APPLICATION NOTE

Neuroinflammation study on TREM2 and SYK signaling in human iPSC-Derived macrophages using HTRF.

This note demonstrate that the use of physiologically relevant human iPSCderived macrophages combined with TREM2 and Phospho / Total SYK HTRF assays offer a robust, semiquantitative and easy-to-implement platform to study microglia-induced neuroinflammation.

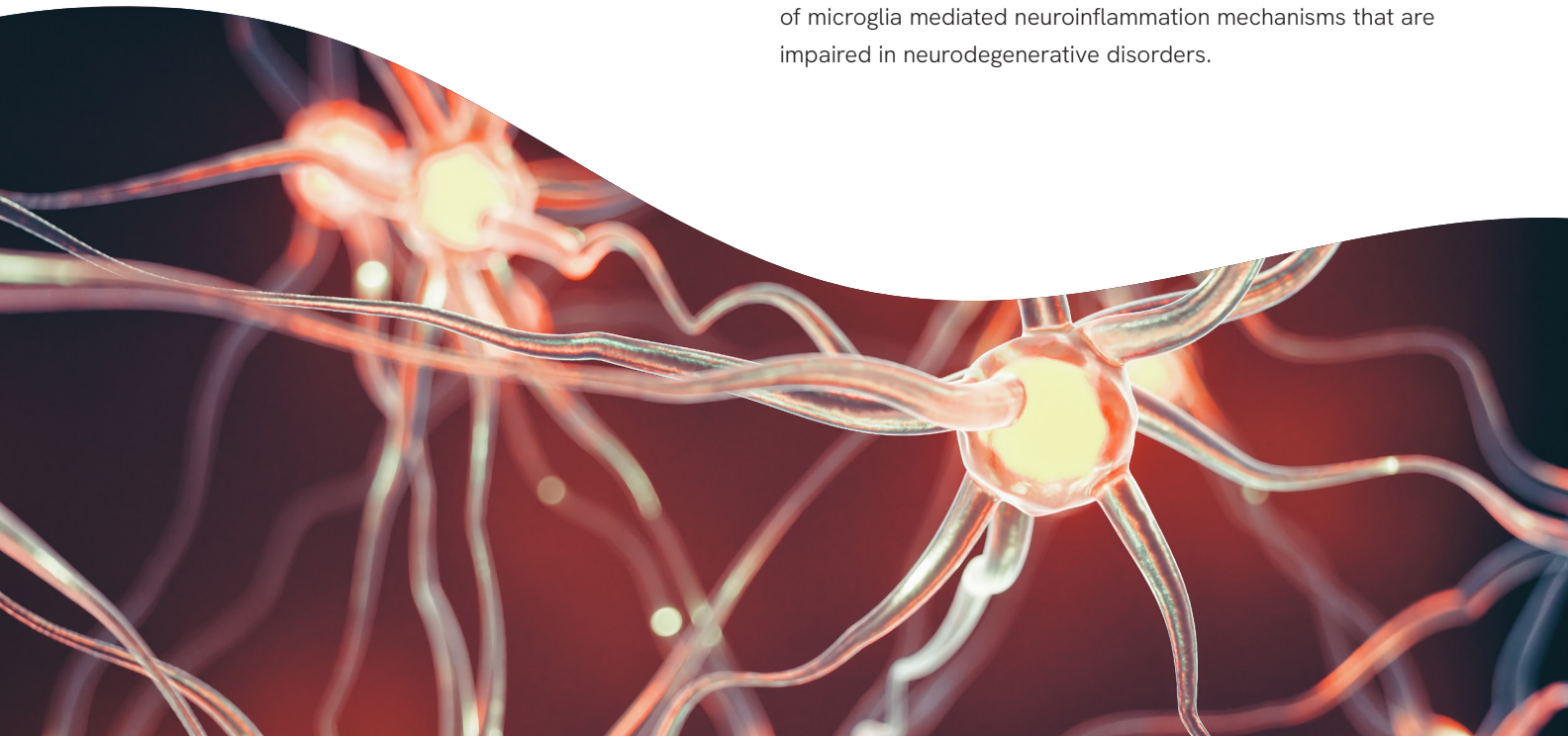
Abstract

There is emerging evidence demonstrating that chronic inflammation and activation of the brain's immune system is a crucial player in neurodegeneration. Sustained inflammation and dysregulation of microglia and astrocytes adversely impact homeostasis in the brain and have been shown to exacerbate the pathogenesis of neurodegenerative diseases.

The Alzheimer's Research UK Oxford Drug Discovery Institute (ODDI) couples the deep disease knowledge and biology expertise of the academic community with high quality, innovative drug discovery technologies. They aim to accelerate the discovery of novel, effective therapeutics for Alzheimer's disease and other neurodegenerative diseases. To that aim, the ODDI utilizes human induced pluripotent stem cell (hiPSC)-based cellular models, and we were able to work with their physiologically relevant hiPSC derived macrophages to study neuroinflammation pathways.

Revvity offers HTRF® (Homogeneous Time - Resolved Fluorescence) cell-based assay kits dedicated to neuroscience research. These assays enable the rapid and accurate measurement of key intracellular or secreted markers, while saving precious samples thanks to their low volume consumption.

This collaborative work demonstrates that hiPSC-derived macrophages combined with HTRF TREM2 and Phospho / Total SYK cellular assays represent high-value solutions for the study of microglia mediated neuroinflammation mechanisms that are impaired in neurodegenerative disorders.



Microglia roles and functions

Microglia, often referred to as the nervous system's immune cells, monitor neuronal health. Neuronal injury or infection triggers a change in microglial transcriptomic signature, causing a dynamic shift in phenotype from homeostatic, to a spectrum of phagocytic anti-inflammatory, and cytotoxic pro-inflammatory states. Maintaining the right balance of an acute pro-inflammatory response to promote clearance of debris and aggregated proteins, followed by resolution and the production of anti-inflammatory mediators, which allow tissue repair and healing, is essential to maintain a healthy brain. The activation of microglia in both Parkinson's disease and Alzheimer's disease seems to be tilted towards chronic activation leading to an exacerbation of inflammation and an acceleration of disease progression.

TREM2 / SYK pathway

Triggering receptor expressed on myeloid cells 2 (TREM2 mediates neuroprotection when physiologically regulated. TREM2 expressed at the cell surface of microglial cells is involved in the phagocytosis of damaged neurons and Aβ aggregates, thereby maintaining the brain homeostasis.

As shown in the schematic below, TREM2 stimulation triggers the phosphorylation of SYK that further activates BTK and PLCγ2, resulting in an increase in intracellular calcium flux that modulates functions such as phagocytosis and survival. Furthermore, TREM2 signalling also affects the secretion of cytokines, antagonizing NFkB mediated cytokine release. Microglial activation, through stimulation of other receptors (such as Toll-like receptors (TLRs) or Fc receptors), results in SYK phosphorylation leading to Erk and Akt activation and NFkB signalling and secretion of pro- inflammatory cytokines.

In pathological conditions, TREM2 loss of function mutations reduce the ability of macrophages to clear cellular debris and aggregates. Furthermore, TREM2 undergoes proteolytic cleavage to release soluble TREM2 (sTREM2), and elevated levels of sTREM2 have been observed in the CSF of patients with Alzheimer's Disease, Multiple Sclerosis and Amyotrophic Lateral Sclerosis. As TREM2 signalling is reduced in neurodegenerative conditions, signalling through TLRs becomes predominant, contributing to the release of pro-inflammatory cytokines. As a result, neuroinflammation becomes sustained in a vicious circle that is linked to the pathogenesis of several neurodegenerative diseases.

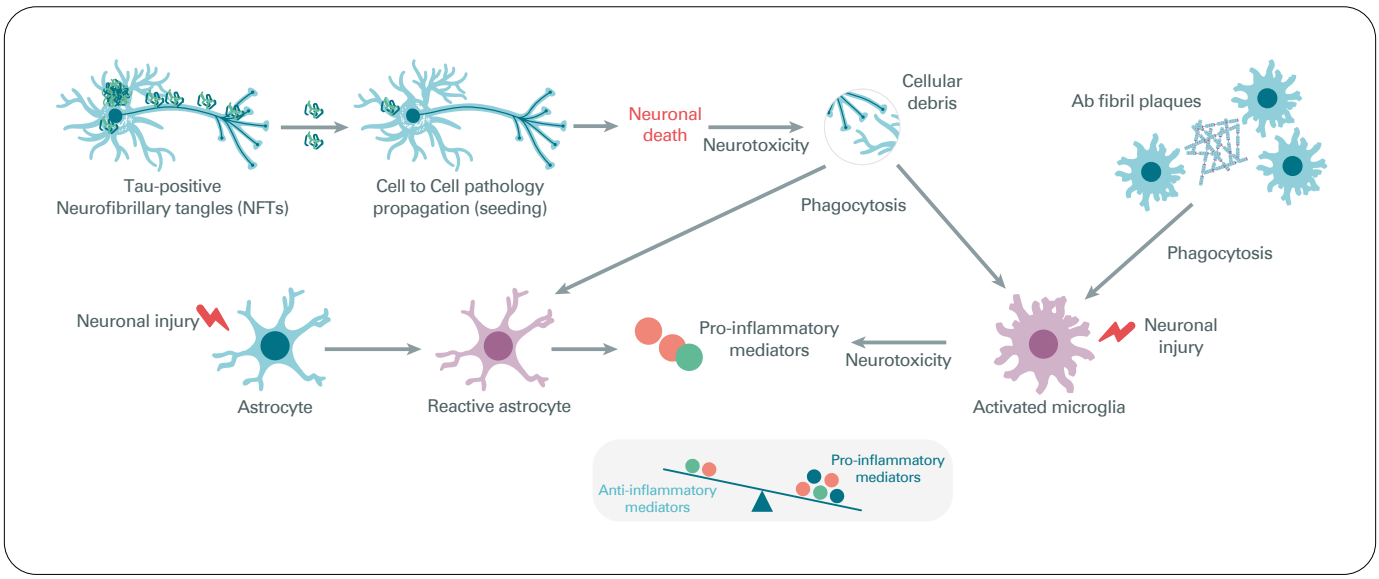


Figure 1: Molecular pathways in Alzheimer's Disease.

The aim of our work was to use the TREM2 and p / T-SYK HTRF kits to study the TREM2 signalling pathway. We assessed the levels of intracellular and shed TREM2 in a HEK-TREM2 / DAP12 overexpressing cell line and human iPSC macrophages, then investigated SYK phosphorylation upon treatment of human iPSC-macrophages with an anti-TREM2 agonist antibody.

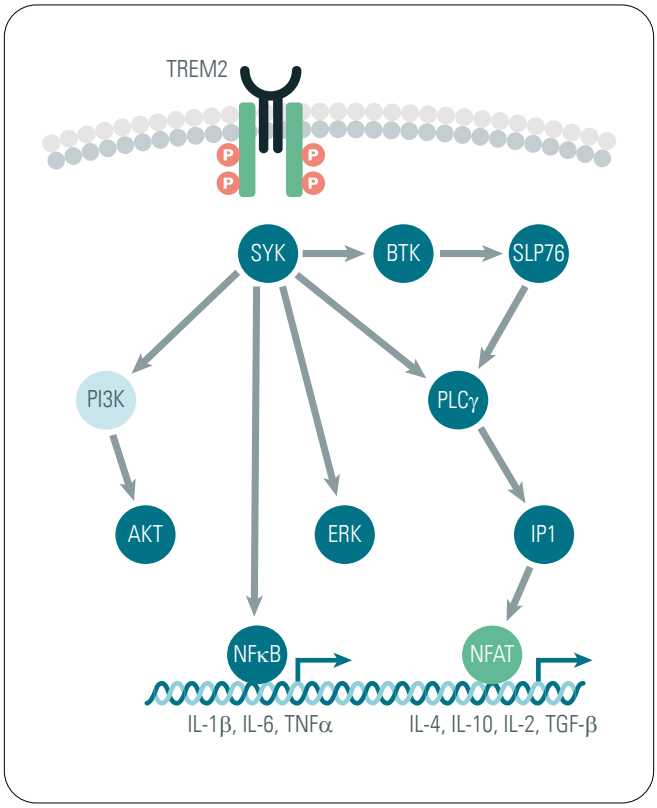


Figure 2: TREM2 signalling pathway.

Materials and methods

Experimental flowchart

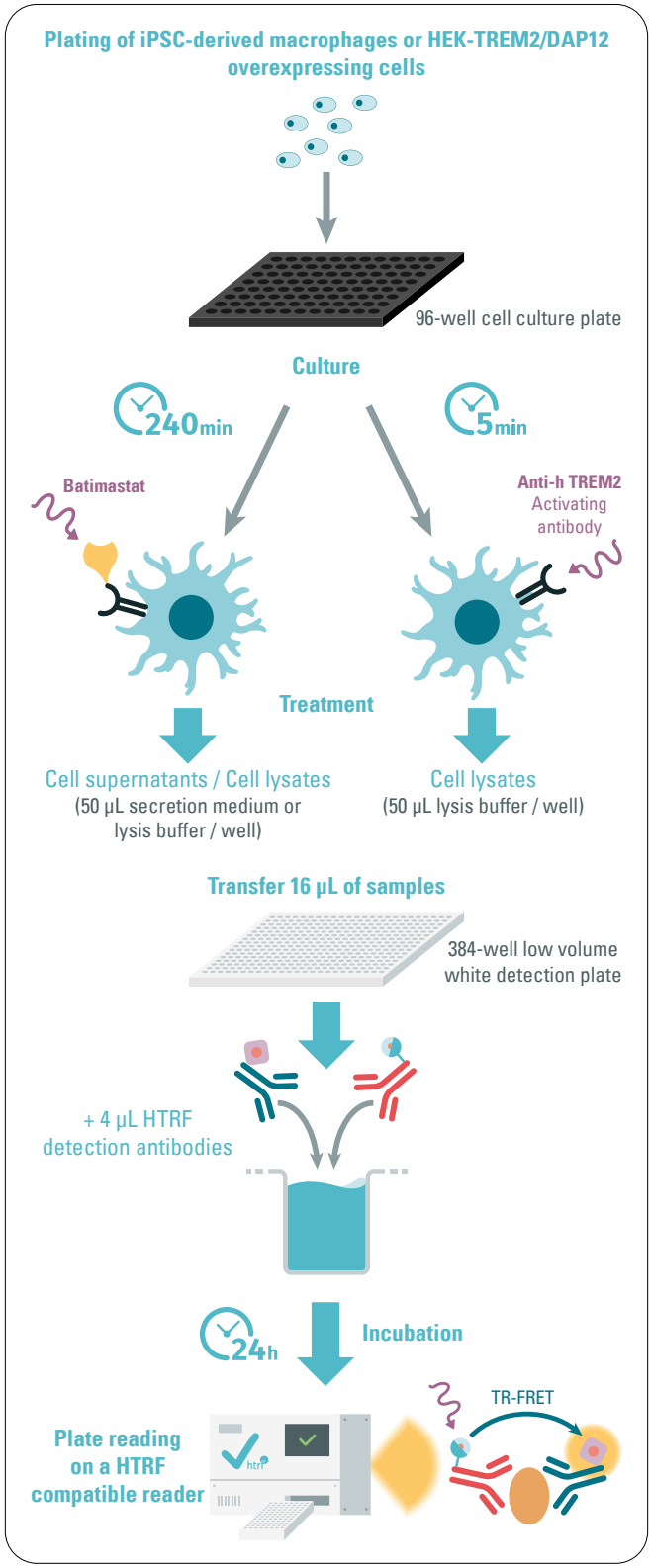


Figure 3: TREM2 assay protocole.

Cell models and reagents

Parental (BIONi010-C, EBiSC #66540023) and TREM2-KO (BIONi010-C-17, EBiSC #66540632) human iPSC-derived macrophages, differentiated as described in Van Wilgenburg *et al.*, 2013 (1), were plated in 96-well plates at 40k cells/well.

HEK293 cells and HEK293 cells overexpressing human TREM2 and DAP12, a kind gift from Professor Peter St. George Hyslop, were cultured in high sucrose DMEM supplemented with 10% FBS, 1 µg/mL puromycin and plated in 96-well plates at 40k cells / well.

Cells were treated with either a titration of Batimastat for 4 hrs to assess TREM2 levels; with 5 µg/mL of an anti-TREM2 antibody for 5 mins, or with the corresponding isotype control (goat IgG) for 5 mins. HTRF cellular assay kits (Revvity-Revvity) used for this study were:

- Human TREM2 part number #63ADK099PEG
- Phospho-SYK (Tyr525 / 526) part number #64SYKY525PEG
- Total SYK part number #64SYKTPEG

Experimental conditions

Immunofluorescence staining

Cells plated in 96-well plates were fixed in 4% PFA for 10 min at RT. Cells were washed in PBS before blocking with 5% BSA for 1 hr. Cells were then incubated with the anti-TREM2 antibody (1:30) overnight at 4°C followed by incubation with the secondary donkey anti-goat IgG-Alexa Fluor 488 (Invitrogen A32814, 1:1000) and Hoechst (1:1000) for 1 hr. Cells were imaged using an Opera Phenix High Content Imaging System (Revvity).

Cell treatments and sample generation

To assess intracellular and shed TREM2 levels, cells were incubated with a titration of Batimastat for 4 hrs at 37°C. Activation of the SYK kinase was achieved in iPSC-macrophages through TREM2 surface receptor stimulation with the anti-TREM2 antibody at a final concentration of 5 µg/mL in 50 µL cell culture medium for 5 min at 37°C. A goat IgG isotype control antibody was used at the same concentration as a negative control.

Following stimulation, cell culture medium was either removed from the wells and retained (stored at -80°C until use for assessment of sTREM2) or discarded and replaced by 50 µL of HTRF lysis buffer. After a 10 min incubation at room temperature under gentle shaking, plates were frozen at -80°C until analysis. Samples for WB analysis were lysed in RIPA buffer.

HTRF cellular assays

HTRF cellular assays are no-wash sandwich immunoassays based on TR-FRET, where the fluorescent signal intensity is proportional to the analyte concentration in the sample. The detection is achieved using analyte-specific antibodies conjugated to donor and acceptor fluorophores. In the particular case of a phospho-protein, one of the antibodies recognizes specifically the phosphorylated residue(s) of interest.

To perform HTRF assays, 16 µL of sample (cell lysate or supernatant) were transferred into a 384-well low volume white plate, and 4 µL of pre-mixed HTRF detection reagents were added. After incubation following manufacturer's instructions, the signal was recorded on a HTRF compatible microplate reader.

To ensure operating in the linear range of each assay, samples were measured neat and diluted. The results presented hereafter correspond to the optimal detection conditions.

HTRF data handling

The measure of TREM2 protein in cell supernatants and cell lysates are absolute quantifications done by interpolating the HTRF specific signal (delta ratio) from the assay standard curve performed in the same matrix as the samples (cell culture medium for soluble TREM2 or lysis buffer for cellular TREM2).

The measure of intracellular phospho- & total SYK proteins is a relative quantification where the HTRF signal intensity (HTRF Ratio) is directly proportional to the concentration of the phosphorylated or total protein in the cell lysate.

Statistical analyses were performed with GraphPad Prism software using the ordinary two-way ANOVA test and the Tukey's multiple comparisons post-test. Histobars represent the mean +/- SD (Standard Deviation) of each experimental triplicate.

Results

Human iPSC-derived macrophages were confirmed to express macrophage surface markers such as CD11b, CD14 and CD45 as described in Van Wilgenburg *et al.*, 2013 (1).

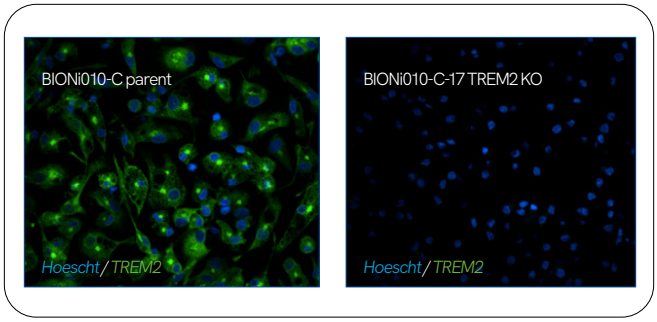
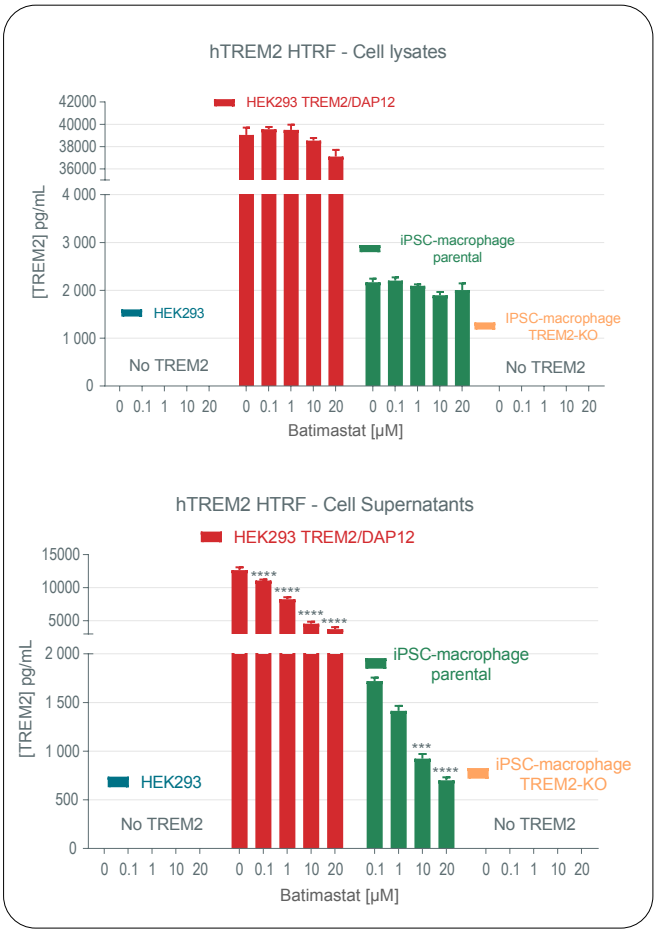


Figure 4: IF analysis of iPSC-derived macrophages (acknowledgments to Hazel Hall-Roberts)

The expression of the TREM2 receptor was assessed by immunofluorescence (IF) to highlight cell morphology and in situ expression. As shown in Figure 1, cells elicit a macrophage / microglia cell pattern and the TREM2 receptor is visible in the parental cells (A) whereas the staining is completely ablated in the corresponding KO cells (B).

First, TREM2 expression and shedding was further confirmed and quantitatively measured in lysates (Figure 5) or supernatants (Figure 6) from the the HEK-TREM2/DAP12 overexpressing cells and the iPSC-macropaghes (parental vs TREM2 KO lines) using the TREM2 HTRF kit. The kit is highly versatile and compatible with many lysis buffers and cell culture media, a standard curve was built in each biological matrix. As shown in Figure 5 and 6, both cell types used express high levels of TREM2.

We next sought to determine whether TREM2 shedding could be inhibited with the pan MMP inhibitor, Batimastat. As shown in Figure 5, TREM2 content is unchanged in HEK+TREM2/DAP12 and iPSC parental cells following treatment, whilst levels of sTREM2 in the cell culture media were dose-dependently and significantly reduced following treatment with batimastat (Figure 6).



Figures 5 and 6: HTRF quantitation of TREM2 in cell lysates and cell supernatants (acknowledgments to Tom Smith and Juliane Obst).

We then aimed to assess the phosphorylation status of SYK upon activation of the TREM2 receptor with an activating antibody. Analyses of SYK was first evaluated by WB, which confirmed SYK phosphorylation when iPSC-macrophage parental cells were treated for 5 minutes with the anti-TREM2 activating antibody (Figure 7). No SYK phosphorylation was observed in the TREM2 KO line,

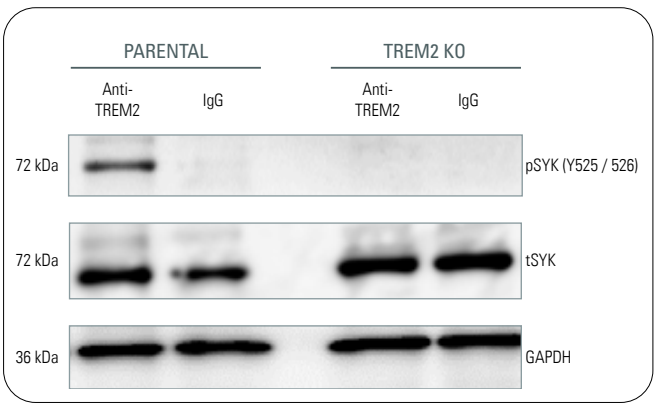


Figure 7: WB analysis of iPSC-macrophage lysates. (acknowledgments to Juliane Obst).

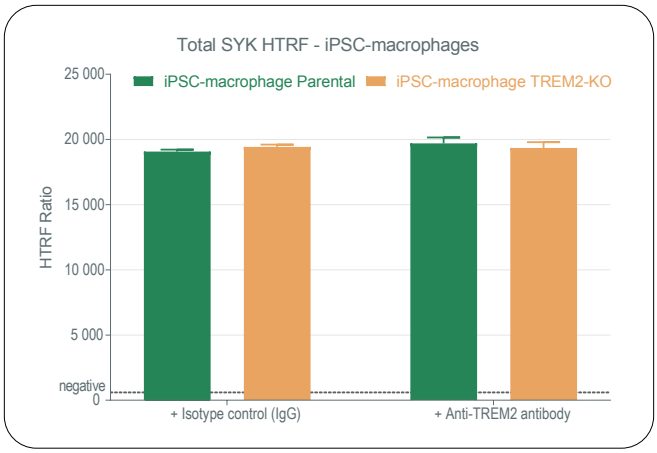


Figure 8: HTRF analysis of Total SYK levels in iPSC-macrophages.

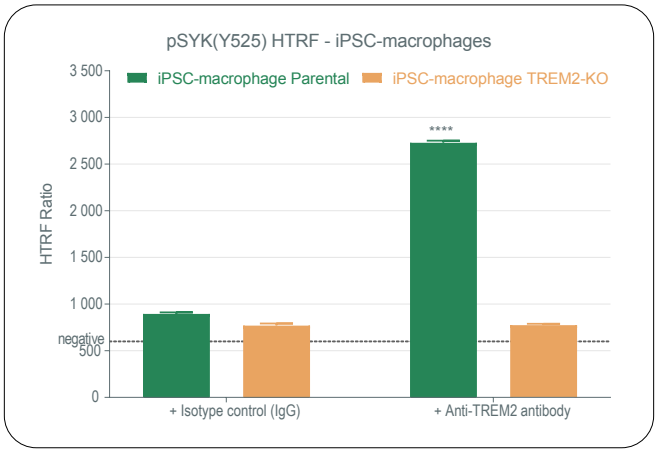


Figure 9: HTRF analysis of phospho SYK levels in iPSC-macrophages.

however total SYK was present. Further investigations of SYK phosphorylation in cell lysates was carried out using HTRF for a semi-quantitative analysis of both total-SYK and phospho-SYK.

Total SYK levels were unchanged in the parent and TREM2 KO line in cells treated with either the IgG control or TREM2 activating antibody (Figure 8). As expected, the anti-TREM2 antibody induced activation of the pathway in the parental cells, as highlighted by the increase in SYK phosphorylation at Tyr525 / 526 (Figure 9), while the treatment did not modulate the phosphorylation in the TREM2 KO cells (Figure 6).

Conclusion

The data presented here demonstrate that the use of physiologically relevant human iPSC-derived macrophages combined with TREM2 and Phospho / Total SYK HTRF assays offer a robust, semiquantitative and easy-to-implement platform to study microglia-induced neuroinflammation.

Reference

1. Van Wilgenburg *et al.*, Efficient, long term production of monocyte derived macrophages from human pluripotent stem cells under partly defined and fully defined conditions; 2013; PLOS One; Vol. 8, Issue 8.1

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