Characterization of iPSC-derived human microglial activation using Automated, Multiplex Capillary Western analysis

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INTRODUCTION

Alzheimer's Disease (AD) is one of the most common neurodegenerative diseases worldwide. Clinically, it is characterized by the presence of extracellular amyloid plaques and intracellular neurofibrillary tangles, resulting in neuronal dysfunction and cell death. Activation of microglia, the native immune cells of the brain, are keen responders and critical players in numerous neurodevelopmental conditions, including an increasingly recognized role in AD pathology and neurodegeneration. Triggering receptor expressed on myeloid cells 2 (TREM2), a receptor protein localized at the membrane of innate immune cells, including microglia in the brain, has been genetically linked to AD, with specific variants increasing disease risk by as much as threefold (1-3). Investigating the role of microglial TREM2 and downstream signaling cascades within human-relevant *in vitro* models has been historically challenging but is now accessible using human induced pluripotent stem cell (iPSC) technology and protocols for differentiating into microglia.

In this study we leveraged commercially available iPSC-derived microglia (iCell® Microglia) to investigate mechanisms of TREM2 signaling using high-throughput Simple Western™ technology and specific antibodies. We first validated iPSC-microglia by probing cell extracts with antibodies generated against established microglial markers (TREM2, DAP12, Iba1, CD33, PU.1). TREM2, upon ligand binding and activation, interacts with the tyrosine kinase-binding protein DNAX-activating protein 12 (DAP12, TYROBP) forming a receptor-signaling complex and activating downstream Syk-associated cell signaling pathways (3-5). To investigate signaling pathways in activated microglia in more detail, we treated normal (AHN), TREM2 homozygous (HO), and heterozygous (HZ) knockout iPSC-derived microglia with pervanadate, lipopolysaccharide (LPS), and IFNγ / TNFα. We then analyzed the effects that these treatments had on proteins downstream of TREM2 activation. We also directly and specifically stimulated TREM2, treating AHN and TREM2 HO iCell microglia with a TREM2 agonist antibody and analyzing cell extracts with a Phospho-Syk FastScan™ ELISA kit.

The identification and implementation of antibodies for neuroscience research is not a trivial task. The antibodies used and validated in this study can be leveraged to further characterize iPSC-derived human cultures. Together, these data demonstrate the utility of high-throughput Simple Western and iPSC-derived microglia for investigating the TREM2-signaling cascade and can be applied to AD therapeutic research, targeting the benefits of upregulating or downregulating TREM2-dependent microglial activation to attenuate AD pathology.

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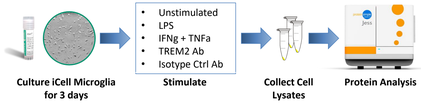
METHODS

Figure 1: Workflow for characterization of iCell Microglia



Human iPSC-derived neural cells from FUJIFILM Cellular Dynamics were cultured according to manufacturer's protocol for 3-7 days prior to stimulation with pervanadate, LPS (100 ng/mL), IFNγ (20 ng/mL) + TNFα (50 ng/mL), TREM2 Ab, or Isotype Ab. Cells were harvested at 10 min (TREM2 Ab), 30 min (pervanadate), or 24 hours (LPS, IFNγ + TNFα) post stimulation and lysed on ice using RIPA lysis buffer containing 1X DNase buffer, 1X Halt Protease/Phosphatase Inhibitor Cocktail, and 20 µg/mL Nuclease A. Samples were frozen until analysis.

Cell lysates analyzed by the Simple Western Jess™ system using between 0.125-4.5 µg lysate per well, depending on target using materials listed in Table 2.

Table 1: Cell Signaling Technology antibodies used in the study.

Key Antibodies Used	Catalog #
TREM2 (D8I4C) Rabbit mAb	91068
TREM2 (E4J7A) Rabbit mAb	55739
DAP12 (D7G1X) Rabbit mAb	12492
CD33 Antibody	77576
Syk (D3Z1E) XP® Rabbit mAb	13198
Phospho-Syk (Tyr525/526) (C87C1) Rabbit mAb	2710
Iba1/AIF-1 (E4O4W) XP® Rabbit mAb	17198
PU.1 (9G7) Rabbit mAb	2258
PLCγ2 (E5U4T) Rabbit mAb	55512
Phospho-PLCγ2 (Tyr759) (E9E9Y) Rabbit mAb	50535
FastScanTM Phospho-Syk (Tyr525/526) ELISA Kit	51426
Additional western protocol reagents can be found at cellsignal.com	

Table 2: Simple Western materials used in the study.

Item	Part Number
RePlex™ Module	RP-001
12-230 kDa Separation Module	SM-W001
Anti-Rabbit Detection Module	DM-001
Total Protein Detection Module	DM-TP01
Jess System	004-650

Table 3: FUJIFILM Cellular Dynamics materials used in the study.

Product	Abbreviation	Donor	Catalog #
iCell Microglia	MGL	01279	C1110
iCell Microglia TREM2 HZ KO	MGL TREM2 HZ	01279	C1134
iCell Microglia TREM2 HO KO	MGL TREM2 HO	01279	C1136
iCell Astrocytes 2.0	ASC	01279	C1249
iCell Induced Excitatory Neurons	IEN	01279	C1251
iCell GABANeurons	GABA	01434	C1012
iCell GlutaNeurons	Gluta	01279	C1033

iCell Microglia Validation using Simple Western and CST antibodies

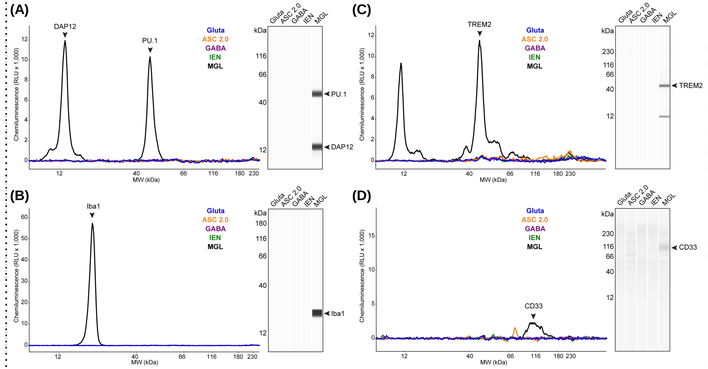


Figure 2: Expression of Key Markers in iCell Microglia (MGL) Cell Line. Simple Western analysis of Gluta, ASC, GABA, IEN, and iCell Microglia cell lines using **(A)** A multiplex of PU.1 (9G7) Rabbit mAb Rabbit mAb and DAP12 (D7G1X) Rabbit mAb. Left, electropherogram view and right, lane view, (**B**) Iba1/AIF-1 (E4O4W) XP® Rabbit mAb, **(C)** TREM2 (D8I4C) Rabbit mAb, and **(D)** CD33 Antibody. All 4 markers are specifically expressed in the MGL.

TREM2 HZ, HO Validation and Stimulation of iCell Microglia

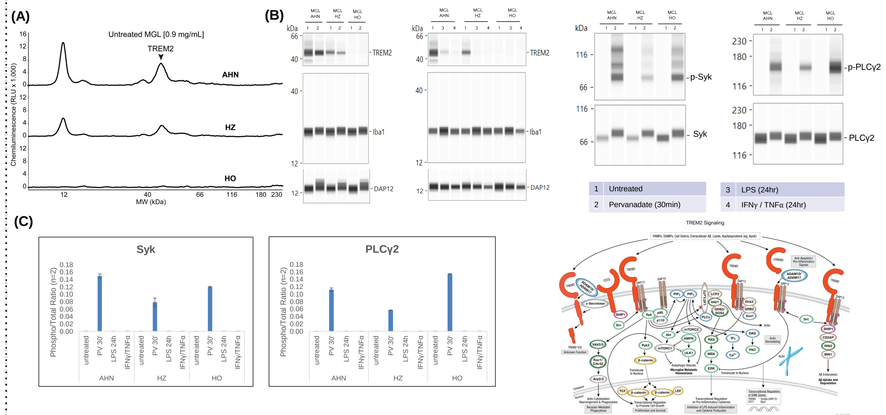
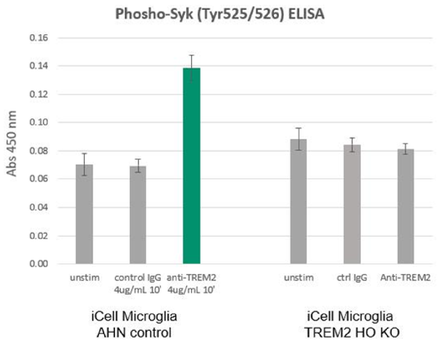


Figure 3: Characterization and Activation of TREM2 HZ & HO iCell Microglia. Simple Western analysis of cell extracts from AHN, HZ, and HO iCell Microglia. Untreated cells were characterized using TREM2 (D8I4C) Rabbit mAb (**A**). Cells untreated or treated with pervanadate, LPS, or IFNγ/TNFα were then validated for expression levels of TREM2, DAP12, Iba1, Syk, phospho-Syk (Tyr525/526), PLCγ2, and phospho-PLCγ2 (Tyr759) (**B**). Quantified ratios of phospho-Syk to total Syk, and phospho-PLCγ2 to total PLCγ2 were generated to further highlight the efficacy of each treatment to activate Syk-associated pathways (**C**). TREM2 expression was successfully eliminated in the HO line and reduced by roughly half in the HZ line, while expression levels of proteins downstream of TREM2, including DAP12 were unaffected. Pervanadate treatment confirmed phosphorylation state of several proteins downstream of TREM2.

Direct TREM2 Stimulation of iCell Microglia



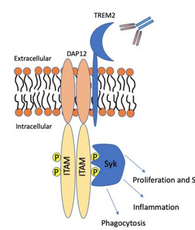


Figure 4: TREM2 Dependent Syk Stimulation of iCell Microglia. AHN and HO KO TREM2 iCell Microglia were untreated, treated with control rabbit IgG, or treated with TREM2 (E4J7A) Rabbit mAb. Cell extracts were then analyzed by ELISA using the FastScan Phospho-Syk (Tyr525/526) ELISA Kit.

CONCLUSIONS

- CST antibodies together with Simple Western is an efficient method for evaluating cell signaling mechanisms in human iPSC-derived microglia (iCell Microglia), offering up to 60-720 assay runs per vial of iCell Microglia (Table 4)
- Human iPSC-derived microglia (iCell Microglia) express microglial cell markers
- AHN, HO, and HZ iCell Microglia express expected levels of TREM2 protein
- iCell Microglia respond to known microglia stimuli and are amenable to high-throughput Simple Western characterization
- pSyk is a reliable readout for TREM2-dependent microglial activation *in vitro* in line with previous findings (3-9)
- Indirect stimulation of iPSC-derived microglia may be insufficient or requires optimization to visualize Syk-associated signaling cascades in the context of TREM2 activation and other pathways
- pSyk induction is more apparent with TREM2 agonist treatment, suggesting that direct stimulation of TREM2 is a superior option to visualize TREM2-dependent microglial activation.
- Complementary data interrogating iCell Microglia using CST antibodies in high-content immunofluorescence can be found in SFN Poster #B68 (Abstract #5782)

Table 4. Efficiency of Simple Western with iPSC-derived Samples

iCell Microglia/vial	1x10^6
Exp. conditions/vial	2 (0.5x10^6 cells)
Cell lysate volume	200 µL
Protein [ ] per condition	~2 mg/mL
# of assays/condition on Simple Western *(in duplicate)*	30-180
# of assays/condition RePlex/Multiplex *(in duplicate – 2 targets/condition)*	60-360
Total # of Simple Western assays/vial of Microglia	**60 - 720**

Future Directions

- Leverage iCell AHN Microglia, Simple Western, and CST's portfolio of monoclonal antibodies to investigate Syk-associated signaling cascades in the context of direct TREM2 activation
- Further investigate the role TREM2 activation may play in promoting Syk-associated cellular processes (phagocytosis, inflammation, proliferation and survival, etc.)
- Use TREM2 R47H iCell Microglia to examine the effect that this AD associated variant may have on TREM2 activation and downstream signaling cascades

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