TREM2 knockout, but not the R47H Alzheimer’s variant, reduces neural phagocytosis and survival of human iPSC-derived macrophages

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Abstract
BACKGROUND: TREM2 is a microglial cell surface receptor, with risk mutations linked to Alzheimer’s disease (AD), including R47H. Binding of ligands to TREM2 triggers Syk-dependent signalling through the DAP12 co-receptor, leading to phagocytosis, survival, and changes to microglial activation state. In biochemical assays, R47H impairs TREM2 binding to phosphatidylserine, a lipid “eat-me” signal exposed by apoptotic neurons. The effect of R47H TREM2 upon phagocytosis of apoptotic neurons by human microglia has not yet been reported.

METHODS: We generated human microglia-like iPSC-macrophages (pMac) from isogenic induced pluripotent stem cell (iPSC) lines with homozygous R47H mutation or TREM2 knockout (KO). To assess microglial phenotypic function in the mutants, we measured: (1) pro-inflammatory cytokine responses by ELISA; (2) survival after growth factor-withdrawal; (3) phagocytosis by novel high-content imaging assays, using two neuron-derived cargoes that expose phosphatidylserine (fixed SH-SY5Ys and synaptosomes). Downstream signalling resulting from TREM2 activation was additionally assessed by assaying Syk phosphorylation and calcium flux.

RESULTS: We demonstrated that TREM2 KO strongly diminished both pMac survival and neural phagocytosis, while having little impact on inflammatory cytokine response. R47H TREM2 modified surface expression and shedding of TREM2, but did not impair TREM2-mediated signalling, survival, or phagocytosis.

CONCLUSIONS: Under healthy conditions in culture, the R47H mutation is not sufficient to cause defects in phagocytosis or survival of human pMac, unlike more severe T66M or W50C TREM2 loss-of-function mutations. We hypothesise that R47H TREM2 affects other microglia phenotypes yet to be explored, and/or that pathogenic manifestation requires other stressors relating to neurodegenerative disease.

Background
Microglia are strongly implicated in the pathogenesis of late-onset Alzheimer’s disease (LOAD). LOAD risk genes, identified by genome-wide association studies (GWAS), include TREM2, PLCG2, INPP5D, and APOE, the protein products of which share common signalling pathways in microglia (1,2).
Furthermore, non-coding LOAD risk variants are largely confined to microglia-specific enhancer regions of the genome (3). “Disease-associated” microglia have been identified in multiple mouse neurodegenerative disease models, which have a common transcriptional signature that is largely dependent on expression of Triggering Receptor Expressed in Myeloid cells 2 (TREM2) (4). TREM2 is a myeloid cell surface receptor, and requires the co-receptor DNAX-Activation Protein 12 (DAP12/TYROBP) for signal transduction. TREM2-DAP12 receptor activation causes activation of spleen-associated tyrosine kinase (Syk). Syk phosphorylates and activates PI3K and PLC\(\gamma\)2, inducing multiple downstream signalling events, resulting in intracellular calcium flux, actin mobilisation, Akt and ERK activation (5). TREM2 signalling ultimately regulates many major microglial functions, including: phagocytosis (6), chemotaxis (7), survival and proliferation (8,9), autophagy (10), and proinflammatory cytokine production(11). TREM2 has a broad-specificity ligand-binding domain with minor preference for anionic substrates, therefore it behaves like a scavenger receptor with a long list of putative ligands: various phospholipids and sphingomyelin (12), lipidated apolipoproteins including ApoE (13), bacterial lipopolysaccharides (14), nucleic acids (15), and oligomeric amyloid-\(\beta\) (16). Two confirmed AD risk-modifying mutations of TREM2, R47H and R62H, occur within the ligand binding domain (17). R47H TREM2 increases the risk of AD by 2 to 4.5-fold, which makes this rare variant second only to ApoE-\(\varepsilon\)4 in the strength of association to sporadic AD (18,19). The R47H mutation of TREM2 alters the structure of the ligand binding region, and significantly reduces binding of phosphatidylserine (PtdSer), phosphatidylethanolamine, and sphingomyelin in biochemical assays (20,21). In cells, reduced intracellular signalling of the R47H mutation was confirmed in response to PtdSer (12,22). PtdSer is well-known as an “eat-me” signal on apoptotic cells, and one study has shown that the TREM2-specific signal from contact with apoptotic N2a cells was effectively blocked by a PtdSer-binding protein (22).

Mouse R47H TREM2 knock-in models have been studied, and in the context of AD pathology these have appeared to phenocopy TREM2 knockout or haploinsufficiency models. Namely, there are fewer amyloid plaque-associated microglia, plaques are more diffuse, and microglia exhibit subtle defects in cytokine responses, survival, proliferation and migration (23,24). However, R47H knock-in mouse
models have reduced TREM2 mRNA, whereas levels of R47H TREM2 mRNA are similar to wildtype in human iPS-derived microglia-like cells and AD patient brains. This difference is the result of a particular sensitivity of the mouse gene to aberrant splicing, therefore it is a poor model for the human R47H mutation (25). Mouse models expressing human TREM2 should not manifest the same defect. Indeed, humanized R47H TREM2-expressing mice crossed with the 5xFAD model have already been studied, and exhibited reduced microglia clustering around amyloid plaques relative to human WT TREM2-5xFAD mice, (26). The biological mechanisms underlying this phenotype are unexplained. Given the questions over the validity of different R47H TREM2 mouse models, it is important to explore the cell phenotypes of R47H TREM2 in an authentic human model. Human induced pluripotent stem cells (iPSC) differentiated to iPSC-macrophages (pMac) display close transcriptional similarity to human fetal microglia (27,28). The differentiation protocol is simple, generating cell populations of high purity and yield. To examine the effect of the R47H mutation on TREM2-specific functions, we have performed phenotypic assays on isogenic wildtype, biallelic R47H and TREM2 KO pMac lines. Specifically, we show that TREM2 KO has minimal impact on LPS-induced inflammation, however, deficits are observed in survival and phagocytosis. R47H TREM2 had no effect on survival or phagocytosis.

Methods

iPSC culture

iPSC lines BIONi010-C (control, Bio Sample ID: SAMEA3158050), BIONi010-C-7 (R47H TREM2, Bio Sample ID: SAMEA4454010), and BIONi010-C-17 (TREM2 KO, Bio Sample ID: SAMEA104386270) were obtained from Bioneer, and are available on ECACC. The parent line BIONi010-C was re-programmed by Bioneer with a non-integrating episomal vector, using normal adult human skin fibroblasts sourced from Lonza (#CC-2511). For the supplementary data, pMac differentiated from SFC840-03-03 iPSC were used, a line derived from dermal fibroblasts from a disease-free donor recruited through the Oxford Parkinson’s Disease Centre. iPSC were cultured in mTeSR™1 media (STEMCELL Technologies), on hESC-qualified Geltrex-coated plates (Gibco), passaging as clumps using 0.5 mM EDTA in PBS. Large-scale SNP quality-controlled batches were frozen at p15-25 and used for experiments within a minimal number of passages post-thaw to ensure consistency. An Illumina Omniexpress 24 v1.2 SNP microarray analysis was performed to verify genomic integrity, as previously described in (28).
Human iPSC differentiation to pMac

iPSC were differentiated to primitive, tissue-type macrophages as previously described (27). In brief, 4 x 10^6 iPSC were seeded into an Aggrewell-800 well (STEMCELL Technologies) to form embryoid bodies, in mTeSR™1 media (STEMCELL Technologies) and fed daily with medium plus growth factors: 50 ng/mL BMP4 (Peprotech), 50 ng/mL VEGF (Peprotech), and 20 ng/mL SCF (Miltenyi Biotec). In a modification to the previously published protocol, the embryoid bodies were cultured for 5-6 days in growth factors instead of 4 days, and after the first 2 days they were transferred into low-adherence 6-well plates. Embryoid bodies were then differentiated in T175 flasks (150 per flask), known as ‘differentiation factories’. iPSC-macrophage precursors (pMacpre), emerging into the supernatant after approximately 2-3 weeks, were harvested weekly, plated in their final assay format and differentiated to pMac for 6-10 days at 37 °C/5% CO_2, in X-VIVO15 with 100 ng/mL M-CSF, 2 mM Glutamax, 100 U/mL penicillin and 100 μg/mL streptomycin.

Cell surface protein purification

pMacpre were seeded at 2 x 10^6 cells/well in 6 well plates and differentiated for a week. Cell surface proteins were extracted using a Pierce Cell Surface Protein Isolation kit (Thermo Fisher). In brief, the plates of pMac were placed on ice and washed twice with cold PBS with Ca^{2+} and Mg^{2+}. Cell surface proteins were biotinylated with 0.5 mg/well of sulfo-NHS-SS-biotin for 30 minutes on ice. Free biotin was quenched with Quenching Buffer (Thermo Fisher), the cells were gently scraped into tubes, spun down, and washed with Tris-buffered saline. The cell pellets were lysed in RIPA buffer (50 mM Tris-HCl (pH7.4), 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulphate, 150 mM sodium chloride, 2 mM EDTA, 50 mM sodium fluoride), briefly sonicated, and protein content measured by Bradford protein assay (Bio-Rad). Total protein concentrations between samples were normalized, and an aliquot of normalized whole-cell lysate saved for western blotting. The remaining homogenates were incubated with twice the volume of Neutravidin beads (Thermo Fisher) overnight at 4°C with rotation. Beads were washed three times by centrifugation and re-suspension in Wash Buffer (Thermo Fisher). Cell surface proteins were eluted from the beads by boiling for 15 minutes with 1 x ‘LDS Sample buffer’ (Thermo Fisher) with 50 mM DTT. Cell surface proteins and whole-cell lysates (containing proteins soluble in 1% Triton X-100) were analysed by western blotting, including a negative control of non-biotinylated cells.

Western blotting

pMacpre were seeded in either 12-well plates (8 x 10^5 pMac per well) or 6-well plates (1.5 x 10^6 pMac per well) and differentiated for a week in macrophage media. Stimulations with a TREM2-activating
antibody (goat polyclonal antibody against human TREM2 AF1828 from R&D Systems) used a concentration of 2.4 μg/1 x 10⁶ cells (3.84 μg/mL) for 10 minutes. Stimulations with dead SH-SY5Ys used a ratio of 3:1 SH-SY5Ys to pMac, with an initial 1 hour incubation at 4°C to allow cells to settle, followed by incubation at 37°C for the time periods indicated. After washing with PBS, pMac were lysed directly in modified RIPA buffer (50 mM Tris-Cl (pH7.4), 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulphate, 150 mM sodium chloride, 2 mM ethylenediaminetetraacetic acid, 50 mM sodium fluoride) with protease and phosphatase inhibitor cocktails (Roche). Lysates were sonicated for 30 seconds at medium power, and centrifuged at 14000xg for 3 minutes to pellet insoluble material. Triton X-100-soluble proteins were boiled with 1 x LDS sample buffer with reducing agent (Thermo Fisher), and samples were resolved by denaturing SDS-PAGE with 8-16% Tris-glycine gels (Thermo Fisher). Subsequently, the separated proteins were transferred to low-fluorescence 0.2 μM-pore PVDF membrane (Thermo Fisher) using a Pierce Power Blotter semi-dry transfer apparatus (Thermo Fisher). Membranes were blocked for 0.5-2 hours with iBind FD solution (Thermo Fisher), and incubated with primary antibodies in iBind solution overnight at 4°C. Primary antibodies for TREM2, phospho-Syk (Y525/Y526), total Syk, and GAPDH were purchased from commercial sources and are listed in Table 1. Membranes were washed 3 x 10 minutes with Tris-buffered saline and 0.05% Tween-20 (TBS-T), and probed for 1 hour at room temperature (RT) with secondary antibodies in iBind FD solution. Membranes were washed 4-6 x 10 minutes with TBS-T and developed with an Odyssey detection system (LI-COR). Optical densities of immunoreactivity were quantified using ImageStudio Lite 5.2 software, and normalised to the GAPDH control.

Immunocytochemistry

pMacpre were seeded at 4 x 10⁴ cell/well in optically-clear bottom CellCarrier 96-well plates (Perkin Elmer), and differentiated in macrophage media for a week. For cell surface staining of live pMac: cells were washed with cold PBS, and incubated on ice for 20 minutes with primary antibody in a live-cell blocking buffer (PBS with 5% BSA and 10 μg/mL human IgG). Cells were washed three times for 5 minutes with cold PBS, on ice, and incubated with secondary antibody in live-cell blocking buffer on ice for 15 minutes. After three washes with cold PBS, cells were fixed with 2% paraformaldehyde in PBS for 20 minutes at RT, then washed with PBS. The cells were permeabilised and counterstained with DAPI as below. For total staining of permeabilised cells: cells were fixed in 2% paraformaldehyde in PBS (Alfa Aesar) for 20 minutes at RT, washed with PBS, permeabilised with 0.1% Triton X-100 in PBS for 10 minutes at RT, and blocked overnight at 4°C in blocking buffer (PBS, 0.05% Triton-X100, 10% normal donkey serum, 5% BSA, 0.01% NaN₃). Cells were incubated with primary antibodies for 1 hour at RT, washed 3 x 15 minutes with 0.3% Triton X-100 in PBS, and incubated with secondary
antibodies for 1 hour at RT. Primary antibody for TREM2 (AF1828, 1:30, R&D Systems) was used in combination with either of the following primary antibodies for sub-cellular markers: TGN-46, Calnexin, Rab11, and LAMP1. Secondary antibodies were donkey anti-goat IgG-Alexa Fluor 488 and donkey anti-rabbit IgG-Alexa Fluor 568. The antibodies used are listed in Table 1. A DAPI nuclear counterstain (1:2000, Sigma) was incubated for 15 minutes at RT, and the cells washed 4 x 15 minutes. Images were acquired with an Opera Phenix High Content Screen System (Perkin Elmer) with a 63x water objective, Z-stacks of 9 fields per well, and 3 wells per condition. Images were processed and co-localisation of TREM2 with intracellular markers quantified with Columbus 2.7 software (Perkin Elmer).

Cytokine ELISAs
pMacpre were seeded at 4 x 10^4 cells/well in optically-clear bottom CellCarrier 96-well plates (PE), and differentiated in macrophage media for a week. In triplicate wells, cells were stimulated for 24 hours ±100 ng/mL IFNγ. All wells were given a full media change to 70 μL macrophage media ± E. coli lipopolysaccharide (LPS) and incubated for 4 hours at 37°C/ 5% CO2. Supernatants were spun down and stored in aliquots at -80°C. For cell counting, cells were stained with NucBlue Live ReadyProbes Reagent (Invitrogen), and nuclei counting performed using an EVOS FL Auto automated microscope at 4x, 4 fields per well, and CellProfiler 2.2 software (29). Supernatant TNF was quantified using a TNFα Human Uncoated ELISA Kit (Invitrogen), in accordance with the manufacturer’s protocol. An in-house ELISA was used to measure IL-6 and sTREM2, from the same supernatants measured for TNF, except with triplicate wells pooled. In-house IL-6/TREM2 ELISA procedure: Greiner high-bind 96 well plates (Sigma) were coated with IL-6 antibody (Life technologies) overnight at 4°C. Plates were washed with PBS + 0.05% Tween20 and incubated with blocking buffer (PBS, 0.05% Tween20 and 1% BSA) to block non-specific binding sites. A standard curve was generated using human recombinant IL-6 or TREM2 (Sino Biologicals). Standard and diluted supernatants were incubated for 2 hours at RT. After washing, plates were incubated with a biotinylated antibody against IL-6 (Life technologies) or TREM2 (R&D Systems) for 1 hour at RT, followed by incubation with HRP-conjugated streptavidin (Thermo Fisher Scientific) for 1 hour at RT. Plates were washed and incubated with 1-Step Ultra TMB ELISA substrate solution (Thermo Fisher Scientific). The reaction was stopped with 2N H₂SO₄ and the chemiluminescent signal was measured on a plate reader at 450 nm. Data from each well was normalised to the average cell count for that condition, and further normalised to the average pg/mL/cell for the whole ELISA plate. The in-house ELISA antibodies are listed in Table 1.

Survival assay
pMacpre were seeded at 4 x 10^4 cells/well in three optically-clear bottom black 96-well plates (Costar), and differentiated in macrophage media. After 7 days a full media change was performed to 100 μL macrophage media ± M-CSF, triplicate wells for each condition on each plate. Plates were incubated at 37°C/5% CO₂ for 3, 7, or 10 days, and the 10-day plate received a 50% media change at 7 days. At the end of each incubation, cells were stained 20 minutes (37°C/5% CO₂) with the ReadyProbes Cell Viability Imaging Kit (Invitrogen). Nuclei counting was performed using an EVOS FL Auto automated microscope, 4x with DAPI and GFP light cubes, 4 fields/well, and CellProfiler 2.2 software (29). Data was presented as (mean number of dead cells/mean number of total cells) x 100 for each condition.

Calcium assay
pMacpre were seeded at 1 x 10^4 cells/well in optically-clear bottom CellCarrier 384-well plates (Perkin Elmer) and differentiated in macrophage medium for 7 days. The stimuli used were 0.5 mM ATP (Sigma) and 10 μg/mL TREM2 antibody (R&D Systems). A 384-well plate containing stimuli was prepared for transfer onto the pMac. pMac were loaded with 25 μL of 4 μM calcium-sensitive dye Fluo4-AM (Thermo Fisher Scientific) in the presence of 0.5% pluronic acid (Life technologies) diluted in HBTS buffer (HEPES Buffered Tyrode’s Solution: NaCl 135 mM, KCl 5 mM, MgCl₂ 1.2 mM, CaCl₂ 2.5 mM, HEPES 10 mM, glucose 11 mM, pH 7.2) for 1 hour at RT. pMac were washed with HBTS before the plates of pMac and stimuli were loaded onto the FLIPR Tetra (Molecular Devices), a high-throughput cell-based screening system with robotic pipettor. Each condition was run in quadruplicate. Relative fluorescent units (RFU) of the assay plate were read with the excitation/emission pairs 470-495 nm LEDs and 515-575 nm emission filters. Settings were adjusted in order to have values of ~ 1000 RFUs at baseline. Basal fluorescence was measured for 1 minute and following injection of stimuli, the response was recorded for 5 minutes at reading intervals of 5 seconds using the ScreenWorks software. Data was exported as maximum-minimum signal and RFU normalised to baseline values set to 100%.

Generation of dead SH-SY5Ys
SH-SY5Ys (ATCC) were cultured in T75 flasks with DMEM/F12 media (Gibco) with 10% FBS (Sigma) and penicillin/streptomycin (Invitrogen), and maintained at 37°C/5% CO₂. Cells were harvested with TrypLE Express (Gibco), washed with Hank’s Balanced Salt Solution (HBSS, Gibco), centrifuged at 400xg for 5 minutes, and re-suspended in 2 mL Live Cell Imaging Solution (LCIS, Invitrogen). Paraformaldehyde (Alfa Aesar) was added to a final concentration of 2%, and the cells fixed for 10 minutes at RT. The cells were washed again with HBSS and centrifuged at 1200xg for 7 minutes.
Generation of rat cortical synaptosomes

Two wildtype female ex-breeder Sprague-Dawley rats (Charles River) were sacrificed using a CO₂ procedure, in accordance with the approved humane killing protocols detailed in Schedule 1 of the Animals in Scientific Procedures Act, 1986, and the brain cortices dissected. Synaptosomes were purified from the fresh cortices using a previously described method of Percoll gradient fractionation, with four Percoll gradients per rat (Dunkley, Jarvie, & Robinson, 2008). An aliquot of the purified synaptosomes was dissolved in 1% NP-40 and the protein concentration determined by Bradford assay. Accordingly, the synaptosomes were diluted to 1 mg/mL of their total protein content with HEPES-buffered media (pH 7.4, 140 mM NaCl (VWR), 5 mM KCl (VWR), 5 mM NaHCO₃ (Sigma), 1.2 mM NaH₂PO₄ (Sigma), 1 mM MgCl₂,·6H₂O (Sigma), 10 mM glucose (VWR), 1 mg/ml BSA (Sigma), 10 mM HEPES (Sigma)) with 5% (v/v) DMSO, and frozen in single-use aliquots at -80°C. Thawed synaptosomes were characterised by negative staining transmission electron microscopy, performed by the Sir William Dunn School of Pathology Electron Microscopy Facility. Upon thawing, synaptosomes were centrifuged at 3000 x g for 10 minutes at 4°C, and washed once with Live Cell Imaging Solution (Invitrogen), to remove residual BSA before pHrodo-labelling.

Annexin V-FITC staining for phosphatidylserine

Phosphatidylserine exposure of phagocytic cargo was visualised using an Annexin V-FITC Apoptosis Detection Kit (Abcam). One day prior to staining, SH-SY5Ys were seeded to 50% confluence in a 24-well plate. Synaptosomes were thawed, washed and re-suspended in Annexin binding buffer, and approximately 0.3 µg per well added to empty wells of the plate, allowing an hour to settle at 37°C/5% CO₂. SH-SY5Ys were washed with HBSS and some wells fixed with 2% paraformaldehyde in Live Cell Imaging Solution for 10 minutes, before another wash, replacing with Annexin binding buffer containing NucBlue. Both SH-SY5Ys and synaptosomes, except for unstained controls, were stained with 1:70 annexin V-FITC and 1:70 propidium iodide for 5 minutes. Propidium iodide stains nuclei of permeable cells, controlling for annexin V staining of the plasma membrane inner leaflet. The plate was imaged at 37°C/5% CO₂ on the EVOS FL Auto with on-stage incubator at 40X with phase, and using the DAPI, GFP and Texas Red light cubes.

Phagocytosis assays

pMacpre were seeded at 2 x 10⁴ cells/well in optically-clear bottom CellCarrier 96-well plates (Perkin Elmer), and differentiated in macrophage media for a week. Cells were stained for 45 minutes at
37°C/5% CO₂ with 1 μM CellTracker Deep Red (Invitrogen) and 1 drop/mL NucBlue Live ReadyProbes Reagent (Invitrogen). Cells were washed with PBS, and then incubated for 1 hour at 37°C/5% CO₂ with 100 μL of Live Cell Imaging Solution (LCIS, Invitrogen) ± phagocytosis inhibitors, before addition of phagocytic cargo. Phagocytosis inhibitors used for validation were 10 μM cytochalasin D (Cayman), 1 μM bafilomycin A1 (Abcam), 1 μM jasplakinolide (Santa Cruz), and 2 μg unlabelled human recombinant annexin V (BD Biosciences). Annexin V was added to well immediately prior to addition of phagocytic cargo. The phagocytic cargo- synaptosomes or dead SH-SY5Ys- were stained with pHrodo iFL Red STP Ester (Invitrogen), using 20 μg of dye per 1 mg synaptosomes, or 12.5 μg of dye per 1 x 10⁶ SH-SY5Ys, aiming for a final concentration of 40 μg/mL. pHrodo-labelling was performed for 30 minutes at RT, protected from the light, in a low protein-binding tube. Cargo was washed twice with HBSS, (centrifugation: 3000 xg synaptosomes, 1200 xg dead SH-SY5Ys), and re-suspended in LCIS to a concentration of 0.6 μg/μL synaptosomes or 8 x 10⁵ cells/mL SH-SY5Ys, and 50 μL/well added to the pMac. Phagocytosis was performed at 37°C/ 5% CO₂ for 0.5-5 hours, in triplicate wells. Cells were fixed with 2% paraformaldehyde in PBS (Alfa Aesar) for 15 minutes at RT, and washed with PBS before imaging. Images were taken with an INCell Analyzer 6000 high-content imaging system (GE Healthcare Life Sciences) with a 40x objective, 9 fields/well on a single plane. Images were quantified with Columbus 2.7 software (Perkin Elmer). The parameters measured for each field were average number of spots/cell, the sum of the spot areas, and the % spot positive cells. Data was averaged for the technical replicates, and normalised to the overall plate average, to adjust for differences between plates.

Flow cytometry for macrophage biomarkers
pMac were lifted from 6-well plates by incubation with StemPro Accutase (Gibco) for 10 minutes at 37°C. The cells were washed with PBS and blocked in FACS buffer (PBS, 1% FCS, 10 μg/mL human IgG) for 10 minutes at RT. 2 x 10⁵ cells per sample were stained with directly-conjugated primary antibodies against CD11b, CD14, and CD45, for 30 minutes at RT. Cells were then washed twice with FACS buffer and fixed with 4% paraformaldehyde in PBS (Alfa Aesar) for 10 minutes at RT. Cells were washed with PBS, and analysed immediately on a FACS Calibur flow cytometer (BD Biosciences). Fluorophore-conjugated isotype controls from the same manufacturers were used. The antibodies are listed in Table 1.

DNA sequencing of R47H mutation
DNA was extracted from a cell pellet of iPSC using the DNeasy Blood & Tissue kit from QIAGEN. A PCR reaction in 25 μL was performed using Phusion HF buffer, 0.5 units Phusion HF DNA polymerase, 200 μM
dNTPs, 500 nM forward primer (AAACACATGCTGTGCCATCC), 500 nM reverse primer (CACAGACGCCAAAACATGAG), and genomic DNA (50-100 ng). PCR reaction: 1 x (98°C, 30 seconds), 30 x (98°C, 5 seconds; 60.7°C, 10 seconds; 72°C, 15 seconds), 1 x 72°C, 5 minutes. PCR products were sent for sequencing (Eurofins) with the reverse primer: TGATGCTGTGCTCCCATTC.

Statistical analysis

Statistical analysis of the data was performed in GraphPad Prism software (version 7), using means from three or more independent repeats, with paired t-tests, one-way or two-way ANOVAs performed where appropriate, and Bonferroni, Sidak or Dunnett corrections for multiple comparison. P values <0.05 were considered to be significant, and are indicated as: *p < 0.05, **p < 0.01, ***p < 0.001.

### Table 1: Antibodies

| Target       | Species raised in | Antibody conjugate | Identifier | Supplier | Conc.  | Use            |
|--------------|-------------------|--------------------|------------|----------|--------|----------------|
| TREM2        | Goat              | -                  | AF1828     | R&D Systems | 3.84 μg/mL / 1:30 | Stimulus/ICC |
| TREM2        | Rabbit            | -                  | EPR20243   | Abcam    | 1:500  | WB             |
| Syk phospho-Y525/Y526 | Rabbit | -                  | MA5-14918  | Thermo Fisher | 1:200  | WB             |
| Total Syk    | Mouse             | -                  | 4D10       | CST      | 1:500  | WB             |
| GAPDH        | Rabbit            | -                  | G9545      | Sigma    | 1:2000 | WB             |
| Rabbit IgG   | Goat              | IRDye 800CW        | 926-32211  | LI-COR   | 1:3000-1:5000 | WB     |
| Mouse IgG    | Donkey            | IRDye 680RD        | 926-68072  | LI-COR   | 1:3000-1:5000 | WB     |
| Calnexin     | Rabbit            | -                  | ab22595    | Abcam    | 1:1500 | ICC            |
| TGN-46       | Rabbit            | -                  | ab50595    | Abcam    | 1:100  | ICC            |
| LAMP1        | Rabbit            | -                  | 9091       | CST      | 1:200  | ICC            |
| Goat IgG     | Donkey            | Alexa Fluor 488    | A11055     | Thermo Fisher | 1:1000 | ICC            |
| Rabbit IgG   | Donkey            | Alexa Fluor 568    | A10042     | Thermo Fisher | 1:500  | ICC            |
| IL-6         | Rat               | -                  | 14-7069-81 | Thermo Fisher | 4 μg/ml | ELISA capture |
| IL-6         | Rat               | Biotinyl-ated      | 13-7068-81 | Thermo Fisher | 2 μg/ml | ELISA detection |
| sTREM2       | Rabbit            | -                  | ab209814   | Abcam    | 8 μg/ml| ELISA capture |
| sTREM2       | Goat              | Biotinyl-ated      | BAF1828    | R&D Systems | 1.5 μg/ml | ELISA detection |
| CD11b        | Mouse (IgG1κ)     | APC                | 301309     | Biolegend | 1:25  | FC             |
| Control      | Mouse (IgG1κ)     | APC                | 400119     | Biolegend | 1:50  | FC             |
| CD14         | Mouse (IgG1κ)     | PE                 | 21620144   | Immuno-tools | 1:25  | FC             |
| Control      | Mouse (IgG1)      | PE                 | 21335014   | Immuno-tools | 1:25  | FC             |
| CD45         | Mouse (IgG1)      | APC                | 21335014   | Immuno-tools | 1:25  | FC             |
| Control      | Mouse (IgG1)      | APC                | 21275516   | Immuno-tools | 1:25  | FC             |

List of antibodies used in this study. WB, Western blot; ICC, Immunocytochemistry; FC, Flow
Results
R47H TREM2 has reduced cell surface expression compared with WT, but equivalent total protein levels

Human microglia can be effectively modelled in vitro by the differentiation of human iPSC, via our previously published protocol for primitive, tissue-type macrophages, pMac (27,30). pMac have a similar transcriptional signature to iPSC-microglia co-cultured with neurons, and express high levels of microglial genes (28). We used three iPSC lines generated by Bioneer with the R47H TREM2 mutation inserted homozygously, and a TREM2 knockout, both on the same genetic background as the control WT iPSC line. We independently validated the R47H mutation by DNA sequencing, and the TREM2 knockout by Western blotting (Additional files 1 and 2: Figure S1 and Figure S2). After differentiating the iPSC lines to pMac we confirmed that a similarly high efficiency of differentiation was achieved between the three lines (Additional file 2: Figure S2). Altered morphology of the TREM2 KO pMac relative to the WT line was observed, and quantification confirmed that KO pMac were significantly smaller and rounder across multiple differentiations (Additional file 2: Figure S2).

In this study, we aimed to characterise the effect of the R47H mutation on TREM2 protein expression, and on cell phenotypes, as illustrated in the diagram in Fig 1A. TREM2 total protein levels in the R47H TREM2 line were unchanged relative to the WT line (Additional file 2: Figure S2). TREM2 functions at the cell surface, therefore cell surface proteins were purified by cell surface biotinylation and TREM2 detected by Western blotting, with comparison to the input cell homogenate (Fig 1B). Surprisingly, the R47H TREM2 mutation showed significantly reduced cell surface levels of TREM2 by an average of 52% in this assay, compared to the WT line (Fig 1C). This difference in surface TREM2 was not detected by immunocytochemistry or flow cytometry of intact pMac (Additional file 2: Figure S2), using an antibody that we confirmed is specific to TREM2 (unlike others, see Additional file 3: Figure S3). Additionally, we measured levels of the soluble TREM2 ectodomain (sTREM2) by ELISA of conditioned media. Shedding of cell surface TREM2 occurs constitutively by the action of sheddases (31,32). The R47H TREM2 pMac produced significantly higher levels of sTREM2 than WT in four hours,
displaying an inverse relationship to cell surface biotinylation (Fig 1D). Finally, we investigated TREM2 intracellular localisation: immunocytochemistry of permeabilised pMac was used to measure colocalisation of TREM2 with markers for the endoplasmic reticulum (ER), trans-Golgi network (TGN), and lysosomes. High levels of TREM2 in the TGN, and weak localisation to the ER and lysosomes was observed, however TREM2 localisation between the WT and R47H pMac was similar, apart from a small 14% reduction in R47H TREM2 localisation to the TGN (Fig 1E,F). Taken together, the data suggests that R47H does not significantly alter TREM2 protein trafficking or maturation, however shedding is elevated in pMac.

TREM2 KO and R47H TREM2 mutation have little effect on LPS-mediated inflammation in pMac
TREM2 deficiency has been reported to potentiate bacterial lipopolysaccharide (LPS)-induced inflammation in mouse bone marrow-derived macrophages, but not human microglia-like cells (33–35). Challenge with E. coli LPS dose-dependently upregulated secretion of the pro-inflammatory cytokines TNF and IL-6, in pMac from all three genotypes (Fig 2A, B). Only at the highest dose of LPS, the TREM2 KO line produced significantly less TNF than the WT line, whereas IL-6 secretion was unaltered. When the pMac were primed with IFNγ for 24 hours before stimulation with LPS (Fig 2C, D), clear potentiation of TNF and IL-6 responses was observed as expected (34). Here, cytokine secretion of the TREM2 KO cells was unchanged versus the WT line, whereas in the R47H TREM2 line there was a 25% reduction of TNF secretion. Together these results suggest that TREM2 expression is not acting as a brake on inflammation in pMac, but the R47H mutation may have a gain-of-function effect under specific conditions.

Loss of TREM2, but not the R47H mutation, reduces survival of pMac
TREM2 activates Akt and β-catenin-mediated pro-survival signalling, and is proposed to have tonic activity in the absence of any specific damage-associated or pathogen-associated signals (5,36). The growth factor M-CSF, used for macrophage differentiation, stimulates the same pro-survival signalling pathways, therefore should compensate for TREM2 deficiency unless depleted. After 7 days of
differentiation with M-CSF, M-CSF was withdrawn from pMac by a full media change, and cell death analysed after a further 3-10 days in comparison to complete media. Interestingly, WT and R47H TREM2 pMac did not lose viability in response to M-CSF withdrawal, even after 10 days (Fig 3A). There was a small but significant enhancement of cell death following M-CSF withdrawal in TREM2 KO pMac that increased over time (Fig 3A). In full media there was little change in viability over time (Fig 3B), therefore cell death was largely driven by M-CSF withdrawal.

Syk-mediated signalling during phagocytosis of dead SH-SY5Ys is partially TREM2-mediated, and not reduced by R47H TREM2

Activation of TREM2-DAP12 activates the kinase Syk (5), which can be identified by Western blotting with an antibody specific for double phosphorylation of Y525 and Y526 (37). Additionally, Syk activation leads to release of intracellular calcium stores via phospholipase C, which can be measured by a live-cell fluorescent assay for calcium flux. To investigate functionality of the TREM2-DAP12 complex, a specific TREM2-activating antibody was used to stimulate downstream signalling in pMac. The TREM2 antibody induced both Syk phosphorylation and calcium flux, which was absent in TREM2 KO pMac but unaffected by R47H TREM2, demonstrating that the R47H mutation does not alter TREM2 activity (Fig 4A-C, Additional file 4: Figure S4). For further studies, a more physiological ligand was sought. As a model of apoptotic neuron membranes, SH-SY5Ys were fixed with paraformaldehyde, inducing phosphatidylserine exposure of the SH-SY5Ys (Additional file 5: Figure S5), a phenomenon reported in platelets (38). Addition of dead SH-SY5Ys to the pMac in a time-course, with an unstimulated control for each time-point, produced a strong Syk phosphorylation signal in the WT line peaking around 30 minutes (Fig 4D). Syk phosphorylation was not significantly different in the R47H line, however the TREM2 KO had a significant 64% decrease in peak Syk phosphorylation relative to WT (Fig 4E). Together this shows that the Syk response of pMac to dead SH-SY5Ys is largely TREM2-dependent, but the R47H TREM2 mutant does not reduce Syk signalling in response to SH-SY5Ys.
TREM2 KO but not R47H TREM2 impairs phagocytosis of dead SH-SY5Ys and synaptosomes

Loss of functional TREM2 has been previously reported to reduce microglial clearance of apoptotic neurons (6). Phagocytosis of neurons was modelled using two different phagocytic cargoes (for time-lapse videos, see Additional files 7 and 8): fixed undifferentiated SH-SY5Ys, and rat cortical synaptosomes, which are a synapse-enriched fraction of rat cortex (Additional file 6: Figure S6). Both fixed (dead) SH-SY5Ys and synaptosomes expose surface phosphatidylserine, as detected by annexin V staining (Additional files 5 and 6: Figure S5 and Figure S6). An acid-sensitive dye, pHrodo iFL Red, was used to label the phagocytic cargoes to improve specific detection by high-content microscopy. Robust inhibition of the phagocytosis signal was achieved with actin and lysosome inhibitors, and significant enhancement by opsonising the cargo, which demonstrates that the assay is sufficiently sensitive to discriminate alterations in phagocytic capability (Additional files 5 and 6: Figure S5 and Figure S6). Addition of recombinant annexin V to mask the exposed phosphatidylserine on the dead SH-SY5Ys led to a significant 30% reduction in phagocytosis, indicating that a large proportion of phagocytosis may be phosphatidylserine-independent (Additional file 5: Figure S5).

The involvement of TREM2 in phagocytosis was investigated by performing ICC for TREM2 on control pMac phagocytosing pHrodo-labelled dead SH-SY5Ys. Strikingly, TREM2 was observed to be strongly concentrated at pMac phagocytic cups (Fig 5A). Internalised SH-SY5Ys in Rab9+ endosomes had weak TREM2 signal, as would be expected for a phagocytic receptor that is quickly recycled back to the plasma membrane (Fig 5B). TREM2 KO pMac had a 63% and 55% reduction in phagocytosis of dead SH-SY5Ys and synaptosomes respectively (Fig 5C-F), in relation to the quantity of cargo consumed over 5 hours (measured as average spots per well), indicating that these processes require TREM2. Additionally, a lower proportion of the TREM2 KO pMac phagocytosed dead SH-SY5Ys. With respect to synaptosome phagocytosis, the proportion of macrophages taking up cargo was saturated very quickly, therefore was not significantly reduced in the TREM2 KO line, likely due to the small size of the cargo (approximately 1.5 μm, measured by electron microscopy in Figure S6, Additional file 6). The R47H mutation of TREM2 did not significantly impair phagocytosis of dead SH-SY5Ys or synaptosomes, consistent with the finding in Fig 4E that phagocytosis-induced Syk phosphorylation
was unaffected by the mutation.

Discussion

In a human stem cell macrophage model, we have shown for the first time that phagocytosis of dead neurons and synaptosomes is largely TREM2-dependent, but not impaired by the R47H Alzheimer’s risk variant. We have also shown that TREM2 has a role in pro-survival signalling, but not TLR4-mediated pro-inflammatory activation.

Supporting the data in the current study, Garcia-Reitboeck et al used human microglia-like cells harbouring the T66M and W50C mutations in TREM2 that impede cell surface expression (approximately 80-90% reduction in TREM2 CTFs and sTREM2), to demonstrate reduced phagocytosis of apoptotic neurons and poorer survival after M-CSF withdrawal (35). Interestingly, in the same T66M and W50C TREM2 lines, phagocytosis of E. coli, zymosan, and acetylated-LDL was unaffected, with the implication that TREM2 is less important in phagocytosis of these cargoes (35,39). Claes et al generated the first published R47H mutation in human embryonic stem cells, and showed that it had no impact on phagocytosis of ex-vivo amyloid plaques or E. coli by microglia-like cells, whereas TREM2−/− and −/+ had defective phagocytosis of these cargoes, agreeing with the current study findings that R47H does not phenocopy TREM2 deficiency in human microglia models (40). The effect of R47H on phagocytosis has not been studied in mouse models.

We also tested pro-inflammatory activation by LPS. TREM2 deficiency had no effect on LPS-stimulated TNF and IL-6 secretion, except for TNF release at the highest LPS concentration. This agrees with the two aforementioned studies of T66M and W50C TREM2 mutations, showing that LPS-stimulated inflammation was unaltered (35,39). The R47H mutation did not impair cytokine release induced by LPS alone, but curiously it attenuated IFNγ-primed LPS inflammation, an effect not seen in the TREM2 KO line. Recently, Piers et al identified a “locked immunometabolic switch” in the same R47H homozygous iPSC line used in this manuscript, namely a defect in upregulating glycolytic energy production in response to sudden energy demands placed on the cell, for example pro-inflammatory stimulation (41). Cytokine secretion, phagocytosis and migration require the cells to alter their metabolism rapidly, increasing glycolysis to supply energy. The authors noted that concurrent LPS
and IFNγ stimulation produced a weaker TNF response in the R47H, T66M and W50C lines compared with wildtype controls (41), which supports our own observation in the current study. Importantly, in the Piers et al study the identified metabolic defects in R47H cells were uncovered using manipulations with LPS + IFNγ and glycolysis inhibitors, the cells were similar to the isogenic control when unstimulated (41). This suggests that pathogenic manifestation of the mutation requires cell stress or high levels of activation.

Our findings of TREM2 loss-of-function phenotypic effects in iPS-microglia have relevance to Nasu-Hakola disease (NHD), a rare autosomal-recessive genetic disease presenting with early-onset dementia. Nasu-Hakola disease is linked to mutations in TREM2, such as T66M and W50C, which prevent complex glycosylation and cause TREM2 to be trapped in the ER. The TREM2 KO model lacks the ER stress caused by misfolded TREM2 in T66M and W50C lines, yet the impact on apoptotic neuron phagocytosis and survival was similar. In contrast to the NHD mutations, the AD mutation R47H TREM2 had no reduction in cell surface expression and no Golgi accumulation, in HEK293T, T cells, or mouse bone-marrow-derived macrophages (12,23,32,42). However, alterations to TREM2 complex glycosylation, and reduced stability and retromer-mediated receptor recycling have been observed with the R47H mutation (21,43,44). In our study, the R47H mutation appeared to reduce cell surface expression of TREM2 measured by cell surface biotinylation, and increase shedding of TREM2, without affecting total cell expression and without causing a significant accumulation of TREM2 in the TGN. Nevertheless, the reduction in cell surface TREM2 of the R47H TREM2 pMac appears to have been too slight to impair TREM2-dependent phagocytosis and survival. The main pathogenic effect of the R47H mutation is hypothesised to be a reduction in ligand-binding capability. Although this is certainly observed in vitro with biochemical assays, the effect may not be quantitatively significant for the interaction of microglia with their natural cargoes.

Conclusions
In a model of human microglia, our findings support the literature showing that TREM2 regulates phagocytosis and survival, but not LPS-inflammation. This study introduces two novel phagocytosis assays for the uptake of annexin V-positive dead SH-SY5Ys and synaptosomes by human iPS-
macrophages, and is the first to apply these to studying the R47H TREM2 variant, a mutation shown to perturb recognition of the apoptosis “eat me” signal PtdSer in biochemical assays. Although phagocytosis of these cargoes was demonstrated to be largely TREM2-dependent and partially PtdSer-dependent, the R47H mutation did not impair phagocytosis in this human system, nor the Syk-dependent signalling that leads to cell survival and phagocytosis. Given that the R47H mutation leads to a 2-to-4.5-fold increased risk of AD (17,19), it is worth continuing to seek microglial phenotypes affected by the risk allele, but we anticipate that additional cell stressors may be required to reveal its true impact.

List Of Abbreviations
KO, knockout; iPSC, induced pluripotent stem cell; pMac, iPSC-macrophage; LPS, lipopolysaccharide; PtdSer, phosphatidylserine

Declarations
Ethics approval and consent to participate
The parent line BIONi010-C was generated by Bioneer from normal adult human skin fibroblasts sourced from Lonza (#CC-2511), who provide the following ethics statement: ‘These cells were isolated from donated human tissue after obtaining permission for their use in research applications by informed consent or legal authorization.’ For Figure S5 (Additional file 5), pMac differentiated from SFC840-03-03 iPSC were used. SFC840-03-03 was derived from dermal fibroblasts from a disease-free donor recruited through the Oxford Parkinson’s Disease Centre, having given signed informed consent, which included derivation of hiPSC lines from skin biopsies (Ethics Committee: National Health Service, Health Research Authority, NRES Committee South Central, Berkshire, UK (REC 10/H0505/71)).

Consent for publication
Not applicable.

Availability of data and materials
All data generated or analysed during this study are included in this published article and its supplementary information files.

Competing interests
The authors declare that they have no competing interests.
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Authors contributions
S.A.C., E.D. and J.B.D. conceived the study, designed the project and proof-read the manuscript; H.H-R. performed most of the experiments and analysis of the data and wrote the manuscript; J.O. performed the calcium assays and IL-6 ELISAs and analysed that data; T.B.S. performed and analysed the sTREM2 ELISA; E.M. and W.S.J. gave scientific input and advice, and proof-read the manuscript.

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Supplementary Information
Additional file 1

File format: TIFF
Title of data: Figure S1

Description of data: Validation of R47H genotype. (A) Donor template used for CRISPR insertion of R47H mutation by Bioneer. (B) Chromatograms from sequencing of parent line BIONi010-C and R47H TREM2 line BIONi010-C-7. Red asterisk indicates the R47H mutation, black asterisks are silent mutations.

Additional file 2

File format: TIFF

Title of data: Figure S2

Description of data: Validation of R47H TREM2 and TREM2 KO iPSCs and pMac. (A) Chromosome karyograms from Illumina microarray SNP analysis. (B) Morphology of pMac determined by loading cells with 1 μM CellTracker Deep Red for 45 minutes, fixed cells were imaged on INCell 6000 microscope (GE). (C) Mean cell area and mean cell roundness quantified from (B). N=3 experiments from different harvests, error bars represent SEM. Statistical analysis was performed using one-way ANOVA. * p < 0.05, *** p < 0.001 versus WT, Dunnett’s post-hoc test. (D) Surface TREM2 and macrophage surface markers CD11b, CD14, and CD45 measured by flow cytometry, compared to relevant isotype IgG, n=1. (E) To visualise cell surface TREM2, live pMac were stained with TREM2 antibody, followed by fluorescent secondary antibody, and subsequently fixed. To visualise total TREM2, pMac were fixed and permeabilised before staining with the same antibodies. Images are maximum projections from a z-stack of 5 slices, 1-5 μm, taken on an Opera Phenix microscope (Perkin Elmer). (F) Cell surface TREM2 immunofluorescence staining quantified from three independent harvests, three wells per repeat, and normalised to whole-cell TREM2 staining in fixed and permeabilised wells on the same plate. Not significant, paired t-test. (G) Representative western blot showing total levels of TREM2 protein.

Additional file 3

File format: TIFF

Title of data: Figure S3

Description of data: Validation of antibodies for TREM2 immunocytochemistry. Fixed and
permeabilised WT, R47H, and TREM2 KO pMac were stained for 1 hour at RT with three different TREM2 antibodies at the concentrations indicated, followed by staining with Alexa Fluor 488-conjugated secondary antibody (1:1000, Invitrogen). Cells were counterstained with DAPI nuclear dye and imaged on an EVOS FL Auto microscope (Thermo Fisher). Ab209814 showed cytoplasmic staining in all three genotypes, 13483-1-AP showed nuclear staining in all three genotypes, whereas AF1828 stained cytoplasm and plasma membrane in WT and R47H TREM2 pMac but not TREM2 KO pMac. Scale bar is 100 μm.

Additional file 4

*File format:* TIFF

*Title of data:* Figure S4

*Description of data:* Calcium assay kinetics. Kinetics of pMac calcium responses to (A) 0.5 mM ATP, and (B) 10 μg/mL TREM2 antibody. N=3-4 experiment repeats with different harvests, error bars represent SEM.

Additional file 5

*File format:* TIFF

*Title of data:* Figure S5

*Description of data:* Validation of dead SH-SY5Y phagocytosis assay. (A) Freshly-fixed SH-SY5Ys stain uniformly for phosphatidylserine exposure (Annexin V-FITC), but have limited cell permeability (propidium iodide). Live SH-SY5Ys do not stain for Annexin V-FITC or propidium iodide, except for focal staining present on the few dead cells in culture. (B) Dose-dependent uptake of dead SH-SY5Ys after 5 hours of phagocytosis with WT line BIONi010-C, means quantified from three independent experiments for % of spot positive (phagocytic) cells per well. N=3 experiments from different harvests, error bars represent SEM. Statistical analysis was performed using two-way ANOVA, controlling for repeated measures over time. * p < 0.05, ** p < 0.01 versus parent line for each time-point, Dunnett’s post-hoc test. (C) Phagocytosis of 3 hours is inhibited with 10 μM cytochalasin D, 1 μM bafilomycin A1, 1 μM jasplakinolide, all with 1 hour pre-treatment, and 13 μg/mL recombinant annexin V added simultaneously to the dead SH-SY5Ys. Data was normalised to mean for each
genotype per experiment. N=3-6 experiments from different harvests and two WT cell lines (SFC840-03-03, the characterisation of this line is described in (Fernandes et al (2016), Stem Cell Reports. 6 (3): 342-356), and BIONi010-C), error bars represent SEM. Statistical analysis was performed using one-way ANOVA. * p < 0.05, *** p < 0.001 versus untreated cells, Dunnett’s post-hoc test.

Additional file 6
File format: TIFF

Title of data: Figure S6

Description of data: Validation of synaptosome phagocytosis assay. (A) Two whole synaptosomes surrounded by cell debris in the cryopreserved prep, visualised by negative staining electron microscopy. White asterisks label the pre-synaptic termini, with many pre-synaptic vesicles, whereas purple asterisks label the post-synaptic termini. A dark post-synaptic density can be seen between connected pre- and -postsynaptic termini. (B) Synaptosomes stain uniformly for phosphatidylserine exposure (Annexin V-FITC), comparison is with unstained synaptosomes. (C) Phagocytosis at 3 hours is dose-dependent, reaching saturation above 30 μg. (D) Phagocytosis is inhibited by 10 μM cytochalasin D and 1 μM bafilomycin A1, and increased by prior opsonisation of synaptosomes for 30 minutes with 20% human serum. Data was normalised to mean for each genotype per experiment, and is represented as sum of spot areas (μm²) per cell. N=3-4 experiments from different harvests, error bars represent SEM. Statistical analysis was performed using one-way ANOVA. * p < 0.05, ** p < 0.01 versus untreated cells, Dunnett’s post-hoc test.

Additional file 7
File format: AVI

Title of data: Video 1: Time-lapse video of dead SH-SY5Y phagocytosis assay

Description of data: WT pMac phagocytosing pHrodo-labelled fixed SH-SY5Ys, displaying an increase in red fluorescence of the SH-SY5Ys after engulfment. In 96wp, 2 x 10⁴ pMac were given 2 x 10⁴ SH-SY5Ys, and images taken every 10 minutes at 20x for 3 hours, using an EVOS FL Auto microscope.

Video is 2 frames per second.

Additional file 8
File format: AVI
Title of data: Video 2: Time-lapse video of synaptosome phagocytosis assay

Description of data: WT pMac phagocytosing pHrodo-labelled synaptosomes, displaying an increase in red fluorescence of the synaptosomes after engulfment. In 96wp, 2 x 10⁴ pMac were given 1 µg of synaptosomes, and images taken every 10 minutes at 20x for 3 hours, using an EVOS FL Auto microscope. Video is 2 frames per second.

Figures
Figure 1
Figure 1

Characterising the surface expression and subcellular localisation of R47H TREM2 in pMac

(A) Schematic of microglia phenotypes investigated in this study. (B) Cell surface proteins on pMac were biotinylated and pulled down, the level of TREM2 protein enrichment was measured by Western blotting vs whole cell lysate, probed on separate blots. (C) Cell surface biotinylation quantified from five independent harvests on separate Western blots, error bars represent SEM. Two-tailed paired t-test: ** p = 0.0011 for R47H versus WT. (D) sTREM2 were measured from unstimulated pMac supernatants by ELISA, average from three independent harvests, for each harvest two well replicates were pooled. Samples were assayed on the same ELISA plate, data for each harvest was normalised to the average cell count, error bars represent SEM. One-way ANOVA, with Dunnett’s post-hoc test: p = 0.0005 R47H versus WT (**), p = 0.0066 KO versus WT (**). (E) Co-localisation of TREM2 with subcellular compartment markers in fixed and permeabilised pMac, images are a confocal slice at 4 μm, taken on an Opera Phenix microscope (Perkin Elmer). Calnexin used as a marker for ER, TGN46 for TGN, and LAMP1 for lysosomes. (F) Co-localisation of TREM2 with subcellular compartments quantified from three independent repeats, three wells per repeat, expressed as a ratio of TREM2 intensity to compartment marker intensity, in regions automatically segmented by high marker staining. Error bars represent SEM. Repeated-measures two-way ANOVA, with Bonferroni’s post-hoc test: p = 0.136 for ER region in R47H versus WT (ns); p = 0.048 for TGN region in R47H versus WT (*); p = 0.098 for lysosome region in R47H versus WT (ns).
Figure 2

A  TNF release after LPS stimulation

B  IL-6 release after LPS stimulation

C  TNF release after IFNg-primed LPS stimulation

D  IL-6 release after IFNg-primed LPS stimulation
Figure 2

Inflammatory response of R47H TREM2 and TREM2 KO pMac to LPS is unchanged compared to WT. (A) Secretion of TNF in response to 4 hours of 0.1-1 μg/mL LPS. N=3 experiments from different harvests, error bars represent SEM. (B) Secretion of IL-6 in the same supernatants as (A). (C) Secretion of TNF in response to 4 hours of 100 ng/mL E.coli LPS ± 24 hours pre-incubation with 100 ng/mL interferon-γ (IFNg), measured by ELISA and normalised to cell number. N=4 experiments from different harvests, errors bars represent SEM. (D) Secretion of IL-6 in the same supernatants as (C). Statistical analysis was performed using two-way ANOVA, with Dunnett’s post-hoc test. Comparisons with the coloured annotations are stimulations versus untreated cells (None) for each genotype: * p < 0.05, ** p < 0.01, *** p < 0.001. Comparisons with the black annotations are R47H or KO versus the parent line for each stimulation: p = 0.0097 for TNF secreted from R47H versus WT with LPS ± IFNg stimulation (**), all unannotated comparisons are not significant.
Figure 3
Figure 3

Survival of TREM2 KO pMac without M-CSF is impaired relative to WT. (A) Percentage cell death of M-CSF-deficient condition, after 3, 7, and 10 days. (B) Percentage cell death of full media condition, after 3, 7, and 10 days. N=3 experiments from different harvests, error bars represent SEM. Repeated-measures two-way ANOVA, with Dunnett’s post-hoc test, pairwise comparisons to WT for each time: M-CSF-deficient KO versus WT at 3 days p = 0.0051 (**), at 7 days p = 0.003 (**), at 10 days p = 0.0001 (***); Full media KO versus WT at 3 days p = 0.035 (*). All unannotated comparisons are not significant.
Figure 4

A. TREM2 Ab and Syk levels in WT, R47H TREM2, and TREM2 KO.

B. pSyk after TREM2 antibody stimulation.

C. Calcium response after TREM2 antibody stimulation.

D. Western blots showing pSyk, Syk, TREM2, and GAPDH levels.

E. pSyk levels after phagocytosis of dead SH-SY5Ys.
Figure 4

Syk activation during contact with dead SH-SY5Ys is blunted in TREM2 KO pMac, but not R47H TREM2 pMac. (A) TREM2-activating antibody stimulation (2.4 μg/1E6 cells and 3.84 μg/mL for 10 minutes) caused Syk phosphorylation in both WT and R47H TREM2 pMac, measured by Western blotting. No response seen in TREM2 antibody-stimulated TREM2 KO cells, or cells treated with a goat IgG isotype control. (B) Syk phosphorylation in response to TREM2 antibody quantified for three independent harvests on separate Western blots, error bars represent SEM. Repeated-measures two-way ANOVA, with Sidak’s multiple comparison test, pairwise comparisons to WT for each treatment: p = 0.001 for KO stimulated with TREM2 Ab versus WT (**), all other comparisons are not significant. (C) Calcium response in response to TREM2 antibody was measured by peak Fluo4-AM fluorescence, normalised to minimum fluorescence and cell number. N=3 experiments from different harvests, error bars represent SEM. Two-way ANOVA, with Dunnett’s multiple comparison test, pairwise comparisons to WT for each treatment: p = 0.0049 for KO stimulated with TREM2 Ab versus WT (**), all other comparisons are not significant. (D) Phagocytosis of dead SH-SY5Ys results in Syk phosphorylation, which is unaffected in R47H TREM2 cells but attenuated in TREM2 KO line, measured by Western blotting at 0.5, 1, and 2 hours after phagocytosis initiation. (E) Syk phosphorylation in response to dead SH-SY5Ys quantified for three independent repeats, error bars represent SEM. Repeated-measures two-way ANOVA, with Dunnett’s post-hoc test, pairwise comparisons to the WT for each time: p = 0.0008 at 0.5 hours stimulation for KO versus WT (**), all other comparisons are not significant.
Figure 5
Figure 5

Phagocytosis of dead SH-SY5Ys and synaptosomes is reduced in TREM2 KO pMac relative to WT. (A) After 3 hours of phagocytosis of pHrodo-labelled dead SH-SY5Ys, immunofluorescence staining shows that TREM2 is highly recruited to the phagocytic cup (marked by white arrow) during engulfment of cells expressing the neuronal marker Tuj1, whereas in (B) TREM2 is lost before maturation to Rab9+ endosomes (marked by white arrow). (C) Phagocytosis of SH-SY5Ys (yellow) by pMac (red), representative images taken at 3 hours with INCell 6000. Inset is a section of the image magnified 3-fold. (D) Reduced rate of uptake of dead SH-SY5Ys (4 x 10^4 cells per well) in TREM2 KO pMac. (E) Phagocytosis of synaptosomes (yellow) by pMac (red), representative images taken at 3 hours with INCell 6000. Inset is a section of the image magnified 3-fold. (F) Reduced rate of uptake of synaptosomes (30 ng per well) in TREM2 KO pMac. (D) & (F) Means were quantified for the parameters: number of spots per cell, sum of spot areas (μm²) per cell, percentage of cells containing phagocytosed particles per field. Data was normalised to mean for each genotype per experiment. N=3 experiments from different harvests, error bars represent SEM. WT shown as grey circles, R47H TREM2 as orange squares, and TREM2 KO as burgundy triangles. Statistical analysis was performed using repeated-measures two-way ANOVA, Dunnett’s post-hoc test, pairwise comparisons to the WT for each time: * p < 0.05, ** p < 0.01, *** p < 0.001, unannotated comparisons are not significant.

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