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TREM2: Gene Expression and Role in Alzheimer's Disease

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TREM2: Gene Expression and Role in Alzheimer's Disease

THESIS

A thesis submitted in partial fulfillment of the
requirements for the degree of Master of Medical Science in the
College of Medicine
at the University of Kentucky

By

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2022

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ABSTRACT OF THESIS

TREM2: Gene Expression and Role in Alzheimer's Disease

Genetic mutations in the *TREM2* gene are highly correlated with risk of Alzheimer's disease, but alternative splicing patterns of *TREM2* transcripts have not been fully described. Characterization of *TREM2* alternative splicing will be of significant use to the scientific community as the field of Alzheimer's disease research progresses.

The goal of this study was to fully describe splicing patterns in *TREM2*, as different splicing isoforms of genes can alter express and/or function of the final protein. Human blood and anterior cingulate cortex brain tissue from 61 individual donors was processed and used for PCR and quantitative PCR as well as western blotting in order to identify and quantify novel *TREM2* isoforms.

Previously described transcripts of *TREM2* which lacked exons 3 or 4, or which retained part of intron 3 were replicated in this study. Additionally, we identified a novel isoform lacking exon 2, *D2-TREM2*, as well as several novel isoforms lacking multiple exons. *D2-TREM2* mRNA in the brain comprised approximately 10% of total *TREM2* RNA in the brain. Quantitative expression of *TREM2* and frequency *D2-TREM2* were compared between subjects with and without Alzheimer's disease, revealing no significant difference between the two groups. The novel splice isoforms identified in this study were found across multiple tissue types. *D2-TREM2* was found with similar frequency in non-brain tissues, ranging from 5.3-13.0%. *D2-TREM2* was found to be translated to protein and shares localization and cell trafficking patterns with full-length *TREM2*. Both D2-TREM2 and full-length TREM2 are predominantly retained in the Golgi complex. Additionally, D2-TREM2 was confirmed to be expressed in the brains of subjects with Alzheimer's disease as well as those without Alzheimer's disease.

Exon 2 of *TREM2* encodes for the ligand-binding domain, which is essential for its function as a receptor and thus function of cells on which it is present, such as microglia in the central nervous system. As *D2-TREM2* lacks exon 2, we hypothesize that *D2-TREM2* may inhibit full-length *TREM2* function and that targeting *TREM2* splicing may offer a novel therapeutic approach for Alzheimer's disease.

KEYWORDS: *TREM2*, Alzheimer's Disease, Gene Splicing, Gene Expression, Microglia

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TREM2: Gene Expression and Role in Alzheimer's Disease

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CHAPTER 1. TREM2: BACKGROUND

Abstract

Polymorphisms in the *TREM2* gene are considered one of the strongest risk factors for Alzheimer's disease. Several mutations have been identified and described but splicing of the *TREM2* gene has not been fully characterized previously. *TREM2* splicing isoforms with exon 3 or 4 skipped or intron 3 retained have been reported, and in this study, we describe novel isoforms. TREM2 remains a significant topic in the field of Alzheimer's disease research, and characterization of TREM2 may prove essential to the ongoing mission in the medical field of developing treatments for Alzheimer's disease. The purpose of this introduction is to provide background information on the genetics of the *TREM2* gene, the physiology of the TREM2 protein, and the role these play as a mechanism in human disease.

TREM2 Physiology

TREM2, or *triggering receptor expressed on myeloid cells 2*, is a protein in the immunoglobulin family of proteins expressed on myeloid lineage cells including osteoclasts, microglia, and other phagocytes[1]. The TREM2 protein presents both as a transmembrane receptor protein and as soluble TREM2 (sTREM2) after proteolytic cleavage or expression of the transcript lacking the transmembrane domain[2]. The *TREM2* gene contains 5 exons, each coding for a portion of the 230-amino acid full-length protein. Exon 1 codes for the cell-translocation signal peptide, prompting the cell to move the full-length protein to the endoplasmic reticulum. Exon 2 codes for an immunoglobulin-like V-set (IgV) domain, responsible for interaction of TREM2 with ligands. Exon 3 codes for a short stalk, connecting the IgV domain with the transmembrane domain, encoded by exon 4. Finally, exon 5 codes for the cytosolic tail of TREM2[3]. Each exon and its corresponding amino acid sequence is vital for proper function of full-length TREM2.

In its transmembrane form, TREM2 binds ligands and regulates several crucial physiological roles, including cell survival and proliferation, phagocytosis, and immune cell function. TREM2 is of particular significance in its role in the central nervous system (CNS) via regulation of microglial functions[4]. In particular, TREM2 stimulation in microglia is a strong activator for phagocytosis. Interestingly, TREM2 can stimulate phagocytosis in microglia without inducing inflammation via the NF-κB pathway, which promotes expression of pro-inflammatory cytokine and chemokine genes. This is contrary to other activators of phagocytosis, such as toll-like receptors (TLRs), which are often strong activators of the NF-κB pathway. Experimentally, overexpression of TREM2

in microglia has been shown to induce phagocytosis of apoptotic neurons while simultaneously reducing transcription of pro-inflammatory cytokines such as IL-1β and TNF-α[5].

TREM2 signaling is transmitted primarily through the DAP12 co-receptor signaling subunit, while also displaying interactions with DAP10. When TREM2 binds a ligand, TREM2 and DAP12 interact in the membrane via oppositely charged residues. Upon initiation of this interaction, DAP12 cytosolic residues in its Immunoreceptor Tyrosine-Based Activation Motifs (ITAMs) are phosphorylated and facilitate intracellular transduction of the TREM2 signal[6]. This interaction of TREM2 with DAP12 results in mobilization of calcium, activation of protein kinases, and remodeling of actin[7].

TREM2 in Human Disease

Nasu-Hakola Disease

Polymorphisms in the *TREM2* gene are strongly associated with several human diseases. One of the first diseases implicating *TREM2* mutations as a cause is polycystic lipomembranous osteodysplasia with sclerosing leukoencephalopathy, also known as PLOSL or Nasu-Hakola disease. Nasu-Hakola disease presents as early-onset dementia associated with microglial dysfunction and presence of bone cysts due to chronic dysfunction of osteoclasts, a key cell type in the bone which mediates bone remodeling[8]. The polymorphisms in *TREM2* associated with Nasu-Hakola disease include missense mutations, which are changes in a single base pair within a codon, changing the amino acid which is formed during translation of the transcript. The specific mutations for Nasu-Hakola disease include Y38C, W50C, T66M, and V126G. These

deviations from normal the normal *TREM2* gene alter the structure of the final TREM2 protein, causing steric issues and disrupting normal ligand binding. Dysfunction of the TREM2 protein due to these mutations prevents normal functioning of microglia and osteoclasts, resulting in characteristic Nasu-Hakola disease pathology[9].

Alzheimer's Disease

TREM2 mutations have also been implicated as a risk factor for Alzheimer's disease as well as contribution to the severity of the disease. As TREM2 is a key receptor in mediating microglial phagocytosis, mutations in the *TREM2* gene are associated with reduced ability to clear neuronal debris, including beta-amyloid (Aβ). This leads to build-up of Aβ, resulting in formation of Aβ "plaques", a key feature of Alzheimer's disease pathology[10]. Genome-wide association studies indicate that a particular *TREM2* mutation, R47H, increases late-onset Alzheimer's disease risk by 3-fold[11]. A 3-fold risk increase is similar to the risk of the apolipoprotein E (APOE) ε4 allele, a heavily implicated and widely studied Alzheimer's disease risk factor. The R47H mutation, similar to the mutations implicated in Nasu-Hakola disease, is proposed to disrupt normal ligand binding in TREM2, preventing activation and proper function of microglia in the CNS, including the aforementioned clearance of Aβ. In cell culture, the R47H mutation has been shown to disrupt proper cell surface expression of TREM2 and modify expression of other genes regulating immunity and proliferation. These effects overlapped significantly with *TREM2* knockout cells in culture, providing further evidence of the disruptive nature of the mutation[12]. Since the discovery of the R47H mutation and its role as a risk factor for Alzheimer's disease, a variety of other mutations in the *TREM2* gene have been discovered. The effects of many of these mutations remain

unknown. However, some mutations, such as R62H and rs7759295, have been found in subjects displaying significant Alzheimer's disease pathology[13]. Alternative splicing of *TREM2* has also been reported, focusing on specific splicing isoforms. While the majority of Alzheimer's disease-associated *TREM2* mutations are found on exon 2 of the *TREM2* gene, others have been found on exons 3 and 4 as well, and more may yet be undiscovered[14]. This led us to further investigate *TREM2* splicing patterns and seek to fully characterize splicing variations in the *TREM2* gene.

CHAPTER 2. MATERIALS AND METHODS

Characteristics and Preparation of Samples

Samples of blood and anterior cingulate cortex autopsy tissue from 61 donors were obtained from the University of Kentucky's Sanders-Brown Alzheimer's Disease Center. The characteristics and cDNA synthesis strategies for these samples have been previously described[15]. All research methodology was conducted under protocol number 48095 of the University of Kentucky Institutional Review Board. The samples obtained included both individuals with confirmed Alzheimer's disease pathology and individuals with no significant Alzheimer's disease pathology. Alzheimer's disease blood and tissue samples were from individuals aged 81.7 ± 6.2 years, while non-Alzheimer's disease samples were obtained from individuals aged 82.4 ± 8.7 years. Postmortem interval was 3.4 ± 0.6 hours for Alzheimer's disease subjects and 2.8 ± 8 hours for non-Alzheimer's disease subjects. Alzheimer's disease subjects were 55% female and 45% male, while non-Alzheimer's disease subjects were 46% female and 54% male. Previous (premortem) Mini-Mental State Exams were conducted on patients, and average scores were 11.9 ± 8 for Alzheimer's disease subjects and 28.4 ± 1.6 for non-Alzheimer's disease subjects. RNA samples were prepared using Qiagen RNeasy Lipid Tissue Mini Kits (Qiagen #74804) according to included protocols. Reverse transcription of RNA into cDNA was conducted using SuperScript IV (Invitrogen #18091050) according to included protocols. For the cross-tissue *TREM2* splicing analysis, fetal RNA from the various tissue types were obtained from Stratagene and cDNA preparation methods have been previously described[16]. Protein homogenate was obtained from hippocampal tissue of three Alzheimer's disease and three non-Alzheimer's disease subjects. Approximately 100mg of tissue was added to

sucrose buffer: 0.25M sucrose, 20mM EDTA, 20mM EGTA, 100mM Tris, pH of 7.4. The tissue was then homogenized with pestle in a microcentrifuge tube. One volume of 2X RIPA buffer (300mM NaCl, 2% NP-40, 2% w/v deoxycholate, 0.2% sodium dodecyl sulfate, 50mM Tris-HCl, pH of 7.4) was added to homogenized samples. Sucrose and RIPA buffers contained a final concentration of protease and phosphatase cocktails of 1X.

Cell Culture

HMC3 human microglial cell line was obtained from American Type Culture Collection (ATCC CRL-3304). Both HEK293 and HMC3 cells were maintained in Eagle's Modified Minimum Essential Medium, ATCC modification (ATCC 30-2003) with 10% fetal bovine serum defined (HyClone, GE Healthcare SH30070.03); 50U/mL penicillin, 50µg/mL streptomycin (Gibco 22400-089). Cells were grown at 37°C in 5% CO_2 and air atmosphere.

TREM2 Splice Isoform Identification: PCR and Sequencing

cDNA samples obtained from anterior cingulate cortex were amplified using *TREM2* exon 1 primers (5'-CCTGACATGCCTGATCCTCT-3') and exon 5 primers (5'-GTGTTCTTACCACCTCCCC-3') using Q5 high-fidelity hot-start polymerase (NEB #M0493L). Parameters for PCR thermocycling were as follows: 98°C 30 s; 98°C 5 s, 67°C 5 s, 72°C 45 s, 30 cycles; 72°C 2 min, 25°C hold. PCR products were run on 8% acrylamide gel and subsequently imaged on a BioRad ChemiDoc XR imager. Bands were extracted from these gels for amplification, using the same settings as above, and purified with Monarch PCR Cleanup Kits (NEB #T1030L). Purified PCR products were sequenced by

ACGT (Wheeling, IL) and compared to reference transcript NM_018965.4 to identify splicing patterns.

TREM2 **Transcript Quantification**

Quantitative PCR was used to quantify expression of *TREM2* transcripts. Primers corresponding to sequences within exons 1 and 2 were used for expression quantification of *TREM2* exon 2 (forward, 5'-CCTTGGCTGGGGAAGGG-3'; reverse, 5'-GGGCATCCTCGAAGCTCT-3') along with primers corresponding to exon 1-3 junction and within exon 3 to ultimately quantify the *TREM2* exon 2 skipped (*D2-TREM2*) isoform (forward, 5'-TTACTCTTTGTCACAGACCC-3'; reverse, 5'-GGGCATCCTCGAAGCTCT-3'). PCR was performed using initial 2 min incubation at 95°, followed by 40 cycles of 10 s at 95°C, 20 s at 60°C, and 20 s at 72°C. 20μL reactions contained 1μM of each primer, 1X PerfeCTa SYBR Green Super Mix (Quanta Biosciences), as well as 20ng cDNA. Samples were amplified in parallel using serially diluted standard generated by PCR of cDNA with indicated primers and subsequently purified and quantified by UV absorbance. Sample results were compared to standard curve to determine number of copies per sample. Overall *TREM2* expression was sum of copy numbers of *TREM2* exon 2 present and exon 2 skipped. All assays were performed in duplicate and normalized to *Iba1* expression as the housekeeping gene. Iba1 was used due to the fact that CNS TREM2 is expressed exclusively in microglia. For cross-tissue fetal cDNA comparisons, percent exon 2 skipping was calculated by dividing exon 2 skipped copies by sum of exon 2 skipped and mean exon 2 present copies. Cross-tissue comparison utilized six technical replicates.

TREM2 **Transcript Cloning**

TREM-2 and *D2-TREM2* cDNA transcripts for immunofluorescence experiments were amplified using PCR with primers corresponding to DNA sequences within exons 1 and 5, as above. For D2-TREM2 size standard used for Western Blot, *D2-TREM2* transcripts were cloned with the aforementioned exon 1 primers and reverse primer corresponding to DNA sequence in the 3' untranslated region (5'-CCAGCTAAATATGACAGTCTTGGA-3') in order to preclude an epitope tag. For PCR amplification, Platinum Taq (Invitrogen #10966034) was used with the following cycle parameters: 2 min at 94°C; 30 sec at 60°C, 2 min at 72°C (30 cycles), 7 min at 72°C, 25°C hold. For cloning, pcDNA 3.1-V5/His TOPO-TA cloning kit (Invitrogen #K480001) was used according to the included protocol. Clones were sequenced for verification by ACGT (Wheeling, IL) and grown at midi-scale production and purification with Qiagen Plasmid Plus Midiprep kits (Qiagen #12943).

Transfection of HMC3 and HEK293 Cells

Transfection of HMC3 human microglia and/or HEK293 cells was performed using Lipofectamine 3000 with Plus reagent (Invitrogen #L3000001) according to included protocols. Quantities used were: 0.8μL Lipofectamine, 1μL Plus reagent, 250ng plasmid per well in 8 well glass chamber slides (MatTek CCS-8). All cells were incubated for 24 hours, then HMC3 cells were processed for microscopy and HEK293 cells were processed for Western Blot.

Confocal Immunofluorescence Microscopy

HMC3 cells, after transfection, were fixed using 10% neutral buffered formalin (Fisher Scientific SF100-4) for 30 minutes and then blocked and permeabilized for 30 minutes using 10% goat serum (Sigma S26-LITER), 0.1% Triton X-100 (Fisher Scientific BP151-500) in PBS (Fisher BioReagents BP665-1). Primary and secondary antibodies were diluted in the above blocking and permeabilization buffers, then incubated at room temperature for 90 minutes. Cells were washed three times in the above blocking and permeabilization buffers in between primary and secondary antibody treatment, then three times in the above PBS prior to slide mounting with Prolong Glass using NucBlue mounting media (Invitrogen #P36981) and high-tolerance No. 1.5 cover glass (ThorLabs CG15KH1). Imaging was performed using a Nikon A1R HD inverted confocal microscope with 60X oil objective lens and NIS Elements AR software.

Western Blot

Protein quantification was performed using a Pierce 660 assay (Invitrogen #1861426) and 40µg loaded per well on 10-20% Tricine gel (Invitrogen #EC6625BOX) then transferred to a 0.22µm PVDF membrane. This membrane was blotted overnight with antibody against sequence within the TREM2 cytosolic domain (Cell Signaling #91068) and probed with goat and anti-rabbit secondary antibody AlexaFluor 800 (Invitrogen #A32735).

Statistical Analysis

Statistical analyses were performed using GraphPad Prism 8.4.2 software. Quantitative data were checked for normality by the D'Agostino & Pearson test. The normally distributed data were analyzed using Welch's t-test, and data not normally distributed were analyzed using a two-tailed Mann Whitney test[17].

CHAPTER 3. ANALYSIS OF *TREM2* SPLICING AND EXPRESSION

TREM2 Alternative Splicing in the Human Brain

TREM2 cDNA from anterior cingulate cortex was PCR-amplified with primers corresponding to sequence within exons 1 and 5. A gene map of *TREM2* is shown in Figure 1 (top), displaying introns and exons of the gene. Significant alternative splicing was found in the brains of both Alzheimer's disease and non-Alzheimer's disease individuals, shown on acrylamide gel in Figure 1. Each splicing isoform was identified and sequenced directly to confirm the nature of alternative splices. This study identified several novel splicing isoforms of *TREM2*, including isoforms with single exons skipped, multiple exons skipped, and intron sequence retained.

The study suggested that the *TREM2* isoform with exon 2 skipped (*D2-TREM2*) was the most abundant alternative splicing isoform. Thus, *TREM2* and *D2-TREM2* were quantified in an assay using multiple brain samples. The number of copies of *TREM2* was normalized to AIF1 (allograft inflammatory factor 1), a protein highly expressed in microglia, where *TREM2* is exclusively found in the CNS. The study suggests that total *TREM2* expression does not differ significantly between Alzheimer's disease and non-Alzheimer's disease individuals. However, in high-pathology vs. low-pathology Alzheimer's disease brains, *TREM2* expression appears to increase significantly with pathology (shown in Figure 2B; $p = 0.0014$, Welch's t-test). *D2-TREM2* expression was also investigated with respect to Alzheimer's disease status. *D2-TREM2* expression was found to be correlated with total *TREM2* expression (shown in Figure 2C), but no

significant difference was observed in *D2-TREM2* expression between Alzheimer's disease and non-Alzheimer's disease individuals.

Analysis of *TREM2* Alternative Splicing Across Tissue Types

Fetal cDNA libraries from several tissues; aorta, lung, kidney, heart, skeletal muscle, brain, and liver; were PCR amplified using primers from *TREM2* exons 1 and 5 to determine if *TREM2* alternative splicing occurs across tissue types. Each splicing isoform was observed in the study, despite individual tissues not all containing every isoform (Figure 3). Quantitatively, a high abundance of the isoform lacking exons 2 and 3 as well as the isoform lacking exons 2 and 4 were observed. It is worth noting that shorter DNA fragments are amplified more efficiency and thus PCR may have biased this result. *D2-TREM2* was abundant across all tissue types, similar to the findings in the adult brains. *D2-TREM2* frequency was similar across each tissue type (Figure 4).

Analysis of D2-TREM2 Localization

D2-TREM2 and full-length *TREM2* were cloned into expression vectors and transfected into HMC3 human microglial cells. Confocal microscopy was used to determine if D2-TREM2 is trafficked and localized in the same manner as full-length TREM2. Staining patterns were similar between full-length TREM2 (shown in Figure 5A) and D2-TREM2 (shown in Figure 5B). The pattern of staining has been previously reported, and the assay performed in this study showed consistent results[18]. Thus, D2-TREM2 and full-length TREM2 appear to localize in the same manner. Both D2-TREM2

and full-length TREM2 are primarily retained in the Golgi complex (D2-TREM2 shown in Figure 5C, full-length TREM2 shown in Figure 5D).

D2-TREM2 Protein in Human Hippocampus Samples

Hippocampal protein from Alzheimer's disease and non-Alzheimer's disease samples was subjected to Western blotting using antibody against the cytosolic tail of TREM2 (also present in D2-TREM2), shown in Figure 6. This assay was performed in order to determine if the D2-TREM2 protein is present in the human brain. Short exposure time of the blot (shown in Figure 6A) revealed proteins labeled by TREM2 antibody in transfected cells but not in non-transfected cells. This confirms that the bands on the Western blot are products of D2-TREM2. Long exposure of the blot (shown in Figure 6B) displays a doublet of bands at 12 and 13 kDa, matching the pattern of *D2-TREM2* transfected cells. The carboxyl-terminal fragment of TREM2 has a predicted molecular weight of 8 kDa and the predicted molecular weight of D2-TREM2 is 11 kDa. Based on this information, both carboxy-terminal fragments and D2-TREM2 migrate at a higher molecular weight than predicted, by 3 kDa. It can be concluded, based on the assay performed, that D2-TREM2 protein is present in both Alzheimer's disease and non-Alzheimer's disease brains.

Figure 1. Extensive Alternative Splicing of *TREM2*.

TREM2 undergoes significant alternative splicing. A diagram of the *TREM2* gene and its corresponding role in the mature protein is shown. Several novel splicing isoforms were identified, skipping one or more exons (numbered 1 through 5), or retaining part of an intron. cDNA from Alzheimer's disease subjects was compared to non-Alzheimer's disease subjects. Exons shown in grey (right) represent sequence after a frameshift mutation.

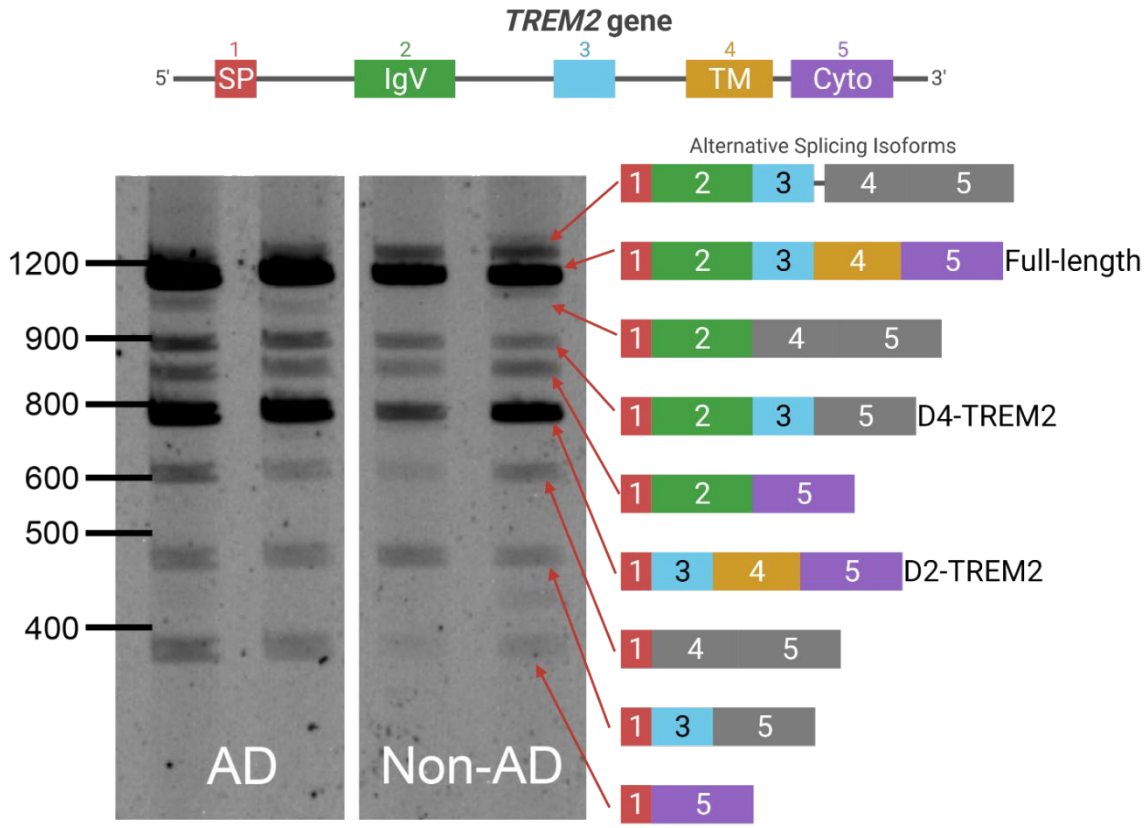


Figure 2. Quantification of *TREM2* and *D2-TREM2* in Human Brain Tissue.

qPCR was used to quantify *TREM2* transcripts. *TREM2* is normalized to AIF1 (expressed on microglia) to compare copy number of *TREM2* in Alzheimer's disease vs. non-Alzheimer's disease subjects (A). *TREM2* expression is compared between low-pathology and high-pathology subjects (B), p = 0.0014, Welch's t-test. Number of *D2-TREM2* copies and full-length *TREM2* copies is compared between Alzheimer's disease and non-Alzheimer's disease subjects (C). The percent of total *TREM2* copies that were *D2-TREM2* copies were compared between AD non non-AD subjects(D).

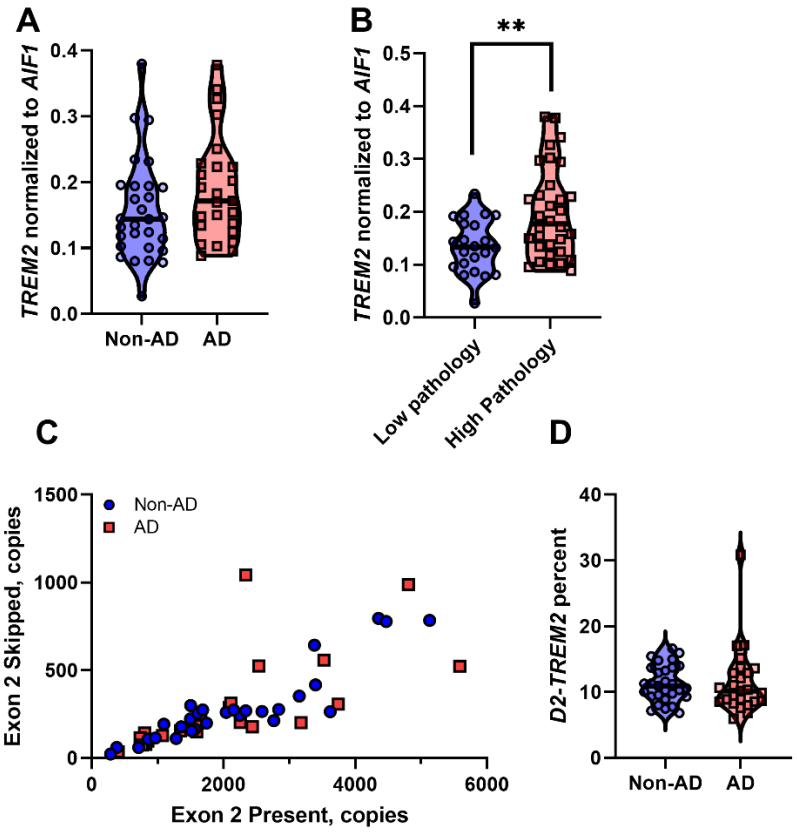


Figure 3. *TREM2* Alternative Splicing Patterns are Present Across Tissue Types. cDNA from fetal aorta, lung, kidney, heart, skeletal muscle, brain, and liver were analyzed for *TREM2* splicing. The splicing isoforms found in adult human brain tissue were present across tissues.

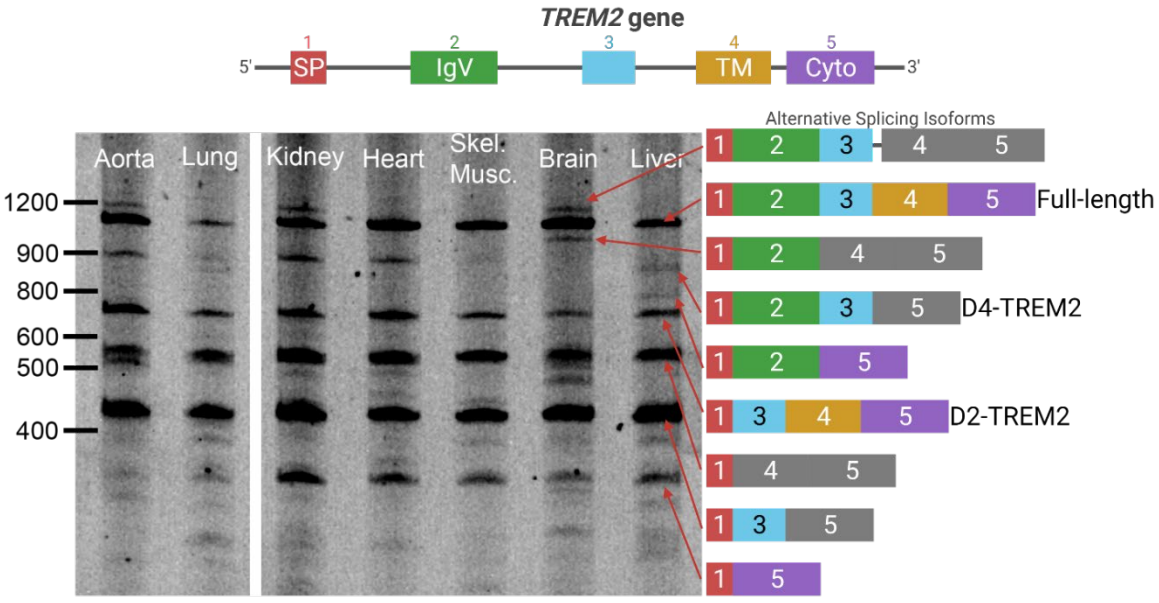


Figure 4. Quantification of *TREM2* and *D2-TREM2* Across Tissue Type.

cDNA from multiple fetal tissue types was quantified to investigate percent of *D2-TREM2* skipped among total *TREM2* copies. *D2-TREM2* was found to be an abundant splicing isoform across tissue types. Exon 2 is skipped at a frequency between 5.3-13%. Data points reflect technical replicates from pooled cDNA libraries.

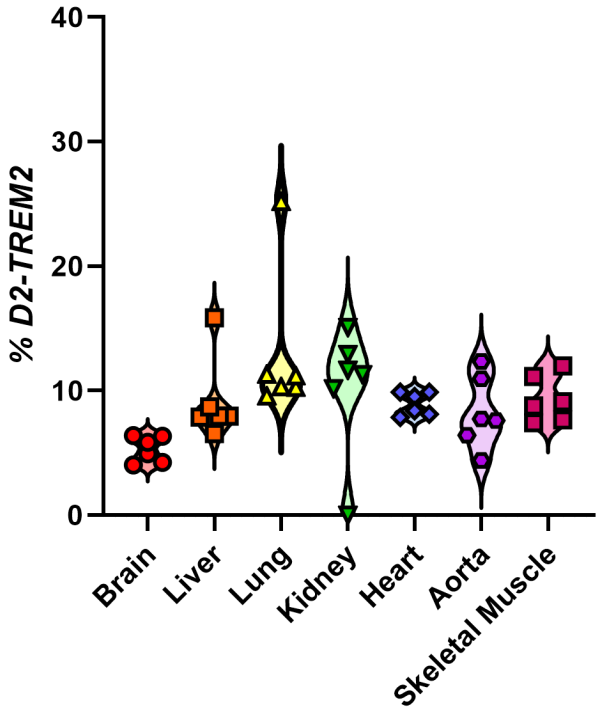


Figure 5. TREM2 and D2-TREM2 Share Localization Patterns.

Transfected HMC3 human microglial cells were analyzed with confocal immunofluorescence microscopy. Full-length TREM2 (A) and D2-TREM2 (B) exhibit similar staining patterns. Both D2-TREM2 (C) and full-length TREM2 (D) appear to be primarily retained in the Golgi complex.

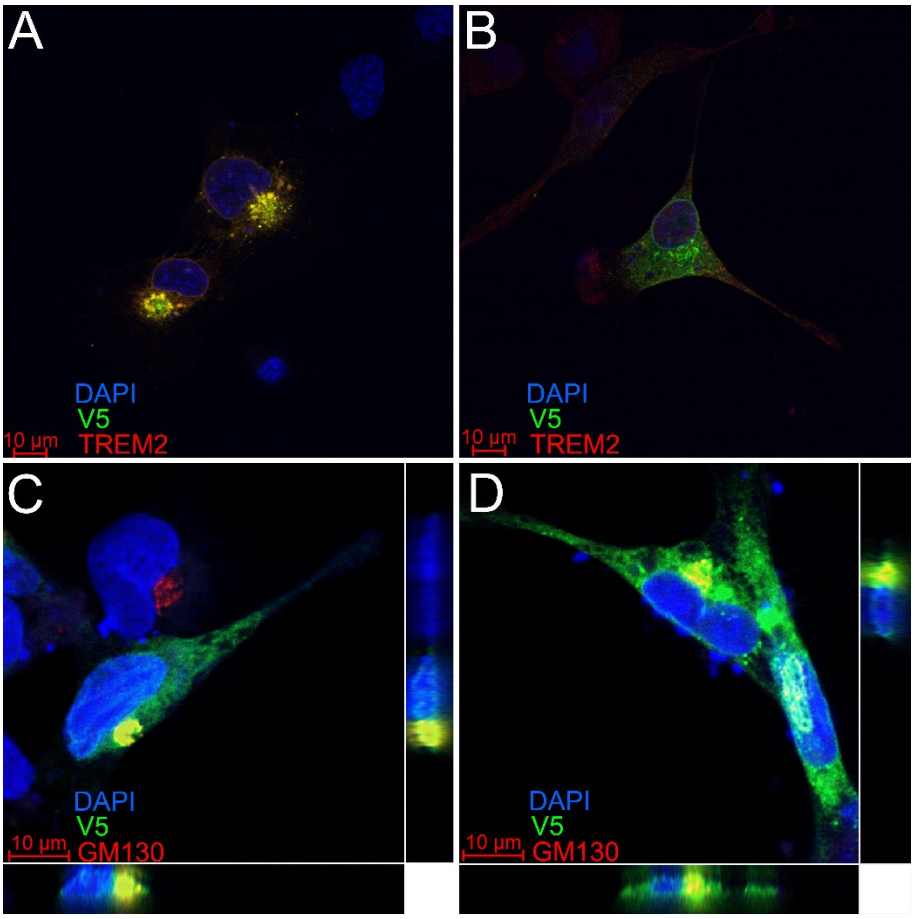
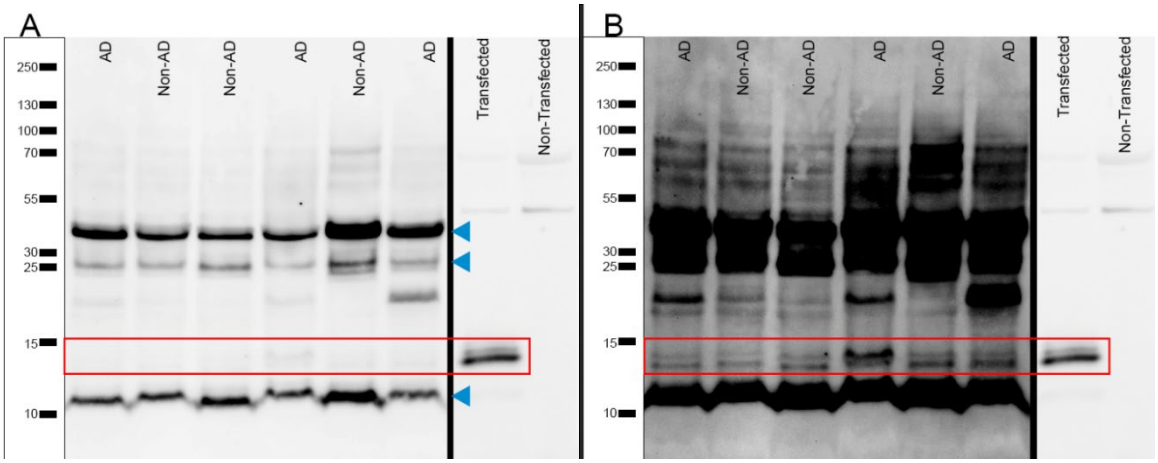


Figure 6. Western Blot of Human Brain Indicates Presence of D2-TREM2 Protein. Western blotting using an antibody tag recognizing the cytosolic tail of TREM2 was performed. Short exposure (A) shows two proteins labeled by TREM2 antibody in transfected cells, but not non-transfected cells, confirming presence of D2-TREM2. Longer exposure (B) shows a doublet of bands at 12 and 13 kDa, matching the pattern in *D2-TREM2* transfected cells. The bands are again present in transfected cells but not non-transfected cells, confirming presence of D2-TREM2.



CHAPTER 4. DISCUSSION AND FUTURE DIRECTION

Summary of Findings

The results of this study provide new insights into the splicing patterns of the *TREM2* gene. The findings show that *TREM2* undergoes more extensive splicing patterns than was previously published. Previously available literature has described a number of alternative splicing isoforms, such as sTREM2 lacking the transmembrane domain, exons 3 and 4 skipped individually, and intron 3 retained, but this study characterized the full range of *TREM2* splicing isoforms in the CNS. The *D2-TREM2* splicing isoform has been reported on recently, providing similar findings to the ones described in this study, including lack of immunoglobulin domain expression causing a loss of function in the final protein and similar cell trafficking characteristics to full-length TREM2[19 20]. This study identifies several novel splicing isoforms of *TREM2*, shown in Figure 1, which were not listed on gene library websites such as Ensembl. This provides the scientific community with a better understanding of the extent to which the *TREM2* gene undergoes alternative splicing and offers a potential basis on which to further investigate these isoforms.

The study also included investigation of whether a correlation exists between the common *D2-TREM2* splicing isoform and Alzheimer's disease pathology. Ultimately, it was determined that there was no significant correlation between frequency of the *D2-TREM2* isoform and Alzheimer's disease related pathology. This provides meaningful insight, as investigation of the TREM2 protein and underlying genetic mechanisms is considered an important current topic in the field of Alzheimer's disease research[21].

While there are still a wide range of questions to be answered regarding the mechanism behind the involvement of TREM2 in the progression of Alzheimer's disease pathology, establishing a strong understanding of the genetic background of *TREM2* is paramount.

Localization and cell trafficking patterns of the TREM2 protein were also investigated in this study. Presence of TREM2 on the cell surface and its receptor activity have been previously described[22], but localization of splicing isoforms of *TREM2* in comparison to the full-length protein have not been fully characterized. This study utilized confocal microscopy and immunofluorescent staining in order to determine if D2-TREM2 localized similarly to full-length TREM2. It was discovered that the D2-TREM2 isoform shares highly similar localization with the full-length protein: both were primarily located in the intracellular Golgi complex (shown in Figure 5C and 5D). Cell culture experiments indicate that TREM2 exhibits a "feed-forward" mechanism which elicits cellular activity in response to a stimulus. TREM2 within the transmembrane Golgi pool is trafficked to the cell surface due to calcium flux[18]. We speculate that D2-TREM2 may interfere with this mechanism, as it lacks the immunoglobulin domain which is vital for signaling that may lead to TREM2 trafficking within the cell.

Western blot also revealed that the D2-TREM2 isoform is present in the human brain as protein (shown in Figure 6). While the preliminary findings of this study confirmed that *D2-TREM2* mRNA is present and abundant in the human brain, the presence of the mature D2-TREM2 protein is a novel finding. The correlation between amount of D2-TREM2 mRNA and protein, when compared to full-length TREM2, is significantly lower. This may indicate that the D2-TREM2 protein is not readily

translated as efficiently in the cell, or that the protein itself loses stability when lacking the amino acid sequence corresponding to exon 2.

Future Direction

TREM2 as a Therapeutic Target

Genetic mutations in the *TREM2* gene have been known to be significant risk factors for Alzheimer's disease for many years[23]. Certain *TREM2* variants have some of the highest odds-ratios for late-onset Alzheimer's disease risk, surpassed only by the APOE4 gene, making it a heavily investigated topic in the field of Alzheimer's disease research[24 25]. In particular, the ligand-binding disruption, thought to be a partial loss-of-function, caused by the R47H and R62H mutations in *TREM2* are considered high-risk mutations for developing late-onset Alzheimer's disease[26 27]. We speculate that D2-TREM2 is functionally null and thus disrupts normal cell signaling and trafficking when present on the cell surface, which may lead to complications contributing to generation of Alzheimer's disease-related pathology. This would be similar to what has been shown in studies related to the CD33 gene, which also has a splicing isoform which lacks the ligand-binding domain[28].

Currently, there is no FDA-approved therapeutic for Alzheimer's disease targeting TREM2. There is one ongoing clinical trial targeting TREM2 with monoclonal antibodies, AL002 (Alector, NCT04592874), which is currently in phase 2 and has garnered the interest of the scientific community. It is possible that modulation of *TREM2* splicing patterns could attenuate onset and progression of Alzheimer's disease, though more research into the mechanisms behind TREM2 and its role in the disease will be

necessary. According to existing literature, the possibility also exists that sTREM2 is protective for Alzheimer's disease[29 30]. We report in this study that the *TREM2* isoform lacking exon 4 (*D4-TREM2*) lacks the transmembrane domain, making it likely to be secreted as sTREM2. This finding, combined with the possibility that sTREM2 is protective against Alzheimer's disease, suggests that targeting *TREM2* mRNA to enhance expression of the *D4-TREM2* isoform may have potential for therapeutics.

We speculate that the *D2-TREM2* isoform may act similarly to R47H and R62H variants, as D2-TREM2 lacks the immunoglobulin domain, which is essential for signaling, and the R47H and R62H mutations alter the immunoglobulin domain and change its function. *In vivo* studies have shown in mice that targeting TREM2 may serve to reduce Alzheimer's disease-related pathology. Targeting TREM2 with monoclonal antibodies in an R47H mouse model reduced amyloid plaques, neurite dystrophy, and inflammatory response over time[31]. These results suggest potential for a therapeutic that may slow the progression of Alzheimer's disease by targeting *TREM2* in patients with mutations to the gene that increase Alzheimer's disease risk. Interestingly, TREM2 function as it relates to Alzheimer's disease appears to change as the disease progresses. It has been suggested that TREM2 and sTREM2 are beneficial in the early stages of Alzheimer's disease progression but exacerbates pathology later in the disease[32]. TREM2 microglial function plays a significant role in the immune inflammatory response, thus exacerbation of inflammation in later stages of Alzheimer's disease likely worsens related pathology. If the role of TREM2 in Alzheimer's disease indeed changes over the course of the disease, targeting *TREM2* splicing in a patient-specific manner may

prove to be a valuable therapeutic strategy. A model of a potential way to exploit *TREM2* as a therapeutic is shown in Figure 7.

mRNA Therapeutics

Targeting intracellular mRNA with therapeutic drugs has become a prominent topic of biomedical research. Antisense oligonucleotide (ASO), antisense RNA (asRNA), short interfering RNA (siRNA), and micro-RNA (miRNA) are blossoming as classes of drugs that target mRNA sequence in order to interfere with the processes which allow it to be translated into protein[33]. These drugs are DNA or RNA molecules themselves and contain sequence complementary to the targeted mRNA. When interacting with the target mRNA, the drug-mRNA complex effectively prevents gene expression by preventing translation or via induction of RNase activity which cleaves the double-stranded RNA or DNA-RNA complex[34]. In particular, antisense oligonucleotides have shown promising results in treating genetic diseases such as spinal muscular atrophy and age-related macular degeneration.

There is a growing list FDA-approved drugs targeting mRNA in the United States. One of the most prominent is nusinersen, marketed under the brand name Spinraza®. Nusinersen is an antisense oligonucleotide used to treat 5q spinal muscular atrophy (SMA), which is caused by mutations in the survival motor neuron 1 (*SMN1*) gene. These mutations prevent the production of functional protein from *SMN1* transcripts and leave only the survival motor neuron 2 gene (*SMN2*, which has a near-identical sequence to *SMN1*) to produce the survival motor neuron protein. SMN2 is expressed at significantly lower rates than SMN1 in healthy individuals due to splicing differences causing truncation of *SMN2* mRNA. Thus, SMN1 protein is produced at

insufficient levels in individuals with *SMN1* mutations, leading to widespread muscle atrophy which is fatal without treatment. Nusinersen targets *SMN2* pre-messenger (pre-splice) RNA in order to alter splicing and ultimately produce more mature full-length mRNA which can be translated into survival motor neuron protein[35]. Effectively, this drug makes up for the lack of protein produced from *SMN1* transcripts by increasing the protein production viability of *SMN2* transcripts. Nusinersen significantly increases quality of life of SMA patients, including positive outcome criteria such as walking independently and ability to swallow.

While there is currently no FDA-approved drug targeting *TREM2* mRNA transcripts, the potential exists for an oligonucleotide-based therapeutic. Though more research is necessary to pinpoint if and when splicing variants of *TREM2* modulate Alzheimer's disease related pathology, antisense oligonucleotide technology could be used to silence particular variants of the *TREM2* transcript. If TREM2 signaling is protective in early Alzheimer's disease, decreasing expression of *D2-TREM2* may be beneficial to slow progression of the disease. If TREM2 signaling exacerbates Alzheimer's disease pathology later progression, decreasing expression of *FL-TREM2* may also slow this stage of progression by limiting the number of functional TREM2 receptors on cells. An example of antisense oligonucleotides accomplishing these goals is shown in Figure 8.

Figure 7. Model to Exploit *TREM2* Splicing as a Potential Alzheimer's Disease Therapeutic.

Early in Alzheimer's disease, TREM2 signaling may be protective. Thus, decreasing *D2-TREM2* and increasing full-length *TREM2* may be beneficial (left). Conversely, TREM2 may be detrimental later in Alzheimer's disease, which may make decreasing full-length *TREM2* and increasing *D2-TREM2* beneficial by increasing the number of functionally null receptors (right).

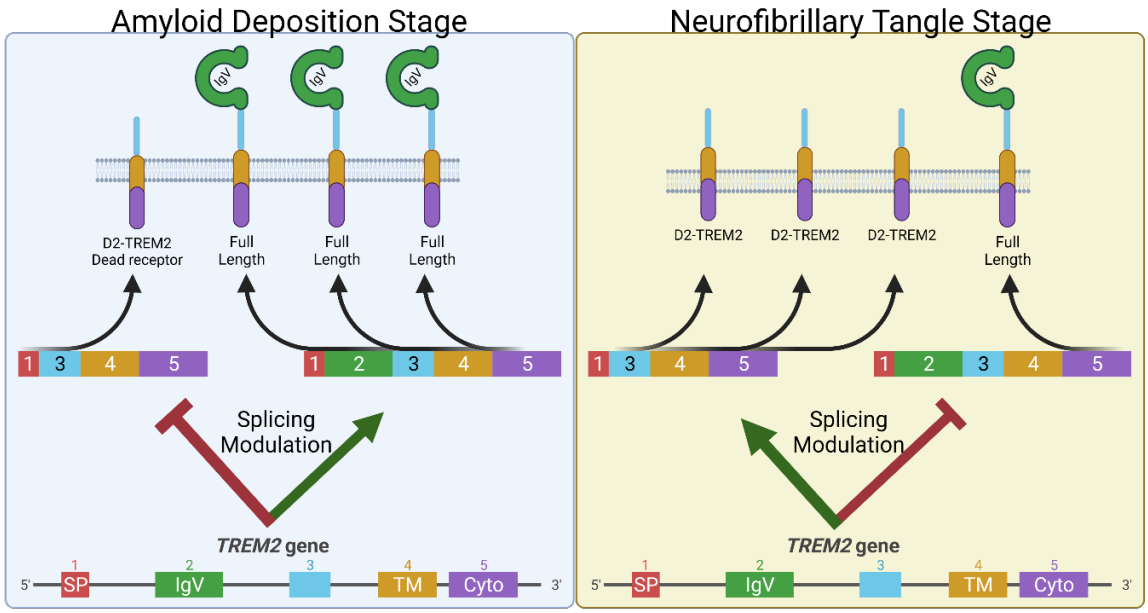
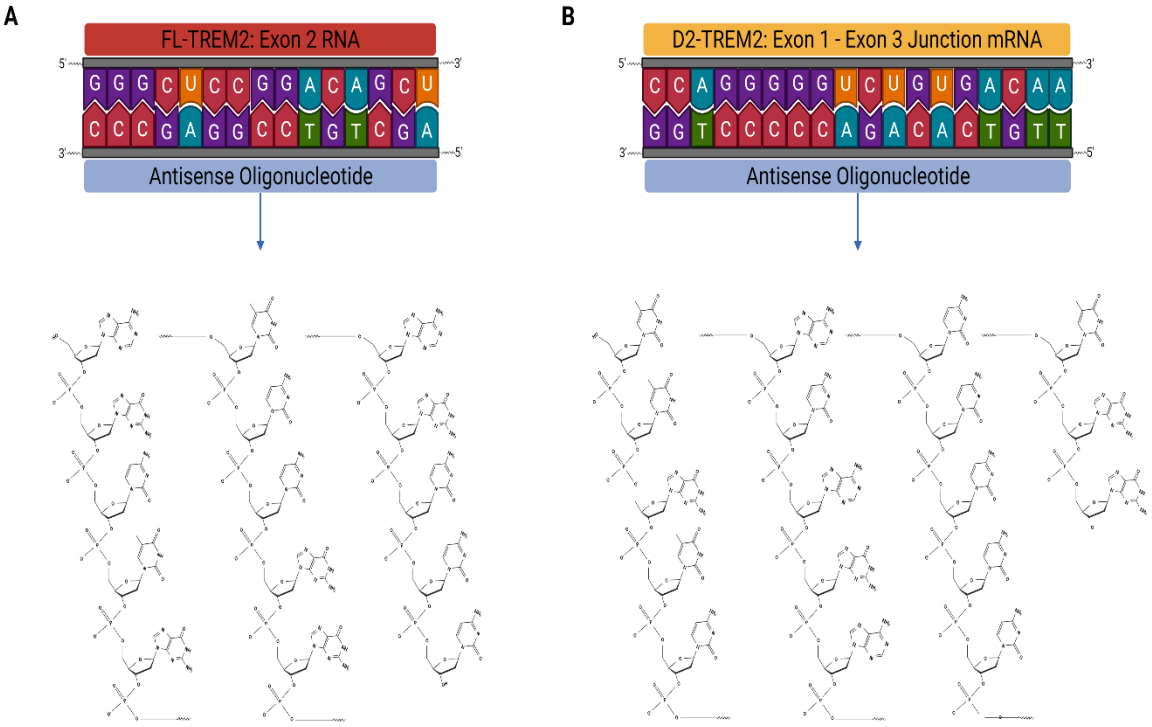


Figure 8. Antisense Oligonucleotide Model Targeting *FL-TREM2* or *D2-TREM2*

Model using antisense oligonucleotides to target *FL-TREM2* or *D2-TREM2*. An oligonucleotide against *FL-TREM2* targets sequence at the beginning of exon 2 RNA (A) in order to reduce *FL-TREM2* expression. An oligonucleotide against *D2-TREM2* targets sequence at the exon 1 – exon 3 junction (B), a feature not present in *FL-TREM2*, in order to reduce *D2-TREM2* expression.



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REFERENCES

1. Ford JW, McVicar DW. TREM and TREM-like receptors in inflammation and disease. Curr Opin Immunol 2009;**21**(1):38-46 doi: 10.1016/j.coi.2009.01.009.
2. Zhang XM, Liu J, Cao M, et al. TREM2: A Novel Potential Biomarker of Alzheimer's Disease. Biomed Environ Sci 2021;**34**(9):719-24 doi: 10.3967/bes2021.099.
3. Feuerbach D, Schindler P, Barske C, et al. ADAM17 is the main sheddase for the generation of human triggering receptor expressed in myeloid cells (hTREM2) ectodomain and cleaves TREM2 after Histidine 157. Neurosci Lett 2017;**660**:109-14 doi: 10.1016/j.neulet.2017.09.034.
4. Yang J, Fu Z, Zhang X, Xiong M, Meng L, Zhang Z. TREM2 ectodomain and its soluble form in Alzheimer's disease. J Neuroinflammation 2020;**17**(1):204 doi: 10.1186/s12974-020-01878-2.
5. Takahashi K, Rochford CD, Neumann H. Clearance of apoptotic neurons without inflammation by microglial triggering receptor expressed on myeloid cells-2. J Exp Med 2005;**201**(4):647-57 doi: 10.1084/jem.20041611.
6. Deczkowska A, Weiner A, Amit I. The Physiology, Pathology, and Potential Therapeutic Applications of the TREM2 Signaling Pathway. Cell 2020;**181**(6):1207-17 doi: 10.1016/j.cell.2020.05.003.
7. Kober DL, Brett TJ. TREM2-Ligand Interactions in Health and Disease. J Mol Biol 2017;**429**(11):1607-29 doi: 10.1016/j.jmb.2017.04.004.
8. Paloneva J, Manninen T, Christman G, et al. Mutations in two genes encoding different subunits of a receptor signaling complex result in an identical disease phenotype. Am J Hum Genet 2002;**71**(3):656-62 doi: 10.1086/342259.
9. Dash R, Choi HJ, Moon IS. Mechanistic insights into the deleterious roles of Nasu-Hakola disease associated TREM2 variants. Sci Rep 2020;**10**(1):3663 doi: 10.1038/s41598-020-60561-x.
10. Tiwari S, Atluri V, Kaushik A, Yndart A, Nair M. Alzheimer's disease: pathogenesis, diagnostics, and therapeutics. Int J Nanomedicine 2019;**14**:5541-54 doi: 10.2147/IJN.S200490.
11. Guerreiro R, Wojtas A, Bras J, et al. TREM2 variants in Alzheimer's disease. N Engl J Med 2013;**368**(2):117-27 doi: 10.1056/NEJMoa1211851.
12. Hall-Roberts H, Agarwal D, Obst J, et al. TREM2 Alzheimer's variant R47H causes similar transcriptional dysregulation to knockout, yet only subtle functional phenotypes in human iPSC-derived macrophages. Alzheimers Res Ther 2020;**12**(1):151 doi: 10.1186/s13195-020-00709-z.
13. Qin Q, Teng Z, Liu C, Li Q, Yin Y, Tang Y. TREM2, microglia, and Alzheimer's disease. Mech Ageing Dev 2021;**195**:111438 doi: 10.1016/j.mad.2021.111438.
14. Del-Aguila JL, Benitez BA, Li Z, et al. TREM2 brain transcript-specific studies in AD and TREM2 mutation carriers. Mol Neurodegener 2019;**14**(1):18 doi: 10.1186/s13024-019-0319-3.
15. Zou F, Gopalraj RK, Lok J, et al. Sex-dependent association of a common low-density lipoprotein receptor polymorphism with RNA splicing efficiency in the brain and Alzheimer's disease. Hum Mol Genet 2008;**17**(7):929-35 doi: 10.1093/hmg/ddm365.

16. Burchett ME, Ling IF, Estus S. FBN1 isoform expression varies in a tissue and development-specific fashion. Biochem Biophys Res Commun 2011;**411**(2):323-8 doi: 10.1016/j.bbrc.2011.06.140.
17. Shaw BC, Snider HC, Turner AK, Zajac DJ, Simpson JF, Estus S. An Alternatively Spliced TREM2 Isoform Lacking the Ligand Binding Domain is Expressed in Human Brain. J Alzheimers Dis 2022;**87**(4):1647-57 doi: 10.3233/JAD-215602.
18. Prada I, Ongania GN, Buonsanti C, Panina-Bordignon P, Meldolesi J. Triggering receptor expressed in myeloid cells 2 (TREM2) trafficking in microglial cells: continuous shuttling to and from the plasma membrane regulated by cell stimulation. Neuroscience 2006;**140**(4):1139-48 doi: 10.1016/j.neuroscience.2006.03.058.
19. Han S, Na Y, Koh I, Nho K, Lee Y. Alternative Splicing Regulation of Low-Frequency Genetic Variants in Exon 2 of TREM2 in Alzheimer's Disease by Splicing-Based Aggregation. Int J Mol Sci 2021;**22**(18) doi: 10.3390/ijms22189865.
20. Kiianitsa K, Kurtz I, Beeman N, et al. Novel TREM2 splicing isoform that lacks the V-set immunoglobulin domain is abundant in the human brain. J Leukoc Biol 2021;**110**(5):829-37 doi: 10.1002/JLB.2HI0720-463RR.
21. Wolfe CM, Fitz NF, Nam KN, Lefterov I, Koldamova R. The Role of APOE and TREM2 in Alzheimer's Disease-Current Understanding and Perspectives. Int J Mol Sci 2018;**20**(1) doi: 10.3390/ijms20010081.
22. McQuade A, Kang YJ, Hasselmann J, et al. Gene expression and functional deficits underlie TREM2-knockout microglia responses in human models of Alzheimer's disease. Nat Commun 2020;**11**(1):5370 doi: 10.1038/s41467-020-19227-5.
23. Jonsson T, Stefansson H, Steinberg S, et al. Variant of TREM2 associated with the risk of Alzheimer's disease. N Engl J Med 2013;**368**(2):107-16 doi: 10.1056/NEJMoa1211103.
24. Karch CM, Goate AM. Alzheimer's disease risk genes and mechanisms of disease pathogenesis. Biol Psychiatry 2015;**77**(1):43-51 doi: 10.1016/j.biopsych.2014.05.006.
25. Wang Z, Zhang Q, Lin JR, et al. Deep post-GWAS analysis identifies potential risk genes and risk variants for Alzheimer's disease, providing new insights into its disease mechanisms. Sci Rep 2021;**11**(1):20511 doi: 10.1038/s41598-021-99352-3.
26. Gratuze M, Leyns CE, Sauerbeck AD, et al. Impact of TREM2R47H variant on tau pathology-induced gliosis and neurodegeneration. J Clin Invest 2020;**130**(9):4954-68 doi: 10.1172/JCI138179.
27. Dean HB, Roberson ED, Song Y. Neurodegenerative Disease-Associated Variants in TREM2 Destabilize the Apical Ligand-Binding Region of the Immunoglobulin Domain. Front Neurol 2019;**10**:1252 doi: 10.3389/fneur.2019.01252.
28. Estus S, Shaw BC, Devanney N, Katsumata Y, Press EE, Fardo DW. Evaluation of CD33 as a genetic risk factor for Alzheimer's disease. Acta Neuropathol 2019;**138**(2):187-99 doi: 10.1007/s00401-019-02000-4.
29. Zhong L, Xu Y, Zhuo R, et al. Soluble TREM2 ameliorates pathological phenotypes by modulating microglial functions in an Alzheimer's disease model. Nat Commun 2019;**10**(1):1365 doi: 10.1038/s41467-019-09118-9.

30. Ewers M, Biechele G, Suarez-Calvet M, et al. Higher CSF sTREM2 and microglia activation are associated with slower rates of beta-amyloid accumulation. EMBO Mol Med 2020;**12**(9):e12308 doi: 10.15252/emmm.202012308.
31. Wang S, Mustafa M, Yuede CM, et al. Anti-human TREM2 induces microglia proliferation and reduces pathology in an Alzheimer's disease model. J Exp Med 2020;**217**(9) doi: 10.1084/jem.20200785.
32. Gratuze M, Leyns CEG, Holtzman DM. New insights into the role of TREM2 in Alzheimer's disease. Mol Neurodegener 2018;**13**(1):66 doi: 10.1186/s13024-018-0298-9.
33. Yu AM, Choi YH, Tu MJ. RNA Drugs and RNA Targets for Small Molecules: Principles, Progress, and Challenges. Pharmacol Rev 2020;**72**(4):862-98 doi: 10.1124/pr.120.019554.
34. Di Fusco D, Dinallo V, Marafini I, Figliuzzi MM, Romano B, Monteleone G. Antisense Oligonucleotide: Basic Concepts and Therapeutic Application in Inflammatory Bowel Disease. Front Pharmacol 2019;**10**:305 doi: 10.3389/fphar.2019.00305.
35. Hoy SM. Nusinersen: A Review in 5q Spinal Muscular Atrophy. CNS Drugs 2018;**32**(7):689-96 doi: 10.1007/s40263-018-0545-1.

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