seeding of Aβ plaques and therefore disease progression.

Other gene variants that are associated with neurodegeneration have also been linked to sporadic EOAD (sEOAD) incidences such as the predicted gene variant in the microtubule associated protein Tau (MAPT) gene, which provides instructions on how to make Tau protein (such as p.A469T variant) (Barber et al., 2017). Mutations in MAPT is thought to help promote AD progression similarly to APP, PSEN1 and PSEN2 in the sense that MAPT is associated with increased risk of sporadic tauopathies (Myers et al., 2005), therefore contributing neurodegeneration. This accumulation of hyperphosphorylated tau protein is known as a

tauopathy and is common to 80% of dementias (Scholl et al., 2018). While mutations in these genes can confer a large genetic risk for EOAD they may also be partially responsible in the development of LOAD depending on the severity of the mutation.

EOAD and LOAD share many commonalities; however, LOAD is significantly more common and aside from the genes previously mentioned, often involves other complex genetic relationships and interactions, environmental factors, and general senescence that all contribute uniquely to LOAD progression throughout the duration of one's life. Like previously stated, the seemingly most logical explanation for developing this condition is the Aβ theory but it is not that simple. This is a disease that its progression starts from a young age and develops to a point where it is visible and at that point it is too late to treat or preventatively diagnose. Therefore, it seems that the best way to attack AD is to decipher its origin and what drives disease initiation and progression past the point of return beyond that of conventional medicine or cellular therapy.

In the last few decades, other genes of interest have begun to be highlighted as top genetic risk factors in the development of LOAD such as the polymorphism in the Apolipoprotein E (ApoE) gene, ApoE4 (Kamboh et al., 1995; Liu et al., 2013). APOE was first identified as one of the protein components of plasma very-low-density lipoprotein (LDL) (Shore and Shore, 1973) and was found to play a critical role in plasma cholesterol metabolism and homeostasis and soon after realized to be a risk factor for hypercholesterolemia and atherosclerosis (Mahley, 1988; Davignon et al., 1988). ApoE is a multifunctional protein expressed in several tissues with its highest levels being expressed in liver and brain while also being expressed at relatively higher levels in adrenal glands, testes, and ovaries (Blue et al., 1983 Srivastava and Srivastava, 1996).

Although ApoE is expressed and plays roles in peripheral cholesterol metabolism, it was found to be the primary lipid and cholesterol transporter in the CNS (Boyles et al., 1985). Its primary role is involved in lipid and cholesterol metabolism where the secreted ApoE binds cholesterol and phospholipids from cellular efflux mediated by ATP-binding cassette (ABC) transporters (Vance, 2012), in which have also been quite extensively linked to AD (Wolf et al., 2012). ApoE mediates the binding of lipoproteins and lipid complexes in the plasma, interstitial fluid, or CSF to specific cell-surface receptors such as LDLR (Huang and Mahley, 2014), therefore, facilitating lipid transportation throughout the body and in CNS. Similarly in neurons, uptake of astrocyte-derived ApoE-containing lipid particles is mediated by the LDL receptor family including LDLR, LDLR related protein (LRP) and ApoE receptor 2 (APOER2) (Boyles et al., 1989; Posse de Chaves et al., 2000). Once bound, these receptors internalize ApoE-containing lipoprotein particles, thus intracellular ApoE may also modulate various other cellular processes physiologically and pathophysiologically such as cytoskeletal assembly and stability or dendritic morphology and function (Huang and Mahley, 2014). In the nervous system, ApoE is the primary transporter of cholesterol (Boyles et al., 1985; Weisgraber et al., 1994) and is synthesized by nearly all cell types under different conditions. Under normal physiological conditions, astrocytes secrete the majority of ApoE (Boyles et al., 1985) while under certain pathological and injury conditions ApoE is produced by microglia as well as neurons (Boyles et al., 1985).

As mentioned, ApoE is a polymorphic gene existing as three isoforms within the human population, including ApoE2, ApoE3 and ApoE4, encoded by ε2, ε3, and ε4 alleles, respectively (Corder et al., 1993). ApoE is a polypeptide consisting of 299 amino acid residues and the different alleles result in a structural difference between the three isoforms in their respective

lipid binding regions. This is due to amino acid substitutions in the 112th and 158th positions where ApoE2 contains two cysteine (Cys) residues at these positions, ApoE3 has a Cys and arginine (Arg) respectively at these positions, and ApoE4 has two Arg residues at these positions. The Arg residue substitutions affect sulfide bonding resulting in ApoE4 having a different tertiary structure than both ApoE3 and ApoE2 (Hatters et al., 2006). Specifically, the Cys112 to Arg112 change in ApoE4, that is not part of the LDLR binding site (amino acids 138-150), is strongly associated with increase in cholesterol and LDL in plasma (Eto et al., 1987; Saito et al., 2004). Not only do these isoforms result in differences in their respective lipid binding regions but each isoforms affinity for LDLRs is also different. In cell, culture-based binding assays have shown that ApoE3 and ApoE4 bind to LDLR with high affinity whereas ApoE2s binding affinity is significantly weaker (Yamamoto et al., 2008). ApoE and cholesterol homeostasis is critical for many processes in the CNS including development, membrane stability, and in neuronal health and physiology (Zhang and Liu 2015). Due to the impermeable blood-brain barrier, cholesterol metabolism in the brain is independent of peripheral cholesterol metabolism, and defects in brain metabolism have been linked to NDDs including the development of AD (Vazquez et al., 2020). Specifically, that high LDL cholesterol levels are significantly linked to AD (Vazques et al., 2020). Given ApoE's physical relationship and partial responsibility in mediating lipid metabolism, mainly cholesterol in the CNS, this captures the importance of maintaining lipid homeostasis for proper neuronal functioning.

It is estimated that about 10-15% of people carry one copy of ApoE ε4 allele, and that two to three percent carry two copies of the allele. ApoE3, the most common isoform, is found in about 70% of the population, and ApoE2, the rarest isoform, can be found in 5-10% of the

population (Guo et al., 2001). Since everyone carries two copies of the ApoE alleles, people with ε2/ ε2 are said to be at the lowest risk for LOAD and those with ε4/ ε4 are at the greatest risk while those with ε3/ ε3 are considered neutral for the risk of LOAD. The risk factor between one copy of ApoE ε4 versus two copies is exponential where one copy is estimated to confer a 3-fold and two copies is estimated to have a 12-fold risk of development in LOAD relative to those who carry two copies of ε3 (Corder et al., 1993; Holtzman et al., 2012). For this purpose, ApoE4 is thought to be neurodestructive and promotes neurodegeneration, and on the other hand, ApoE2 is thought to be neuroprotective against neurodegeneration and LOAD development and to reduce AD risk by nearly 50% (Conejero-Goldberg et al., 2014). ApoE has been shown to be extensively involved in the immune response as evident through recent transcriptomic studies (Keren-Shaul et al., 2017; Krasemann et al.,2017). In amyloid models, ApoE upregulation is a major molecular signature of the subtype of microglia known as disease-associated microglia (DAM) (Li et al., 2020; Keren-Shaul et al., 2017; Krasemann et al., 2017). One thing is clear, ApoE is very much so an integral part to AD pathogenesis, but its full extent of each of the different isoform's contribution to either the progression or restriction remains to be fully characterized.

The mechanisms underlying the neuroprotective effects of ApoE2 are less defined than ApoE4s neurodestructive effects; however, the goal is not necessarily to show that ApoE2 is per se protective but rather, not destructive, or offers less a risk than the common ApoE3. ApoE2 gene carriers have a reduced risk of AD of nearly 50% (Conejero-Goldberg et al., 2014) but the mechanisms by which ApoE2 confers neuroprotection has yet to be determined. Studies of ApoE protein abundance in human postmortem cortex follows an isoform-dependent pattern of ApoE2>ApoE3>ApoE4 (Conjero-Goldberg et al., 2014). This suggests that ApoE2 abundance

coupled with reduced ability to bind LDLRs (Yamamoto et al., 2008; Conjero-Goldberg et al., 2014) may act to promote Aβ clearance (Li et al., 2020). The evidence surrounding ApoE4's neurodestructive properties is undeniable and it is clear why ApoE4 would be a top risk factor for AD. A recent study examining ApoE4 copy number-dependent proteomic changes in cerebrospinal fluid (CSF) using target proteomic data from the Alzheimer's Disease Neuroimaging Initiative (ADNI) CSF samples concluded that increased APOE4 copy number was associated with a decrease in C-reactive protein (CRP), a marker of inflammation, CSF levels across all models analyzed that was also accompanied by increased Aldolase A (ALDOA), a critical enzyme involved in glycolysis , CH3LI, a CSF biomarker that increases with age and early in AD as well as FABPH (fatty acid binding protein) CSF levels when controlling for Aβ (Berger et al., 2021). Decreased CRP levels in periphery serum (Nilsson et al., 2011) and in CSF (Gabin et al., 2018) have been previously shown to correlate with increased cognitive dysfunction and AD progression in an ApoE4-dependent manner. It is also established that there is ApoE isoform specific effects on the Aβ pathway (Kim and Holtzman, 2009) and that ApoE4 is associated significantly with an increase in Aβ plaques at earlier ages compared to ApoE3 or ApoE2 individuals (Deane et al., 2008; Kim et al., 2011). Due to ApoE4 unique structure it is susceptible to proteolysis, resulting in the generation of neurotoxic fragment that cause pathological mitochondrial and cytoskeletal alterations (Mahley and Huang, 2012). Moreover, it was shown that ApoE2 had less neurotoxic effects than APOE3 following proteolysis. ApoE4 promotes the progression of AD through several different mechanisms compared to that of ApoE3 or ApoE2 and that ApoE2, while maybe not having any directly identifiable protective features against AD, likely contributes to AD protection by strengthening and reinforcing the networks that are susceptible to failure in AD such as Aβ

synthesis as well as tau accumulation. It has been shown that ApoE is involved in the innate immune response and ApoE4 promotes inflammatory response evoked by Aβ while ApoE2 inhibits the inflammatory response through the vitamin D receptor pathway (Dorey et al., 2017).

ApoE has been a long standing top genetic risk factor for LOAD but recently a new gene has come to light and some of its variants confer similar risks to LOAD as ApoE4, known as the Triggering Receptor Expressed on Myeloid Cells – 2 (TREM2) (Guerreiro et al., 2013; Korvatska et al., 2015). Not only do variations in TREM2 confer an increased risk of AD but it was shown that the ApoE isoforms are a ligand for TREM2 (Atagi et al. 2015). This mechanistic and likely functional relationship between two of the top genetic risk factors for AD prompts further investigation to uncover how these two genes may interplay throughout one's lifespan to contribute to AD development and progression.

TREM2 is an innate immune receptor expressed on cells of the myeloid lineage such as dendritic cells and osteoclasts but most notably highly expressed in macrophages and microglia, the latter are resident macrophages of the CNS. Belonging to the immunoglobulin (Ig) superfamily, TREM2 is a 230 amino acid polypeptide consisting of a single-pass transmembrane receptor that also contains a single extracellular V-type Ig domain followed by a short stalk leading to a single transmembrane helix (Bouchon et al., 2000) where it interacts with DNAX-activation protein of 12 (DAP12), encoded by *TYROBP*, to mediate downstream signaling pathways (Kober and Brett, 2017). Upon ligand recognition, TREM2 is associated with DAP12 which contains an immunoreceptor-tyrosine activation motif (ITAM). Upon ligand recognition by TREM2, tyrosine residues within ITAM become phosphorylated recruiting spleen tyrosine kinase (Syk) to activate downstream signaling molecules such as extracellular signal regulated protein

kinase (ERK), phosphatidylinositol 3-kinase (PI3K) and phospholipase Cγ (PLCγ) (Takahashi et al., 2005). TREM2 terminates with a short cytoplasmic tail lacking any known trafficking motifs (Colonna and Wang, 2016). Although expressed primarily on the membrane of myeloid-derived cells, TREM2 can be shed its extracellular domain from cell membrane as a soluble form (sTREM2), but its complete functional outcome remains unknown. Shedding of membrane-bound TREM2 is mediated by α-secretases A Disintegrin and Metalloproteinase 10/17 (ADAM10/17) to cleave TREM2 at H157-S158 bond releasing the N-terminal fragment of TREM2, sTREM2, into the extracellular space (Wunderlich et al., 2013). The physiological functions of TREM2 are relatively well characterized and its roles in NDDs is being uncovered at a fast pace; however, the complete roles of its soluble counterpart both physiologically and pathologically remain elusive. Interestingly it was shown that TREM2 and sTREM2 bind oligomeric Aβ and that TREM2 deficiency impairs Aβ degradation in primary microglial cultures and mouse brains (Zhao et al., 2018), suggesting sTREM2 may act as a scavenger receptor capable of recruiting cells and acting as a sort of chemokine.

Under normal physiological conditions TREM2 signaling in microglia promote cell survival, proliferation, activation, phagocytosis, and inflammation regulation (Painter et al., 2015). TREM2 can bind a diverse array of ligands including bacterial lipopolysaccharides (LPS) and damage associated markers (DAMPs) such as phospholipids (Daws et al., 2003), lipids exposed during axonal injury (Wang et al., 2015), as well as previously stated ApoE2, ApoE3 and ApoE4 (Atagi et al., 2015). TREM2 in mice is also involved in synaptic pruning (Filipello et al., 2018) and mice deficient in TREM2 showed reduced numbers of synapse at 4 weeks of age (Jay et al., 2019). Atagi et al., uncovered a functional relationship between TREM2 and ApoE that ApoE binds to

apoptotic N2a cells and increase microglial phagocytosis in a TREM2 dependent manner (Atagi et al., 2015). It was also shown that each isoform binds TREM2 with high affinity and induces differentially levels of phosphorylated Syk, with ApoE2 and ApoE3 bind more readily to TREM2 than ApoE4 (Atagi et al., 2015). Contrary to this, ApoE4 stimulates Syk activation to a greater extent than either ApoE2 or ApoE3 (Yao et al., 2019).

Given its widespread binding ability and diverse functions, in recent years, TREM2 and its variants have been increasingly linked to NDDs including AD (Zhou et al., 2019), Parkinson's disease (PD) (Guo et al., 2019), Frontotemporal dementia (FTD) (Kleinberger et al., 2017), and amyotrophic lateral sclerosis (ALS) (Cady et al., 2014). Moreover, it was discovered early on that loss of function mutations in either TREM2 or TYROBP, DAP12s encoding gene, results in Polycystic lipomembranous osteodysplasia with sclerosing leukoencephalopathy (PLOSL), also known as Nasu-Hakola disease (NHD) (Lanier et al 1998; Paloneva et al., 2000; Kaneko et al., 2010). This rare recessively inherited disease is characterized by a combination of rapidly progressing to presenile dementia and bone cysts restricted to the wrists and ankles (Hakola, 1972; Hakola 1990). TREM2's involvement in several neurodegenerative diseases suggests TREM2 is critically involved in shared disease mechanisms (Jay et al 2017), highlighting the importance of intact TREM2 signaling.

TREM2's role in AD, however, is much more apparent than other diseases and offers researchers exciting and promising path to follow. Like previously stated, the R47H TREM2 variant shares a risk of developing LOAD like that of ApoE4. The single nucleotide polymorphism (SNP) rs75932628-T encoding the R47H TREM2 variant was shown to confer a significantly increased risk of LOAD with odds ratio of 5.05 (Guerriero et al., 2013). This loss of function

mutation has been shown to lead to microglial dysregulation in neuroinflammation, and a study showed that a TREM2 antibody (Ab) was able to restore TREM2 functioning in microglia (Cheng et al., 2018). It was also shown that R47H TREM2 greatly impairs ApoE's ability to bind (Atagi et al., 2015). While the R47H TREM2 variant is becoming linked to AD, several other variants have been linked to the development of AD and other NDDs. Mutations in TREM2 such as the T66M variant (Le Ber et al., 2014) and Q198X (Guerriero et al., 2013) in the development of FTD as well as other variants being linked to AD like the W198X variant (Giraldo et al., 2013). Although its polymorphisms are associated with a risk for LOAD (Guerreiro et al., 2013), TREM2's role in neurodegeneration is controversial and is likely a double-edged sword in the context of NDDs (Konishi and Kiyama, 2018). Wang et al., showed that deletion of TREM2 in 5XFAD mice reduced Aβ accumulation due to dysfunctional response of microglia Aβ plaques (Wang et al., 2015). On the other hand, Jay et al., showed that TREM2 deficiency resulted in reduced infiltration of inflammatory myeloid cells and ameliorated AD pathology (Jay et al., 2015) at early stages and exacerbated it at later stages (Jay et al., 2017). Subsequently, Wang *et al*., showed that amyloid-associated myeloid cells are derived from brain-resident microglia rather than from recruited peripheral monocytes (Wang et al., 2016). It was found that genetic targeting of *Trem2* in APP-PS1 mice at early stages restored homeostatic microglia associated with the reduction of Aβ plaques (Krasemann et al., 2017).

Mentioned earlier, TREM2 has a soluble form, sTREM2, and not only has TREM2 begun to be increasingly linked to the development of AD but sTREM2 is being identified to also play critically important roles in the progression and development of AD. Although the actions of sTREM2 remain undefined, researchers are uncovering the complex functions sTREM2 may carry

out in normal homeostasis as well as in the context of AD and contribution to either progression or prevention. Some studies have shown that sTREM2 is neuroprotective by stimulating microglial recruitment, activation, and phagocytosis of Aβ plaques and by blocking, reversing, or preventing neurotoxicity (Vilalta et al., 2021). sTREM2 has also been shown to reduce Aβ pathology and improve synaptic functions by modulating microglial activity in 5xFAD mouse model (Zhong et al., 2019). Not only has the research shown that sTREM2 directly interacts with AD pathologies, but its levels in CSF were reported elevated in AD patients compared to controls as well as sharing a correlation to total tau protein as well as phosphorylated tau but interestingly not with Aβ (Heslegrave et al., 2016; Suarez-Calvet et al., 2019). Moreover, sTREM2 was seen to increase in the preclinical stages of AD (Zhong et al., 2017; Suarez-Calvet et al., 2019) and then decreases at early symptomatic stages (Suarez-Calvet et al., 2019), supporting the idea that sTREM2 plays a critical role in AD disease progression. sTREM2 may act as a second messenger to allow crosstalk between microglia to coordinate an appropriate response. Interestingly CSF sTREM2 differed between TREM2 variants, whereas the R47H variant had higher CSF sTREM2, L211P had lower CSF sTREM2 (Suarez-Calvet et al., 2019) suggesting that mutations in TREM2 that are not part of the cleavage site influence TREM2 release or that shedding may be mediated by intrinsic factors. Moreover, sTREM2 signaling in microglia was shown to increase survival (Zhong et al., 2017) suggesting that sTREM2 be capable of initiating key transcription machinery in other cells possibly acting under chemotactic mechanisms.

Aβ plaques and neurofibrillary tangles have been under the main scope of AD research for several decades now but more recently, another major key player in the pathogenesis of AD has come to focus. Another hallmark of AD that accompanies Aβ and neurofibrillary tangles is

chronic neuroinflammation in response to these pathologies that is mediated by microglia. Microglia are responsible for initiating and mediating the immune response to pathogenic stimuli such as Aβ and p-tau. In recent years, extensive research has been conducted into microglia for its key roles in neuroinflammation across several NDDs.

Microglial cells are of mesenchymal or myeloid origin (Chan et al., 2006) and are the resident macrophages in the CNS. Besides their classic role as an immune cell, microglial cells protect the brain by stimulating phagocytic clearance and providing trophic sustenance to preserve cerebral homeostasis and support tissue repair (Hampel et al., 2020). In the event of loss in homeostasis, microglia become activated resulting in a highly dynamic responsive state (Sarlus and Heneka, 2017). This may include cellular morphology changes, increased secretion of molecular mediators like cytokines and chemokines, as well as increased proliferative responses (Streit et al., 2014). Imbalances in the brain such as Aβ and neurofibrillary tangles can trigger a step in activation referred to as “Priming” (Perry et al., 2014). Priming in microglia is influenced by acute alterations in their microenvironment (Hampel et al., 2020), releasing molecules that enhance their proliferation. This is a mechanism that allows microglia to be more inclined to respond to secondary therefore eliciting amplified inflammatory reactions (Perry et al., 2014). Activated microglia is a common feature of AD and other forms of neurodegeneration and there is increasing evidence for a high degree of heterogeneity between activated microglial subpopulations (Heneka et al., 2020; Wang and Colonna et al., 2019).

Mainly two distinct and considered activated populations exist in a dynamic state of opposite polarization for macrophages or microglia; the M1 or pro-inflammatory phenotype, and the M2 or anti-inflammatory (tissue repair) phenotype (Tang and Le, 2016; Ransohoff 2016). The

M1 phenotype occurs through classical activation in response to cytokines such as interferon γ (IFNγ), LPS, DAMPs (damage-associated molecular patterns) including Aβ and apoptotic debris (Abbas et al., 2016). The M1 phenotype is primarily involved in initiating and sustaining the inflammatory cascade in response to neurological insults or tissue injuries. Alternative activation results in the M2 phenotype which typically occurs in response to growth factors, and anti-inflammatory signals like Interleukin-4 (IL-4) and IL-13 (Abbas et al., 2016). This phenotype involved in initiating tissue repair and inflammatory response resolution. While these phenotypes are generally well-accepted models for microglia and macrophage activation, it is not as clear cut in vivo and has several complex underlying mechanisms that govern microglial response to different stimuli. However, in the context of chronic neuroinflammation and AD, studies have shown that in pre-clinical models, M1-like microglia seem to prevail at the injury site during the early stage of disease, and once inflammation resolution and repair completion, M2 microglia are diminished (Tang and Le, 2016). This helps show the persistence of pro-inflammatory cells and stimuli contributing to chronic neuroinflammation throughout the onset, development, and progression of AD. Although increasing evidence is compiling showing the dynamic activation states of microglia and shifts between them, the extent of the real-life relationship between microglia and phenotypes, or subtypes, is likely much more complex spectrum than just M1 and M2.

Experimental models of AD have shown that microglia cluster around Aβ plaques, possibly due to chemotactic mechanism, and may contribute in both Aβ clearing and in preventing further accumulation or seeding of plaques (Edwards et al., 2019). It is also possible that microglial dysregulation, including dystrophic microglia, may trigger or be a worsening factor in Aβ seeding

(Hampe et al., 2020; Edwards et al., 2019). At the molecular level, Aβ oligomers and fibrils are the driving force behind neuroinflammation in AD (Forloni et al., 2018; Glass et al., 2010). Although microglia are able to bind Aβ through surfaces receptors such as A1 scavenger receptor (SCARA1) or TREM2 (Zhao et al., 2018), whether the resulting effects are beneficial towards the progression of the disease is up for debate. While microglia have been shown to interact with Aβ, there is much debate to whether their actions are beneficial for the course of the disease or whether they exacerbate AD. For instance, starting at an early age Aβ clearance is sufficient via phagocytosis and degradation from activated microglia. Despite the advantageous early activation, their chronic activation is detrimental to disease progression and therefore facilitates chronic neuroinflammation thus accelerating neurodegeneration and initiating disease progression at an earlier state.

Microglia dysregulation is thought to be one of the driving factors behind neuroinflammation in several NDDs, not just persistent activation by Aβ in AD or other diseases and associated pathogenic stimuli. To help define the mechanisms by which microglial dysregulation in the context of AD and other NDDs neuroinflammation, Krasemann et al., isolated microglia and analyzed transcriptomes during aging and disease progression in APP-PS1 AD mouse model, as well as ALS and Multiple sclerosis (MS) models which revealed two distinct microglia populations. Cluster 1 was associated with loss of 68 homeostatic microglial genes including *P2ry12, Tmem119, Jun, Csf1, Tgfbr1, and Tgfb1* and transcriptions factors *Mef2a, Jun and Egf1* (Krisemann et al., 2017) which are all enriched in adult microglia (Butovsky et al., 2014; Buttgereit et al., 2016). Cluster 2 was associated with upregulation of 28 inflammatory molecules including, Spp1, Csf1 and ApoE, of which ApoE was one of the most upregulated genes

(Krisemann et al., 2017). This microglial phenotype termed MGnD, is resemblant of the expected neurodegenerative microglial responsible for promoting neurodegeneration and sustaining disease progression. In fact, MGnD microglia were associated with Aβ plaques and diffuse neuritic dystrophy in the AD cortex of APP-PS1 mice (Krisemann et al., 2017). This study shows that a distinct microglia subtype is likely developed through dysregulation and contributes to disease progression through self-reinforced mechanisms. This can be better interpreted as a loss of homeostatic mechanisms which result in a pro-inflammatory environment.

ApoE4 and TREM2 are major genetic risk factors for AD, but how they interact to affect microglial responses to Aβ remains largely uncharacterized. Using a preclinical AD mouse model, Fitz et al., reported an APOE isoform-specific phospholipid signature with correlation between human *ApoEε3/3* and *ApoEε4/4* AD brain and lipoproteins from astrocyte conditioned media of ApoE3 and ApoE4 mice (Fitz et al., 2021). It was shown that ApoE3 lipoproteins induce faster microglia migration to Aβ, facilitate Aβ uptake and ameliorate Aβ effects on cognition compared to ApoE4, and moreover this trend was augmented in vitro by TREM2 deficiency, suggesting interaction (Fitz et al., 2017). Not only do ApoE4 microglia show reduced ability to respond to Aβ but it was recently shown that lipid accumulation due to ApoE4 induces a lipid accumulated state that renders microglia weakly responsive to neuron activity (Victor et al., 2022). Victor et al., exposed ApoE3 and ApoE4 immortalized microglia-like cells (iMGLs) to conditioned neuronal media to test the isoform-dependent differences of ApoE on microglia neuronal activity surveillance. Interestingly, ApoE4 iMGLs response to conditioned media was significantly larger transcriptional response with nearly double the genes both up and down-regulated compared to that of ApoE3 with ApoE4 iMGLs being significantly enriched in HIF-1 signaling, JAK-STAT signaling

and cytokine-cytokine receptor interaction suggesting a strong pro-inflammatory response. Consistent with Krisemann et al.' work, ApoE4 iMGLs were also found to express significantly less levels of *P2ry12* compared to ApoE3 iMGLs, and upregulation of *P2ry6,* which is associated with a hypervigilant microglial state (Koizumi et al., 2013), was only experienced in ApoE3 iMGLs and not ApoE4 (Victor et al., 2022). ApoE isoforms have been shown to differentially affect different aspects of AD including cell migration to neurodegenerative lesions, survival, proliferation as well as inflammation but since Atagi et al., in 2015 identified that ApoE is a novel ligand with high affinity for TREM2, the TREM2-dependant mechanisms by which ApoE mediates microglial response to Aβ, neuronal activity, inflammation, phagocytosis and more in the context of AD or even other NDDs remain to be explored.

Chronic neuroinflammation is thought to fuel disease progression from an early stage by initiating microglial activation, which inadvertently contributing to disease progression is a self-reinforced manner. Biomarkers of chronic neuroinflammation are detected in the CSF of AD patients and is extensively being studied. Concentrations of several cytokines and other inflammatory biomarkers associated with microglia such as sTREM2, monocyte chemoattractant protein-1 (MCP-1), and YKL-40 (Swardfager et al., 2010; Lai et al., 2017). A recent meta-analysis reports higher concentration of YKL-40, sTREM2, MCP-1, and TGF-β in the CSF of AD patients compared to controls (Shen et al., 2019). In particular, robust evidence from several studies focus on CSF YKL-40 that shows a fair classificatory capability in differentiating between AD individuals and controls; however, distinguishing from other forms of dementia would be difficult (Balacci et al., 2019). Biomarkers provide good insight into what might be going on; however, it is not

precisely indicative and really offers no remedy. Some advancements in the field however have been made towards producing a viable treatment for patients living with AD.

Currently few drugs are in clinically trials aimed at targeting AD pathologies to alleviate symptoms but little progress has been made. Wang et al., showed that an anti-human TREM2 agonistic monoclonal antibody (mAb), AL002c, induced microglial proliferation of both the common variant and R47H variant in transgenic mice (Wang et al., 2020). Prolong exposure showed reduced filamentous plaques and neurite dystrophy, impacted behavior and tempered microglial inflammatory response (Wang et al., 2020). This is the first time a mAb was shown to relieve AD pathologies in a well-tolerated manner. Furthermore, they showed that AL002c was safe, and well tolerated in a Phase-1 clinical trial of 56 healthy adult participants with no adverse effect recorded. AL002c is currently undergoing Phase-2 clinical trials. Another antibody, known as aducanumab is a human IgG1 anti-Aβ mAb approved by FDA in 2021 and is selective for Aβ aggregates on the basis of slowing down cognitive decline in clinical trials but has not yet shown conclusive results and is under some controversy since its approval (Tampi et al., 2021). Other drugs are attempting similar strategies aimed at targeting and removing excess Aβ, however, at the time of clinical diagnosis, the patients' cognitive functions as well as physical molecular structure is already significantly damaged to the point that removing Aβ may just not be enough to allow the patient to recover completely or even partially.

Alzheimer's disease is a complex multifaceted disease existing of a complex web of gene interactions that all contribute progressively from early life to the accumulation of Aβ plaques and hyperphosphorylated tau in neurofibrillary tangles which clinically presents itself alter in life. Currently, there is no effective treatments to stop or reverse disease progression with limited

therapies to even help cope. Few available drugs in current trial offer limited help but are none the less exciting. Given the devastation cause by AD, the complexity in diagnosis, as well as ongoing research efforts, all contributions to the field help build the puzzle required to understand and ideally cure one day, are greatly beneficial.

1.1 Hypothesis and Aims

Neuroinflammation is a hallmark in AD as the body tries to process these insults and the mechanisms by which TREM2 and sTREM2 modulate neuroinflammation remain unknown. Given that the exact relationship and mechanisms between the different ApoE isoforms and TREM2 signaling during microglia activation in Alzheimer's neuroinflammation has yet to be fully established and my project aims to determine this.

Since TREM2 is largely expressed in microglia and macrophages and can bind ApoE isoforms. Understanding in-depth the relationship that exists between different ApoE isoforms, sTREM2 and macrophage phenotype states in inflammation can help provide insight into the roles and mechanisms of sTREM2 and ApoE isoforms in the context of inflammation and AD. Identifying the underlying causes of dysregulation of inflammation will ultimately help leading to the development of the therapies for targeting and mitigating chronic neuroinflammation, thereby stopping or reducing the rate of disease progression.

Macrophages are similar to microglia and can be activated to display M1 and M2 phenotypes similarly. Here they will be used as a cheaper and disposable model for this study. THP-1 cells are a myeloid-derived human monocytic leukemia cell line that are easily

differentiated into macrophages known to express membrane bound TREM2 and will be used to exemplify the relationships aimed to be characterized in this study.

TREM2 can be shed from the membrane, releasing as soluble sTREM2. Since sTREM2 remains to be fully characterized especially in the context of inflammation and it is currently not known whether sTREM2 is capable of inducing inflammation. This project aims to identify whether sTREM2 can induce an inflammatory response in myeloid-derived cells. ***My hypothesis is that sTREM2 may induce an inflammatory response or affect cytokine expression likely via MAPK-JNK or NFkB signaling pathway in this model***.

Since ApoE exists as 3 different isoforms and has been shown to bind TREM2, this project is aimed at identifying whether ApoE isoforms are able to differentially regulate the expression of cytokines in a THP-1/Macrophage model. ***My hypothesis is that ApoE2 may elicit an anti-inflammatory response in THP-1 differentiated macrophages while ApoE4 may induce an inflammatory response***.

2.0 Materials and methods

2.1. Cell Cultures

THP-1 (Human leukemia monocytic cell line) cells were obtained from the Human Health Therapeutics Research Center (HHT) at the National Research Council of Canada (Ottawa, ON). Cells were cultured in suspension in RPMI 1640 (Wisent Bioproducts) supplemented with 10% FBS, 1X penicillin/streptomycin (ThermoFisher Scientific), and 1X L-glutamine (ThermoFisher Scientific). Cells maintained in an incubator at 37°C and 5% CO_2. Media was replaced every 2-3 days by fresh complete RPMI 1640 media, and cells were passaged once cell concentrations

exceeded $1x10^6$/mL. Cells were discarded after passage 25. Passages 5-15 were used in throughout this study.

2.2 Treating THP-1 cells with sTREM2

THP-1 monocytes were counted on a hemocytometer. 3.6 million THP-1 cells were centrifuged at 300g for 7 minutes. Cell culture media was then removed and cells resuspended in RMPI1640 containing 1% fetal bovine serum (FBS). THP-1 cells were then pipetted onto a 24-well plate at 150,000 cells per well.

Human soluble TREM2 (sTREM2 protein) (Sino Biological, #11084-H08H) was purchased and reconstituted according to manufacturer's instructions in sterile water and stored at -20°C. Plated cells were then treated with 0.1µg/mL or 1.0µg/mL of sTREM2. Negative control cells were treated with sterile water, and 0.1µg/mL LPS was used as a positive control to induce inflammatory response. Cells were harvested at 2-, 4- ,6- and 8-hours post treatment as described in Section 2.4 and supernatant was collected and stored at -80°C for later cytokine ELISA.

Rat monoclonal IgG_{2B} antibody against human TREM2 was purchased from R & D systems (#MAB17291). Antibody was reconstituted according to manufacturer and was stored at -20°C. THP-1 cells were plated as described previously and cells were treated with either 0.1µg/mL antibody, 0.1µg/mL sTREM2, or both 0.1µg/mL sTREM2 and antibody. Antibody and sTREM2 were preincubated together at 37°C in 1% FBS RPMI1640 for 30 minutes prior to treatment. Cells were harvested at 2-, 4- ,6- and 8-hours post treatment and as described in Section 2.4, and supernatant was collected and stored at -80°C for later ELISA.

Chemical inhibitors were purchased from Selleck Chemical and reconstituted according to manufacturer's instructions in order to target major inflammatory signaling pathways. Prior to treatment, IC50 of each inhibitor was tested on cultured cells for 8 hours to ensure it did not affect cell viability using the dye exclusion test. SP600125 (#S1460), a broad spectrum JNK inhibitor for JNK1, JNK2 and JNK3 with an IC50 of 40nM, and 90nM, respectively, was used to target JNK-MAPK pathway. Piceatannol (S3026), a natural stilbene, and selective Syk inhibitor with an IC50 of 15µM. MG-132 (#S2619), a potent well known proteasome inhibitor with an IC50 of 0.1 µM, was used to target NFκB activation. Cells were plated as previously described in 1% FBS RPMI1640. Cells were then treated with either 0.1µg/mL sTREM2, IC50 of either SP00125, MG-132 or Piceatannol, or both 0.1µg/mL sTREM2 and IC50 of either inhibitor. Prior to the addition of sTREM2, cells were pre-incubated with inhibitor for 10 minutes. Control cells were treated with DMSO. Cells were harvested at 6 hours post treatment as described in Section 2.4 and supernatant was collected and stored at -80°C for later ELISA assays.

MCC950 (Selleck chemicals, #S7809) was purchased to inhibit NLRP3 inflammasome activation. 7.5nM of MCC950 was incubated with THP-1 cells for 10 minutes prior to the addition of 0.1µg/mL sTREM2, 0.1µg/m mAb or both. At 6 hours, cells were harvested and expression of IL-1β was assessed using RT-qPCR.

2.3. Differentiation and Polarization of THP-1 Monocytes into Macrophages

This protocol was generously provided by Patrick Salois at the Human Health Therapeutics Research Center, National Research Council of Canada (Montreal, QC). THP-1 cells were counted using a hemocytometer. Desired number of THP-1 monocytes were centrifuged at 300g for 7

minutes at room temperature and resuspended in monocyte attachment media (PromoCell, Stemcell technologies) to a final concentration of $1.5x10^6$ cells/mL. 100µL (150,000) cells was pipetted per well in a 96- or 24-well flat bottom tissue culture treated plate and allowed to sit at room temperature for 10 minutes (Sigma Aldrich). The plate was then transferred to an incubator and allowed to incubate for 1.5 hours at 37°C and 5% CO_2. Following this brief incubation, attachment media was replaced with fresh RPMI1640 substituted with 10% heat inactivated (HI) FBS (FBS was heat-inactivated at 56°C with moderate mixing for 30 minutes) and supplemented with 25ng/mL PMA (Sigma Aldrich). The plate was transferred to an incubator at 37°C and 5% CO_2 for 72 hours for THP-1 cells to differentiate. Differentiated cells were then washed twice with RPMI1640 10% HI FBS and allowed to rest in media for 24 hours at 37°C and 5% CO_2. Following a brief resting period, cells were then polarized for 48 hours at 37°C and 5% CO_2 to M0, M1 and M2 macrophages. M0 macrophages using RPMI1640 as described above supplemented with no additional cytokines. M1 macrophages were polarized using RMPI1640 supplemented with 10pg/mL LPS (Sigma Aldrich) and 25ng/mL IFN-γ (Stemcell Technologies, #78141). M2 macrophages were polarized using 25ng/mL of IL-4 (Stemcell Technologies, #78045) and IL-13 (Stemcell Technologies, #78029), respectively. Polarization lasted 48 hours at which time the supernatant was either stored at -80°C for later experiments or cells were washed and treated, then prepared for RT-qPCR. Images were taken using Bio-rads ZOE to show morphological changes between undifferentiated THP-1 cells and polarized macrophages (Supplemental Figure 1.)

2.4 Reverse Transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated from treated THP-1 monocytes or macrophages using 100µL TRIzol reagent (Invitrogen) following manufacturer's instructions. For THP-1 monocytes, cells in suspension were removed from treated plate and centrifuged at 300g for 5 minutes, supernatant was removed and stored at -80°C for future experiments, and TRIzol reagent was added to cell pellet. For macrophages due to their adherence, supernatant was removed from the plate and stored at -80°C for future experiments, and TRIzol reagent was able to be added directly to wells. Manufacturers protocol was followed except that all centrifugations were carried out at 14,000g with the addition of an extra chloroform wash step which included diluting the fractionated aqueous layer to 500µL in dimethyl pyrocarbonate (DEPC)-treated dH_2O and fractionating another aqueous using equal parts chloroform. The separated aqueous layer volume was then precipitated using 450µL isopropanol and 45µL 3M Sodium Acetate (pH 5.2) for 30 minutes at room temperature. RNA Pellet was washed three times with ice-cold 75% ethanol and solubilized in 15µL DEPC-treated dH_2O at 55°C for 10 minutes. RNA was quantified using ThermoFisher Scientific NanoDrop spectrophotometer and was then either used directly for reverse transcription or stored at -80°C for later.

Reverse transcription (cDNA synthesis) was performed using M-MLV reverse transcription kit 200U/µL (Invitrogen) supplied with 2.5µM dNTP mix, 5X First strand buffer and 0.1M DTT. 100ng of isolated total RNA was mixed with 200ng random hexamer primer (Invitrogen, #SO142), 0.5µM dNTP, and DEPC-treated dH_2O and heated at 65°C for 5 minutes and then directly placed on ice. Samples were centrifuged and then combined with 1X first strand buffer and 10mM DTT and heated at 37°C for 2 minutes. 200U M-MLV reverse transcriptase was finally added to yield

a 20µl reaction and was allowed to incubate at room temperature for 10 minutes before incubating at 37°C for 50 minutes. The reaction was inactivated at 70°C for 15 minutes. Prepared complementary DNA (cDNA) was diluted 10-fold for use in subsequent qPCR reaction.

qPCR Amplification was performed in a final volume of 10µL using PowerTrack SYBR Green Master Mix (ThermoFisher, #A46012) following manufacturer's cycling protocol for 45 cycles. Samples were run in duplicates and optimal annealing temperature for each primer was determined using a gradient style assay and used the annealing temperature that yielded the best amplification curve. The expression of several cytokines was assessed using specific primers for TNF-α, IL-1β, IL-10, TARC/CCL17 and housekeeping gene β-actin. Relative quantification using delta ct method outlined by Ganger et al. 2017 was used to quantify gene expression, normalized with β-actin.

2.5. Enzyme linked-immunosorbent assay (ELISA)

RayBio Human TREM2 ELISA kit (#ELH-TREM2) was used to detect sTREM2 in differentiating macrophages medium. Following differentiation like previously described, cell culture supernatants were harvested at 24 hours and 48 hours of polarization and stored at -80°C. Manufacturer's protocol was followed except that samples were incubated overnight at 4°C with shaking. Samples were diluted 2-fold in 1X assay diluent, standards were prepared using fresh cell culture media.

RayBio Human IL-1β ELISA kit (#ELH-IL1β) and Human TNF-α ELISA kit (#ELH-TNFα) were used to detect levels of IL-1β protein and TNF-α expression, respectively, in ApoE protein-treated macrophages and sTREM2–treated THP-1 monocytes. Supernatants stored at -80°C from

experiments previously described were thawed to room temperature and diluted 2-fold in 1X assay diluent, standards were prepared using fresh cell culture media. Manufacturer's protocol was followed. Absorbance of each well was analyzed at 450nm using Synergy HTX microplate reader. Standard curves used for cytokine quantification included in appendices section.

Anogen Multiplex Human Cytokine ELISA Kit (#EM10001) was used in the semi-quantitative determination of pro-inflammatory cytokines expression in polarized M0, M1 and M2 macrophages being treated by sTREM2. Pro-inflammatory cytokines analyzed included IL-1α, IL-1β, IL-6, IL-8, IFN-γ, GM-CSF, MCAF, and TNF-α. Standards were diluted in RPMI1640 supplemented with FBS. Two concentrations of each standard were used to generate standard curves, a high concentration provided by manufacturer and a 1:32 dilution. Manufacturer's protocol was followed. Synergy HTX microplate reader. Standard curves used for cytokine quantification included in appendices section.

2.6. Western blot analysis

THP-1 monocytes were differentiated to macrophages as previously described and treated with 3µM of either ApoE2 protein (Fitzgerald, #30R-AA019), ApoE3 protein (Fitzgerald, #30R-AA016) or ApoE4 protein (Fitzgerald, #30R-2382), for 48 hours during the polarization period. Following polarization, supernatants were removed, and macrophages were lysed using whole cell extract (WCE) buffer containing 10% glycerol, 50mM Tris-HCl pH 7.6, 400mM NaCl, 5mM EDTA, 1mM PMSF and 1mM DTT. Cells were lysed for 30 minutes with moderate shaking at 4°C and centrifuged at 14,000rpm for 20 minutes at 4°C. Immediately after centrifugation, WCE from

treated macrophages were quantified using Bradford assay. Standard was prepared from serial dilution of BSA.

25μg of WCE protein was prepared with 6X Lammaeli buffer and ran on 10% SDS-PAGE gel. Proteins were then transferred to PVDF membrane following standard western blot procedure. Primary rabbit antibodies for human β-actin (D6A8) (Cell Signaling Technology, #8457) and human TREM2 (D814C) (Cell Signaling Technology, #91068) were used to probe PVDF membrane. Protein of interest was probed first, stripped and then probed by β-actin antibody. Membrane was stripped at 50°C in stripping buffer prepared with 10% SDS, 0.5M Tris-HCl pH 6.8, 14M β-mercaptoethanol and dH_2O.

Primary antibodies were diluted 1:1000 as per manufacturer's instructions and incubated overnight at 4°C in 5% milk and TBS supplemented with 1% Tween. Anti-rabbit secondary antibody (Cell signaling technology, #7074) was diluted 1:10000 and incubated for 1 hour at 4°C in 5% milk and TBS 1% Tween before developing. Bio-Rads Clarity Western ECL substrate was used to develop protein bands for chemiluminescence detection using Bio-Rad gel-doc. Western blot densitometry quantification was carried out using Bio-rads Image Lab software.

2.7. Live cell imaging

Live cell imaging to observe differentiation and polarization morphology changes from THP-1 monocytes to M0, M1 and M2 macrophages was completed by using Bio-Rads ZOE fluorescent cell imager.

2.8. Statistical analysis

Data were presented as Mean ± SD. Statistical analysis for single comparison was performed by Student's *t*-test where each experiment was repeated at least 3 times (n= 3). For multiple comparisons, one way ANOVA was performed. The criterion for statistical significance was $p<0.05$. All analysis was carried out using GraphPad Prism 9 software.

3.0 RESULTS

Part A: sTREM2 induces cytokine expression in THP-1 monocytes through MAPK-JNK Signaling

i. sTREM2 Induces Expression of Cytokine in THP-1 Monocyte Model.

To characterize the role of sTREM2 in the inflammatory response, THP-1 monocytes were treated with 0.1µg/mL or 1.0µg/mL recombinant human sTREM2, and cytokine expression was assessed at 2, 4, 6, and 8 hours post treatment using RT-qPCR. ELISAs for TNF-α and IL-1β were conducted to confirm the expression at the protein level. Negative control cells were treated with equal volume sterile H_2O. Bacterial LPS was used as a positive control to exemplify induction of inflammatory response in this model since it is known to strongly stimulate expression of inflammatory cytokines in monocytes. 0.1µg/mL LPS strongly stimulated the expression of TNF-α and IL-1β and to a lesser extent TARC/CCL17 and IL-10 at 2, 4, 6, and 8 hours post treatment (Figure 1). Overall, LPS served as a strong positive control to ensure functionality of the primers and induction of inflammatory cytokine expression. The controls in the following experiments are negative controls.

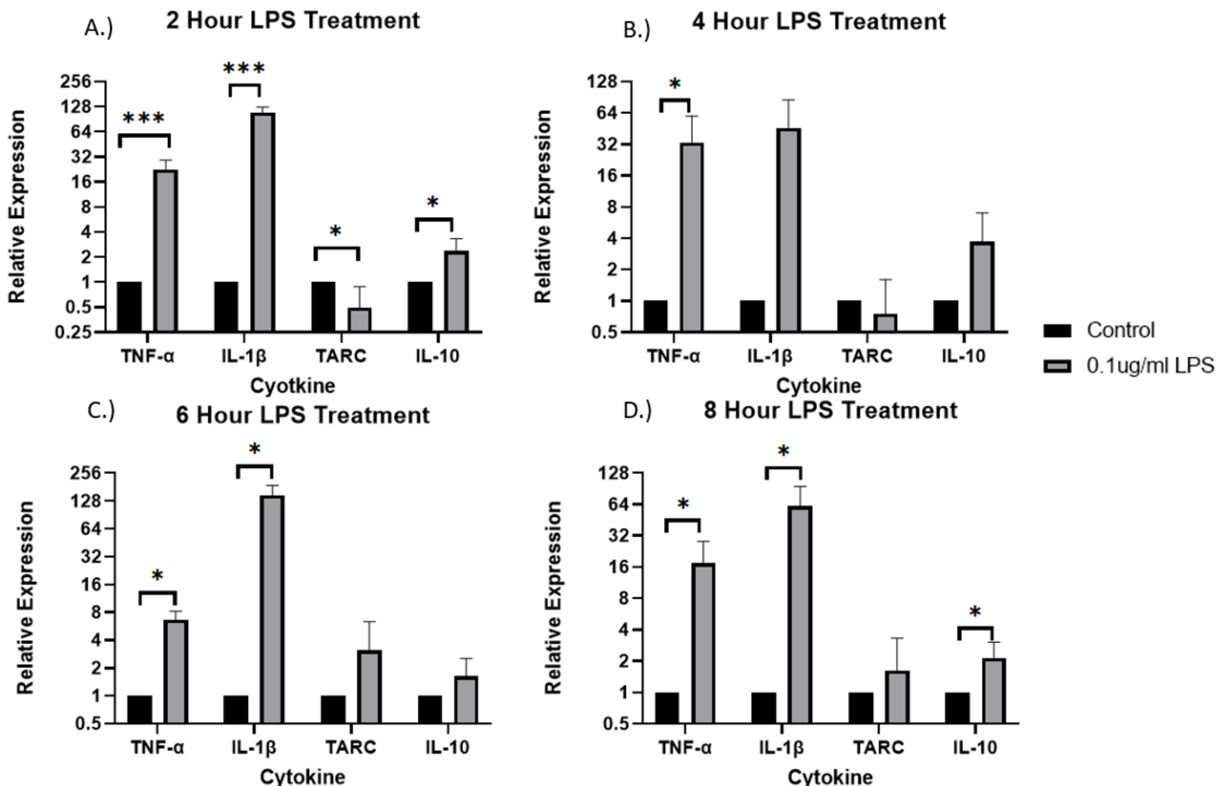


Figure 1. LPS induced cytokine expression in THP-1 cell model: Treating THP-1 monocytes with 0.1µg/mL LPS strongly stimulated expression of TNF-α and IL-1β (***=$p<0.001$; *=$p<0.05$) as well as TARC and IL-10 expression (*=0.05) as compared to negative controls at 2(A), 4(B), 6(C) and 8(D) hours post treatment.

At 2 hours post treatment 0.1µg/mL sTREM2 stimulated the expression of IL-1β (*p=0.03) and TNF-α (**p=0.01) but failed to stimulate expression of TARC or IL-10 compared to untreated control samples. 1.0µg/mL sTREM2 failed to stimulate expression of cytokines at 2 hours post treatment although IL-1β and TARC transcription was slightly elevated (Figure 2).

At 4 hours post treatment, the expression of cytokines induced by addition of 0.1µg/mL sTREM2 remained like 2 hours post treatment where TNF-α ($p<0.0001$) and IL-1β were increased but TARC expression remained similar to control levels. Interestingly at this time point, the expression of IL-10 was induced by both 0.1µg/mL (p=0.05) and 1.0µg/mL sTREM2 was higher

than both TNF-α and IL-1β. sTREM2 at 1.0μg/mL was able to stimulate the expression of TNF-α, IL-1β, TARC, as well as IL-10 compared to untreated controls although none were shown to be statistically significant (Figure 2).

At 6 hours post treatment (Figure 2) both concentrations, 0.1μg/mL and 1.0 μg/mL sTREM2, were able to significantly stimulate the expression of TNF-α, IL-1β, TARC, and IL-10 compared to control THP-1 cells. At 0.1μg/mL, sTREM2 induced the expression of TNF-α ($p=0.05$) and IL-1β ($p=0.01$) to levels consistent with previously seen; however, IL-10 ($p=0.001$) expression was stimulated unlike previously observed. A similar pattern is seen following treatment using 1.0μg/mL sTREM2 where TNF-α ($p=0.0004$), IL-1β ($p=0.02$) and IL-10 ($p=0.01$) were increased as compared to untreated control. At this time point, expression levels for each TNF-α, IL-1β, TARC, and IL-10 expression levels were at the highest and most consistent levels following both 0.1μg/mL and 1.0 μg/mL sTREM2 treatment compared to either of the other time points.

Consistent with shown at previously time points, at 8 hours post treatment (Figure 2), 0.1μg/mL sTREM2 was able to increase the expression of TNF-α ($p=0.03$), IL-1β, TARC ($p=0.02$) and IL-10 ($p=0.02$). At the concentration of 1.0μg/mL, sTREM2 was able to exert similar effects on THP-1 cells as 0.1μg/mL sTREM2 with increased expression of TNF-α ($p<0.0001$).

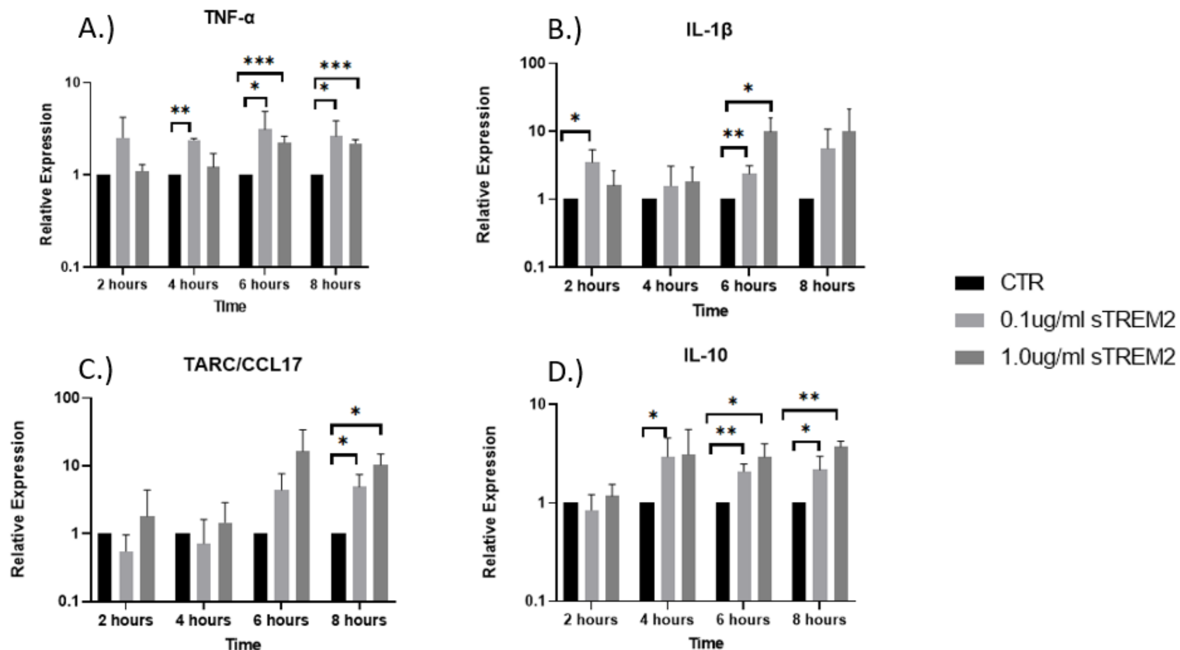


Figure 2. sTREM2 induced cytokine expression in THP-1 cell model: Treating THP-1 cells with 0.1µg/mL and 1.0µg/mL sTREM2 induced the expression of (A) TNF-α, (B) IL-1β, (C) TARC, and (D) IL-10. Data presented as Mean + SD. Individual treatments were assessed using students t-test where *<0.05; **<0.01; ***<0.001. Complete data set was analyzed using one-way ANOVA which showed (A) **p=0.007, (B) p=0.1, (C) p=0.1 and (D) *p=0.03.

Together these results show that 0.1µg/mL sTREM2 can consistently stimulated the expression of major cytokines TNF-α, IL-1β, TARC, and IL-10 with the expression of inflammatory cytokines TNF-α and IL-1β at earlier to later time points and the expression of anti-inflammatory cytokines TARC and IL-10 at later time points. On the other hand, 1.0µg/mL sTREM2 was not as successful at significantly stimulating expression of these cytokines. However, it is interesting that large amounts of sTREM2 does not exponentially increase cytokine production or induce apoptosis, suggesting that sTREM2-dependent signaling may be constant rather than acting in a dose-dependent manner in response to stimuli. One-way ANOVA revealed significant relationship for TNF-α (P=0.007) and IL-10 expression (p=0.03) but did not for IL-1β (p=0.1) or

TARC (p=0.1) expression. ELISA for TNF-α and IL-1β protein levels in treatment supernatant was conducted; however, the results were inconclusive with the majority of samples being below detectable levels of the kit. Identifying that sTREM2 could induce the expression of key inflammatory regulating cytokines in THP-1 monocytes but given the fact that it is not currently known what receptor sTREM2 binds on the membrane of THP-1 cells or how it is recognized prompted us to investigate more into the mechanisms behind sTREM2-dependent cytokine expression. To further investigate, we employed a commercially available TREM2 antibody in an attempt to try to neutralize sTREM2's ability to induce expression of cytokines in THP-1 cells.

ii. TREM2 mAb Partially Alleviated sTREM2-Induced Expression of Cytokines

After showing that 0.1µg/mL sTREM2 was able to stimulate the expression of cytokines in THP-1 cells with consistency, I attempted to neutralize this ability with a commercially available rat anti-human IgG_{2B} TREM2 antibody purchased from R&D systems. To assess the antibody's ability to bind and neutralize sTREM2, I co-incubated recombinant sTREM2 and the antibody at equal concentrations of 0.1µg/mL for 30 minutes at 37°C prior to treating plated THP-1 cells. Like previous, cells were harvested at 2, 4, 6 and 8 hours posted treatment, supernatant was stored at -80°C, and cytokine expression was assessed by RT-qPCR. ELISAs for TNF-α and IL-1β protein expression were conducted later.

At 2 hours post treatment 0.1µg/mL sTREM2 was able to stimulate expression of inflammatory cytokines in THP-1 cells as previously shown (Figure 3). Addition of only the anti-TREM2 mAb to cultured THP-1 cells interestingly also increased expression of TNF-α (p=0.002), IL-1β (p=0.002),

TARC and IL-10. This can likely be attributed to the antibody engaging TREM2 receptor on cell membrane which has been shown to play roles in activation and similar pathways (Yao et al., 2019; Kraserman et al., 2017). Addition of 0.1μg/mL sTREM2 pre-incubated with 0.1μg/mL mAb showed reduced expression of TNF-α and IL-10 as compared to 0.1μg/mL sTREM2 alone or mAb alone; however, it is interesting that sTREM2+mAb increased expression of both IL-1β ($p=0.02$) and TARC to levels higher than either sTREM2 or mAb alone (Figure 3). This may be, as a result, recognized as a complex and being internalized or bound to cell membrane which may trigger an alternative reaction to induce expression of these cytokines.

At 4 hours post treatment (Figure 3B), the effects of 0.1μg/mL sTREM2 was able to induce expression of TNF-α ($p=0.06$) and IL-1β to levels similar to previously shown. Again, the TREM2 mAb alone was able to stimulate increased expression of TNF-α and TARC, likely through activating cell surface bound TREM2. Interestingly, a consistent significant decrease of IL-1β ($p<0.001$) was observed as compared to sTREM2 and untreated groups, which is likely due to TREM2-dependent mechanisms. IL-10 expression remained unchanged by mAb treatment. Consistent with the 2-hour time point, the expression of both TNF-α and IL-10 expression were both decreased by treatment with 0.1μg/mL sTREM2+mAb as compared to cells that were treated with sTREM2 alone. Like previous I also observed that sTREM2+mAb largely stimulated the increased expression of TARC and IL-1β as compared to sTREM2-treated groups (Figure 3B).

At 6 hours post treatment (Figure 3) THP-1 cells treated with 0.1μg/mL sTREM2 showed increased expression of TNF-α ($p=0.04$), IL-1β ($p=0.1$), TARC ($p=0.04$), and IL-10 ($p=0.08$) like seen in previous time points. Treatment with mAb also stimulated expression of all cytokines analyzed. Interestingly, treatment with sTREM2+mAb did not have any large effects on TNF-α ($p=0.001$) at

this time point as compared to sTREM2 or mAb-treated controls but showed decreased expression of IL-10 as compared to sTREM2 treated groups (Figure 3C). Consistent with previous observation, treatment of the cells with sTREM2+mAb stimulated expression of IL-1β (p=0.001) and TARC compared to sTREM2 or mAb treated groups which may be indicating different modes of action on cytokine expression under these conditions.

At 8 hours post treatment (Figure 3), previously described results for 2-, 4-, and 6-hour time points were observed. 0.1µg/mL sTREM2 significantly induced the expression of TNF-α (p=0.01), IL-1β (p=0.1), TARC, and IL-10 (p=0.06) compared to control THP-1 cells. Treatment with mAb also stimulated the expression of TNF-α (p=0.02), IL-10, and TARC (p=0.04) but significantly decreased the expression of IL-10 (p<0.001), which was unseen previously. sTREM2+mAb treatment strongly increased expression of IL-1β and TARC while decreasing expression of IL-10 (p=0.04). Interestingly, effects of mAb pre-incubation with sTREM2 had little effects on TNF-α.

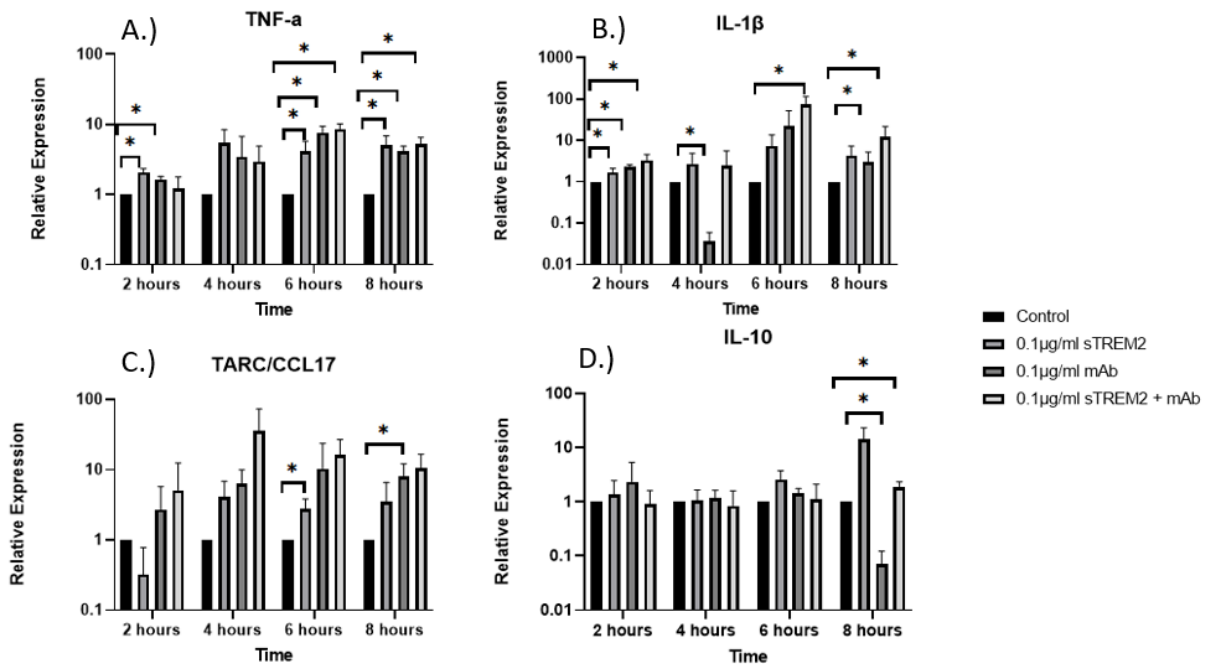


Figure 3. The effects of TREM2 antibody on sTREM2-induced cytokine expression: THP-1 cells were treated with 0.1µg/mL sTREM2, mAb or both sTREM2+mAb for 2, 4, 6 and 8 hours and cytokine expression of (A) TNF-α, (B) IL-1β, (C) TARC, and (D) IL-10 was assessed using qPCR. Data presented as Mean+SD. Individual treatments were assessed using students t-test where *<0.05; **<0.01; ***<0.001. Complete data set was analyzed using one-way ANOVA which showed (A) p=0.1, (B) p=0.2, (C) p=0.1 and (D) p=0.3. sTREM2 treatment preformed similar to previously shown in Figure 2, mAb treatment alone stimulated expression of cytokines probably through TREM2-dependant mechanisms while sTREM+mAb treatment partially reduced sTREM2 induced expression of some cytokines, others were greatly increased.

These results reinforce that sTREM2 is able to stimulate cytokine expression in our THP-1 cell model. Moreover, it was shown that targeting extracellular sTREM2 with mAb was sufficient at alleviating the expression of some cytokines including TNF-α and IL-10 to some degree at some time points, but significantly induced the expression of other cytokines such as IL-1β and TARC. Treatment of THP-1 cells with only mAb also upregulated the expression of TNF-α, IL-1β, TARC, and IL-10 compared to control cells and even at sometimes compared to sTREM2 treatment alone. Since this is an anti-TREM2 mAb, it is assumed that the expression of these cytokines by

mAb treatment was due to engagement of cell membrane TREM2. Together these results suggest that targeting either sTREM2 or TREM2 may elicit a specific cellular response. Here it is important to note that while the mAb incubated with sTREM2 reduced the expression of cytokines, they were not reduced to the levels equal or less than controls but did however reduce expression compared to sTREM2. Although students t-test between control and a single treatment and a give cytokines expression level proved many of these relationships to be significant, one-way ANOVA showed insignificant relationships for TNF-α ($p=0.1$), IL-1β ($p=0.2$), TARC ($p=0.1$) and IL-10 ($p=0.3$). ELISAs for TNF-α and IL-1β were conducted to assess expression of these cytokines at the protein levels; however, results were inconclusive with the majority of samples falling below detectable levels. Future experiments should use a larger sample size when conducting this assay. These results suggest that sTREM2 may induce expression of cytokines through different mechanisms. The mechanisms by which sTREM2 induces inflammatory response in monocytes needs to be explored further. While early expression of TNF-α induced by sTREM2 was reduced by binding of the antibody, later results stray from strongly supporting this. IL-10 expression was noticeably reduced by the binding of antibody to sTREM2. I also observed that the antibody peaked IL-10 expression at the 2-hour time point and as the time points progressed, the expression of IL-10 decreased. This may suggest that prolonged treatment with TREM2 antibody could lead to reduced anti-inflammatory signaling. To try to help identify cellular pathways utilized by sTREM2 to induce cytokine expression in THP-1 cells, several chemical molecular inhibitors were used.

i. Inhibition of JNK blocks sTREM2-induced expression of cytokines in THP-1 monocytes

It remains unknown which pathways sTREM2 may use to induce expression of cytokines in THP-1 monocyte model. Here I used several chemical inhibitors from Selleck Chemicals and employed them in an assay similar to previous sTREM2 treatments. MG-132 was used to inhibit proteasome and therefore NFκB activation. SP600125 was used to inhibit JNK which is downstream in the MAPK signaling pathway. Piceatannol was chosen as a Syk inhibitor which is the major kinase for activating TREM2 signaling. While the IC50's of the inhibitors were provided by the manufacturer and subsequently used in the following experiments, it was not known whether inhibitors would cause cell death in THP-1 cells. In order to explore this possibility, a cell viability test was completed to assess for cell loss over a period longer than the intended treatment time with the IC50s of each inhibitor indicated by the manufacturer. Cells were counted and plated at a concentration of 500,000 cells/mL and treated with an inhibitor. At 8 hours, cells were collected and recounted which all remained within 95% viability. THP-1 cells were pre-incubated with each inhibitor for 15 minutes at 37°C to ensure full diffusion and to allow binding of the inhibitor prior to the administration of 0.1µg/mL sTREM2. Cytokine gene expression was assessed by RT-qPCR at the 6-hour time point to reduce sample size as this time point showed consistent sTREM2-induced cytokine expression in my model.

First, I targeted the Syk using the chemical inhibitor Piceatannol in an attempt to inhibit sTREM2 induced cytokine expression in THP-1 monocytes. Under normal physiological conditions TREM2 signaling has been shown to recruit and phosphorylate Syk enzyme to communicate downstream after ligand binding and association with its adaptor protein DAP12 (Atagi et al., 2015; Kober and Brett 2017; Panter et al., 2015).

Piceatannol was administered to cultured THP-1 cells at an IC50 of 15µM (Figure 4A). Treatment with 0.1µg/mL sTREM2 induced expression of TNF-α, IL-1β, TARC, and IL-10 compared to untreated cell. While treatment with 15µM piceatannol did not induce cell death in the previous cell viability experiment, the inhibitor did not affect sTREM2-induced expression of cytokines. However, it appeared that the inhibitor slightly enhanced expression of these cytokines as compared to both sTREM2-treated and untreated THP-1 cells. It is important to note that this experiment did not show that sTREM2 uses TREM2-Syk signaling pathway to induce cytokine expression. Piceatannol did not inhibit expression of cytokines induced by sTREM2 which is interesting because this suggests that sTREM2 may not bind to or interact with membrane bound TREM2 in the context of inflammation induction and possibly conducts primary function using different signaling enzymes. However, TREM2 signaling has been shown to interact with several different key cellular pathways (Kober and Brett, 2017), which lead me to question whether sTREM2 interacts with the proteasome and whether the expression of cytokines seen in THP-1 cells was the outcome of this especially due to its involvement in the secretion of IL-1β (Yao et al., 2019, Chanput et al, 2010).

Next, I sought to inhibit possible activation of NFκB signaling by using the proteasome inhibitor MG-132 (Figure 4B) which was much more known and documented than piceatannol. It has also previously been shown that TREM2's phagocytic activities communicate with NFκB which suggest sTREM2 may have a functional relationship with the NFκB activation as well. To test this, MG-132 was administered to plated THP-1 cells at an IC50 of 100nM as indicated by manufacturer. It was seen that 0.1µg/mL sTREM2 consistently stimulated expression of TNF-α, IL-1β, TARC, and IL-10 as shown previously. THP-1 cells treated with 100nm MG-132 showed

increased expression in IL-1β and IL-10 that of which only IL-10 was expressed to higher levels than sTREM2 treatment, unlike the Syk inhibitor but TNF-α and TARC expression remained relatively unchanged by 100nM MG-132. This suggests that MG-132 is a good functional inhibitor. Interestingly, 100nM MG-132 appeared to have some effect on sTREM2-induced cytokine expression in THP-1 cells but did not completely inhibit the response. TNF-α and TARC expression induced by sTREM2 treatment was reduced to levels similar to untreated control cells but IL-1β and IL-10 expression was only slightly reduced. Although this was shown to be not statistically significant, a trend is still clearly visible and sTREM2 signaling through the NFκB pathway should be further explored and characterized. The fact that MG-132 did not completely abolish sTREM2-induced cytokine expression supports the complexity of sTREM2 and its ability to differentially regulate cytokine expression.

Lastly, I targeted JNK which is a kinase downstream of MAPK signaling, a major pathway of inflammatory activation. JNK is also known to be activated in response to cellular stress (Vukic et al., 2009). To target JNK, I used SP600125 administered at an IC50 of 90nM to inhibit JNK1, JNK2 and JNK3 as indicated by manufacturer. Consistent with previous, sTREM2 induced expression of cytokines in THP-1 cells (Figure 4C). Addition of only the inhibitor did not alter cytokine expression majorly except for slightly stimulating the expression of IL-1β, indicating that this should function as a good primer in this model. Here I observed that after a short incubation with SP600125 and following addition of sTREM2, the expression of cytokines was nearly completely abolished by the inhibitor (Figure 4C). It was primarily seen that the expression of TNF-α ($p=0.01$), TARC, and IL-10 was inhibited to levels equal to or lower than untreated control samples. IL-1β expression however was not completely reduced but it was partially ($p<0.001$)

which could be attributed to the complexity of communication in cytokine regulating signaling networks as well as the potentially different targets of sTREM2 which could lead to cytokine expression through different pathways. However, given that the majority of cytokine expression was abolished by SP600125 indicates that this may be the primary cellular pathway used by sTREM2 to induce expression of these cytokines in monocytes.

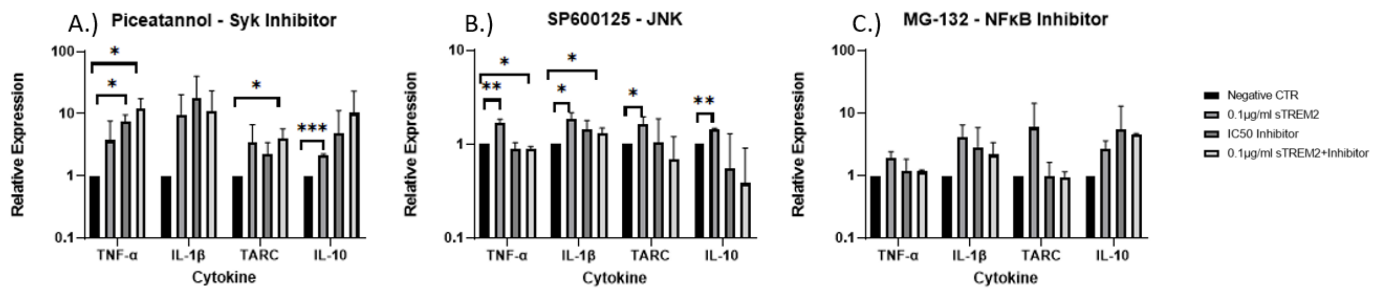


Figure 4. The effects of Syk, NFkB and JNK inhibitors on sTREM2-induced cytokine expression Data presented as Mean+SD. Individual treatments were assessed using students t-test where *<0.05;**<0.01;***<0.001. Complete data set was analyzed using one-way ANOVA which showed (A) p=0.07, (B)* p=0.018 and (C) p=0.2. (A) Piceatannol administered at a concentration of 15µM was shown to have no effect on inhibiting cytokine expression in THP-1 cells induced by sTREM2; (B) 100nM MG-132 failed to completely inhibit expression of cytokines induced by sTREM2 but seems to partially resolve TNF-α a, IL-1β and TARC expression; (C) SP600125 at 90nM was able to mitigate expression of cytokines induced by sTREM2 partially reduced IL-1β expression.

Interestingly it was seen that MG-132 did partially inhibit the expression of both TNF-α and TARC and slightly decreased expression of IL-1β although this relationship was not shown to be statistically significant. One-way ANOVA was unable to show significance between treatments, and the difference in cytokine production was likely to small, however a visible trend exists and should be explored further. Here I showed that SP600125, a well-documented JNK inhibitor (Vukic et al. 2009), was able to inhibit the expression of inflammatory cytokines nearly completely in THP-1 cells induced by 0.1µg/mL sTREM2. One-way ANOVA showed strong statistical

significance in this treatment between treated and control groups (p=01). Together these results indicate that sTREM2 may primarily use MAPK-JNK signaling pathway to induce expression of cytokines in THP-1 cells.

THP-1 monocytes used in above experiments were not differentiated into macrophage. To test whether sTREM2 also induces the expression of cytokines in macrophages, I differentiated THP-1 cells into M0, M1 and M2 macrophages as described below.

ii. sTREM2 induced expression of cytokines in THP-1 differentiated macrophages

Knowing that TREM2 functions primarily in macrophages and microglia during the innate immune response (Painter et al., 2015), I sought to characterize the effects of sTREM2 on macrophages differentiated from THP-1 cells. THP-1 cells were differentiated into macrophages (M0) and then polarized them to M1 or M2 phenotype as described in the Methods section (for the morphology of the polarized macrophages, please see supplementary Figure 1). The protocol for THP-1 cell differentiation and polarization to M0, M1 and M2 macrophages has been validated by FACS analyses of marker expressions on these cells by Patrick Salois at National Research Council of Canada (data not shown). Following polarization, M0, M1 and M2 cells were washed three times with fresh culture media and then treated with either 0.1µg/mL or 1.0µg/mL sTREM2 for 2- and 8-hour time points and control M0, M1, and M2 cells were treated with sterile water. Following treatment cytokine expression was quantified by RT-qPCR. Protein expression of IL-1α, IL-1β, IL-6, IL-8. GM-CSF, IFN-γ, MCAF and TNF-α were also analyzed using a human cytokine multiplex ELISA kit.

At 2 hours post treatment both concentrations of sTREM2 stimulated the expression of cytokines in polarized macrophages. In M0 cells (Figure 5A), 0.1μg/mL sTREM2 induced the largest expressional changes in TNF-α ($p<0.001$) and IL-1β ($p<0.001$), but also stimulated the expression of IL-6 ($p=0.02$) and TARC. sTREM2 at 1.0μg/mL was also able to stimulate the expression of TNF-α and IL-1β ($p=0.03$) as well as IL-10 ($p=0.004$) but not IL-6 or TARC as compared to untreated M0 macrophages (Figure 5A).

Following a 2-hour treatment with 0.1μg/mL sTREM2, M1 macrophages did not display large scale cytokine expression changes like in M0 macrophages except for TNF-α ($p=0.04$) expression which showed a reduction in expression compared to control (Figure 5B). This was also true for 1.0μg/mL sTREM2-treated M1 macrophages where induction of inflammatory cytokine expression was not increased but rather decreased in TNF-α ($p=0.02$), IL-6 ($p=0.07$), TARC ($p<0.0001$) as well as IL-10 ($p=0.007$). This supports that sTREM2 may play a role in initiating inflammation rather than promoting it in M1 cells that are already “programmed” to be inflammatory phenotype. This may be supported by the fact that 1.0μg/mL sTREM2 shows reduced expression of cytokines and therefore accumulation of sTREM2 may result in a reduction in cytokine expression or initiate a different response.

In M2 macrophages (Figure 5C), at 2 hours post treatment, 1.0μg/mL sTREM2 had minimal effects on cytokine expression with levels remaining similar to that of control M2 macrophages except for IL-6 ($p=0.007$) and IL-1β which were decreased. Treatment of M2 macrophages with 0.1μg/mL sTREM2 was much more efficient at stimulating the expression of cytokines. The expression of pro-inflammatory cytokines TNF-α ($p=0.04$), IL-1β ($p=0.08$) and IL-6 was increased in M2 macrophages following treatment with 0.1μg/mL sTREM2. In M0 and M2

macrophages, 0.1µg/mL sTREM2 primarily exerted it effects on pro-inflammatory cytokines, supporting the theory that sTREM2 likely plays important roles in initiating the inflammatory response in neighboring cells at an early stage. On the contrary, 1.0µg/mL sTREM2 was relatively inefficient at stimulating expression of inflammatory cytokines in M0, M1, or M2 macrophages but did not cause any obvious cytotoxic effects from such a large dose.

At two hours post treatment 0.1µg/mL sTREM2 strongly induced the expression of pro-inflammatory cytokines in M0 and M2 cells while having minimal effect on M1 macrophage cytokine expression. sTREM2 at 1.0µg/mL did not induce large changes in cytokine expression in any of the macrophage types. Similarly, to previously shown in THP-1 cells treated with 0. 1µg/mL sTREM2 can induce expression of pro-inflammatory cytokines at early time points. Suggesting that sTREM2 may play key roles in initiating the inflammatory response in myeloid-derived cells.

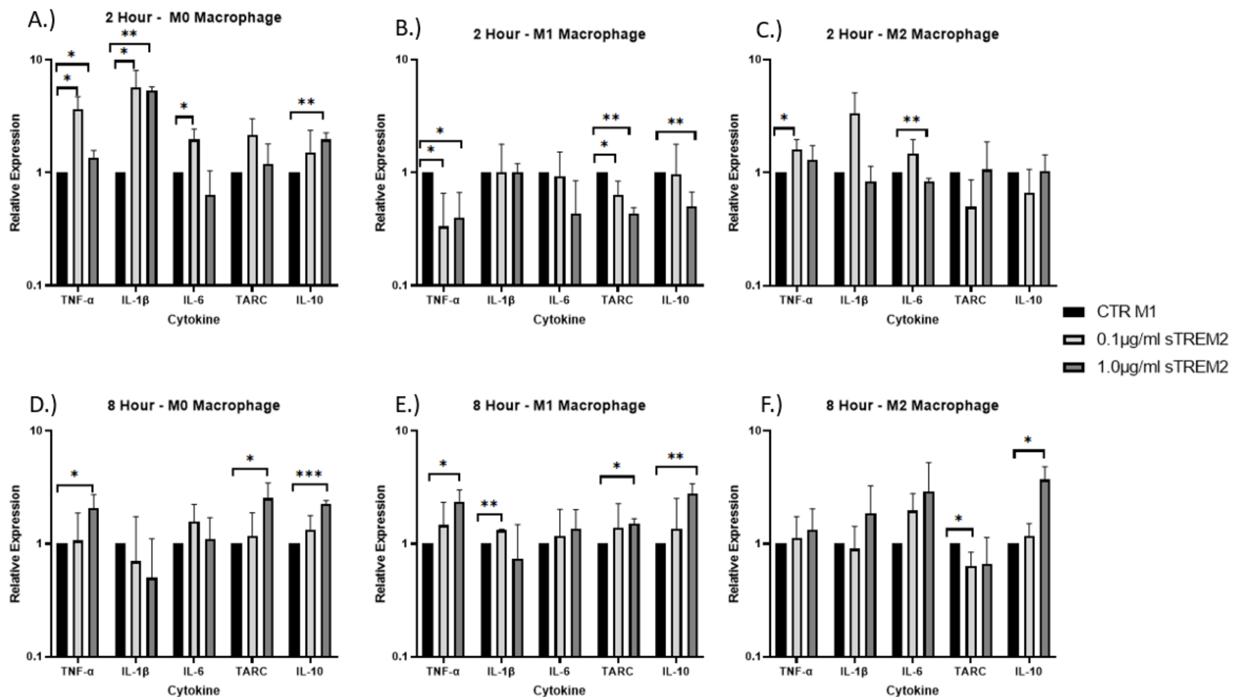


Figure 5. The effects of sTREM2 on cytokine expression in THP-1-derived macrophages: (A) Cytokine expression in M0 macrophages following 2-hour sTREM2 treatment. (B) Cytokine expression in M1 macrophages following 2-hour sTREM2 treatment. (C) Cytokine expression in

M2 macrophages following 2-hour sTREM2 treatment. (D) Cytokine expression in M0 macrophages following 8-hour sTREM2 treatment. (b) Cytokine expression in M0 macrophages following 8-hour sTREM2 treatment. (c) Cytokine expression in M0 macrophages following 8-hour sTREM2 treatment. (*p<0.05; **p<0.01; ***p<0.001) *(Experiment completed by Steven Marshal)*

At 8 hours post treatment the effects of 1.0μg/mL sTREM2 had much more of effect on the expression of inflammatory cytokines in all M0, M1 and M2 macrophages as compared to the effects at 2 hours post treatment. Interestingly the effects of 0.1μg/mL sTREM2 on cytokine expression in M0, M1 and M2 cells was reduced as compared to effects on cytokine expression at 2 hours post treatment. This may indicate that sTREM2s effects are relatively quick acting.

In M0 macrophages following treatment with 0.1μg/mL sTREM2 slightly induced expression of IL-6, TARC, and IL-10 in comparison to control M0 macrophages at 8 hours post treatment. Unlike previously, TNF-α and IL-1β expression remained relatively unchanged at the 8 hour time point in M0 macrophages (Figure 5D) as compared to controls. Interestingly, 1.0μg/mL sTREM2 significantly induced the expression of TNF-α (p=0.05), TARC (p=0.04) and IL-10 (p<0.0001) with mean relative changes of 2-, 1.5-, and 1.5- fold, respectively. It is shown that at 8 hours, the expression of anti-inflammatory cytokines TARC and IL-10 was significantly upregulated with high levels of expression by 1.0μg/mL sTREM2 treatment. Here we see that at higher concentrations and longer exposure to sTREM2, M0 macrophages expressed high levels of anti-inflammatory cytokines as compared to control M0 macrophages.

In M1 cells at the 8-hour time point (Figure 5E), the effects of both 0.1μg/mL and 1.0μg/mL sTREM2 were greater than at the 2-hour time point in M1 macrophages. Treatment of M1 macrophages with 0.1μg/mL sTREM2 was able to stimulate the expression of IL-1β (p<0.001)

but slightly induce the expression of TNF-α, IL-6, TARC, and IL-10 as compared to untreated M1 macrophages. In M1 macrophages at 8 hours post treatment, 1.0μg/mL sTREM2 stimulated the expression of TNF-α ($p=0.02$), IL-6, TARC ($p=0.007$), and IL-10 ($p=0.008$) compared to control M1 macrophages. It is important to note here that M1 macrophages are polarized towards a pro-inflammatory state, and therefore for sTREM2 to strongly induce the expression of IL-10 at 8 hours in this cell type supports the idea that prolonged exposure to sTREM2 may initiate anti-inflammatory response.

Similarly, to M1 macrophages, in M2 macrophages (Figure 5F), 1.0μg/mL sTREM2 also strongly stimulated the expression of IL-10 ($p=0.01$) in comparison to control M2 macrophages, which coincides with what we have previously presented earlier. IL-1β and TARC expression was also increased following treatment although this was not shown to be significant. sTREM2 at 0.1μg/ml showed minimal effects on cytokine expression in M2 macrophages at the 8-hour time point compared to control macrophages.

In the context of our model at early time points, low concentrations of sTREM2 can stimulate the expression of pro-inflammatory cytokines TNF-α and IL-1β consistently and to a lesser extent IL-6 in M0, M1 and M2 macrophages. At the later time point, higher concentrations of sTREM2 were able to stimulate the expression of anti-inflammatory cytokines IL-10 strongly and to a lesser extent TARC. With respect to the fact that sTREM2 has a short half-life, this relationship may be seen in that duration of exposure to sTREM2 may play a more prominent role in cytokine expression rather than the actual concentration having the more driving effect on cytokine expression in macrophages.

To test whether the elevated levels of expression in pro-inflammatory cytokines translated to expression at the protein level in treated M0 (Table 1A), M1 (Table 1B), and M2 (Table 1C) macrophages, a human cytokine multiplex kit was used to detect the levels of IL-1α, IL-1β, IL-6, IL-8, GM-CSF, IFN-γ, MCAF (monocyte chemotactic and activating factor) and TNF-α using the supernatant from the 2 hour treatment of 0.1μg/mL or 1.0μg/mL sTREM2 treatment (Table 1) (for standard curves of the multiplex ELISA, please see supplementary Figure 4). Like previously shown (Figure 5A) M0 macrophages treated with 0.1μg/mL sTREM2 increased the expression of IL-1β and TNF-α as compared to control. Although IL-1β expression was significantly upregulated in Figure 5A, it is not directly translated to the protein level, although increased protein expression was seen. TNF-α protein expression in M0 macrophages treated with 0.1μg/mL sTREM2 showed a quite large 20pg/mL increase in protein level as compared to untreated M0 macrophages. Like qPCR results in Figure 2A, 1.0μg/mL sTREM2 treatment increased IL-1β by about 8pg/mL which is not as large as the RNA expression levels of IL-1β. The only other cytokines that seemed to be increased by 0.1μg/mL sTREM2 treatment in M0 macrophages compared to controls seemed to be INF-γ and MCAF which showed approximately 30pg/mL and 10pg/mL, respectively. Consistent with qPCR results, 1.0μg/mL sTREM2 did not largely increase the protein expression of any cytokines measured by the human multiplex kit.

M1 treated macrophages (Table 1B) showed relatively not increase in cytokine expression following 0.1μg/mL or 1.0μg/mL sTREM2 treatment compared to untreated M1 macrophages. This was consistent with the qPCR results shown previously (Figure 5B).

In M2 macrophages (Table 1C), treatment with 0.1μg/mL sTREM2 did not majorly alter the expression of any cytokines although consistent with previously shown (Figure 5C), but it did

slightly increase the expression of TNF-α by about 7pg/mL compared to untreated control M2 cells. Interestingly an increase in IFN-γ protein expression was also seen in 0.1μg/mL sTREM2 treated M2 macrophages. The large increase in IL-1β expression following 0.1μg/mL sTREM2 was not seen at the protein level; however, the qPCR data for this cytokine showed large variability in expression. Interestingly, 1.0μg/mL sTREM2 treatment increased the expression of several proteins detected by this cytokine kit. The largest expression changes were in IL-8, IFN-γ, and GM-CSF which showed increases of 18pg/mL, 35pg/mL, and 32pg/mL, respectively. Other cytokines were also slightly increased in M2 macrophages following 1.0μg/mL sTREM2 treatment including IL-1α, IL-1β, and MCAF. Together this data helps support that sTREM2 is a key player in activating cells and initiating cytokine expression.

Table 1. Human cytokine Multiplex ELISA results for (A) M0 macrophages, (B) M1 macrophages, and (C) M2 macrophages treated for 2-hours by 0.1μg/mL and 1.0μg/mL sTREM2.

A.)

	IL-1α [pg/ml]	IL-1β [pg/ml]	IL-6 [pg/ml]	IL-8 [pg/ml]	GM-CSF [pg/ml]	IFN-γ [pg/ml]	MCAF [pg/ml]	TNF-α [pg/ml]
M0 CTR	25.58	28.01	9.47	183.83	111.45	28.14	31.08	101.89
M0 0.1μg/ml sTREM2	23.25	35.29	12.99	142.27	78.72	57.9	41.21	119.21
M0 1.0μg/ml sTREM2	29.644	36.09	10.93	165.38	99.53	41.45	31.58	104.44

B.)

	IL-1α [pg/ml]	IL-1β [pg/ml]	IL-6 [pg/ml]	IL-8 [pg/ml]	GM-CSF [pg/ml]	IFN-γ [pg/ml]	MCAF [pg/ml]	TNF-α [pg/ml]
M1 CTR	24.99	28.02	9.76	188.8	121.12	35.97	36.14	113.41
M1 0.1μg/ml sTREM2	24.99	33.67	9.17	135.57	86.16	29.71	31.58	109.87
M1 1.0μg/ml sTREM2	24.99	35.28	9.47	151.9	98.05	43.81	32.09	107.21

C.)

	IL-1α [pg/ml]	IL-1β [pg/ml]	IL-6 [pg/ml]	IL-8 [pg/ml]	GM-CSF [pg/ml]	IFN-γ [pg/ml]	MCAF [pg/ml]	TNF-α [pg/ml]
M2 CTR	24.99	36.9	10.64	110.74	87.64	43.8	125.28	117.84
M2 0.1μg/ml sTREM2	26.16	38.52	9.76	109.32	77.97	53.98	125.29	124.04
M2 1.0μg/ml sTREM2	34.28	41.75	10.94	128.48	119.63	79.83	133.38	120.5

Together the increased expression of pro-inflammatory cytokines in mainly M0 and M2 macrophages both at the transcriptional and protein levels shows that sTREM2 can induce an

inflammatory response in myeloid-derived cells. Interestingly sTREM2 did not show any effects on the expression levels in M1 macrophages which supports that sTREM2 may be important in activating cells and programming them towards a pro-inflammatory state. Although increased expression pro-inflammatory cytokines were readily detectable by qPCR (Figure 5) and ELISA (Table 1), anti-inflammatory cytokines IL-10 and chemokine TARC expressions were also increased, at least at the transcriptional level which suggest that sTREM2 has some anti-inflammatory actions as well. More studies will need to be conducted to investigate sTREM2 ability to induce expressional changes in not only cytokines but other genes as well that are responsible for inflammatory regulation and initiation. ELISA data for the 8-hour time point was not collected due to reagent cost and time constraints to complete this project. Part of the Section iv work was completed by Steven Marshall, Hon. B. Sci., under my supervision.

iii. Polarized macrophages differentially shed sTREM2

While it is widely known that TREM2 is expressed on myeloid cells like macrophages and microglia (Painter et al., 2015) and studies have found that M2 macrophages express larger quantities of TREM2 than either M0 or M1 cells (Turnbull et al., 2006) but whether TREM2 on the cell membrane can be shed into culture media has yet to be definitively confirmed in our THP-1/Macrophage model. To figure out whether differentiating macrophages shed TREM2 into culture media, macrophages were differentiated as previously described in the Methods section and at 24- and 48-hours during polarization, supernatants from polarized M0, M1 and M2 macrophages were collected and stored at -80°C. Later a human TREM2 ELISA kit was used to detect levels of sTREM2 in culture supernatant.

In all samples, sTREM2 was readily detectable under current model conditions in cell culture media of polarized M0, M1 and M2 macrophages. After generating a standard curve (Figure 6) and interpolating values of sTREM2 concentration expressed by polarizing macrophages showed that M2 macrophages shed sTREM2 to the highest degree followed by M0 and M1 macrophages. M2 macrophages shed nearly double the amount of TREM2 at 24 hours compared to M1 macrophages and over triple the amount at 48 hours (Table 2). The levels of sTREM2 increased approximately two-fold between 24 hours and 48 hours in M0 and M2 macrophages but not M1 macrophages. In M0 macrophages at 24 hours, approximately 6ng/mL sTREM2 was shed as compared to around 12ng/mL at 48 hours. On the contrast, sTREM2 level in culture media of M1 macrophages at 24 hours was around 4ng/mL and only increased by about 1.5ng/mL at 48 hours. M2 cells expressed the highest levels like previously mentioned, expressing around double that of M1 at 24 hours with 8ng/mL detectable in culture media and 17ng/mL at 48 hours. It is important to note that levels of TREM2 expression in culture media for M2 macrophages at 48 hours is likely to be slightly over estimated due to the fact that it is outside the range of concentration generated by standard curve. These results clearly indicate that M2 macrophages shed the largest amount of TREM2 in culture media, followed by M0 and then M1 macrophages. It is currently not known whether sTREM2 affects expression of surface TREM2 in M0, M1 or M2 macrophages or whether other factors may affect TREM2 expression and shedding in macrophages and should be a primary focus of future experiments. The above work was conducted by Patrick Salois at the National Research Council of Canada.

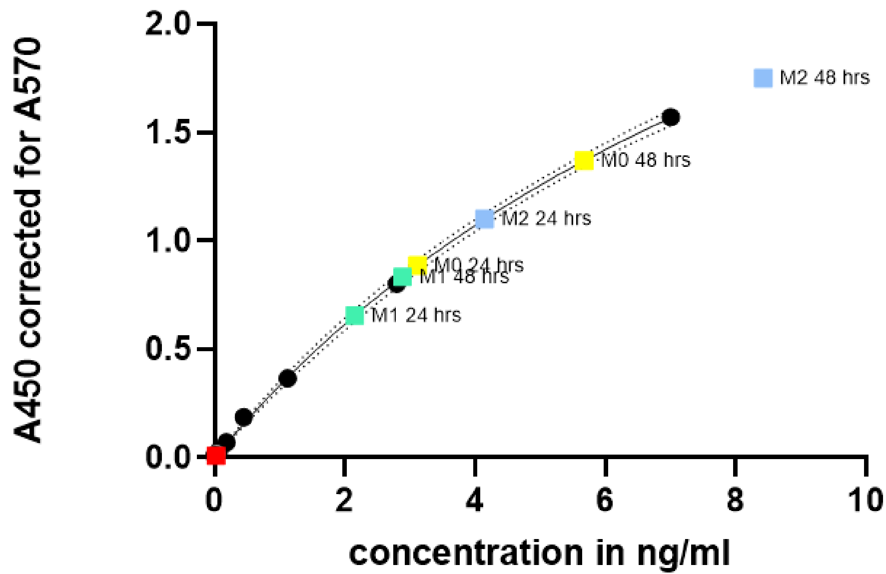


Figure 6: Standard curve generated to detect human TREM2 in polarized macrophage culture supernatant

Table 2: Interpolated values from Figure 6 for sTREM2 concentrations in macrophage culture supernatant.

Sample	Concentration (ng/mL)
M0 (24hrs)	6.23
M0 (48hrs)	11.35
M1 (24hrs)	4.31
M1 (48hrs)	5.77
M2 (24hrs)	8.30
M2 (48hrs)	16.85

Part B: ApoE isoforms differentially affect cytokine and TREM2 expression in THP-1 derived macrophages.

i. ApoE isoforms differentially induced the expression of inflammatory cytokines in polarized macrophages.

ApoE is known as a ligand of TREM2 receptor (Atagi et al., 2015) and both play crucial roles in normal neurophysiology. As ApoE exists in three isoforms, ApoE2, ApoE3 and ApoE4; while ApoE4 is a top risk factor of AD, on the other hand ApoE3 is considered neutral, and ApoE2 is thought to be neuroprotective (Huang et al., 2017). The effect of ApoE-TREM2 interaction on macrophage polarization and cytokine expression is still largely unknown. Here I employed a similar assay as the previous sTREM2 and macrophages treatment. Although instead of treating with sTREM2, I added 3µM of either ApoE2, ApoE3 or ApoE4 to culture media (Dorey et al., 2017) during the 48-hour polarization phase to investigate whether ApoE isoform proteins have any effect on macrophage polarization and cytokine expression profiles. Following polarization M0, M1 and M2 macrophages were harvested, and RT-qPCR was conducted to assess the difference in cytokine profiles attributed to either of the ApoE isoforms. Supernatants were collected after treatment at stored at -80°C for later to assess the expression of the cytokines at the protein level.

In M0 macrophages, treatment with 3µM of either isoform strongly stimulated the expression of pro-inflammatory cytokines (Figure 7). ApoE2 strongly stimulated the expression of TNF-α ($p=0.001$), and IL-1β ($p=0.005$) as compared to control. ApoE4 also strongly stimulated the expression of TNF-α ($p=0.06$) and IL-1β ($p=0.06$) to levels similar to ApoE2 and significantly higher than control. Interestingly, ApoE3 induced the lowest amount of pro-inflammatory

cytokines but still stimulated TNF-α (p=0.04) and IL-1β in M0 macrophages as compared to control. The effects of the ApoE isoform proteins on anti-inflammatory cytokine expression was significantly less significant with only ApoE4 stimulating the expression of IL-10 to levels higher than control M0 macrophages; while ApoE2 and ApoE3 treated macrophages showed no change in IL-10 transcript expression. The levels of TARC were slightly elevated by treatment with ApoE2 and ApoE4 stimulating expression to similar levels at around 2-fold while ApoE3 stimulated the expression of TARC slightly more than untreated M0 macrophages (Figure 7). Together these results indicate that ApoE isoforms differentially induce the expression of inflammatory cytokines in THP-1 differentiated M0 macrophages.

In M1 macrophages the differential effects of the ApoE isoforms are much more prominent than M0 (Figure 7B). This is likely because these cells are transcriptionally active for inflammatory phenotype due to the culture media containing cytokines to polarize the cells rather than M0 cells which contain no additional cytokines to promote polarization. Here I see that ApoE2 strongly stimulated TNF-α and IL-1β (p=0.02) compared to control M1 macrophages, like what was seen regarding its effects on M0 macrophages. Unlike previously however, ApoE2 also stimulated the expression of IL-10 and TARC to levels significantly higher than control M1 macrophages. It is also very important to mention that ApoE2 stimulated levels of expression in both IL-10 and TARC to levels nearly double that of ApoE4 which will be discussed below. ApoE3 was able to moderately induce the expression of TNF-α (p=0.03) and IL-1β (p=0.02), as well as TARC compared to control M1 macrophages but failed to stimulate the expression of IL-10. All levels of cytokine expression induced by ApoE3 treatment were to a lesser extent than either ApoE2 or ApoE4. ApoE4 stimulated the expression of TNF-α (p=0.01), IL-1β (p=0.005), TARC

(p=0.02) as well as IL-10 (p=0.03) compared to control macrophages. Here I see that both ApoE2 and ApoE4 stimulated pro-inflammatory signaling in M1 polarized macrophages similar to each other; however, ApoE2 is able to stimulate the expression of anti-inflammatory cytokines IL-10 and TARC to a significantly greater extent than ApoE4 (Figure 7). This is especially important as this cell type is persuaded towards pro-inflammatory responses, therefore expressing anti-inflammatory cytokines in the context of chronic inflammation would help in resolution.

Cytokine expression in M2 macrophages was much like M1 macrophage cytokine expression following treatment with either of the ApoE isoforms (Figure 7). ApoE2 strongly stimulated IL-1β (p=0.01) and TARC (p=0.02) as compared to control M2 macrophages. TNF-α and IL-10 expression following 3μM ApoE2 treatment was also moderately increased in M2 macrophages compared to control cells, displaying a similar profile to ApoE2-treated M1 macrophages. ApoE3 strongly stimulated the expression of TARC (p=0.002) and moderately stimulated the expression of TNF-α and IL-1β compared to control macrophages, which also displays a similar profile as ApoE3-treated M1 macrophages. ApoE4 strongly stimulated the expression of IL-1β (p=0.04) and TARC (p=0.007), but to levels slightly less than ApoE2-treated M2 macrophages. ApoE4 also moderately stimulated the expression of TNF-α (p=0.02) while IL-10 was not expressed as compared to control macrophages (Figure 7).

These results show that each ApoE isoform may differentially regulate the expression of these cytokines in THP-1 differentiated macrophages of different phenotypes. All isoforms were able to increase the expression of inflammatory cytokines in M0, M1 and M2 macrophages. When looking at the effects of ApoE4 on cytokine expression, it is interesting to consider that both ApoE2 and ApoE4 induced the expression of TNF-α and IL-1β to similar degrees in M0, M1,

and M2 macrophages compared to control macrophages while ApoE3 stimulated the least expression of these cytokines. The difference however is that ApoE2 stimulated the expression of anti-inflammatory cytokines IL-10 and TARC in M0, M1 and M2 macrophages to levels significantly higher than controls and higher than ApoE4- or ApoE3-treated macrophages. Although ApoE was previously shown to bind TREM2 (Atagi et al., 2015), it cannot be said for certain that the expression of these cytokines is solely due to an ApoE-TREM2 interaction. ApoE can also bind other receptors on cell membrane, such as LDLR, LRP-1, VLDLR and apoE receptor 2 (Zhao et al. 2018) and trigger cellular responses. While it can be speculated this might be occurring to some degree, this would need to be confirm for certain that ApoE-induced inflammation in macrophages occurs due to TREM2 dependent mechanisms. TREM2-knockout THP-1 cell line will help to answer the question. The induction of the inflammatory cytokines TNF-α and IL-1β by either of the isoforms across all macrophages may be indicative of ApoE's inflammatory nature, meaning that ApoE itself may stimulate the expression of inflammatory cytokines in this cell model at least at the transcriptional level.

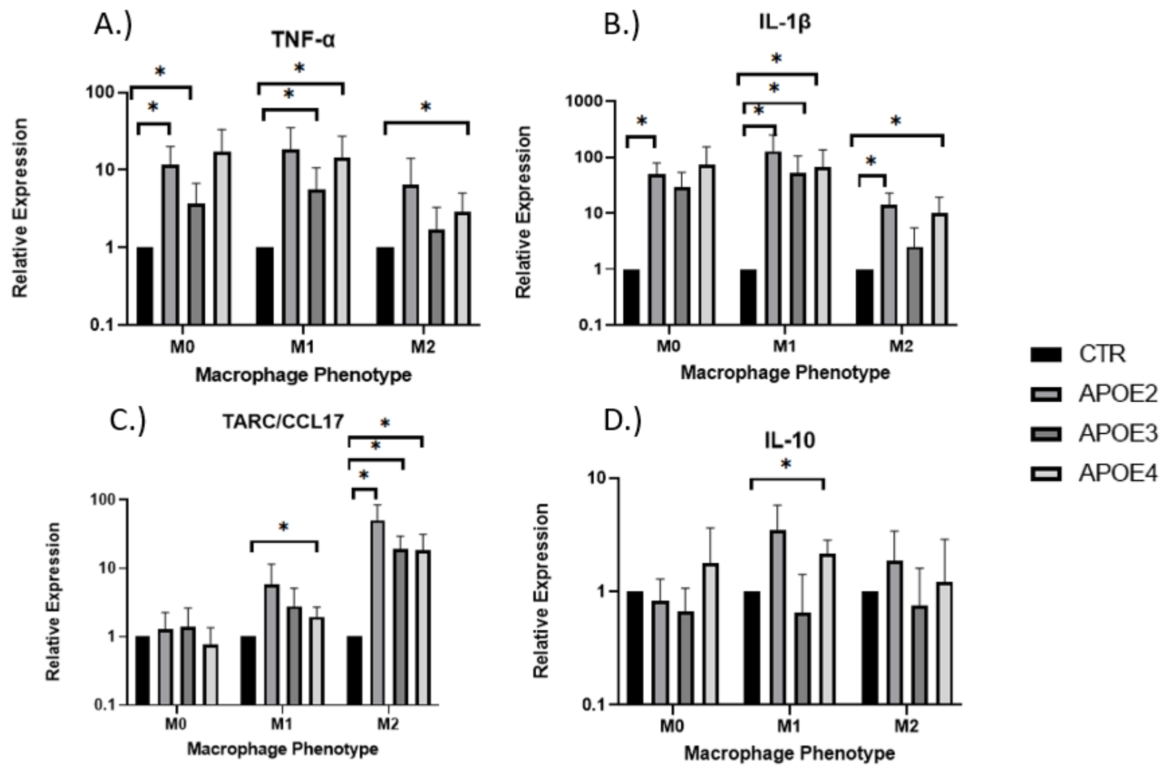


Figure 7. The effects of different ApoE isoforms on cytokine expression in M0, M1 and M2 macrophages: Cells were polarized in the presence of 3µM APOE isoform during the polarization phase and cytokine expression of (A) TNF-α, (B) IL-1β, (C) TARC, and (D) IL-10 was analyzed by RT-qPCR. Data presented as Mean + SD. Individual treatments were assessed using students t-test where *<0.05; **<0.01; ***<0.001. Complete data set was analyzed using one-way ANOVA which showed (A) p=0.07, (B) p=0.18, (C) p=0.36 and (D) *p=0.23.

To identify whether this increased expression of TNF-α (Table 3) and IL-1β (Table 4) induced by different ApoE isoforms translated to increased protein in macrophages, a human cytokine ELISA for each was conducted. Consistent with what was shown previously, all ApoE isoforms were able to stimulate the expression of TNF-α at the protein level in M0 (Table 3A), M1 (Table 3B), and M2 macrophages (Table 3C). Interestingly in M0 macrophages, unlike previous where ApoE2 and ApoE4 stimulated expression to similar levels, it is seen that the

isoforms stimulated the expression of TNF-α in a pattern of ApoE2 < ApoE3 < ApoE4. It is also seen that ApoE2 stimulated the expression of TNF-α to a lower degree than control in M0 macrophages. Similarly, to M0 macrophages, M1 and M2 macrophages followed a similar trend in ApoE-induced inflammatory cytokine expression where ApoE2 (p=0.02) < ApoE3 (p=0.06) < ApoE4 (p=0.06) stimulated the expression of TNF-α compared to control. Not only was the expression increased but ApoE4 nearly doubled the level of expression of TNF-α in all macrophages compared to controls or ApoE2 (Table 2). While control macrophages had a somewhat high level of TNF-α expression, ApoE3 treated macrophages showed levels similar to control except for in M1 macrophages.

For IL-1β, the expression at the transcriptional level was almost replicated at the protein level in ApoE-treated M0, M1, and M2 macrophages (Table 3). ApoE2 (p=0.005) and ApoE4 (p=0.003) strongly stimulated the expression of IL-1β compared to control M0 macrophages (Table 3A); while ApoE3 (p=0.02) stimulated the expression to a lesser degree than both. Similar in M1 macrophage (Table 3B), ApoE2 (p=0.0006) and ApoE4 (p=0.02) largely stimulated the expression of IL-1β while ApoE3 (p<0.0001) stimulated the expression the least. M2 macrophages followed this trend as ApoE2- and ApoE4- (p=0.02) treated M2 macrophages strongly expressed IL-1β, and ApoE3 (p=0.06) also induced the expression. For IL-1β expression induced by ApoE treatment in qPCR results (Figure 7), ApoE2 and ApoE4 stimulated transcript level expression to similar degrees but at the protein level, while both strongly increased its expression compared to ApoE3, but ApoE4-treated macrophages expressed nearly 50% more IL-1β than ApoE2-treated macrophages. Together these results confirm that ApoE isoforms differentially induced the expression of inflammatory cytokines in our model. While the exact

mechanism remains to be determined, it will be important for future efforts to identify exactly how this occurs whether through TREM2 signaling-dependent mechanisms or other. To try to help characterize how ApoE isoforms may affect TREM2 in the context of inflammation and our model, the level of TREM2 following M0, M1 or M2 macrophage treatment with either ApoE2, ApoE3 or ApoE4 was assessed using western blot.

A.)

M0 Macrophage	Concentration (pg/mL)
CTR Untreated	1835.12
ApoE2 Treated	1222.77
ApoE3 Treated	1946.44
ApoE4 Treated	3282.47

B.)

M1 Macrophage	Concentration (pg/mL)
CTR Untreated	610.28
ApoE2 Treated	1501.1
ApoE3 Treated	1612.45
ApoE4 Treated	2892.79

C.)

M2 Macrophage	Concentration (pg/mL)
CTR Untreated	3115.468
ApoE2 Treated	4228.82
ApoE3 Treated	6349.85
ApoE4 Treated	7290.53

Table 3. The effect of ApoE isoforms on TNF-α production in macrophages: (A) Concentration of TNF-α protein expressed by M0 macrophages after ApoE treatment. (B) Concentration of TNF-α protein expressed by M1 macrophages after ApoE treatment. (C) Concentration of TNF-α protein expressed by M2 macrophages after ApoE treatment. (For standard curve of TNF-α ELISA, please see supplementary Figure 2).

A.)

M0 Macrophage	Concentration (pg/mL)
CTR Untreated	2.1
ApoE2 Treated	45.32
ApoE3 Treated	35.61
ApoE4 Treated	56.59

B.)

M1 Macrophage	Concentration (pg/mL)
CTR Untreated	2.5
ApoE2 Treated	42.14
ApoE3 Treated	29.80
ApoE4 Treated	57.79

C.)

M2 Macrophage	Concentration (pg/mL)
CTR Untreated	1.5
ApoE2 Treated	26.16
ApoE3 Treated	16.32
ApoE4 Treated	39.6

Table 4. The effect of ApoE isoforms on IL-1β production in macrophages (A) Concentration of IL-1β protein expressed by M0 macrophages after ApoE treatment. (B) Concentration of IL-1β protein expressed by M1 macrophages after ApoE treatment. (C) Concentration of IL-1β protein expressed by M2 macrophages after ApoE treatment. (For standard curve of IL-1β ELISA, please see supplementary Figure 2).

ii. ApoE isoforms differentially regulated TREM2 expression in polarized macrophages.

It is unknown whether ApoE isoforms would differentially regulate TREM2 expression in M0, M1 or M2 macrophages. To test this, I repeated the previous experiment but whole cell extracts (WCE) were retrieved after treatment, quantified, and ran on a 9% SDS-PAGE gel. An anti-human TREM2 rabbit monoclonal antibody (D814C) was purchased from Cell Signaling Technology and used for western blot to assess levels of TREM2 expressed in M0, M1 and M2 macrophages treated by either ApoE2, ApoE3 or ApoE4 and compared to control untreated macrophages.

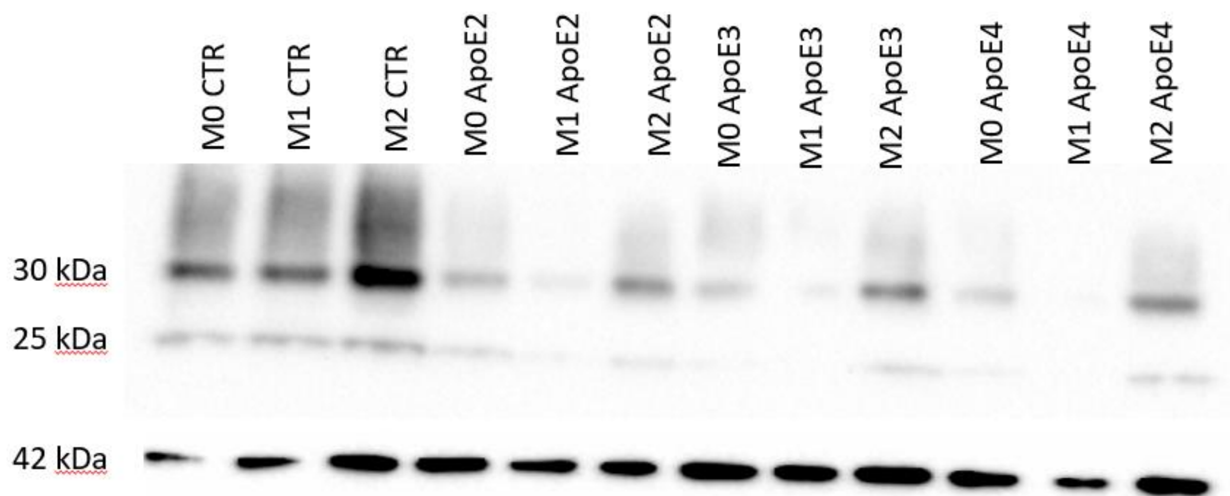


Figure 8: Secondary western blot for TREM2 expression in M0, M1, and M2 macrophages following ApoE treatment compared to β-actin as reference

Control macrophages express TREM2 consistent with their expression of sTREM2 in culture media. M2 macrophages clearly express the highest amount of TREM2 followed by M0 and then M1 macrophages. Compared to control cells, ApoE isoforms seeming decreased expression of TREM2 in M0, M1 and M2 macrophages. Nevertheless, the trend of ApoE inducing expression of TREM2 in M1<M0<M2 is replicated. Again, it also seems that the ApoE4<ApoE3<ApoE2 trend in affecting TREM2 expression is also followed. These results reinforce that ApoE isoforms differentially affected the expression of TREM2 as compared to control macrophages. Consistent with literature where M2 cells express the highest amount of TREM2 compared to M0 or M1 macrophages which is clearly seen here

sTREM2 has been shown to have a molecular weight ranging from 24kDa to 40kDa depending on the degree of glycosylation (Piccio et al., 2008). Below the readily detectable TREM2 protein a faint band at around 25kDa (Figure 9) exists which is likely unglycosylated sTREM2 or incompletely processed TREM2 that has not yet reached the membrane as it is whole cell extract. However, this does not exclude the possibility that sTREM2 would be detectable in the cytosol of macrophages. This should be confirmed in future studies and would give insight towards the how sTREM2 is able to induce expression of cytokines in macrophages. This would also likely provide important information on other signaling pathways that sTREM2 is involved in.

Together these results indicate that ApoE isoforms differentially affected the expression of cytokines in macrophages of different phenotypes as compared to control macrophages and differentially regulated TREM2 expression in these cells. Like mentioned previously, although ApoE isoforms can induce differential expression of TREM2, it was not

confirmed whether this was happening due to a TREM2-dependent mechanism which should be primary focus in future experiments. It will also be important to identify how ApoE isoforms differentially induce the expression of cytokines and TREM2 in macrophages of different phenotypes.

4.0 Discussion

4.1. sTREM2 induced cytokine expression in THP-1 monocytes

In recent years, the roles of sTREM2 in normal microglia function (Vilalta et al., 2021) and in AD are becoming increasingly apparent. Importantly sTREM2 has been shown to be increased in CSF, with variable levels dependent on the variants, just before the clinical onset of symptoms in AD (Zhong et al., 2017; Suarez-Calvet et al., 2019). Microglia is considered as a resident macrophage in CNS. Studies show that microglia is derived from macrophage during CNS development and macrophage can also migrate into CNS as well (London et al. 2013). The goal of this study was to determine whether sTREM2 could induce an inflammatory response in myeloid-derived cell model. THP-1 cell line is a human monocytic leukemia line of myeloid origin and used as a model in this study. The cells were treated with different concentrations of sTREM2, and the cytokine expression and relevant signaling pathways involved were investigated.

It was shown that sTREM2 can induce the expression of several pro-inflammatory and anti-inflammatory cytokines in our THP-1 model. As THP-1 cells are monocytes, this may suggest that sTREM2 can induce large scale transcriptional changes in these cells. This could indicate that sTREM2 plays crucial roles in innate immune and inflammatory response. The positive control used in this study, 0.1µg/mL LPS, was used to show strong induction of inflammatory cytokines

in THP-1 cells as compared to sTREM2-treated THP-1 cells. This suggests that sTREM2 has a strong regulatory function, likely involved in process of innate immune and inflammatory response. This is also supported by the fact that sTREM2 is readily detectable in both CSF and plasma. sTREM2 detectable in plasma of AD patients, non-AD individuals and other NDDs has been reported to range anywhere from 10-50ng/mL (Ferri et al., 2021) and is significantly lower in CSF at around 10ng/mL with cognitively impaired individuals typically displaying slightly higher levels of CSF compared to unimpaired individuals (Knapskog et al., 2020).

In this model, the concentrations of sTREM2 used were relatively higher than under normal conditions but still encapsulated that sTERM2 can induce inflammatory cytokine expression in myeloid-derived cells, reinforcing that it probably plays important roles in initiating inflammation and activating cells. Interestingly given that 1.0μg/mL sTREM2 was used to treat cells and is over 20 times more concentrated than what is found in plasma and over a hundred more than in CSF but did not cause observably obvious cytotoxic effects such as apoptosis or significantly aberrant inflammatory cytokine expression. This supports that increased sTREM2 may not be recognized as pathogenic stimuli in this THP-1 model but rather may have important regulating functions which remain to be fully characterized further as well as in vivo. Also, in support of this was sTREM2's ability to induce the expression of pro-inflammatory cytokines in THP-1 cells at earlier time points but also significantly stimulating the expression of anti-inflammatory chemokine TARC and cytokine IL-10 at later time points.

Could large doses of sTREM2 lead to inconsistent modulation? It has been shown that sTREM2 has a relatively short half-life of about 4 hours in mouse brains (Henjum et al., 2016) which supports 0.1μg/mL sTREM2 being capable of inducing expression of cytokines more so

than 1.0μg/mL. Although culture conditions and in vivo conditions differ largely due to lack of proteolytic secretases required for sTREM2, therefore its half-life is probably significantly longer in culture conditions, but nonetheless should be a consideration for future experiments as these enzymes may be released in response to large doses of sTREM2. Another thing to consider would be that at the 8-hour time point would sTREM2 that was added still be influencing cells in culture or were we observing lasting effects say from the 2-hour time point? This could be tested in the future by assessing sTREM2 in culture media following treatment, but it may be hard to distinguish between sTREM2 that was added and sTREM2 that could have been shed from the cell membrane in culture.

While the addition of sTREM2 was able to induce the expression of key inflammatory cytokines, the underlying mechanisms that cause specific cytokine expression remain to be defined. Such that it is not currently known how sTREM2 is physically modulating the innate immune response. Could sTREM2 be binding surface TREM2 or other receptors to elicit such cellular response or could it be bound and internalized by scavenger receptors? It is also important to consider whether sTREM2-mediated response is dependent on the receptor(s) bound which is likely the case. Could sTREM2 bind to a component in the media or released by these cells as THP-1 cells are cancerous so there may be some endogenous factors released into the culture media that may have alternative effects on the outcome of cytokine expression induced by sTREM2. Could sTREM2 binding membrane receptors to induce these expressional changes or is it in complex with other factors being recognized by cell receptors in solution to induce cellular response? Future efforts should be directed towards identifying targets of sTREM2.

4.2. The effects of TREM2 antibody on sTREM2-induced cytokine expression

Recently, a TREM2 antibody known as AL002c, which is an agonist antibody, has been shown to activate microglia in 5XFAD mice and that administration of AL002c showed induction of microglial proliferation in wildtype and R47H TREM2 mice (Wang et al., 2020) and that it also reduced neurotoxicity and inflammatory signaling. This shows that targeting TREM2 may be beneficial to modulating AD progression. With this in mind, the goal of this experiment was to see whether a commercially available mAb would be able to neutralize the ability of exogenous sTREM2 to induce expression of inflammatory cytokines in our myeloid model.

Pre-incubation of sTREM2 with a rat anti-human TREM2 IgG_{2B} mAb was able to partially reduce sTREM2-induced expression of inflammatory cytokines in THP-1 cells but significantly increased the expression of others. This antibody can bind to TREM2 but there is no evidence whether this antibody can activate TREM2 signaling in cells. Reduction in sTREM-induced expression of TNF-α and IL-10 mediated by the TREM2 antibody may suggest reduced inflammatory response as a result of neutralizing sTREM2-induced expression of the cytokines but an increased expression in IL-1β and TARC suggests that other mechanisms may be at play here. The complex of sTREM2/antibody may bind to certain receptors on cell membrane activating relevant signaling. This instance may be enough to highlight the complexity involved in sTREM2 signaling and inflammation. TREM2 and sTREM2 possess a V-set domain which allows binding to a large variety of substrates; therefore, it is likely that sTREM2 signaling is strongly dependent on the substrates or receptors bound. Moreover, the effects of the antibody on sTREM2 are likely dependent on the epitope it binds. Importantly it should be noted that a larger decrease in cytokine expression may have been seen if the antibody and sTREM2 were incubated

together at equal molar concentrations to account for binding ability and atomic mass. Interestingly, it was seen that the antibody alone was able to induce expression of inflammatory cytokines which was likely due to its engagement of TREM2 receptor on cell membrane, supporting its roles in activation of cellular signaling. Throughout the duration of the treatment with mAb, IL-10 showed its highest level of expression at the initial time point and showed progressive decline throughout, suggesting that TREM2 is important in regulating and maintaining a proper response. A proposed mechanism for this may be that once TREM2 recognizes a ligand, dependent on the nature of the ligand, it may be responsible for inhibiting an over exaggerated response or to ensure that the response remains "in check".

Contrary to reduced expression of TNF-α and IL-10, the mAb strongly stimulated expression of IL-1β and TARC when bound to sTREM2. The mechanism for which this occurs is unknown but could possibly be explained by sTREM2+mAB being recognized as an immune complex and stimulating the activation of the NLRP3 inflammasome to release IL-1β. sTREM2's role in NLRP3 activation has yet to be described but should be investigated in the future. I wanted to test whether the NLRP3 inflammasome was involved in the increased expression of IL-1β being observed in the sTREM2+mAb treated groups (Supplementary Figure 5). I used a known NLRP3 inhibitor MCC950 to target NLRP3 activation. The experiment was only completed once but showed that the inhibitor was able to partially alleviate IL-1β expression induced by 0.1μg/mL sTREM+mAb. This suggests that the NLRP3 inflammasome may be involved in sTREM2 induced inflammation.

This work highlights the functional complexity of sTREM2 in the context of inflammatory regulation and shows that sTREM2 can regulate inflammatory cytokine expression in myeloid-

derived cells. Moreover, targeting TREM2 and sTREM2 with a mAb showed increased expression of several cytokines which suggests that targeting TREM2 may modulate the inflammatory response. Phase 2 clinical trials for AD treatment using a TREM2 antibody AL002 are currently underway but the immune modulating function of TREM2 activation need to continue to be defined in order to fully take advantage of this receptor for the development molecular therapies.

4.3. The signaling pathways involved in sTREM2-induced cytokine expression.

We've shown that sTREM2 can induce expression of cytokines in myeloid-derived cells and that targeting sTREM2 could prove to be beneficial in terms of reducing inflammation. However, the cellular pathways sTREM2 utilizes to induce this expression has not been identified. The intention of this study was to define the cellular signaling pathways that sTREM2 may use to induce the expression of cytokines in our model. To test this, inhibitors for several well-defined signaling pathways were used to determine a particular pathway sTREM2 may use in myeloid cells.

Membrane bound TREM2 has been shown to signal through second messenger Syk enzyme to mediate downstream signaling which is recruited and phosphorylated by ITAMS with TREM2's adaptor protein, DAP12 (Peng et al., 2010). Moreover, TREM2 has been shown to antagonize toll like receptor (TLR) mediated inflammatory response through modulation of JNK and NFκB (Takashi et al., 2005; Hamerman et al., 2006). Since several studies have shown that TREM2 signals through several key inflammatory networks as previously discussed, we aimed to

identify whether sTREM2 induces the expression of the cytokines in THP-1 cells using either Syk, MAPK-JNK, or NFκB. MG-132 was used to inhibit proteasome-mediated IkB degradation and therefore NFκB activation. SP600125 was used to inhibit JNK which is downstream in the MAPK signaling pathway. Piceatannol was chosen as an Syk inhibitor which is the major signaling enzyme for TREM2 receptor activation.

Syk inhibitor had no effect on cytokine expression induced by sTREM2 treatment. This is important as it shows that sTREM2 has distinct signaling mechanisms differing from TREM2. This however should continue to be explored because like stated previously, sTREM2 probably has diverse effects and is dependent on the substrates or receptors bound and therefore may function through in a TREM2 independent manner. There is also the possibility that this inhibitor was just inefficient as it is relatively less documented as compared to the other inhibitors tested.

SP600125, a well-documented JNK inhibitor, has previously been shown to inhibit JNK-AP1 singalling pathway in brain endothelial cells in response to Aβ peptide (Vukic et al., 2009). Here we showed that SP6000125 inhibited the expression of the cytokines induced by sTREM2 in THP-1 cells to levels similar to controls, with the exception of IL-1β. Incomplete inhibition of IL-1β expression by the inhibitor suggests that another pathway may be active and also partially responsible for the expression of IL-1β. This indicates that in myeloid cells, sTREM2 may primarily use the JNK-MAPK pathway to induce cytokine expression. It will be very important for future experiments to determine whether this translates to microglia in the AD brain although JNK inhibitor was shown to inhibit Aβ-induced inflammatory response in vitro (Vukic V et al. 2009). It would likely be expected that MAPK-JNK signaling would be significantly activated in AD patients at early stage since sTREM2 levels were shown to rise at this time (Suarez-Calvet et al., 2019).

Previously the JNK-AP1 pathway has been linked to the expression of inflammatory genes in human AD brain and in human brain endothelial cells treated with Aβ (Vukic et al., 2009). Moreover, there is supporting evidence suggesting that JNK cascade is activated in AD neurons in response Aβ (Morishima et al., 2001). It was also shown that JNK activation is associated with intracellular Aβ accumulation (Shoji et al., 2000). Together these results suggest that the MAPK-JNK signaling pathway may be one of the main culprits behind chronic neuroinflammation in AD, and sTREM2 may signal through this pathway to activate the expression of inflammatory cytokines.

The transcription factor NFκB has been shown to play important mediating roles in the innate immune response and as a crucial regulator of IL-1β expression (Liu et al., 2017; Hwang et al., 2019). IL-1β has also been shown to increase neuronal susceptibility to degeneration in NDD (Koprich et al., 2008). Given that IL-1β was significantly upregulated in THP-1 monocytes following sTREM2 treatment and its implications in NDD, we expected some degree of NFκB signaling would be active under these treatment conditions. MG-132 is a well-known molecular inhibitor of NFκB and was used in this experiment. Similarly, to SP600125, MG-132 was able reduce the expression of some cytokines but did not decrease expression of IL-10. Consistent with previously mentioned, IL-1β expression was not completely reduced but was partially reduced suggesting multiple pathways may be activated by sTREM2. The extent whether NFkB and JNK-MAPK pathways are involved in sTREM2-induced inflammation in vivo is not defined and should be a primary focus in future work. Moreover, it will be important to identify the degree to which these pathways may interact to regulate inflammatory response should be explored.

Together these results suggest that sTREM2 mediates inflammation likely through multiple networks including mainly through JNK-MAPK pathway as well as partially through NFκB in this cell model. These results should be confirmed in a primary human microglial or iPSC-derived microglial model to see if this relationship exists. The above experiments show that sTREM2 can induce cytokine expression in THP-1 cells. To test whether sTREM2 induces a similar pattern of cytokine expression in neuroinflammation, human microglial model with an appropriate microenvironment should be used. This alternatively could be verified in a mouse AD model. Future work should also look at TREM2 variant dependent abilities to induce inflammation. For instance, comparing R47H sTREM2 to wild-type sTREM2 in mediating inflammation in microglia.

4.4. The effects of sTREM2 on cytokine expression in polarized macrophages

Consistent with what was shown in sTREM2-treated THP-1 cells, macrophages differentiated from THP-1 cells also showed an increase in expression of several inflammatory cytokines. THP-1 differentiated macrophages were then polarized to display a neutral or homeostatic phenotype (M0), a pro-inflammatory phenotype (M1) or an anti-inflammatory phenotype (M2). These macrophages were then treated with sTREM2, and cytokine expression was assessed.

At early time points, the lower concentration of sTREM2 was able to stimulate the expression of pro-inflammatory cytokines in M0 and M2 macrophages but not M1 macrophages. TREM2 has been showed to play roles in macrophage activation in response to cerebral lesions and pathological stimuli (Cignarella et al., 2020). While sTREM2's effects on macrophage activation is largely uncharacterized, here my results show that sTREM2 may promote a pro-

inflammatory response in cell types that are not “pro-inflammatory” at early stages of treatment. This suggests that sTREM2 likely plays important roles in activating cells and initiating inflammation. Again, this would likely be highly dependent on the substrate and the mechanism of activation may be variable because of it. Interestingly at early time points, anti-inflammatory cytokines were not readily detectable further supporting the idea that sTREM2 activates macrophages. Literature has shown that TREM2 receptor activation attenuated neuro-inflammation in mouse AD model (Chen et al., 2020) and therefore it would be expected that TREM2 can induce the expression of anti-inflammatory cytokines or modulate microglial phenotype to help resolve primary inflammation. Here it is shown that sTREM2 induced an increased expression in TARC and IL-10 in M0, M1, and M2 cells relative to control macrophages at the later time points. This suggests that while sTREM2 is capable of inducing the expression of inflammatory cytokines and initiates the inflammatory cascade at early stage, sTREM2 may also be able to upregulate the expression of anti-inflammatory cytokines at a later stage for resolution of inflammatory response or tissue repair. My study also suggests that sTREM2 may be an important surveillance factor/chaperone that is required for the activation and phenotype modifications in macrophages and possibly microglia.

The model used in this study may be more indicative of sTREM2 ability to regulate inflammation in the periphery compared to the CNS, and therefore should be validated in microglia. Also, this project only verified pro-inflammatory cytokines at the protein level, further analysis of anti-inflammatory protein expression is highly recommended. As TREM2 is also involved in lipid metabolism (Li et al, 2022), it would be interesting to investigate whether microglial sTREM2 enters peripheral circulation through the blood-brain barrier (BBB) to

modulate functions or vice versa, if macrophages sTREM2 passes through the BBB to exert effects in the CNS. This should be considered in the future.

While we showed that sTREM2 can induce inflammatory response in myeloid-derived cells such as THP-1 monocytes and macrophages, it would be beneficial to also characterize possible effects of sTREM2 on other cell types in the context of AD, such as neurons, astrocytes and brain vascular cells. The reasons for which TREM2 are shed releasing sTREM2 are still largely unknown; however, it is speculated that sTREM2 may be continuously shed or that specific stimuli may induce TREM2 cleavage dependent on the degree of expression or other unknown factors.

4.5 Shedding of sTREM2 by M0, M1 and M2 macrophages

It is widely accepted that M2 macrophages express the highest levels of TREM2 followed by M0 macrophages and lastly M1 macrophages expressing the least amount of TREM2. It is currently unknown whether these phenotypes actively shed sTREM2 into their surrounds. The goal of this study was also to identify which polarized macrophage phenotypes shed the highest levels of TREM2, which would also suggest the highest level of expression.

Here we showed that M2 macrophages shed the highest levels of sTREM2 into their surrounding microenvironment, followed by M0 macrophages and lastly by M1 macrophages. M0 and M2 macrophages showed an approximate doubling in sTREM2 release from 24- 48 hours compared to M1 macrophages. While this confirms that the non-proinflammatory phenotypes express and shed the highest levels of sTREM2, the mechanism for this is poorly understood. Like previously mentioned, sTREM2 may be an important surveillance molecule in identifying and

initiating immune response. To support this, if M0 and M2 macrophages are acting in a homeostatic or restorative manner respectively, the continuous active shedding of sTREM2 into the surrounding environment, to act as a scavenger receptor and a type of “alarm” which can recognize, initiate, and terminate an appropriate immune response in neighboring macrophages to a particular stimulus. This would be a relatively good adaptive mechanism for cells to actively survey an environment while carrying out actions that are transcriptionally pro-inflammatory. Although sTREM2 was being shed in the culture media to different degrees during our treatment, the percentage compared to our treatment was minimal, therefore we can likely rule out that it had any major effect on my experimental results.

4.6. The effects of ApoE isoforms on cytokine expression in macrophages

ApoE4 has been a long standing top genetic risk factor for AD (Kamboh et al., 1995) and its role in innate immunity and in neuroinflammation is continuously being defined. As mentioned previously, ApoE4 is known to contribute to Alzheimer’s neurodegeneration while it has been shown that ApoE2 is neuroprotective (Coonjero-Goldberg et al., 2005; Li et al., 2020) against developing AD, and ApoE3 is considered to be neutral. To further characterize the roles of the ApoE isoforms in inflammation, macrophages were differentiated from THP-1 cells as previously described, polarized and treated with ApoE isoforms.

Surprisingly my results show that ApoE isoforms strongly stimulated the expression of pro-inflammatory cytokines in all macrophage phenotypes. However, the expression of TNF-α and IL-1β differed between isoforms, with ApoE4 and ApoE2 stimulating the highest expression

and ApoE3 still stimulating the expression of these cytokines relative to control but significantly less than either of the other isoforms. Given that ApoE2 is neuroprotective, it would be expected that following ApoE2 treatment, macrophage inflammatory cytokine production would be somewhat attenuated but that is not the case here.

Although all isoforms strongly stimulated the expression of pro-inflammatory cytokines in M0, M1 and M2 cells, especially ApoE2 and ApoE4 inducing around the same expression levels, it is very important to note that anti-inflammatory cytokine production was attenuated in ApoE4 treated macrophages. While treatment with ApoE isoforms induced the expression of pro-inflammatory cytokines, the isoforms were also seen to induce expression of anti-inflammatory TARC and IL-10. Importantly, ApoE2 strongly stimulated the expression of these cytokines in M2 and M1 macrophages compared to ApoE4 treated macrophages. It would be expected that ApoE isoforms might stimulate the expression of anti-inflammatory cytokines in an ApoE2 > ApoE3 > ApoE4 fashion. When analyzing the protein expression of pro-inflammatory cytokines in ApoE treated macrophages, we do in fact see that expression of these cytokines follows an ApoE2 > ApoE3 > ApoE4 fashion, suggesting that another mechanism may be induced by ApoE2 to promote the resolution of inflammatory response.

Even though ApoE2 induces the expression of pro-inflammatory cytokines to a degree similar to that of ApoE4, the protective abilities of ApoE2 lie within its robust ability to stimulate the expression of anti-inflammatory cytokines as well; whereas ApoE4 does not induce large expression of anti-inflammatory cytokines. This could lead to a completely robust innate immune response in ApoE2-treated macrophages and an aberrant chronic pro-inflammatory response without anti-inflammatory resolution in ApoE4-treated macrophages. Throughout the duration

of life, in ApoE4 carrier individuals this would lead to significant contribution to neuroinflammation and neurodegeneration and progression of diseased state and would like also to lead to increased complications from other factors due to persistent neuroinflammation. Moreover, a proposed mechanism leading to microglial dysregulation in the context of AD could be that aberrant ApoE4-mediated inflammation in early stages of life, which would be invisible without histopathological or post-mortem assessment, could lead to “primed” microglia as discussed in the introduction. This would contribute to microglial dysregulation and be a significant driving factor in disease progression.

It is important to consider that while these cells were treated with different ApoE isoforms, we did not genotype THP-1 cells and therefore do not know whether the cells are, ApoE2, ApoE3, or ApoE4. To circumvent this in the future it may be beneficial to knock out or silence the ApoE gene in microglia, then to transfect them with either of the isoforms. It would probably be best to assess the differential effects of all ApoE isoforms on ApoE2-expressing cells, ApoE3-expressing cells, and ApoE4-expressing cells. This would help to create more realistic models and could help provide insight towards molecularly targeting ApoE.

While the mechanism for which ApoE isoforms induce differential cytokine expression is not fully characterized, ApoE has been shown to bind TREM2 receptor on cells (Atagi et al., 2015). While it is tempting to speculate that ApoE-induced inflammation occurs under these conditions in a TREM2-dependent fashion, it is not known for certain. In the future, precipitation binding assays could be done to determine whether ApoE isoforms induce cytokine expression via TREM2. It would be interesting to see whether sTREM2 cleaved from cell membrane of polarizing macrophages would bind to ApoE isoforms and whether the structural differences between

ApoE4, ApoE3, and ApoE2 results in affected binding of sTREM2. This should be explored in future studies.

4.7. The effects of ApoE isoforms on TREM2 expression in macrophages

Like previously mentioned, it is unknown whether ApoE-induced inflammation occurs due to TREM2-dependent mechanisms which should be identified in the future. This experiment sought to identify whether ApoE isoforms were able to affect the expression of TREM2 in macrophages of different phenotypes. ApoE isoform-treated M0, M1 and M2 macrophages were used in a western blot analysis to determine the level of TREM2 expression.

The initial blots showed almost no TREM2 expression in control macrophages but ApoE isoforms significantly increased expression in M2 macrophages and slightly increased expression in M0 macrophages but not in M1. ApoE4-treated M2 macrophages appeared to express the highest levels of TREM2 compared to control followed by ApoE3- or ApoE2-treated macrophages. In the following blots however, control macrophages displayed a higher level of TREM2 expression which followed a M1<M0<M2 expression pattern consistent with literature. Similar to the previous blot though, it is evident that ApoE isoforms differentially induce the expression of TREM2. Interestingly these blots show that ApoE2 may enhance the expression of TREM2 in macrophages in comparison to ApoE4. Enhanced TREM2 expression is associated with M2 phenotype, and ApoE2 may promote M2 phenotype by enhancing TREM2 expression in cells, which remains further investigated in future.

Yao et al., 2019 has shown that ApoE isoforms differentially stimulate TREM2 receptor evident by the phosphorylation state of Syk. It was shown that ApoE4 stimulates an increase in

levels of total and phosphorylated Syk compared to ApoE2 (Yao et al., 2019). This along with the differential expression induced by ApoE isoforms may indicate that TREM2 expression due to ApoE isoforms may be either TREM2-dependent or-independent and should be explored in future studies.

Regardless of the somewhat inconsistent results, this data shows that ApoE isoforms differentially induced the expression of TREM2. Although inconclusive, the ability for ApoE2 to stimulate expression of TREM2 but does not overly stimulate the receptor shows ApoE2's neuroprotective qualities over the ApoE4 isoform. Not only would ApoE2 promote a robust inflammatory response with strongly regulated pro-inflammatory signaling at the beginning, but anti-inflammatory resolution at the end. ApoE2 would also stimulate the increased expression of TREM2 which has been shown to be an important factor in several NDD and is increasingly being identified to have important implications for restricting the development of AD (Price et al.,2020; Yao et al., 2019; Wang et al., 2016; Zhong et al., 2019).

5.0 Conclusions

TREM2 is an innate immune receptor expressed primarily in microglia and macrophages and is critically involved in recognizing pathogenic stimuli as well as communicating the correct cellular response. Mutations in TREM2 are top genetic risk factors for several NDDs (Krasemann et al., 2017). The more recently identified R47H TREM2 variant confers a risk in developing AD similar to that of another long known top genetic risk factor ApoE4 for AD. Not only are these two top genetic risk factors intricately involved in normal neural homeostasis and innate immunity, but they are also mechanistically linked as TREM2 was shown to bind ApoE isoforms (Atagi et al.,

2015). Given that these two top genetic risk factors play roles throughout normal physiology, but likely collaborate to drive and/or restrict AD development or/and progression. Their relationships remains to be fully defined and this project aims to help add to current literature.

This study has identified that sTREM2 can induce cytokine expression in myeloid-derived cell models which could be used as preliminary data towards a more in-depth, real-life situation model. Not only did I set out to identify sTREM2's roles in inflammation, but I also wanted to characterize the ability for ApoE isoforms to differentially regulate inflammation as well.

In conclusion, sTREM2 at a lower concentration (0.1 μg/mL) seems to sufficiently stimulate the expression of several pro-inflammatory cytokines at early time point; while sTREM2 at both a lower (0.1 μg/mL) and a higher concentration (1μg/mL) appear to stimulate the expression of anti-inflammatory cytokines more consistently at later time point in the THP-1 cell model. This could possibly indicate that different concentrations and incubation times of sTREM2 may have differential effects on affecting cytokine expression profiles in the cell model. Interestingly, co-incubation of sTREM2 with a TREM2 antibody was sufficient to neutralize the effects of sTREM2 on inducing the expression of some cytokines but promoting the expression of others. Data indicates that, since SP600125 inhibited sTREM2-induced cytokine expression and MG-132 played a partial inhibitory role, sTREM2 may regulate the cytokine expression mainly through MAPK-JNK pathway and partially via NFκB pathway. ApoE2 and ApoE4 both strongly stimulated the expression of TNF-α and IL-1β in M0, M1 and M2 macrophages as compared to control and ApoE3. Interestingly, ApoE4 did seem to induce an impaired inflammatory response as both IL-10 and TARC/CCL17 expression were markedly reduced as compared to ApoE2. ApoE isoforms stimulated the expression of TREM2 protein strongly in M2 macrophage, followed by

M0 macrophages and lastly did not largely stimulate expression in M1 macrophages. The results suggest that ApoE isoforms have differential effects in inducing cytokine and TREM2 expressions in M0, M1 and M2 cells. The mechanisms behind these observations remain further investigated.

References

A. Alzheimer, R.A. Stelzmann, H.N. Schnitzlein, F.R. Murtagh. An English translation of Alzheimer's 1907 paper, 'Uber eine eigenartige Erkankung der Hirnrinde', Clin. Anat. 8: 429–431, 1995.

Abbas, A. K., Lichtman, A. H., & Pillai, S. Basic Immunology: Functions and Disorders of the Immune System (5th ed.) 2016; 36. St. Louis, Missouri: ELSEVIER

ALZ. Org (www.alz.org)

Alzheimer's Association. 2019 Alzheimer's disease facts and figures. Alzheimer's Dement. 15: 321–387, 2019.

Atagi Y, Liu CC, Painter MM, Chen XF, Verbeeck C, Zheng H, Li X, Rademakers R, Kang SS, Xu H, Younkin S, Das P, Fryer JD, Bu G. Apolipoprotein E Is a Ligand for Triggering 22 Receptor Expressed on Myeloid Cells 2 (TREM2). J Biol Chem. 290(43): 26043-26050, 2015.

Baldacci F, Lista S, Palermo G, Giorgi FS, Vergallo A, Hampel H. The neuroinflammatory biomarker YKL-40 for neurodegenerative diseases: advances in development. Expert Rev Proteomics. 16: 593–600, 2019.

Barber, I. S., Braae, A., Clement, N., Patel, T., Guetta-Barans, T., Brookes, K., Medway, C., Chappel, S., Guerreiro, R., Bras, J., Hernadez, D., Singletone, A., Hardy, J., & Mann, D. M. Mutation analysis of sporadic early-onset Alzheimer's disease using the NeuroX array. Neurobiology of Ageing, *49*: 215, 2017.

Bateman RJ, Aisen PS, De Strooper B, Fox NC, Lemere CA, Ringman JM, Salloway S, Sperling RA, Windisch M, Xiong C. Autosomal-dominant Alzheimer's disease: a review and proposal for the prevention of Alzheimer's disease. Alzheimers Res Ther. 3(1): 1, 2011.

Berger, M., Cooter, M., Roesler, A. S., Chung, S., Park, J., Modliszewski, J. L., VanDusen, K. W., Thompson, J. W., Moseley, A., Devinney, M. J., Smani, S., Hall, A., Cai, V., Browndyke, J. N., Lutz, M. W., & Corcoran, D. L. Apoe4 copy number-dependent proteomic changes in the cerebrospinal fluid. *J. Alzheimer's Dis. 79*(2): 511–530, 2021.

Blue, M.L., Williams, D.L., Zucker, S., Khan, S.A., and Blum, C.B. Apolipoprotein E synthesis in human kidney, adrenal gland, and liver. Proc. Natl. Acad. Sci. USA 80: 283–287, 1983.

Bouchon, A., J. Dietrich, and M. Colonna. Cutting edge: inflammatory responses can be triggered by TREM-1, a novel receptor expressed on neutrophils and monocytes. J. Immunol. 164: 4991–4995, 2000.

Boyles J. K., Zoellner C. D., Anderson L. J., Kosik L. M., Pitas R. E., Weisgraber K. H., Hui D. Y., Mahley R. W., Gebicke-Haerter P. J., Ignatius M. J., et al. A role for apolipoprotein E, apolipoprotein A-I, and low density lipoprotein receptors in cholesterol transport during regeneration and remyelination of the rat sciatic nerve. *J. Clin. Invest.* 83: 1015–1031, 1989

Boyles, J.K., Pitas, R.E., Wilson, E., Mahley, R.W., and Taylor, J.M. Apolipoprotein E associated with astrocytic glia of the central nervous system and with nonmyelinating glia of the peripheral nervous system. J. Clin. Invest. 76: 1501–1513, 1985.

Brion JP, Couck AM, Passareiro E et al. Neurofibrillary tangles of Alzheimer's disease: an immunohistochemical study. J Submicrosc Cytol 17: 89–96, 1985.

Butovsky O, Jedrychowski MP, Moore CS, Cialic R, Lanser AJ, Gabriely G, Koeglsperger T, Dake B, Wu PM, Doykan CE, et al. Identification of a unique TGF-beta-dependent molecular and functional signature in microglia. *Nature Neuroscience.* 17: 131–143, 2014.

Buttgereit A, Lelios I, Yu X, Vrohlings M, Krakoski NR, Gautier EL, Nishinakamura R, Becher B, Greter M. Sall1 is a transcriptional regulator defining microglia identity and function. Nature immunology. 17: 1397–1406, 2016.

Cady J, Koval ED, Benitez BA, Zaidman C, Jockel-Balsarotti J, Allred P, Baloh RH, Ravits J, Simpson E, Appel SH, Pestronk A, Goate AM, Miller TM, Cruchaga C, Harms MB. TREM2 variant p.R47H as a risk factor for sporadic amyotrophic lateral sclerosis. JAMA Neurol. 71(4): 449-453, 2014.

Campion, D., Dumanchin, C., Hannequin, D., Dubois, B., Belliard, S., Puel, M., Thomas-Anterion, C., Michon, A., Martin, C., Charbonnier, F., Raux, G., Camuzat, A., Penet, C., Mesnage, V., Martinez, M., Clerget-Darpoux, F., Brice, A., & Frebourg, T. Early-onset autosomal dominant alzheimer disease: Prevalence, genetic heterogeneity, and mutation spectrum. The American Journal of Human Genetics, 65(3): 664–670, 1999.

Chan WY, Kohsaka S, Rezaie P. The origin and cell lineage of microglia—New concepts. Brain Res Rev. 53: 344–354, 2007.

Chanput W, Mes J, Vreeburg RA, Savelkoul HF, Wichers HJ. Transcription profiles of LPS-stimulated THP-1 monocytes and macrophages: a tool to study inflammation modulating effects of food-derived compounds. Food Funct. Dec;1(3):254-61, 2010.

Chen S, Peng J, Sherchan P, Ma Y, Xiang S, Yan F, Zhao H, Jiang Y, Wang N, Zhang JH, Zhang H. TREM2 activation attenuates neuroinflammation and neuronal apoptosis via PI3K/Akt pathway after intracerebral hemorrhage in mice. J Neuroinflammation. May 28;17(1):168, 2020.

Cheng-Hathaway PJ, Reed-Geaghan EG, Jay TR, Casali BT, Bemiller SM, Puntambekar SS, von Saucken VE, Williams RY, Karlo JC, Moutinho M, Xu G, Ransohoff RM, Lamb BT, Landreth GE. The Trem2 R47H variant confers loss-of-function-like phenotypes in Alzheimer's disease. Mol Neurodegener. Jun 1;13(1):29, 2018.

Cignarella F, Filipello F, Bollman B, Cantoni C, Locca A, Mikesell R, Manis M, Ibrahim A, Deng L, Benitez BA, Cruchaga C, Licastro D, Mihindukulasuriya K, Harari O, Buckland M, Holtzman DM, Rosenthal A, Schwabe T, Tassi I, Piccio L. TREM2 activation on microglia promotes myelin debris clearance and remyelination in a model of multiple sclerosis. Acta Neuropathol. Oct;140(4):513-534, 2020.

Clemerson G, Walsh S, Isaac C. Towards living well with young onset dementia: An exploration of coping from the perspective of those diagnosed. *Dementia (London)* 13: 451-466, 2014.

Colonna M, Wang Y. TREM2 variants: new keys to decipher Alzheimer disease pathogenesis. Nat Rev Neurosci. 17: 201–207, 2016.

Conejero-Goldberg C, Gomar JJ, Bobes-Bascaran T, Hyde TM, Kleinman JE, Herman MM, Chen S, Davies P, Goldberg TE. APOE2 enhances neuroprotection against Alzheimer's disease through multiple molecular mechanisms. Mol Psychiatry. 19(11): 1243-1250, 2014.

Corder, E. H., Saunders, A. M., Strittmatter, W. J., Schmechel, D. E., Gaskell, P. C., Small, G. W., Roses, A. D., Haines, J. L., and Pericak-Vance, M. A. Gene dose of apolipoprotein E type 4 allele and the risk of Alzheimer’s disease in late onset families. Science 261: 921–923, 1993.

Davignon, J., Bouthillier, D., Nestruck, A.C., and Sing, C.F. Apolipoprotein E polymorphism and atherosclerosis: insight from a study in octogenarians. Trans. Am. Clin. Climatol. Assoc. 99: 100-110, 1988.

Daws, M. R., Sullam, P. M., Niemi, E. C., Chen, T. T., Tchao, N. K., and Seaman, W. E. Pattern recognition by TREM-2: binding of anionic ligands. J. Immunol. 171, 594 –599, 2003

Deane R, Sagare A, Hamm K, Parisi M, Lane S, Finn MB, Holtzman DM, Zlokovic BV. ApoE isoform–specific disruption of amyloid β peptide clearance from mouse brain. *J. Clin. Invest.* 118:4002–4013, 2008.

Dorey E, Bamji-Mirza M, Najem D, Li Y, Liu H, Callaghan D, Walker D, Lue LF, Stanimirovic D, Zhang W. Apolipoprotein E Isoforms Differentially Regulate Alzheimer's Disease and Amyloid-β-Induced Inflammatory Response in vivo and in vitro. J Alzheimers Dis. ;57(4):1265Document2-1279, 2017.

Edwards FA. A unifying hypothesis for Alzheimer’s disease: from Plaques to neurodegeneration. Trends Neurosci. 42: 310–322, 2019.

Eriksson H, Fereshtehnejad SM, Falahati F, Farahmand B, Religa D, Eriksdotter M. Differences in routine clinical practice between early and late onset Alzheimer's disease. J. Alzheimer's Dis. 41: 411-419, 2014.

Eto, M., Watanabe, K., Iwashima, Y., Morikawa, A., Chonan, N., Oshima, E., Sekiguchi, M., and Ishii, K. Increased frequency of apolipoprotein epsilon 4 allele in type II diabetes with hypercholesterolemia. Diabetes 36: 1301–1306, 1987.

FDA.gov (www.FDA.gov)

Ferri E, Rossi PD, Geraci A, Ciccone S, Cesari M, Arosio B. The sTREM2 Concentrations in the Blood: A Marker of Neurodegeneration? Front Mol Biosci. 2021.

Filipello, F., Morini, R., Corradini, I., Zerbi, V., Canzi, A., Michalski, B., Erreni, M., Markicevic, M., Starvaggi-Cucuzza, C., Otero, K., Piccio, L., Cignarella, F., Perrucci, F., Tamborini, M., Genua, M., Rajendran, L., Menna, E., Vetrano, S., Fahnestock, M., Matteoli, M. The microglial innate immune receptor TREM2 is required for synapse elimination and Normal Brain Connectivity. *Immunity*, *48*(5): 979-991, 2018.

Fitz NF, Nam KN, Wolfe CM, Letronne F, Playso BE, Iordanova BE, Kozai TDY, Biedrzycki RJ, Kagan VE, Tyurina YY, Han X, Lefterov I, Koldamova R. Phospholipids of APOE lipoproteins activate microglia in an isoform-specific manner in preclinical models of Alzheimer's disease. Nat Commun. 12(1): 3416, 2021.

Forloni G, Balducci C. Alzheimer's disease, oligomers, and inflammation. J Alzheimer's Dis.

62:1261–1276, 2018.

Gabin JM, Saltvedt I, Tambs K, Holmen J. The association of high sensitivity C-reactive protein and incident Alzheimer disease in patients 60 years and older: The HUNT study, Norway. Immun Ageing 15: 4, 2018.

Giraldo M, Lopera F, Siniard AL, Corneveaux JJ, Schrauwen I, Carvajal J, Munoz C, Ramirez-Restrepo M, Gaiteri C, Myers AJ, et al. Variants in triggering receptor expressed on myeloid cells 2 are associated with both behavioral variant frontotemporal lobar degeneration and Alzheimer's disease. Neurobiol Aging. 34(8): 2077. 2013.

Glass CK, Saijo K, Winner B, Marchetto MC, Gage FH. Mechanisms underlying inflammation in neurodegeneration. Cell. 140: 918–934, 2010.

Guerreiro RJ, Lohmann E, Bras JM, Gibbs JR, Rohrer JD, Gurunlian N, Dursun B, Bilgic B, Hanagasi H, Gurvit H, et al. Using Exome Sequencing to Reveal Mutations in TREM2 Presenting as a Frontotemporal Dementia-like Syndrome Without Bone Involvement. JAMA Neurol. 70: 78–84, 2013.

Guo N, Peng Z. MG132, a proteasome inhibitor, induces apoptosis in tumor cells. Asia Pac J Clin Oncol., 6-11, 2013.

Guo Z, Fratiglioni L, Viitanen M et al. Apolipoprotein E genotypes and the incidence of Alzheimer's disease among persons aged 75 years and older: variation by use of antihypertensive medication? *Am. J. Epidemiol.* 153: 225-231, 2001.

Hakola, H.P.A. Neuropsychiatric and genetic aspects of a new hereditary disease characterized by progressive dementia and lipomembranous polycystic osteodysplasia. *Acta Psychiatr. Scand. Suppl.* 232: 1–173, 1972.

Hakola, H.P.A. Polycystic lipomembranous osteodysplasia with sclerosing leukoencephalopathy (membranous lipodystrophy). A neuropsychiatric follow-up study. (monograph 17) in *Monographs of Psychiatria Fennica* (eds Henriksson, M., Huttunen, M., Kuoppasalmi, K., Lindfors, O. & Lönnqvist, J.) 1–114 (Foundation for Psychiatric Research in Finland, Helsinki, 1990).

Hamerman J. A., Jarioura, J. R., Humphrey, M. B., Nakamura, M. C., Seaman, W. E., and Lanier, L. L. Cutting edge: inhibition of TLR and FcR responses in macrophages by triggering receptor expressed on myeloid cells-2 (TREM2) and DAP12. J. Immunol. 177, 2051-2055, 2006.

Hardy J, Selkoe DJ. The amyloid hypothesis of Alzheimer's disease: progress and problems on the road to therapeutics. *Science.* 297: 353–356, 2002.

Hatters, D. M., Peters-Libeu, C. A., & Weisgraber, K. H. Apolipoprotein E structre: Insights into function. *Trends in Biochemical Sciences*, *31*(8): 445–454, 2006.

Heneka MT, Carson MJ, Khoury J El, Landreth GE, Brosseron F, Feinstein DL, et al. Neuroinflammation in Alzheimer's disease. Lancet Neurol. 14: 388–405, 2015.

Henjum, K., Almdahl, I. S., Årskog, V., Minthon, L., Hansson, O., Fladby, T., & Nilsson, L. N. Cerebrospinal fluid soluble Trem2 in aging and alzheimer's disease. *Alzheimer's Research & Therapy*, *8*(1)., 2016.

Heslegrave, A., Heywood, W., Paterson, R., Magdalinou, N., Svensson, J., Johansson, P., Öhrfelt, A., Blennow, K., Hardy, J., Schott, J., Mills, K., Zetterberg, H. Increased cerebrospinal fluid soluble trem2 concentration in Alzheimer's disease. *Molecular Neurodegeneration*, *11*(1): 3, 2016.

Holtzman, D.M., Herz, J., and Bu, G. Apolipoprotein E and apolipoprotein E receptors: normal biology and roles in Alzheimer disease. Cold Spring Harb. Perspect. Med. 2: a006312, 2012.

Huang Y, Mahley RW. Apolipoprotein E: structure and function in lipid metabolism, neurobiology, and Alzheimer's diseases. Neurobiol Dis. 72 Pt A: 3-12, 2014.

Huang YA, Zhou B, Wernig M, Südhof TC. ApoE2, ApoE3, and ApoE4 Differentially Stimulate APP Transcription and Aβ Secretion. Cell. Jan 26;168(3):427-441.e21. doi: 10.1016/j.cell.2016.12.044. Epub Jan 19, 2017.

Hwang Ju C, Choi DY, Park MH, Hong JT. NF-κB as a Key Mediator of Brain Inflammation in Alzheimer's Disease. CNS Neurol Disord Drug Targets. ;18(1):3-10, 2019

Jack, C. R., Jr., Knopman, D. S., Jagust, W. J., Petersen, R. C., Weiner, M. W., Aisen, P. S., Shaw, L. M., Vemuri, P., Wiste, H. J., Weigand, S. D., Lesnick, T. G., Pankratz, V. S., Donohue, M. C., and Trojanowski, J. Q. Tracking pathophysiological processes in Alzheimer's disease: an updated hypothetical model of dynamic biomarkers. Lancet Neurol. 12: 207–216, 2013.

Jay TR, Hirsch AM, Broihier ML, Miller CM, Neilson LE, Ransohoff RM, Lamb BT, Landreth GE. Disease Progression-Dependent Effects of TREM2 Deficiency in a Mouse Model of Alzheimer's Disease. *J. Neurosci.* 37: 637–647, 2017.

Jay TR, Miller CM, Cheng PJ, Graham LC, Bemiller S, Broihier ML, Xu G, Margevicius D, Karlo JC, Sousa GL, et al. TREM2 deficiency eliminates TREM2+ inflammatory macrophages and ameliorates pathology in Alzheimer's disease mouse models. *J. Exp. Med.* 212: 287–295, 2015

Jay, T. R., von Saucken, V. E., & Landreth, G. E. Trem2 in Neurodegenerative Diseases. *Molecular Neurodegeneration*, *12*(1): 56, 2017.

Jay, T.R., von Saucken, V.E., Mun˜ oz, B., Codocedo, J.F., Atwood, B.K., Lamb, B.T., and Landreth, G.E. TREM2 is required for microglial instruction of astrocytic synaptic engulfment in neurodevelopment. Glia 67: 1873–1892, 2019.

Kamboh MI, Sanghera DK, Ferrell RE, DeKosky ST. APOE4-associated Alzheimer's disease risk is modified by alpha 1-antichymotrypsin polymorphism. Nat Genet. 10(4): 486-488, 1995.

Kaneko M, Sano K, Nakayama J, Amano N. Nasu-Hakola disease: The first case reported by Nasu and review: The 50th Anniversary of Japanese Society of Neuropathology. Neuropathology. 30(5): 463-470, 2010.

Keren-Shaul H, et al. A Unique Microglia Type Associated with Restricting Development of Alzheimer's Disease. Cell. 169(7):1276–1290.e17, 2017.

Kim J, Basak JM, Holtzman DM. The role of apolipoprotein E in Alzheimer's disease. *Neuron.* 63: 287–303, 2009.

Kim J, Jiang H, Park S, Eltorai AE, Stewart FR, Yoon H, Basak JM, Finn MB, Holtzman DM. Haploinsufficiency of human APOE reduces amyloid deposition in a mouse model of amyloid-β amyloidosis. *J. Neurosci.* 31: 18007–18012, 2011.

Kleinberger G, Brendel M, Mracsko E, Wefers B, Groeneweg L, Xiang X, Focke C, Deußing M, Suárez-Calvet M, Mazaheri F, Parhizkar S, Pettkus N, Wurst W, Feederle R, Bartenstein P, Mueggler T, Arzberger T, Knuesel I, Rominger A, Haass C. The FTD-like syndrome causing TREM2 T66M mutation impairs microglia function, brain perfusion, and glucose metabolism. EMBO J. 36(13): 1837-1853, 2017.

Knapskog AB, Henjum K, Idland AV, Eldholm RS, Persson K, Saltvedt I, Watne LO, Engedal K, Nilsson LNG. Cerebrospinal fluid sTREM2 in Alzheimer's disease: comparisons between clinical presentation and AT classification. Sci Rep. Sep 28, 2020.

Kober, D. L., & Brett, T. J. TREM2-ligand interactions in health and disease. J.Mol.Biol, 11: 1607-1629, 2017.

Koizumi, S., Ohsawa, K., Inoue, K., Kohsaka, S. Purinergic receptors in microglia: functional modal shifts of microglia mediated by P2 and P1 receptors. Glia 61: 47–54, 2013.

Konishi H, Kiyama H. Microglial TREM2/DAP12 Signaling: A Double-Edged Sword in Neural Diseases. Front Cell Neurosci. 12: 206, 2018.

Koprich JB, Reske-Nielsen C, Mithal P, Isacson O. Neuroinflammation mediated by IL-1beta increases susceptibility of dopamine neurons to degeneration in an animal model of Parkinson's disease. J Neuroinflammation. Feb 27; 5: 8, 2008

Korvatska, O., Leverenz, J. B., Jayadev, S., McMillan, P., Kurtz, I., Guo, X., Rumbaugh, M., Matsushita, M., Girirajan, S., Dorschner, M. O. R47H variant of TREM2 associated with Alzheimer disease in a large late-onset family: clinical, genetic, and neuropathological study. JAMA Neurol. 72: 920 –927, 2015.

Krasemann S, Madore C, Cialic R, Baufeld C, Calcagno N, El Fatimy R, Beckers L, O'Loughlin E, Xu Y, Fanek Z, Greco DJ, Smith ST, Tweet G, Humulock Z, Zrzavy T, Conde-Sanroman P, Gacias M, Weng Z, Chen H, Tjon E, Mazaheri F, Hartmann K, Madi A, Ulrich JD, Glatzel M, Worthmann A, Heeren J, Budnik B, Lemere C, Ikezu T, Heppner FL, Litvak V, Holtzman DM, Lassmann H, Weiner HL, Ochando J, Haass C, Butovsky O. The TREM2-APOE Pathway Drives the Transcriptional Phenotype of Dysfunctional Microglia in Neurodegenerative Diseases. Immunity. 47(3): 566-581.e9, 2017.

Lai KSP, Liu CS, Rau A, Lanctôt KL, Köhler CA, Pakosh M, et al. Peripheral inflammatory markers in Alzheimer’s disease: a systematic review and metaanalysis of 175 studies. J Neurol Neurosurg Psychiatry. 88: 876–882, 2017.

Lanier, L.L., Corliss, B.C., Wu, J., Leong, C. & Phillips, J.H. Immunoreceptor DAP12 bearing a tyrosine-based activation motif is involved in activating NK cells. *Nature* 391: 703–707, 1998.TREM2

Le Ber I, De Septenville A, Guerreiro R, Bras J, Camuzat A, Caroppo P, Lattante S, Couarch P, Kabashi E, Bouya-Ahmed K, et al. Homozygous TREM2 mutation in a family with atypical frontotemporal dementia. Neurobiol Aging. 35(10): 2419, 2014.

Levy-Lahad E, Wijsman EM, Nemens E et al. A familial Alzheimer's disease locus on chromosome 1. Science 269: 970–973, 1995.

Li, RY., Qin, Q., Yang, HC. *et al.* TREM2 in the pathogenesis of AD: a lipid metabolism regulator and potential metabolic therapeutic target. *Mol Neurodegeneration* 17, 40, 2022.

Li, Z., Shue, F., Zhao, N. *et al.* APOE2: protective mechanism and therapeutic implications for Alzheimer's disease. *Mol Neurodegeneration* **15**: 63, 2020.

Liu CC, Liu CC, Kanekiyo T, Xu H, Bu G. Apolipoprotein E and Alzheimer disease: risk, mechanisms and therapy. Nat Rev Neurol. 9(2): 106-18, 2013.

Liu T, Zhang L, Joo D, Sun SC. NF-κB signaling in inflammation. Signal Transduct Target Ther.;2:17023, 2017.

Liu, C.-C., Kanekiyo,T., Xu, H., and Bu, G. ApolipoproteinE and Alzheimer disease: risk, mechanisms and therapy. Nat. Rev. Neurol. 9: 106 –118, 2013.

London A, Cohen M, Schwartz M. Microglia and monocyte-derived macrophages: functionally distinct populations that act in concert in CNS plasticity and repair Front. Cell. Neurosci. 7:34, 2013.

Mahley RW, Huang Y. Apolipoprotein e sets the stage: response to injury triggers neuropathology. Neuron. 76(5):871-885, 2012.

Mahley, R.W. Apolipoprotein E: cholesterol transport protein with expanding role in cell biology. Science 240: 622, 1988.

McQuade, A., & Blurton-Jones, M. Microglia in alzheimer's disease: Exploring how genetics and phenotype influence risk. Journal of Molecular Biology, *431*(9): 1805–1817, 2019.

Mendez MF. Early-Onset Alzheimer Disease. Neurol Clin. 35(2): 263-281, 2017.

Moors MA, Mizel SB. Proteasome-mediated regulation of interleukin-1beta turnover and export in human monocytes. J Leukoc Biol. Jul;68(1):131-6, 2000.

Morishima Y, Gotoh Y, Zieg J, Barrett T, Takano H, Flavell R, Davis RJ, Shirasaki Y, Greenberg ME. Beta-amyloid induces neuronal apoptosis via a mechanism that involves the c-Jun N-terminal kinase pathway and the induction of Fas ligand. J Neurosci Oct 1;21(19):7551-60, 2001.

Myers A.J, M. Kaleem, L. Marlowe, A.M. Pittman, A.J. Lees, H.C. Fung, J. Duckworth, D. Leung, A. Gibson, C.M. Morris, R. de Silva, J. Hardy, The H1c haplotype at the *MAPT* locus is associated with Alzheimer's disease. *Human Molecular Genetics*, 14(16): 2399–2404, 2015.

Nilsson K, Gustafson L, Hultberg B. C-reactive protein level is decreased in patients with Alzheimer’s disease and related to cognitive function and survival time. Clin Biochem 44: 1205-1208, 2011.

Nyberg J, Aberg MA, Schioler L, et al. Cardiovascular and cognitive fitness at age 18 and risk of early-onset dementia. *Brain*137(Pt 5): 514–1523, 2014

Painter, M. M., Atagi, Y., Liu, C., Rademakers, R., Xu, H., Fryer, J. D., & Bu, G. TREM2 in CNS homeostasis and neurodegenerative disease. Molecular Neurodegeneration, 10(1): 1, 2015

Paloneva J, Kestilä M, Wu J, Salminen A, Böhling T, Ruotsalainen V, Hakola P, Bakker AB, Phillips JH, Pekkarinen P, Lanier LL, Timonen T, Peltonen L. Loss-of-function mutations in TYROBP (DAP12) result in a presenile dementia with bone cysts. Nat Genet. 25(3):357-361, 2000.

Peng, Q., Malhotra, S., Tochia, J. A., Kerr, W. G., Coggeshall, K. M., and Humphrey, M.B (2010) TREM2- and DAP12 dependent activation of PI3K requires DAP10 and is inhibited by SHIP1. Sci. Signal., 2010.

Perry VH, Holmes C. Microglial priming in neurodegenerative disease. Nat Rev Neurol. 10: 217–224, 2014.

Piccio L, Buonsanti C, Cella M, Tassi I, Schmidt RE, Fenoglio C, Rinker J 2nd, Naismith RT, Panina-Bordignon P, Passini N, Galimberti D, Scarpini E, Colonna M, Cross AH. Identification of soluble TREM-2 in the cerebrospinal fluid and its association with multiple sclerosis and CNS inflammation. Brain. 2008 Nov;131(Pt 11):3081-91. doi: 10.1093/brain/awn217. Epub Sep 12, 2008.

Pollock NJ, Mirra SS, Binder LI et al. Filamentous aggregates in Pick’s disease, progressive supranuclear palsy, and Alzheimer’s disease share antigenic determinants with microtubule-associated protein, tau. Lancet 2: 1211, 1986.

Posse de Chaves E. I., Vance D. E., Campenot R. B., Kiss R. S., Vance J. E. Uptake of lipoproteins for axonal growth of sympathetic neurons. *J. Biol. Chem.* 275: 19883–19890, 2000.

Price BR, Sudduth TL, Weekman EM, Johnson S, Hawthorne D, Woolums A, Wilcock DM. Therapeutic Trem2 activation ameliorates amyloid-beta deposition and improves cognition in the 5XFAD model of amyloid deposition. J Neuroinflammation. Aug 14;17(1):238, 2020

Ransohoff RM. A polarizing question: do M1 and M2 microglia exist? Nat Neurosci. 19: 987–991, 2016.

Roberson ED, et al. Reducing endogenous tau ameliorates amyloid β-induced deficits in an Alzheimer's disease mouse model. *Science.* 316:750–754. Goate A, Chartier-Harlin MC, Mullan M et al. Segregation of a missense mutation in the amyloid precursor protein gene with familial Alzheimer's disease. Nature 349: 704–706, 1991.

Saito, M., Eto, M., Nitta, H., Kanda, Y., Shigeto, M., Nakayama, K., Tawaramoto, K., Kawasaki, F., Kamei, S., Kohara, K., et al. Effect of apoupoprotein E4 allele on plasma LDL cholesterol response to diet therapy in type 2 diabetic patients. Apunts. Medicina de L'Esport 39: 41–42, 2004.

Sáiz-Vazquez O, Puente-Martínez A, Ubillos-Landa S, Pacheco-Bonrostro J, Santabárbara J. Cholesterol and Alzheimer's Disease Risk: A Meta-Meta-Analysis. Brain Sci. 10(6): 386, 2020.

Sarlus H, Heneka MT. Microglia in Alzheimer's disease. J Clin Invest. 127:3240–3249, 2017.

Schöll M, Maass A, Mattsson N, Ashton NJ, Blennow K, Zetterberg H, Jagust W. Biomarkers for tau pathology. Mol Cell Neurosci. 97: 18-33, 2019.

Sherrington R, Rogaev EI, Liang Y, et al.St George-Hyslop PH. Cloning of a gene bearing missense mutations in early-onset familial Alzheimer's disease. Nature 375: 754–760, 1995.

Shoji M, Iwakami N, Takeuchi S, Waragai M, Suzuki M, Kanazawa I, Lippa CF, Ono S, Okazawa H. JNK activation is associated with intracellular beta-amyloid accumulation. Brain Res Mol Brain Res. Dec 28;85(1-2):221-33, 2000.

Shore, V.G., and Shore, B. Heterogeneity of human plasma very low density lipoproteins. Separation of species differing in protein components. Biochemistry 12: 502–507, 1973.

Srivastava RA, Bhasin N, Srivastava N. Apolipoprotein E gene expression in various tissues of mouse and regulation by estrogen. Biochem Mol Biol Int. 38(1): 91-101, 1996.

Streit WJ, Xue Q-S, Tischer J, Bechmann I. Microglial pathology. Acta Neuropathol Commun. 2:142, 2014.

Suárez-Calvet, M., Morenas-Rodríguez, E., Kleinberger, G., Schlepckow, K., Araque Caballero, M. Á., Franzmeier, N., Capell, A., Fellerer, K., Nuscher, B., Eren, E., Levin, J., Deming, Y., Piccio, L., Karch, C. M., Cruchaga, C., Shaw, L. M., Trojanowski, J. Q., Weiner, M., Ewers, M., & Haass, C. Early increase of CSF STREM2 in alzheimer's disease is associated with tau related-neurodegeneration but not with amyloid-β pathology. *Molecular Neurodegeneration*, *14*(1): 1, 2019.

Swardfager W, Lanctôt K, Rothenburg L, Wong A, Cappell J, Herrmann N. A meta-analysis of cytokines in Alzheimer's disease. Biol Psychiatry. 68: 930–941, 2010.

Takahashi K., Rochford C. D., Neumann H. Clearance of apoptotic neurons without inflammation by microglial triggering receptor expressed on myeloid cells-2. J. Exp. Med. 201: 647–657, 2005.

Takahashi, K., Rochford, C. D., and Neumann, H. Clearance of apoptotic neurons without inflammation by microglial triggering receptor expressed on myeloid cells -2. J. Exp. Med. 201, 647-657, 2005.

Tampi RR, Forester BP, Agronin M. Aducanumab: evidence from clinical trial data and controversies. Drugs Context. 10: 2021-7-3, 2021.

Tang Y, Le W. Differential roles of M1 and M2 microglia in neurodegenerative diseases. Mol Neurobiol. 53: 1181–1194, 2016.

Tellechea P, Pujol N, Esteve-Belloch P, Echeveste B, García-Eulate MR, Arbizu J, Riverol M. Early- and late-onset Alzheimer disease: Are they the same entity? Neurologia (Engl Ed). 33(4): 244-253, 2018.

Turnbull IR, Gilfillan S, Cella M, Aoshi T, Miller M, Piccio L, Hernandez M, Colonna M. Cutting edge: TREM-2 attenuates macrophage activation. J Immunol. Sep 15;177(6):3520-4, 2006.

Vance JE. Dysregulation of cholesterol balance in the brain: contribution to neurodegenerative diseases. Dis Model Mech. 5(6): 746-755, 2012.

Victor, M. B., Leary, N., Luna, X., Meharena, H. S., Bozzelli, P. L., Samaan, G., Murdock, M. H., von Maydell, D., Effenberger, A. H., Cerit, O., Wen, H.-L., Liu, L., Welch, G., Bonner, M., Tsai, L.-H. Lipid accumulation induced by APOE4 impairs microglial surveillance of neuronal-network activity. Cell Stem Cell 29: 1197-1212, 2022.

Vilalta, A., Zhou, Y., Sevalle, J., Griffin, J. K., Satoh, K., Allendorf, D. H., De, S., Puigdellívol, M., Bruzas, A., Burguillos, M. A., Dodd, R. B., Chen, F., Zhang, Y., Flagmeier, P., Needham, L.-M., Enomoto, M., Qamar, S., Henderson, J., Walter, J., Brown, G. C. Wildtype strem2 blocks AB aggregation and neurotoxicity, but the Alzheimer's R47h mutant increases AB aggregation. J. Biol. Chem. 296: 100631, 2021.

Vilalta, A., Zhou, Y., Sevalle, J., Griffin, J. K., Satoh, K., Allendorf, D. H., De, S., Puigdellívol, M., Bruzas, A., Burguillos, M. A., Dodd, R. B., Chen, F., Zhang, Y., Flagmeier, P., Needham, L.-M., Enomoto, M., Qamar, S., Henderson, J., Walter, J., … Brown, G. C.. Wild-type strem2 blocks AB aggregation and neurotoxicity, while the alzheimer’s R47H mutant does the opposite., 2020.

Vukic, V., Callaghan, D., Walker, D., Lue, L.-F., Liu, Q. Y., Couraud, P.-O., Romero, I. A., Weksler, B., Stanimirovic, D. B., & Zhang, W. Expression of inflammatory genes induced by beta-amyloid peptides in human brain endothelial cells and in Alzheimer’s brain is mediated by the JNK-AP1 signaling pathway. *Neurobiology of Disease*, *34*(1), 95–106. 2009.

Wang S, Colonna M. Microglia in Alzheimer’s disease: a target for immunotherapy. J Leukoc Biol. 106: JLB.MR0818-319R, 2019.

Wang S, Mustafa M, Yuede CM, Salazar SV, Kong P, Long H, Ward M, Siddiqui O, Paul R, Gilfillan S, Ibrahim A, Rhinn H, Tassi I, Rosenthal A, Schwabe T, Colonna M. Anti-human TREM2 induces microglia proliferation and reduces pathology in an Alzheimer's disease model. J Exp Med. 217(9): e20200785, 2020.

Wang Y, Ulland TK, Ulrich JD, Song W, Tzaferis JA, Hole JT, Yuan P, Mahan TE, Shi Y, Gilfillan S, et al. TREM2-mediated early microglial response limits diffusion and toxicity of amyloid plaques. *J. Exp. Med.* 213: 667–675, 2016.

Wang, Y., Cella, M., Mallinson, K., Ulrich, J. D., Young, K. L., Robinette, M. L., Gilfillan, S., Krishnan, G. M., Sudhakar, S., Zinselmeyer, B. H., Holtzman, D. M., Cirrito, J. R., and Colonna, M. TREM2 lipid sensing sustains the microglial response in an Alzheimer’s disease model. Cell 160: 1061–1071, 2015.

Weber, G. E., Khrestian, M., Tuason, E. D., Shao, Y., Pillai, J., Rao, S., Feng, H., Zhou, Y., Cheng, F., DeSilva, T. M., Stauffer, S., Leverenz, J. B., & Bekris, L. M. Peripheral strem2-related inflammatory activity alterations in early stage Alzheimer’s disease, 2021.

Weisgraber KH Roses AD Strittmatter WJ. The role of apolipoprotein E in the nervous system. Curr. Opin. Lipidol. 5: 110–116, 1994.

Wolf A, Bauer B, Hartz AM. ABC Transporters and the Alzheimer's Disease Enigma. Front Psychiatry. 3:54, 2012.

Wunderlich P, Glebov K, Kemmerling N, Tien NT, Neumann H, Walter J. Sequential proteolytic processing of the triggering receptor expressed on myeloid cells-2 (TREM2) protein by ectodomain shedding and γ-secretase-dependent intramembranous cleavage. J Biol Chem. 288(46): 33027-33036, 2013.

Yamamoto, T., Choi, H. W., & Ryan, R. O. Apolipoprotein e isoform-specific binding to the low-density lipoprotein receptor. *Analytical Biochemistry*, *372*(2): 222–226, 2008.

Yao, H., Coppola, K., Schweig, J. E., Crawford, F., Mullan, M., & Paris, D. Distinct signaling pathways regulate trem2 phagocytic and NFKB antagonistic activities. *Frontiers in Cellular Neuroscience*, *13:* 457, 2019.

Zhang J, Liu Q. Cholesterol metabolism and homeostasis in the brain. Protein Cell. 6(4): 254-264, 2015. Zhao Y, Wu X, Li X, Jiang LL, Gui X, Liu Y, Sun Y, Zhu B, Piña-Crespo JC, Zhang M, Zhang N, Chen X, Bu G, An Z, Huang TY, Xu H. TREM2 Is a Receptor for β-Amyloid that Mediates Microglial Function. Neuron. 97(5): 1023-1031.e7, 2018.

Zheng H, Koo E. Biology and pathophysiology of the amyloid precursor protein. *Mol Neurodegener.* 6: 27, 2011.

Zhong, L., Chen, X.-F., Wang, T., Wang, Z., Liao, C., Wang, Z., Huang, R., Wang, D., Li, X., Wu, L., Jia, L., Zheng, H., Painter, M., Atagi, Y., Liu, C.-C., Zhang, Y.-W., Fryer, J. D., Xu, H., & Bu, G. (2017). Soluble trem2 induces inflammatory responses and enhances microglial survival. *J. Exp. Med. 214*(3): 597–607, 2017.

Zhong, L., Xu, Y., Zhuo, R., Wang, T., Wang, K., Huang, R., Wang, D., Gao, Y., Zhu, Y., Sheng, X., Chen, K., Wang, N., Zhu, L., Can, D., Marten, Y., Shinohara, M., Liu, C.-C., Du, D., Sun, H., Wen L, Xu H, Bu G, Chen, X.-F. Soluble trem2 ameliorates pathological phenotypes by modulating microglial functions in an Alzheimer's disease model. *Nature Communications*, *10*(1): 1365, 2019.

Zhou SL, Tan CC, Hou XH, Cao XP, Tan L, Yu JT. TREM2 Variants and Neurodegenerative Diseases: A Systematic Review and Meta-Analysis. J Alzheimers Dis. 68(3): 1171-1184, 2019.

Contributions of Collaborators

Ryan Arsenault is a student in the HHT (Human Health Therapeutics) Graduate Program at the Human Health Therapeutics Research Centre, National Research Council of Canada (NRC) and is financially supported by HHT-NRC. His research project was supported by funding from HHT-NRC. The research project was conducted in co-supervisor Dr. Qiao Li's laboratory at the University of Ottawa.

Dr. Wandong Zhang helped conceptualize and design the project as well as helping throughout the editorial process and revisions of this work.

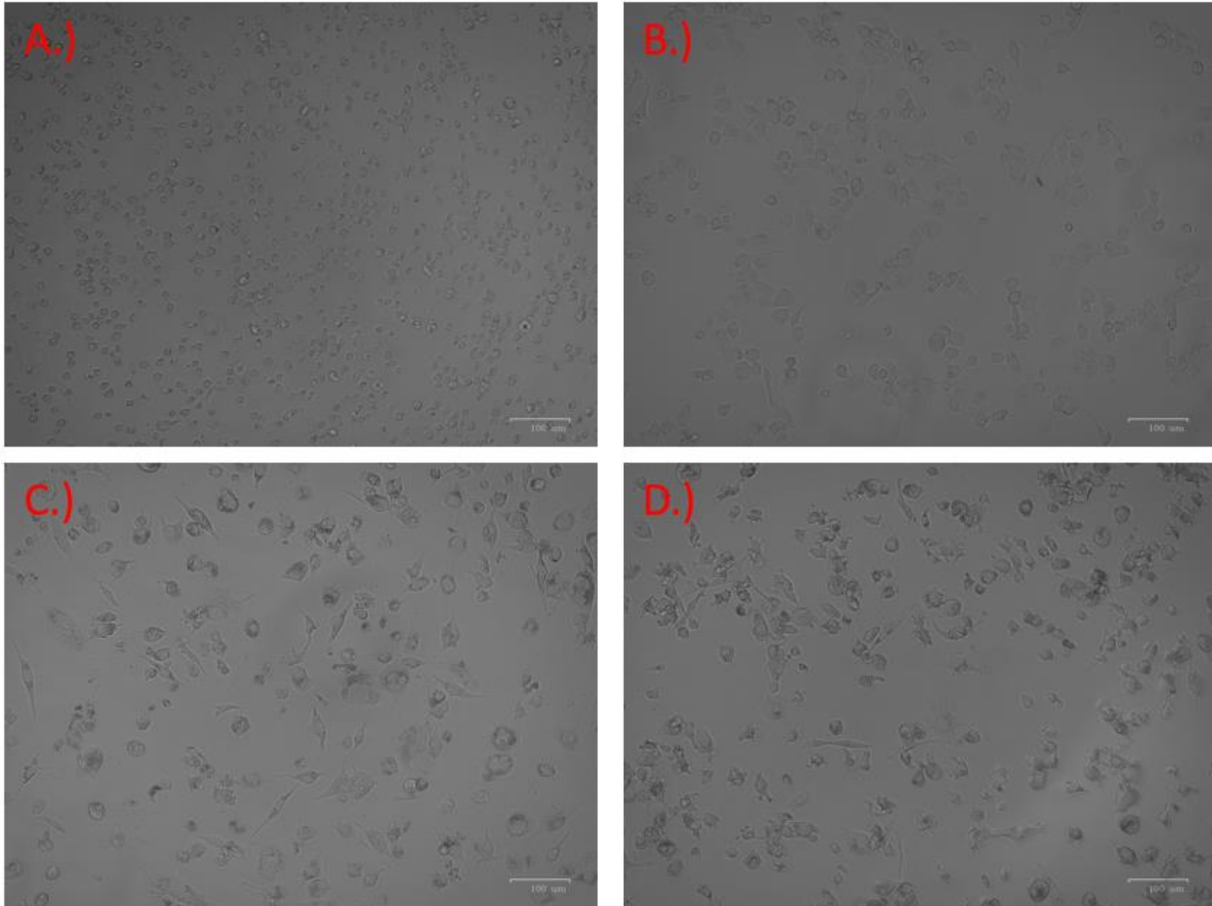
Steven Marshall was a Bachelor of Science student at the University of Ottawa pursuing his honors degree. He completed the qPCR and treatment work in the Part A, section IV of the results section.

Dr. Patrick Salois, Human Health and Therapeutics, National Research Council of Canada, Montreal QC, completed the work assessing the expression of sTREM2 by polarized M0, M1, and M2 macrophages in cell culture supernatant.

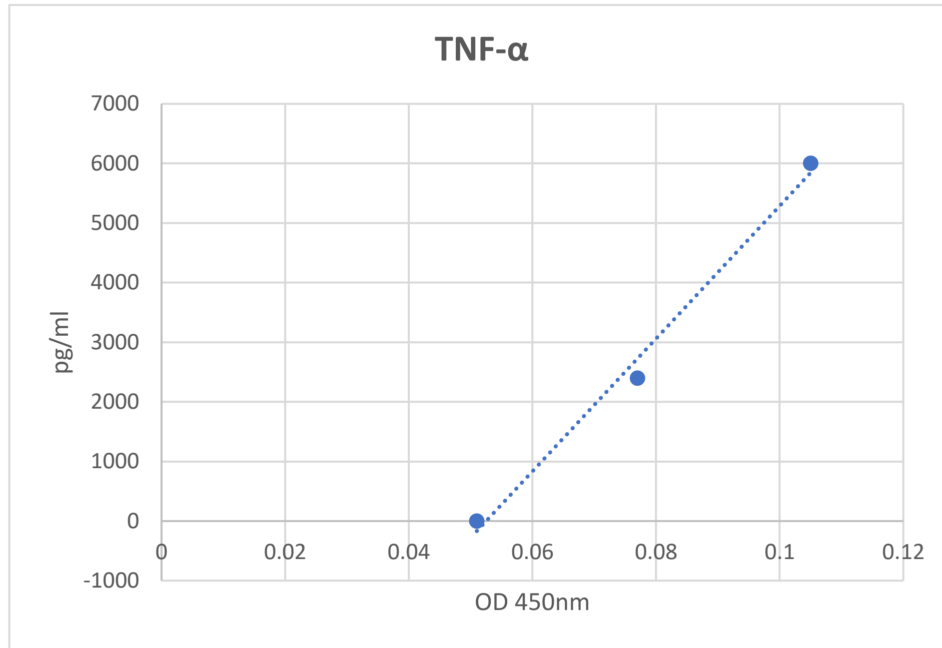
Appendices

Supplementary Table 1: Forward and reverse qPCR primers used for the detection of cytokine expression in this study.

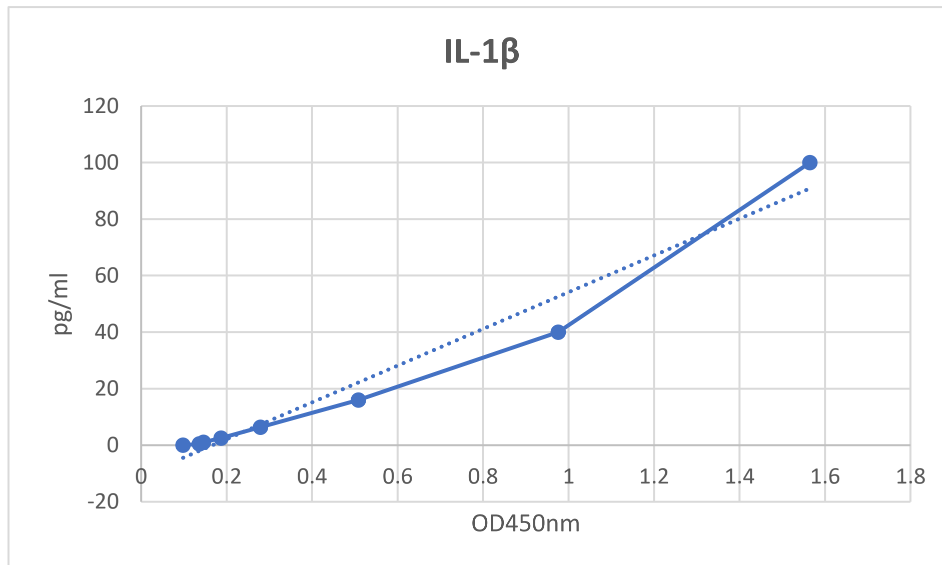
Gene	5’ Primer (sense primer)	3’ Primer (anti-sense primer)
TNF-α	5’-GTGCTCCTCACCCACACC-3’	5’CCCTTCTCCAGCTGGAAGAC-3’
IL-1β	5’-CCTGTGGCCTTGGGCCTC-3’	5’-TGATGTACCAGTTGGGGAACT-3’
IL-10	5’-TATTTATTACCTCTGATACCTC-3’	5’-AATTATAATATTGGGCTTCTTTC-3’
IL-6	5’-AGTGAGGAACAAGCCAGAGC-3’	5’-AGCTGCGCAGAATGAGATGA-3’
TARC	5’-AGTGAGGAACAAGCCAGAGC-3’	5’-AGCTGCGCAGAATGAGATGA-3’
B-actin	5’ – CATGTACGTTGCTATCGAGGC-3’	5’- CTCCTTAATACGCACGAT -3’



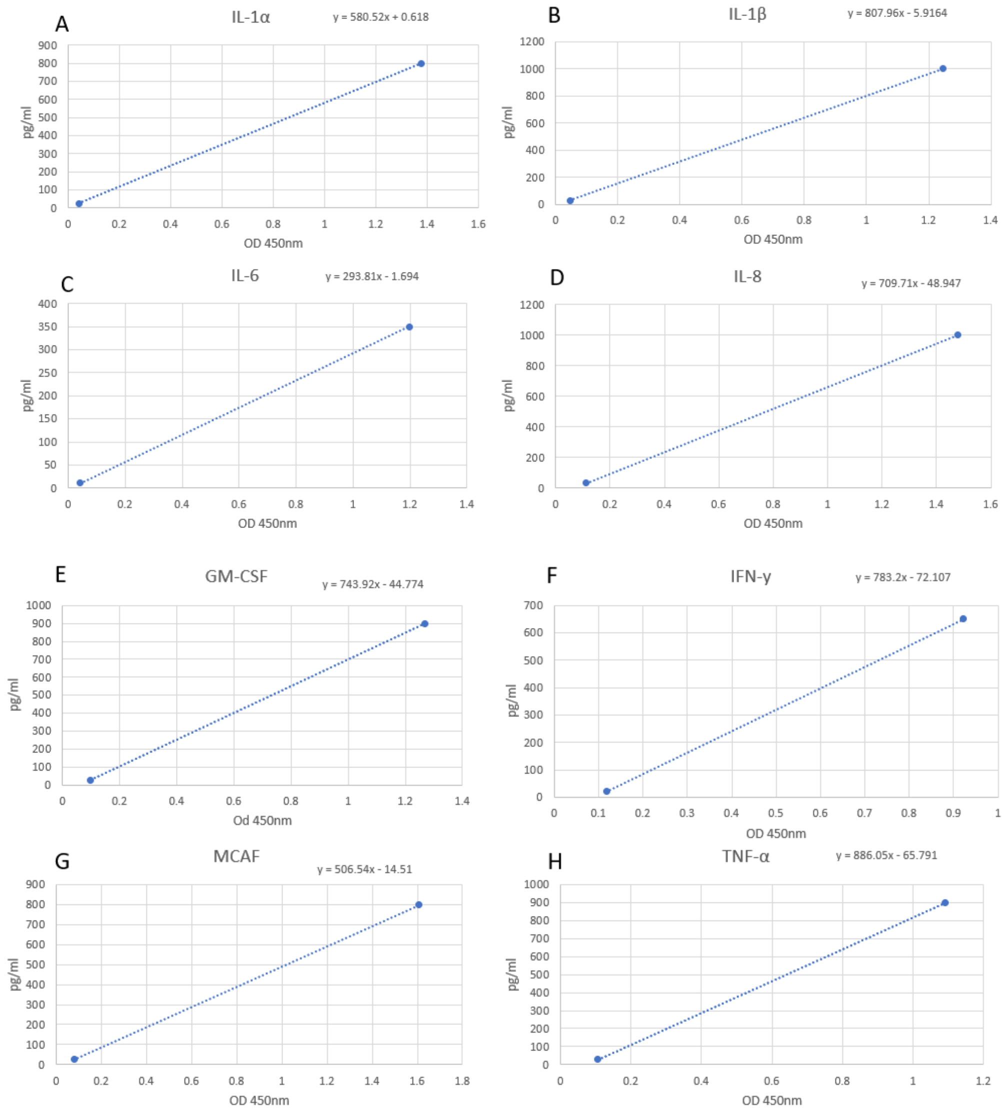
Supplementary Figure 1: Images taken using Bio-Rad's ZOE imager during macrophage differentiation protocol. Frame A shows undifferentiated THP-1 monocytes at the first day of differentiation. Frames B, C, and D show polarized M0, M1 and M2 macrophages respectively at the end of the protocol.



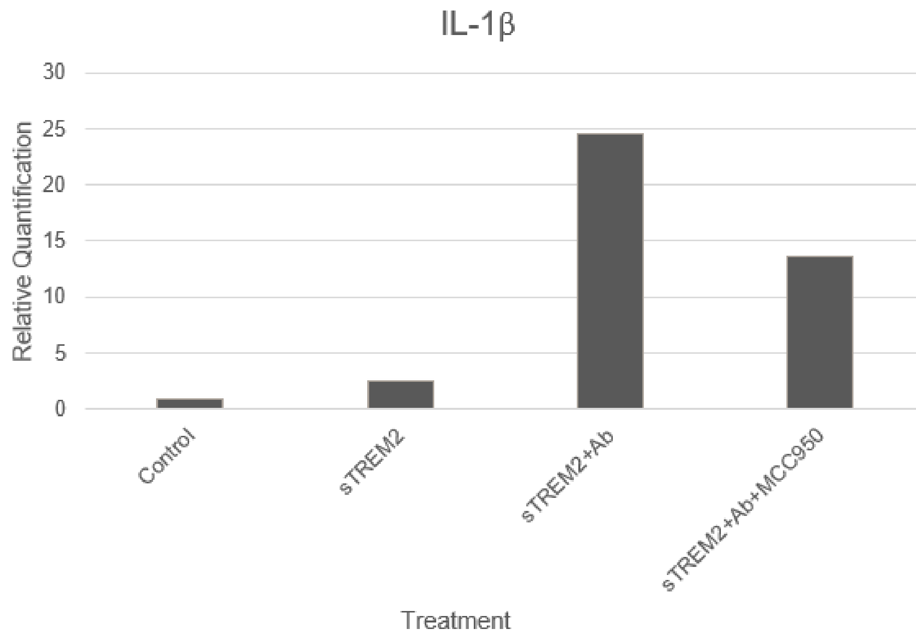
Supplementary Figure 2: ELISA standard curve used for cytokine quantification of TNF-α using RayBio kits.



Supplementary Figure 3: ELISA standard curve used for cytokine quantification of IL-1β using RayBio kits.



Supplementary Figure 4: Multiplex human ELISA standard curves generated for cytokine quantification in this study. (A) IL-1α, (B) IL-1β, (C) IL-6, (D) GM-CSF, (F) IFN-γ, (G) MCAF, (H) TNF-α.



Supplementary Figure 5: THP-1 cells were treated for 6 hours with 0.1µg/mL sTREM2, 0.1µg/mL sTREM2+mAB, and 0.1µg/mL sTREM2+mAB with MCC950. qPCR results show that MCC950 was able to partially inhibit expression of IL-1β.