Novel TREM2 splicing isoform that lacks the V-set immunoglobulin domain is abundant in the human brain.

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Abstract

TREM2 is an immunoglobulin-like receptor expressed by certain myeloid cells, such as macrophages, dendritic cells, osteoclasts and microglia. In the brain, TREM2 plays an important role in the immune function of microglia, and its dysfunction is linked to various neurodegenerative conditions in humans. Ablation of TREM2 or its adaptor protein TYROBP causes Polycystic Lipo-Membranous Osteodysplasia with Sclerosing Leukoencephalopathy (also known as Nasu-Hakola disorder) with early onset of dementia, while some missense variants in TREM2 are associated with an increased risk of late-onset Alzheimer's disease. Human TREM2 gene is a subject to alternative splicing, and its major, full-length canonical transcript encompasses 5 exons. Herein, we report a novel alternatively spliced TREM2 isoform without exon 2 (Δ e2), which constitutes a sizable fraction of TREM2 transcripts and has highly variable inter-individual expression in the human brain (average frequency 10%; range 3.7-35%). The protein encoded by Δ e2 lacks a V-set immunoglobulin domain from its extracellular part but retains its transmembrane and cytoplasmic domains. We demonstrated Δ e2 protein expression in TREM2-positive THP-1 cells, in which the expression of full-length transcript was precluded by CRISPR/Cas9 disruption of the exon 2 coding frame. In “add-back” experiments, overexpression of full-length, but not Δ e2 TREM2, restored phagocytic capacity and promoted interferon type I response in the knockout cells. Our findings suggest that expression of a Δ e2 splice isoform may modify the dosage of full-length transcript potentially weakening some TREM2 receptor functions in the human brain.

Introduction

Triggering receptor expressed on myeloid cells 2 (TREM2) is an immunoglobulin-like molecule expressed by some myeloid cells, such as macrophages, dendritic cells, osteoclasts and microglia. It is an important regulator of the innate immune response and its deficiency is linked to several neurodegenerative disorders in humans (1). TREM2 forms a signaling receptor complex with TYROBP adaptor protein. Loss-of-function (LOF) bi-allelic mutations in TREM2 or TYROBP cause Polycystic Lipo-Membranous Osteodysplasia with Sclerosing Leukoencephalopathy (PLOSL; OMIM # 618193 and # 221770), a recessive disorder that presents with early onset dementia and bone cysts (2), or familial Frontotemporal Dementia without bone cysts (FTD) (3),(4),(5). PLOSL patients appear cognitively and neurologically normal till the 4th decade, highlighting a neuroprotective role of TREM2 in adult/aging microglia. The TREM2 missense variants R47H and R62H are associated with an increased risk of Alzheimer's disease (AD) (OR 2.8-4.6 (6),(7) and 1.4-2.4 (8),(9), respectively), and R47H may also be linked to Parkinson's disorder (10) and Amyotrophic Lateral Sclerosis (11). TREM2 is involved in multiple aspects of myeloid cell function (12). In microglia, it regulates chemotaxis, enhances phagocytosis (13) and influences survival, proliferation and differentiation (14). Recently, we demonstrated a novel role of TREM2 in the regulation of antiviral interferon type I response (IFN I) in myeloid cells and exaggerated response in the brain of R47H carriers with AD (15). TREM2 binds lipids and lipoproteins (16), including the known AD risk factors ApoE and ApoJ/CLU (17). Another TREM2 ligand is amyloid beta peptide (A β) (18), a major constituent of amyloid deposits in AD brains. TREM2 binding to A β activates microglia cytokine production and degradation of internalized peptide. A soluble form of TREM2, a

product of cleavage or alternative splicing, may have a separate function as an extracellular signaling molecule that promotes cell survival (19).

The human TREM2 gene is represented by several alternatively spliced transcripts (Fig. 1A). A major canonical form, ENST00000373113, comprises 5 exons and encodes the full-length transmembrane receptor protein. Two minor isoforms are products of alternative splicing of exon 4 that encodes the transmembrane domain. In ENST00000338469, exon 4 is skipped, while in ENST00000373122 exon 4 has an alternative start that changes its coding sequence. As a result, both isoforms lack the transmembrane domain and are thought to be secreted proteins. All three TREM2 isoforms are expressed in the human cortex (8). Herein, we report a novel alternatively spliced TREM2 transcript lacking exon 2 which constitutes a sizable fraction of TREM2 transcripts in the brain and has functional activities different from the canonical TREM2 receptor.

Materials and Methods

Brain tissues for RNA analyses. The study was approved by the Institutional Review Board of the University of Washington. Postmortem human brain tissues of cognitively normal individuals were provided by the UW Neuropathology Core Brain Bank. Permission was obtained from donors for brain autopsy and the use of tissues for research purposes. The average post-mortem interval was 4.2 hr (range 2.5-6 hr). Tissue samples were flash-frozen at the time of autopsy and stored at -80°C .

Cell lines and derivatives. Human myeloid cell lines THP-1 and MOLM-13 were obtained from the ATCC. Cells were cultured in RPMI supplemented with GlutaMax and 10% fetal

bovine serum. TREM2 knockouts THP-1 (KO) were engineered using CRISPR/Cas9 disruption of the coding frame in exon 2 (15). Coding sequences of human full-length TREM2 (NM_018965.3) and the Δ e2 isoform were synthesized and cloned into the doxycycline-inducible lentiviral pCW57-MCS1-2A-MCS2 vector (Addgene, #71782). For “add-back” experiments, TREM2 KO cells were transduced with the corresponding full-length or Δ e2 lentiviral expression constructs. After 2 weeks of puromycin selection, resistant cells were harvested; ectopic TREM2 expression was induced with doxycycline (100 ng/ml) and validated by qRT-PCR and by Western blot.

RNA isolation and cDNA synthesis. Total RNA from cultured cells was isolated with the RNAeasy kit (Qiagen; #74106). Total RNA from cryopreserved brain autopsies was isolated using TRIZOL reagent (Invitrogen). cDNA was synthesized using SuperScript III First-Strand Synthesis kit with oligo(dT) primers (Invitrogen, #18080051).

TREM2 cDNA cloning. Total RNA was isolated from frontal cortex of four subjects and converted to cDNA. Full coding sequence of all known TREM2 isoforms was amplified using primers positioned within non-translated 5' and 3' regions; forward primer: 5'-gcagttcaagggaagacga-3'; reverse primer: 5'-tccagctaaatatgacagtcttg-3'. PCR products were gel-purified, cloned into pCR-XL-Topo vector (Invitrogen, # K4700) and sequenced.

qRT-PCR assays were performed on a 7500 Real-Time PCR System (Applied Biosystems) in technical duplicates or triplicates. TBP was used as an endogenous control. The following

predesigned TaqMan assays (Life Technologies) were used: TREM2 Hs01010721_m1; TYROBP Hs00182426_m1; TBP Hs00427620_m1; IFNB Hs01077958_s1.

We designed a pair of custom TaqMan assays which specifically measure the $\Delta e2$ transcript (e1/e3: probe spanning exon1/3 junction) vs full-length and other transcripts that retain exon 2 (e2/e3: probe spanning exon 2/3 junction).

Oligo sequences and concentrations for e1/e3 assay were:

forward primer: 5'-TCTTGCACAAGGCACTCT-3' (600 nM);

reverse primer: 5'-GAACCAGAGATCTCCAGCAT-3' (600 nM);

probe: 5'-TGTCACAGACCCCCTGGATCACCG-3' (133 nM).

Oligo sequences and concentrations for e2/e3 assay were:

forward primer: 5'-ACGCTGCGGAATCTACAA-3' (900 nM);

reverse primer: 5'-GAACCAGAGATCTCCAGCAT-3' (900 nM);

probe: 5'-CTGGCAGACCCCCTGGATCACCG-3' (200 nM)

Western blotting. 50 μ g of whole cell lysates were resolved on polyacrylamide gel, transferred to PVDF membrane and stained with monoclonal TREM2 Abs that recognize C-terminal epitope (Cell Signaling Technology, #91068) at 1:1,000 dilution. For gel loading controls, monoclonal β -Tubulin Abs (Cell Signaling Technology, #86298) were used at 1:1,000 dilution.

Phagocytosis assay. *In vitro* aggregation of amyloid peptide ($A\beta_{1-42}$, Bachem #H-1368, 100 μ M in PBS) was carried out by incubation for 4 days at 37°C; insoluble pellet was precipitated and labeled by pHrodo Red according to the manufacturer's protocol (Life Technologies, #P36600). Phagocytosis of aggregated $A\beta$ was measured as described (15). Briefly, THP-1 monocytes were

plated at 5×10^5 /ml density, TREM2 expression was induced by 100 ng/ml doxycycline overnight (16-18 hr). Cells were incubated for 3 hours at 37°C with 0.25 μ g/ml A β -pHrodo that only yields a fluorescent signal in an acidic lysophagosome compartment, collected and washed in ice-cold PBS with 1% BSA. Uptake of A β -pHrodo was measured on the LSRII Flow cytometer (BD Biosciences) and data analyzed by FlowJo. 20,000 events per sample were scored, cells were gated by forward and side scatter to exclude dead ones and doublets. Phagocytosis was calculated from mean fluorescence intensity of internalized pHrodo.

THP-1 differentiation into macrophages and stimulation of IFN I response. Differentiation to macrophages was performed as previously described (15) using 5ng/ml phorbol 12-myristate 13-acetate (PMA) for 48 hr followed by 24 hr of recovery without PMA. IFN I response was induced by high molecular weight poly(I:C) complexes with LyoVec transfection reagent (Invivogen, ttrl-piclv, 500 ng/ml), in combination with IFN β (PeproTech, #300-02, 100 Units/ml) for 24 hr. For “add-back” experiments, TREM2 expression was induced by 100 ng/ml doxycycline 16-18 hr prior to the IFN I response stimulation.

Statistical analysis was performed using Prism version 8 software (GraphPad, La Jolla, CA).

Results and Discussion.

We analyzed the distribution of TREM2 isoforms in human brains using primers to 5' and 3' untranslated regions. RT-PCR products were cloned and sequenced. In addition to the annotated isoforms, we identified a novel splice variant lacking exon 2 (Δ e2, Fig. 1A) that comprised approximately 40% of sequenced clones (Fig. 1B). Exon 2 skipping produces in-frame deletion

of amino acids 14-130 corresponding to a portion of signal peptide and the entire V-set Immunoglobulin (Ig V) domain (aa 29-112).

To confirm $\Delta e2$ is a naturally occurring TREM2 isoform, we tested THP-1 and MOLM-13, two cell lines ranked as top TREM2-TYROBP expressors among 1,443 cell lines

(<https://genevestigator.com>, Fig. 1C). Because both PCR and cloning of fragments of different length may introduce an efficiency bias and therefore cannot reliably quantify isoforms, we designed a pair of TaqMan qRT-PCR assays, in which probes spanned either exon 2/3 or exon 1/3 junctions. The e1/e3 assay measures exclusively the $\Delta e2$ while e2/e3 assay accounts for all TREM2 isoforms that retained exon 2. We used plasmids with cloned full length and $\Delta e2$ TREM2 sequences to verify assay's specificity and to make standard curves for absolute copy number determination. The $\Delta e2$ isoform accounted for ~9% and 7% of total TREM2 transcripts in THP-1 and MOLM-13, respectively (Fig. 1D). In human brain, $\Delta e2$ had highly variable expression ranging 3.5-17% in the frontal lobe and 3.7-35% in the hippocampus (Fig. 1F). Both areas expressed comparable levels of total TREM2 transcript with higher inter-individual variability in the hippocampus (Fig. 1E).

To confirm expression of TREM2 isoforms at protein level, we used unmodified THP-1 and cells in which exon 2 coding sequence was disrupted by CRISPR/Cas9 (TREM2 KO THP-1, (15)). The editing is expected to terminate translation of all isoforms that retain exon 2 while preserving the $\Delta e2$ isoform. Cells were stimulated by IL-4 known to induce TREM2 expression in primary macrophages (20). The membrane was probed with rabbit monoclonal antibodies recognizing an epitope at C-terminus. In unmodified cells, two major bands were observed: the upper one corresponding to full-length protein and the lower one with a predicted size of C-terminal TREM2 fragment (CTF), a membrane-associated product of proteolytic receptor

shedding (Fig.2A). The cleavage occurs at extracellular H157/S158 residues (21), (22). A minor band corresponding to the predicted size of $\Delta e2$ polypeptide was located above the CTF band. As expected, TREM2 KO cells did not express full-length protein. Upon IL-4 stimulation, only the $\Delta e2$ isoform was increased in TREM2 KO, whereas all TREM2 species were increased in unmodified cells.

We recently showed that TREM2 ablation substantially blunted IFN I response and reduced A β phagocytosis in THP-1 cells; these activities were restored upon overexpression of either wild type or R47H variant proteins in TREM2 KO THP-1 cells (15). To see if $\Delta e2$ isoform complements these deficits, we integrated it into TREM2 KO THP-1 under control of a doxycycline-inducible promoter. $\Delta e2$ was unable to restore A β phagocytosis in TREM2 KO; it also had diminished ability to promote an IFN I response (Fig.2 B-C). Thus, $\Delta e2$, a naturally occurring TREM2 splice isoform enriched in the human brain, does not complement some activities of full-length TREM2.

Alternative RNA splicing is an important mechanism that generates protein diversity by reshuffling functional domains of proteins. Variants that affect splicing regulatory motifs are usually deleterious, and about one-tenth of disease-associated variants reported in the Human Gene Mutation Database are splicing mutations (23). Of known loss of function TREM2 variants responsible for PLOSL and FTD about 20% disrupt the coding frame via altered splicing. These include variants that cause intron 2 retention (24) and exon 3 skipping (25) in FTD or PLOSL patients. A variant in intron 1, c.40+3delAGG, responsible for FTD without bone cysts, weakens the donor splice site resulting in 2-fold reduction of TREM2 level (26).

Exon 2 encodes most of the extracellular TREM2 moiety harboring the AD-associated R47 and R62, as well as the majority of residues mutated in PLOSL and FTD (1). Its absence is likely to

affect TREM2 interactions with known ligands and is a plausible cause of $\Delta e2$'s inability to complement the A β phagocytosis deficiency of TREM2 KO cells. Because exon skipping/inclusion are mutually exclusive events, production of the $\Delta e2$ transcript is expected to decrease the levels of canonical transcripts that retain exon 2. Of note, the $\Delta e2$ retains transmembrane and cytoplasmic domains, essentially mimicking the CTF, a membrane-associated product of receptor shedding. Future studies will be required to determine whether $\Delta e2$ has an additional regulatory role, for instance by competing for binding with the TYROBP adaptor and sequestering full length TREM2 from the receptor complex.

TREM2 resides within the TREM gene cluster on chromosome 6p21.1, which also includes structurally similar paralogs TREM1, TREML1, TREML2 and TREML4. Coding and non-coding variants in these genes were found to modify AD risks, independently of TREM2 R47H (27), (28). Interestingly, the TREML1/TLT-1 paralog also has an alternatively spliced transcript (ENST00000437044.2) (29) with skipped exon 2 corresponding to an entire Ig V domain. This suggests that Ig V inclusion/exclusion via alternative splicing is one of nature's tool for functional diversification of some of the Ig-like immune molecules.

The inhibitory CD33 receptor is another microglia-specific AD risk gene (30), (31). Similar to TREM2, CD33 belongs to the superfamily of Ig-like immune receptors, and its Ig V domain is encoded by exon 2 (Fig. 3). Intriguingly, the AD-associated CD33 risk allele, rs3865444(C), instructs inclusion of exon 2 and production of full-length protein, while a protective allele, rs3865444(A), instructs exon 2 skipping and production of $\Delta e2$ CD33 (32), (33). The full-length CD33 exerts an inhibitory effect on some microglia activities, such as cytokine production and/or phagocytosis in response to AD-relevant stimuli, whereas $\Delta e2$ CD33 is unable to suppress microglia activation and amyloid plaque phagocytosis *in vitro* and *in vivo* (34). While the

regulatory variants that change AD risk by instructing inclusion/exclusion of Ig V-encoding exon 2 are known for CD33, the impact of cis-regulatory variation on TREM2 splicing and its contribution to neurodegenerative pathologies remain to be elucidated.

AUTHORSHIP

K. K. designed and performed the experiments, analyzed the results, and wrote the manuscript, I. K., M.M., N. B., N.L. and W-M. C. performed the experiments and analyzed the results; W. H. R. and O.K. supervised the studies, analyzed and interpreted the results, wrote the manuscript and acquired funding.

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DISCLOSURE

The authors declare no conflicts of interest.

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Figure legends

Figure 1. Δ e2 TREM2 splice isoform is enriched in human brain. **(A)** Δ e2 and three annotated (canonical) alternatively spliced TREM2 transcripts that retained exon 2. Blue boxes mark coding sequence in exons. **(B)** Frequencies of isoforms in the cDNA library cloned from human frontal cortex (four subjects, N=75 clones sequenced). **(C)** Relative levels of TREM2 and TYROBP transcripts expressed by TREM2-positive THP-1 and MOLM-13 cell lines. **(D)** fraction of Δ e2 transcript expressed by these cells. **(E)** Relative levels of TREM2 expressed in the frontal lobe (FL, N=6) and hippocampus (HP, N=6) of the human brain. Gene expression in C and E was measured using qRT-PCR with commercial TaqMan assays for TYROBP and for major TREM2 isoforms (specific for e3/e4 junction), expression levels were normalized to TBP expression. **(F)** fraction of Δ e2 transcript expressed in FL (N=8) and HP (N=12). D, F - Levels of Δ e2 and isoforms that retained exon 2 were measured using custom TaqMan assays specific for e1/e3 and e2/e3 junctions, respectively. Absolute copy number of each isoform was calculated using calibration curves of corresponding plasmid standards.

Figure 2. Protein expression and functional activities of Δ e2 TREM2 **(A).** Western blot of THP-1 cell extracts probed with anti-TREM2 Abs, before and after 24 hr stimulation with IL-4. WT – unmodified THP-1; KO1, KO2, KO3 – independent TREM2 knockout clones. FL – full length

receptor encoded by a major canonical isoform; CTF - a membrane-associated C-terminal fragment, a product of full lengths receptor shedding due to proteolysis at the amino acids 157-158 (21). Loading controls were probed with anti-tubulin Abs. **(B)**. Phagocytosis of aggregated pHrodo-labeled Amyloid beta peptide (A β). WT – unmodified THP-1; KO - TREM2 knockout (KO1 clone); oeFL, oe Δ e2 – TREM2 KO1 cells with stably integrated lentiviral doxycycline-inducible constructs expressing full length or Δ e2 isoform, respectively. TREM2 overexpression was induced with doxycycline. Cells were incubated with pHrodo-labeled A β and fluorescence measured by flow cytometry. Shown are means $\pm$ SD of biological triplicates; * - $p < 0.05$; one-way ANOVA (Dunnett's multiple comparison test). **(C)**. IFN I response of *in vitro* differentiated THP-1 macrophages. THP-1 monocytes were differentiated to macrophages by PMA; TREM2 expression was induced with doxycycline prior to stimulation of IFN I response with poly(I:C) + IFN β . After 24 hr stimulation, cells were collected and total RNA isolated. IFN I response was quantified as induction of IFNB mRNA, expression levels were normalized to unstimulated unmodified THP-1. WT – unmodified THP-1; KO - TREM2 knockout (KO1 clone); oeFL, oe Δ e2 – TREM2 KO1 cells with stably integrated lentiviral doxycycline-inducible constructs over-expressing full length or Δ e2 isoform, respectively. Shown are means $\pm$ SD of biological triplicates; **** - $p < 0.0001$; two-way ANOVA, (Tukey's multiple comparison test).

Figure 3. Domain organization of isoforms of microglial receptors TREM2 and CD33.

SP - signal peptide; TM - transmembrane domain; CP - cytoplasmic domain; Ig V, Ig C - Immunoglobulin-like domains; FL - full length; CTF - C-terminal fragment, Δ e2 - lacking exon 2. H157/S158 - proteolytic shedding site; K186 - conserved residue responsible for interaction with TYROBP adaptor. Corresponding exons are depicted by square brackets. The diagram is not drawn to scale.

A

B

C

D

E

F

Figure 1

Figure 2

Figure 3