TREM2 regulates purinergic receptor-mediated calcium signaling and motility in human iPSC-derived microglia

Amit Jairaman^{1†}, Amanda McQuade^{2,3,4†}, You Jung Kang⁵, Alberto Granzotto^{2,6,7}, Jean Paul Chadarevian², Sunil Gandhi², Ian Parker^{1,2}, Ian Smith², Shivashankar Othy^{1,8}, Hansang Cho^{5,9}, Mathew Blurton-Jones^{2,3,8*}, Michael Cahalan^{1,8*}

Affiliations:

¹ Department of Physiology & Biophysics, University of California, Irvine, CA 92697-4561, USA

² Department of Neurobiology & Behavior, University of California, Irvine, CA 92697, USA

³ Sue and Bill Gross Stem Cell Research Center, University of California, Irvine, CA 92697, USA

⁴ UCI Institute for Memory Impairments and Neurological Disorders, University of California, Irvine, CA 92697, USA

⁵ Department of Mechanical Engineering and Engineering Science, Center for Biomedical Engineering and Science, University of North Carolina, Charlotte, NC 28223, USA

⁶ Center for Advanced Sciences and Technology (CAST), University "G. d'Annunzio" of Chieti-Pescara, Chieti 66100, Italy

⁷ Department of Neuroscience, Imaging, and Clinical Sciences (DNISC), University "G. d'Annunzio" of Chieti-Pescara, Chieti 66100, Italy

⁸ Institute for Immunology, University of California, Irvine, CA 92697, USA

⁹ Institute of Quantum Biophysics, Department of Biophysics, Sungkyunkwan University, 2066 Seobu-ro, Jangnan-gu, Suwon-si, Gyeonggi-do 16419, Korea

† Equal contributions

* Co-corresponding authors: Michael D. Cahalan (mcahalan@uci.edu), Mathew Blurton-Jones (mblurton@uci.edu)

Key Words: microglia, motility, Alzheimer's disease, P2Y receptor, Ca²⁺ signaling, store-operated Ca²⁺ entry (SOCE), genetically encoded Ca²⁺ indicator

Abstract

The membrane protein TREM2 (Triggering Receptor Expressed on Myeloid cells 2) regulates key microglial functions including phagocytosis and chemotaxis. Loss-of-function variants of TREM2 are associated with increased risk of Alzheimer’s disease (AD). Because abnormalities in Ca^{2+} signaling have been observed in several AD models, we investigated TREM2 regulation of Ca^{2+} signaling via genetic deletion in human induced pluripotent stem cell-derived microglia (iPSC-microglia). We found that iPSC-microglia lacking TREM2 (TREM2 KO) show exaggerated Ca^{2+} signals in response to purinergic agonists such as ADP that shape microglial injury responses. This ADP hypersensitivity, driven by increased expression of P2Y12 and P2Y13 receptors, results in sustained Ca^{2+} influx and alters cell motility and process extension in TREM2 KO microglia. In a “ Ca^{2+} clamp” assay using iPSC-microglia expressing the genetically encoded Ca^{2+} probe, Salsa6f, we found that cytosolic Ca^{2+} tunes motility to a greater extent in TREM2 KO microglia. Despite showing greater overall displacement, TREM2 KO microglia exhibit reduced directional chemotaxis along ADP gradients. Accordingly, the chemotactic defect in TREM2 KO microglia was rescued by reducing cytosolic Ca^{2+} using a P2Y12 receptor antagonist. Our results show that loss of TREM2 confers a defect in microglial Ca^{2+} response to purinergic signals, suggesting a window of Ca^{2+} signaling for optimal microglial motility.

Main Text

Introduction

As the primary immune cells of the central nervous system, microglia survey their local environment to maintain homeostasis and respond to local brain injury or abnormal neuronal activity. Microglia are strongly implicated in several neurodevelopmental and neurodegenerative diseases^{1–7}, warranting further study of human microglial dynamics. Purinergic metabolites (ATP, ADP, UTP, UDP) in the brain constitute key signals driving microglial activation and chemotaxis^{8–11}. ATP released from both homeostatic and damaged cells is hydrolyzed locally by nucleosidases such as the ectonucleotidase NTPDase1 (CD39) or pyrophosphatase NPP1 to produce ADP^{12–14}. ADP is then detected by P2Y purinergic receptors on microglia, causing IP₃-dependent Ca²⁺ release from the endoplasmic reticulum (ER) lumen. Ca²⁺ depletion from the ER in turn activates ER STIM1 proteins to translocate proximally to puncta where closely apposed plasma membrane (PM) Orai1 channels are activated. This mechanism underlies store-operated Ca²⁺ entry (SOCE) in many cell types¹⁵, including microglia^{16–18}.

Purinergic signaling is central to microglial communication with other brain cell types and has been negatively correlated with the onset of Disease Associated Microglia (DAM) transcriptional states^{19–23}. P2Y₁₂ and P2Y₁₃ receptors are highly expressed by microglia and are activated predominantly by ADP^{14,24}. P2Y₁₂ receptors are essential for microglial chemotaxis and have been implicated in the microglial response to cortical injury^{25,26}, NLRP3 inflammasome activation^{27,28}, neuronal hyperactivity and protection^{25,29}, and blood brain barrier maintenance³⁰. While purinergic receptors have been broadly identified as markers of microglial homeostasis,

mechanisms by which receptor expression may drive or maintain homeostatic microglial states remain incompletely understood.

Neuroinflammatory pathologies are often associated with altered Ca^{2+} signaling³¹. Microglia, in particular, show altered Ca^{2+} responses in mouse models of Alzheimer's Disease (AD) by mechanisms that are not fully understood^{32–34}. Ca^{2+} responses to purinergic metabolites have been extensively studied in cultured murine microglia, acute brain slices, and more recently in anesthetized mice^{8,10,32,35–37}. However, our understanding of how specific patterns of Ca^{2+} signals in microglia correlate with and tune downstream microglial responses such as cell motility or process extension remains incomplete. There is also a paucity of knowledge on how regulators of purinergic Ca^{2+} signals in microglia might play a role in the dysregulation of Ca^{2+} signaling associated with aging and neuroinflammation.

TREM2 encodes a cell surface receptor that binds a variety of ligands including various lipids, apolipoprotein E (ApoE), and amyloid- β peptides and signals through its adaptor protein DAP12 to activate a host of downstream pathways^{21,38–40}. Loss of TREM2 function is thought to lock microglia in a more homeostatic-like state^{21,41,42}. Indeed, microglia lacking TREM2 expression fail to activate against disease pathology, correlating with increased risk of AD^{21,39,43}. Purinergic receptor hyperexpression has been reported at the transcriptome level across multiple TREM2 loss of function models including human patient mutations^{19–21,23,39,44}, although the mechanistic link between purinergic receptor expression and TREM2 function remains poorly understood.

We previously developed methods to generate human induced pluripotent stem cell-derived microglia (iPSC-microglia)^{45,46}, which can be used to model human microglial behavior *in vitro*. While iPSC-microglia are proving increasingly useful to investigate neurodegenerative

disorders ^{47–51}, Ca²⁺ signaling has not yet been extensively profiled in these models. In this study, we examined purinergic Ca²⁺ signaling in human iPSC-microglia and found that sustained Ca²⁺ influx in response to ADP regulates microglial process extension, motility speed, and turning behavior. We show that microglia lacking TREM2 are hyperresponsive to purinergic signaling. Using novel iPSC-microglia lines that express a ratiometric genetically encoded Ca²⁺ probe, Salsa6f ⁵², we found that the motility characteristics of human wild-type and TREM2-knockout microglia are differentially tuned by Ca²⁺ signaling. Informed by these discoveries, we were able to rescue chemotactic deficiencies in TREM2-knockout microglia by dampening purinergic receptor signaling.

Results

Purinergic receptor Ca^{2+} signaling is enhanced in TREM2-knockout human iPSC-microglia

To determine if TREM2 plays a role in microglial Ca^{2+} signaling, we compared cytosolic Ca^{2+} responses to the purinergic agonist ADP in isogenic, CRISPR-modified wild type (WT) and TREM2-knockout (TREM2 KO) human induced pluripotent stem cell-derived microglia (iPSC-microglia). ADP stimulation induced a biphasic Ca^{2+} response – a rapid initial peak followed by sustained Ca^{2+} elevation lasting several minutes, in line with previous observations in mouse microglia^{53,54}. Both phases of the Ca^{2+} response were significantly elevated in TREM2 KO microglia (**Figure 1A, B**). TREM2 KO microglia also showed exaggerated Ca^{2+} responses to other purinergic agonists; ATP and UTP (**Figure 1C, D**). Notably, responses to ATP and UTP decayed faster than responses to ADP in both WT and TREM2 KO cells, indicating that purinergic stimulation by ADP is more potent in inducing sustained Ca^{2+} signals in human iPSC-microglia (**Figure 1E and Figure 1-figure supplement 1A**).

Sustained ADP-evoked Ca^{2+} elevation in TREM2 KO microglia is not due to SOCE enhancement

To determine the mechanism driving higher ADP-evoked Ca^{2+} signals observed in TREM2 KO cells, we investigated the individual steps in the purinergic Ca^{2+} signaling pathway. The initial Ca^{2+} response to P2Y receptor engagement occurs through phospholipase C and IP_3 -mediated ER Ca^{2+} store-release, which triggers sustained cytosolic Ca^{2+} elevation by SOCE (**Figure 1F**). Consistent with this, the sustained Ca^{2+} phase in TREM2 KO iPSC-microglia was blocked by Gd^{3+} and 2-APB, non-specific inhibitors of SOCE (**Figure 1-figure supplement 1B**). To determine if SOCE is increased in TREM2 KO microglia, we directly measured the rate of Ca^{2+} influx following Ca^{2+} re-

addition after maximal store-depletion by the SERCA pump inhibitor, thapsigargin (TG). Instead, we find the rate and amplitude of SOCE were both modestly reduced in TREM2 KO cells (**Figure 1G**), and thus changes in the maximal capacity of SOCE do not account for the elevated response of TREM2 KO microglia to ADP. Moreover, STIM1 and Orai1 mRNA expression, and Orai1 protein expression are similar in WT and TREM2 KO microglia (**Figure 1-figure supplement 2**).

We therefore hypothesized that the exaggerated secondary Ca^{2+} phase in TREM2 KO microglia may be driven primarily by increased ER Ca^{2+} store-release resulting in increased SOCE. To assess this, we plotted Ca^{2+} responses in individual cells, using Ca^{2+} levels 5 min after ADP as a surrogate for SOCE engagement and the initial peak Ca^{2+} as a readout of ER store-release (**Figure 1-figure supplement 3**). Both WT and TREM2 KO microglia showed a similar relationship between SOCE and store-release, suggesting that SOCE is engaged by similar mechanisms in the two cell types, but is recruited to a greater extent in TREM2 KO cells due to increased ER store-release. To examine whether a larger ER store content contributes to increased Ca^{2+} responses in TREM2 KO microglia, we measured Ca^{2+} levels after maximally depleting ER stores with thapsigargin (TG), ionomycin, or by IP_3 uncaging in Ca^{2+} -free solution buffered with EGTA. In all instances, quantification of peak or cumulative cytosolic Ca^{2+} increase suggested that ER store-content and IP_3 receptor activity is not altered in microglia lacking TREM2 (**Figure 1G-I**). These results indicate that TREM2 controls ADP-driven Ca^{2+} signals upstream of IP_3 receptors, ER Ca^{2+} store levels, and SOCE.

Up-regulated P2Y12 and P2Y13 receptor expression drives increased ER Ca^{2+} release in TREM2-KO microglia

To isolate the early events of Ca^{2+} signaling upstream of SOCE, we monitored Ca^{2+} store release in Ca^{2+} -free medium and found that TREM2 KO cells still showed significantly higher Ca^{2+} response to ADP, compared to WT (**Figure 2A, B**). Dose-response curves for the peak Ca^{2+} response showed a steep leftward shift in TREM2 KO cells (**Figure 2C**). The EC_{50} value for WT microglia was 650 nM, whereas TREM2 KO microglia reached their EC_{50} by 15 nM. This stark difference was driven at least in part by a diminished percentage of WT cells responding to ADP at low μM doses (**Figure 2D**). However, limiting the analysis to only those cells that show a Ca^{2+} rise revealed that responding TREM2 KO cells still exhibited higher Ca^{2+} responses to ADP than “responding” WT cells (**Figure 2E**). TREM2 KO microglia are thus significantly more sensitive to ADP than WT cells and exhibit a strong shift in dose-dependent response which may be critical in sensing ADP and in detecting ADP gradients.

In principle, signal amplification in microglia lacking TREM2 could occur at the level of P2Y receptors or IP_3 receptors. Our results with IP_3 uncaging (**Figure 1I**) rule out any major differences in IP_3 receptor expression or activity. This was further corroborated at the transcriptome level, which showed similar expression of IP_3 receptor type 2 (the major IP_3R subtype expressed in iPSC-microglia) mRNA in WT and TREM2 KO cells (**Figure 1-figure supplement 2**)^{39,45}. We therefore focused on P2Y12 and P2Y13 receptors, the main P2Y receptor subtypes in microglia that bind ADP^{45,46}. RNA sequencing showed that expression of P2Y12 and P2Y13 receptors was significantly increased in TREM2 KO cells (**Figure 2F**). To confirm up-regulation at the protein level, we performed immunostaining and found significantly higher expression of both these receptors on the PM of TREM2 KO cells (**Figure 2G, H**). Consistent with this, Ca^{2+} responses to ADP in Ca^{2+} free medium were significantly inhibited by P2Y12 and P2Y13 receptor antagonists (PSB 0739 and

MRS 2211) in both WT and TREM2 KO microglia (**Figure 2I, J**), implicating involvement of both receptor subtypes. In summary, deletion of TREM2 in iPSC-derived microglia leads to up-regulation of P2Y12 and P2Y13 receptors, rendering the cells hypersensitive to ADP signaling.

ADP potentiates cell motility and process extension in human WT iPSC-microglia

ADP is a potent chemoattractant for microglia¹⁰. Analogous to a previous study in fibroblasts⁵⁵, we found that ADP treatment alters cell motility and leads to increased rates of scratch wound closure in WT iPSC-microglia (**Figure 3A**). To investigate the cellular mechanism of accelerated wound closure, we used time-lapse imaging to track open-field microglial cell motility (**Figure 3B**). Mean cell speed and displacement (defined as the overall change in position from the origin at a given time) were both increased after application of ADP, whereas average track straightness was unaltered (**Figure 3C**). These data suggest that ADP-driven changes in motility in WT iPSC-microglia primarily arise from increases in microglial speed, not altered turning behavior. ADP-dependent increases in speed were reversed in the presence of P2Y12 (PSB 0739) and P2Y13 (MRS 2211) receptor antagonists, confirming the role of ADP in microglial cell motility (**Figure 3D**). To determine if Ca²⁺ influx regulates ADP-mediated increases in motility, we measured cell migration with ADP in Ca²⁺-free medium; in this paradigm, a transient Ca²⁺ signal would result from P2Y receptor-induced IP₃ generation and release of Ca²⁺ from internal stores, but without Ca²⁺ entry across the PM. We found that removing extracellular Ca²⁺ significantly decreased cell speed, displacement, and track straightness (**Figure 3E**).

In addition, some microglia responded to ADP addition by extending processes and altering their morphology rather than increasing motility (**Figure 3-figure supplement 1**).

Microglia have been observed to extend processes in response to injury and purinergic stimulation in brain slices^{8,26}. Therefore, we compared process complexity before and 30 min after ADP exposure in WT microglia and observed significant increases in both the number of branches per process and total length of these processes (**Figure 3F**). Similar to effects on cell motility, ADP-mediated process extension was inhibited by P2Y₁₂ (PSB 0739) and P2Y₁₃ (MRS 2211) receptor antagonists. Furthermore, even before process extension was activated with ADP, cells treated with P2Y antagonists showed significantly fewer and shorter processes. Altogether, these results demonstrate that Ca²⁺ influx across the PM is required for optimal ADP-induced migration and extension of processes in WT iPSC-microglia.

ADP-evoked changes in cell motility and process extension are enhanced in TREM2-KO microglia

To characterize differences in motility characteristics between WT and TREM2 KO microglia responding to ADP, we plotted mean squared displacement (MSD) vs time and compared cell track overlays (flower plots) which showed that ADP enhances motility in TREM2 KO cells to a greater extent than in WT microglia (**Figure 4A, B**). Baseline motility characteristics in unstimulated cells, however, were similar in WT and TREM2-KO cells (**Figure 4-figure supplement 1A-C**), consistent with our previously published findings showing no difference in scratch wound closure rates³⁹. To further understand the basis of differences in ADP-induced motility between WT and TREM2 KO cells, we analyzed mean speed, displacement and track straightness. Although mean speeds were similar, TREM2 KO microglia showed greater displacement than WT cells (**Figure 4C, D**), raising the possibility that KO cells may turn with lower frequency. Indeed, an

analysis of track straightness revealed that TREM2 KO microglia move farther from their origin for the same total distance traveled (**Figure 4E**). Vector autocorrelation, an analysis of directional persistence⁵⁷, further confirmed that WT cells turn more frequently than TREM2 KO microglia (**Figure 4-figure supplement 1D, E**). To assess if these differences in TREM2 KO cells require sustained Ca^{2+} influx, we analyzed microglial motility in response to ADP stimulation in the absence of extracellular Ca^{2+} (**Figure 4F-J**). Mean-squared displacement (MSD) and cell-track overlay plots showed that motility is constrained when Ca^{2+} is removed from the external bath in both WT and TREM2 KO cells (**Figure 4 A, B vs. F, G**). In the absence of extracellular Ca^{2+} , TREM2 KO microglia showed similar mean speed, displacement, and track-straightness as WT cells (**Figure 4 C-E vs H-J**). We conclude that increases in microglial motility (speed, displacement, and straightness) require sustained Ca^{2+} influx, and that deletion of TREM2 reduces microglial turning in response to ADP.

We next analyzed how TREM2 deletion affects the extension of cellular processes. Compared to WT cells, TREM2 KO iPSC-microglia showed a more dramatic increase in the number of branches and length of processes extended in response to ADP (**Figure 4 K-M, Figure 4-figure supplement 2A, B**). Some of these differences can be attributed to the reduced morphological complexity of TREM2 KO cells prior to stimulation. Importantly, Ca^{2+} entry across the PM is required in WT microglia for optimal process extension in response to ADP, since ADP stimulation in Ca^{2+} -free medium was insufficient to induce process extension (**Figure 4-figure supplement 2A and B vs C and D**). However, TREM2 KO cells still showed some increase in process branching and extension following ADP addition in the absence of external Ca^{2+} (**Figure 4-figure supplement 2C,**

D). Together, these results indicate that the increase in Ca^{2+} influx regulates microglial motility and process extension to a greater extent in TREM2 KO microglia.

Expression of Salsa6f, a ratiometric Ca^{2+} reporter, does not perturb microglial functions

Our findings so far suggest that patterns of Ca^{2+} signaling in iPSC-microglia regulate cell motility and process extension. To further characterize the effects of sustained Ca^{2+} signals on microglial motility, we developed a novel iPSC line that expresses a genetically encoded, ratiometric Ca^{2+} indicator, Salsa6f, to monitor cytosolic Ca^{2+} and motility simultaneously in individual cells⁵². Salsa6f relies on GCaMP6f to detect Ca^{2+} , which is fused with tdTomato in order to provide a stable Ca^{2+} -independent red fluorescence, allowing cells to be tracked consistently even when Ca^{2+} levels are low. Salsa6f WT iPSC-microglia showed the expected increase in the GCaMP6f fluorescence in response to Ca^{2+} elevation without affecting tdTomato signal, permitting determination of ratiometric Ca^{2+} levels (G/R ratio), and confirming that the probe was functional (**Figure 5A-C and Figure 5-figure supplement 1**). Compared to non-transgenic iPSC-microglia, Salsa6f-expressing cells showed similar expression of microglial identity markers P2RY13 and IBA1, retained their ability for phagocytosis across several substrates, and had similar differentiation yields, indicating that Salsa6f expression does not perturb microglial function or activation (**Figure 5D-G**). Isogenic Salsa6f-lines lacking TREM2 were more responsive to ADP than WT cells, consistent with our data in Figures 1 and 2 (**Figure 5H**). Using this model, we were able to simultaneously track Ca^{2+} signals and cell-motility in response to ADP (**Figure 5I**).

Ca^{2+} levels tune motility in TREM2 KO iPSC-microglia

To isolate the specific effects of sustained Ca^{2+} elevations on microglia motility, we developed a “ Ca^{2+} clamp” protocol that relies on triggering SOCE and varying external Ca^{2+} to stabilize cytosolic Ca^{2+} at “low” or “high” levels in the Salsa6f reporter line (**Figure 6A**), similar to our previous study in T lymphocytes⁵⁹. ER Ca^{2+} stores were depleted by addition of TG in Ca^{2+} -free medium, followed by readdition of 0.2 or 2 mM external Ca^{2+} (**Figure 6B**). Motility measurements were made during the slowly-falling phase of the Ca^{2+} response (**Figure 6C**). In WT cells, lowering extracellular Ca^{2+} from 2 to 0.2 mM predictably decreased the G/R ratio but did not influence mean speed, cell displacement, or track straightness (**Figure 6C, D top**). However, in TREM2 KO microglia, clamping Ca^{2+} to a lower level significantly increased speed, displacement, and track straightness (**Figure 6C, D bottom**). These data suggest that motility characteristics of TREM2 KO microglia are more sensitive to changes in cytoplasmic Ca^{2+} levels than in WT cells. Addition of ADP in this “clamp” paradigm to activate Ca^{2+} -independent signaling pathways that regulate cell motility did not alter the potential for Ca^{2+} to tune cell motility in TREM2 KO microglia (**Figure 6-figure supplement 1A**).

To further analyze the Ca^{2+} dependence of microglial motility, we plotted Salsa6f G/R Ca^{2+} ratios at every time point against the instantaneous speeds for each individual cell (**Figure 6E**). These data revealed a stronger dependence of instantaneous speed on Ca^{2+} levels in TREM2 KO microglia (**Figure 6F**). Furthermore, when stratifying cell speed arbitrarily as “fast” ($> 10 \mu\text{m}/\text{min}$) or “slow” ($< 10 \mu\text{m}/\text{min}$), we observe a marked reduction in the percentage of “fast” cells when Ca^{2+} levels are high in TREM2 KO microglia (**Figure 6G**). Interestingly, cell displacement correlated with Ca^{2+} to the same degree in both WT and KO cells (**Figure 6-figure supplement 1B, C**).

Together, the Ca^{2+} clamp experiments show that TREM2 KO human microglia are more sensitive to tuning of motility by cytosolic Ca^{2+} than WT cells.

Chemotactic defects in TREM2 KO microglia are rescued by dampening purinergic receptor activity

In vivo, directed migration of microglia is driven by gradients of ADP from dying or injured cells^{26,29}. To study chemotaxis toward ADP over a stable gradient, we exposed iPSC-microglia to ADP in two-chamber microfluidic devices. Consistent with previous findings, WT iPSC-microglia directionally migrated up the concentration gradient of ADP resulting in higher numbers of cells within the central chamber^{39,60}. In the absence of a chemotactic cue, this directional migration was lost (**Figure 7A**). This assay revealed a deficit of chemotaxis in TREM2 KO microglia (**Figure 7A**), mirroring reports that TREM2 KO microglia are unable to migrate toward damaged neurons near amyloid plaques in AD^{38,39,61}. Given that ADP hypersensitivity in TREM2 KO cells is driven by increased expression of P2Y receptors, we examined the effects of dampening P2Y signaling to WT levels. Treatment with the P2Y12 antagonist, PSB 0739, reduced Ca^{2+} responses in TREM2 KO cells and rescued the migration deficit in the chemotaxis assay (**Figure 7B-C**). These results link the increased Ca^{2+} signals and altered motility characteristics evoked by ADP in TREM2 KO cells to directed migration toward areas of tissue damage, a vital functional response in microglia.

Discussion

This study focuses on two aims: understanding the roles of purinergic signaling in regulating human microglial motility behavior; and elucidating the impact of TREM2 loss of function on this pathway. We find that the high level of purinergic receptor expression in TREM2 KO iPSC-microglia renders them highly sensitive to ADP-mediated signaling and leads to exaggerated cytoplasmic Ca^{2+} responses. Mechanistically, this increase in Ca^{2+} signals is driven by enhanced IP_3 -mediated store-release coupled to SOCE. Indeed, based on the dose-response curves for peak ADP- Ca^{2+} responses in Ca^{2+} free buffer, TREM2 KO cells have an EC_{50} at least 10-fold lower than WT cells. As a functional consequence, TREM2 KO microglia exhibit a defect in turning behavior. Despite moving in straighter paths, TREM2 KO cells are unable to migrate towards a gradient of ADP as efficiently as WT cells, concordant with previous studies showing reduced migration of TREM2 KO cells towards $\text{A}\beta$ plaques³⁹. We propose a model (**Figure 8**) whereby enhanced ADP signaling abolishes the ability of TREM2 KO cells to distinguish gradations of agonist (in this case ADP). The loss of gradient-sensing results in an inability to perform directed migration. We hypothesize that increased ADP signaling and exaggerated Ca^{2+} responses in TREM2 KO cells result in Ca^{2+} signaling domains that are no longer restricted to the cell region near to the highest ADP concentrations, thereby disrupting the polarity of key signaling molecules that drive directed cell motility.

The amplitude and duration of Ca^{2+} signals shape specificity of downstream cell responses. Our experiments with ADP in Ca^{2+} free medium revealed that a transient Ca^{2+} signal is insufficient to induce microglial motility in either WT or TREM2 KO cells. Previous studies have shown that mouse microglia with genetic deletion of STIM1 or Orai1 also show defects in cell

migration to ATP^{53,62}, likely because diminished SOCE renders them unable to sustain Ca²⁺ signals in response to ATP. The dependence of motility on prolonged purinergic Ca²⁺ signals may thus be a general feature of microglia. In contrast, a Ca²⁺ transient can initiate some process extension in TREM2 KO but not in WT microglia, suggesting a threshold for ADP signaling that is reached in KO but not WT cells, and highlighting subtle differences in the Ca²⁺ requirement for motility and process extension in TREM2 KO microglia.

To directly monitor Ca²⁺ signaling and motility simultaneously in individual cells, we developed a novel iPSC-microglia cell-line expressing a genetically encoded, ratiometric Ca²⁺ indicator Salsa6f, a GCaMP6f-tdTomato fusion protein. Because Salsa6f allows simultaneous measurement of Ca²⁺ signal and tracking of processes, this Salsa6f iPSC line is likely to be a useful tool to dissect the relationship between Ca²⁺ signaling and the function of various iPSC-derived human cell types including neurons, astrocytes, and microglia. In addition, this line may be readily xenotransplanted for use with human/microglia chimeric models to examine functional Ca²⁺ responses to injury and pathology *in vivo*. Using Salsa6f-expressing microglia, we uncovered critical differences in how Ca²⁺ levels tune motility in WT and TREM2 KO microglia. By tracking instantaneous velocity at the same time as Salsa6f Ca²⁺ ratios in individual cells, we found that TREM2 KO cell motility showed a greater sensitivity to changes in cytosolic Ca²⁺ levels with significantly higher speeds than WT cells at lower Ca²⁺ and a more dramatic reduction in cell speed at high Ca²⁺ levels. Accordingly, reducing cytosolic Ca²⁺ in a Ca²⁺ “clamp” assay resulted in increased mean speed, displacement, and straighter paths for TREM2 KO iPSC-microglia, but had no effect on these motility metrics in WT cells. Consistent with this observation, chemotaxis in TREM2 KO cells was restored by partially inhibiting P2RY12 receptors. In response to

neurodegenerative disease, microglia down-regulate P2RY12^{21,23,30}. Active regulation of purinergic receptor expression is critical for sensing ADP gradients and decreasing motility near the chemotactic source. Further understanding of how TREM2 KO cells dysregulate purinergic receptor expression after an insult may prove useful for therapeutic modulation of microglial activation states.

The studies presented here provide evidence that reducing purinergic receptor activity may be clinically applicable in Alzheimer's patients with TREM2 loss of function mutations^{38,50,63}. Pharmacologically targeting P2Y12 receptors to dampen both the Ca²⁺ dependent (PLC) and independent (DAG) arms of the GPCR signaling pathway may be useful to control microglial activation and motility. However, our results suggest that altering downstream Ca²⁺ flux may be sufficient, and thus CRAC (Orai1) channel blockers that would specifically inhibit the sustained Ca²⁺ signals without affecting the initial Ca²⁺ transient or the activation of DAG may provide a more targeted approach.

Currently, TREM2 activating antibodies are being examined in early stage clinical trials for Alzheimer's disease^{64,65}, making it critically important to understand the broad consequences of TREM2 signaling. Therefore, an understanding of how TREM2 influences responses to purinergic signals, and regulates cytosolic Ca²⁺ in human iPSC-microglia is critical. Beyond TREM2, we have found that protective variants in MS4A6A and PLCG2 gene expression also decrease P2Y12 and P2Y13 receptor expression (unpublished data), suggesting this mechanism of microglial activation could be common to several microglial AD risk loci.

In summary, deletion of TREM2 renders iPSC-microglia highly sensitive to ADP, leading to prolonged Ca²⁺ influx which increases cell displacement by decreasing cell turning. Despite this,

TREM2 KO microglia show a defect in chemotaxis that is likely due to their inability to sense ADP gradients. Decreasing purinergic signaling in TREM2 KO microglia is able to rescue directional chemotactic migration. We suggest that purinergic modulation or direct modulation of Ca^{2+} signaling could provide novel therapeutic strategies in many AD patient populations, not solely those with reduced TREM2 function.

Figure Legends

Figure 1: Microglia lacking TREM2 show exaggerated Ca^{2+} responses to purinergic stimulation. (A) Representative images of WT (top) and TREM2 KO (bottom) iPSC-microglia loaded with Fluo-4 (green) and Fura-red (red) showing resting cytosolic Ca^{2+} and peak Ca^{2+} after ADP addition. Bright-field image overlaid, scale bar= 20 μm . (B-D) Average ratiometric traces (left panels) showing changes in cytosolic Ca^{2+} over time in response to 2.5 μM ADP (B), 2.5 μM ATP (C), and 10 μM UTP (D) in 1 mM Ca^{2+} containing buffer (n=39-44 cells, B; 63-71 cells, C; 45-55 cells, D). Quantification of baseline subtracted peak Ca^{2+} response and cytosolic Ca^{2+} levels 5 min after agonist application shown on the right (250-274 cells, B; 165-179 cells, C; 175-269 cells, D; 3-5 experiments; Mann-Whitney test). (E) Ca^{2+} levels in WT iPSC-microglia 5 min after addition of ADP, ATP, and UTP (right, n=17-52 cells from 2-3 experiments). Cells were gated for similar initial peak Ca^{2+} response to agonists (left). (F) Schematic highlighting key downstream Ca^{2+} signaling events in response to ADP. (G) Average trace showing SOCE engaged via emptying ER Ca^{2+} stores with thapsigargin (TG, 2 μM) in Ca^{2+} -free buffer followed by re-addition of 1 mM Ca^{2+} (90-129 cells). Bar-graph summaries of ER store-release quantified as area under the curve, rate of SOCE, and peak SOCE (187-266 cells, 2 experiments, Mann-Whitney test). (H) Average trace (left, 71-117 cells) and summary of ER store-release after 2 μM ionomycin treatment in Ca^{2+} free buffer (right, 146-234 cells, 2 experiments; Mann-Whitney test) (I) Same as H but in response to UV IP_3 uncaging (167-200 cells, nonparametric t-test). Data shown as mean $\pm$ SEM for traces and bar-graphs.

Figure 2: Higher sensitivity of TREM2 KO microglia to ADP is driven by increased purinergic receptor expression

(A) Representative images of WT (top) and TREM2 KO (bottom) iPSC-microglia before and peak Ca^{2+} response after ADP addition in Ca^{2+} free buffer. Overlay of Fluo-4 (green), Fura-red (red) and Brightfield (grey) images. Scale bar= 20 μm . **(B)** Average trace showing Ca^{2+} response to ADP in Ca^{2+} -free buffer (left, 64-83 cells). Quantification of peak signal (right, 264-289 cells, 4 experiments, Mann-Whitney test). **(C-E)** Dose-response curve showing baseline subtracted Peak Ca^{2+} responses to ADP in Ca^{2+} -free buffer **(C)**, percent of “responding” cells **(D)** and peak Ca^{2+} responses only in “responding” cells **(E)**. (Each data-point is average of 84-474 cells, WT; 70-468 cells, 2-5 experiments). **(F)** RNA normalized read counts of P2RY12/13 expression from bulk RNA-sequencing of WT and TREM2 KO iPSC-microglia (n=4, adjusted p-values from DESeq2). **(G-H)** Immunofluorescence and quantification of P2RY12 **(G)** and P2RY13 **(H)** in iPSC-microglia (n=4 independent wells, 3 images per well, student’s t-test). **(I-J)** Peak Ca^{2+} response in Ca^{2+} free buffer after treatment with ADP in the presence of P2RY12 antagonist PSB 0739 **(I)** and P2RY13 antagonist MRS 2211 **(J)**. (72-128 cells, I; 83-117 cells, J; representative of 3 experiments, Mann-Whitney Test). Data shown as mean $\pm$ SEM for traces and bar-graphs.

Figure 3: Nondirectional ADP exposure increases WT microglial speed and process extension.

(A) Average trace showing closure of scratch wound produced with IncuCyte S3 WoundMaker. iPSC-microglia imaged every 30 min after scratch wound with or without ADP stimulation (n=4 wells; 2 images per well). **(B)** Representative image of WT iPSC-microglia motility 30 min after ADP exposure with cell tracks overlain (left). Pseudocolored images (center) across time: 0 min (red), 4 min (orange), 8 min (yellow), 12 min (green), 16 min (cyan), 20 min (blue), 24 min (purple), 28 min (magenta). Scale bar= 100 μm . White boxes zoomed in at right to demonstrate motile (top) and non-motile (bottom) cells. **(C)** Representative color images (top left) and displacement vectors (bottom left) of WT iPSC-microglia at baseline (no ADP, grey) and 30 min after 2.5 μM ADP treatment (red). Summary of Mean Speed ($\mu\text{m}/\text{min}$), Displacement over 10 min ($\mu\text{m}/10 \text{ min}$) and Track straightness (track length/track displacement) (414-602 cells, 2 experiments). **(D)** Representative images, displacement vectors, and quantification of WT iPSC-microglia motility

for 20 min following ADP addition. Cells were pre-treated with vehicle (grey), MRS 2211 (10 μ M, gold), or PBS 0739 (10 μ M, blue) (180-187 cells, 2 experiments). (E) Representative images, displacement vectors, and quantification of WT iPSC-microglia motility after ADP in 1 mM Ca^{2+} (light grey) or Ca^{2+} -free buffer (dark grey) (401-602 cells, 3 experiments). (F) Representative images (left) and process extension (right) of iPSC-microglia (cytoplasmic GFP, grey) before or 30 min after ADP addition. Cells were pre-treated with vehicle (grey), MRS 2211 (10 μ M, gold), or PBS 0739 (10 μ M, blue) (52-163 cells, 3-4 experiments). (C-F) One way ANOVA with Tukey post hoc test. Data shown as mean $\pm$ SEM for traces and bar-graphs.

Figure 4: ADP-driven process extension and cell displacement are increased in TREM2 KO iPSC-microglia. (A-E) Motility of WT (grey) and TREM2 KO (green) iPSC-microglia over 20 min following ADP addition in 1 mM Ca^{2+} -containing buffer. (A) Plots of track displacement in μ m centered from point of origin at (0,0). (B) Mean-squared displacement (MSD) vs time. Mean-cell speeds (C), total track displacement in 10-min interval (D), and track straightness (E) for 130-327 cells, 7 experiments, student's t-test. (F-J). Same as (A-F) but in Ca^{2+} -free medium (125-279 cells, 2 experiments, student's t-test). (K) Representative images of GFP-expressing WT (top) and TREM2 KO (bottom) iPSC-microglia, before and 30 min after 2.5 μ M ADP addition. (L) Quantification of total number of branches per cell before and after ADP treatment (left) and paired dot-plots showing fold change in branch number from pre-ADP levels (right). Each data-point represents an imaging field in the paired-plots. (M) Total process length before and after ADP treatment displayed as raw values per cell (left) and as fold change from baseline conditions per imaging field (right). For L and M, n=151-158 cells, WT; 133-167 cells, KO; 9-10 imaging fields, 3-4 experiments. One-way ANOVA with multiple comparisons for single-cell data, two-tailed paired t-test for the paired-plots.

Figure 5: Validation of Salsa6f transgenic iPSC-microglia. (A) Representative green (GCaMP6f), red (tdTomato) and Green/Red channel overlay images of transgenic Salsa6f expressing iPSC-microglia at low (resting Ca^{2+} , top row) and high (Peak SOCE, bottom row) cytosolic Ca^{2+} levels. Bright-field image shown for comparison. Cells were treated with TG to evoke SOCE. Scale bar =

5 μm . **(B)** Trace of average change in fluorescence intensity of tdTomato (red) and GCaMP6f (green) over time (n=32 cells). **(C)** Ratiometric GCaMP6f/ tdTomato signal (Green/ Red or G/R Ratio) over time calculated from **(B)**. **(D)** Immunofluorescence images of P2RY13 and IBA1 in WT or Salsa6f-transgenic iPSC-microglia (left). Right panel shows quantification of P2RY13 and IBA1 protein expression (n=4 wells, 2 independent images per well, t-test). **(E)** Phagocytosis of Synaptosomes in WT non-transgenic (open circle) and Salsa6f-expressing (closed circle) iPSC-microglia. Cytochalasin D (grey, 10 μM) used as negative control to inhibit phagocytosis. Live cultures imaged on IncuCyte S3 (n=4 wells; 4 images per well). **(F)** Phagocytic load at 24 hrs for synaptosomes, beta-amyloid, zymosan A, and *S. aureus* (n=4 wells; 4 images per well; one way ANOVA with tukey post-hoc test). **(G)** Microglia cell counts at final day of differentiation (n=3 wells, t-test). **(H)** Ca^{2+} response to 2.5 μM ADP in WT vs TREM2 KO Salsa6f-expressing iPSC-microglia (n=41-53 cells). **(I)** Fluorescence intensity of tdTomato (red) and GCaMP6f (green) (left axis) simultaneously measured alongside cell displacement (black) (right axis).

Figure 6: Cytosolic Ca^{2+} levels tune microglial motility in TREM2 KO cells. **(A)** Schematic of traditional SOCE pathway with store-refilling (left) and a “ Ca^{2+} -clamp” method of sustaining cytoplasmic Ca^{2+} to “low” and “high” levels with 0.2 and 2mM extracellular Ca^{2+} and using TG to inhibit store-refilling (right). **(B)** Average SOCE traces in WT Salsa6f iPSC-microglia showing cytoplasmic Ca^{2+} ‘clamped’ at different levels after addition of 0.2 or 2 mM extracellular Ca^{2+} (n=78-110 cells). **(C)** Average change in cytoplasmic Ca^{2+} levels in WT and TREM2 KO microglia over 25 minutes after SOCE activation. **(D)** Comparison of Ca^{2+} levels and microglia motility in WT (top) and TREM2 KO (bottom) microglia. Cytosolic Ca^{2+} levels indicated by instantaneous single-cell G/R Ratio (n=74-158 cells). Mean-speed, displacement and track straightness calculated as before in Figures 3 and 4. Yellow (0.2 mM Ca, TG), green (2 mM Ca, TG). Students t-test **** p < 0.0001; ** p = 0.0062; * p = 0.432; ns > 0.9999. **(E)** Correlation of instantaneous Ca^{2+} and instantaneous speed in WT and KO cells. Red line denotes 10 $\mu\text{m}/\text{sec}$ (cells above this threshold considered “fast-moving”). For WT: p < 0.0001; r = -0.1316; number pairs = 5850. For KO: p < 0.0001; r = -0.1433; number pairs = 6063 (Spearman’s correlation). **(F)** Mean speed of cells binned by instantaneous G/R Ca^{2+} ratio (1-way ANOVA **** p < 0.0001). Each data point is calculated

for a bin increment of 0.5 G/R ratio. (G) Percentage of fast-moving cells quantified as a function of G/R Ca^{2+} ratio. X-axis G/R ratios binned in increments of 0.5 as in F. In E-G, n=78-100 cells.

Figure 7: Migration deficits in TREM2 KO microglia are rescued by inhibition of purinergic signaling. (A) Migration towards ADP in a two-chamber microfluidic device. Representative images of RFP expressing microglia that migrated into the central chamber 3 days after 100 ng/mL ADP addition. Dotted circle delineates separation of inner and outer chamber. Scale bar = 500 μm . Quantification of microglial migration (right panel). Migrated cell counts are normalized to WT cells treated with ADP (n=3-4 experiments; One-way ANOVA with multiple comparisons). (B) Baseline subtracted peak ratiometric Ca^{2+} signal in response to 2.5 μM ADP in 1 mM extracellular Ca^{2+} , and in the presence or absence of 10 μM PSB 0739 (44 cells, WT; 39-43 cells, KO; representative of 3 independent experiments; Students t-test). (C) Two-chamber migration to 100 ng/mL ADP with or without 10 μM PSB 0739. Values are normalized to WT cells with ADP (n=3-4 experiments; One-way ANOVA with multiple comparisons). Representative images shown on the left. Scale bar = 500 μm .

Figure 8: A Model for Ca^{2+} regulation of ADP-mediated chemotaxis in microglia lacking TREM2. (A) Dose-response curve comparing ADP-mediated peak- Ca^{2+} response in WT and TREM2 KO microglia. EC50 for KO cells is more than 10-fold lower than WT microglia. P2Y12 and 13 receptor expression is higher in cells lacking TREM2 rendering them hypersensitive to ADP signaling. (B) Due to exaggerated purinergic ADP signaling, microglia lacking TREM2 exhibit a deficit in moving towards ADP gradients. Despite moving in straighter tracks, TREM2 KO cells migrate in all directions contributing to their chemotactic inefficiency. Partially suppressing P2Y12 receptor signaling would shift the response to ADP to the right (arrow in panel A) and lower cytosolic Ca^{2+} in TREM2 KO cells, restoring their ability to move towards the ADP gradient.

Supplementary Figure Legends

Figure 1-figure supplement 1: Differential engagement of SOCE by purinergic agonists. (A) Ca^{2+} responses in TREM2 KO iPSC-microglia 5 min after application of 2.5 μM ADP (blue), 2.5 μM ATP (red), and 10 μM UTP (yellow) that are gated for similar initial peak Ca^{2+} response to the agonists shown in the left panel ($n=11-65$ cells, pooled from 2 experiments). (B) Ratiometric Ca^{2+} responses in WT iPSC-microglia treated with 2-APB (50 μM) or Gd^{3+} (5 μM) to block store-operated Ca^{2+} entry (SOCE) ($n= 80-148$ cells). P-value calculated for A and B using ordinary one-way ANOVA with multiple comparisons.

Figure 1-figure supplement 2: Expression of STIM1, Orai1, and IP3R2 are not altered in TREM2 KO iPSC-microglia. (A) Volcano plot of differentially expressed genes from bulk RNA-sequencing of WT and TREM2 KO iPSC-microglia ($n=4$). Genes for IP3R, STIM, and ORAI are highlighted. (B) Raw frequency per kilobase million reads (FPKM) in iPSC-microglia. (C) RNA normalized read counts of highly expressed isoforms for (C) IP3R2, (D) STIM1, and ORAI1. Adjusted p-values from DESeq2. (E) Immunofluorescence of Orai1 in WT and TREM2 KO iPSC-microglia. $n=3$ images per well from 4 independent wells; students t-test.

Figure 1-figure supplement 3: SOCE is not altered in TREM2 KO microglia. (A) Representative single-cell trace of Ca^{2+} signal in response to ADP in 1mM extracellular Ca^{2+} buffer showing the scheme for measuring ER store-release as the initial Ca^{2+} peak and SOCE as cytosolic Ca^{2+} level 5 min after ADP application. (B) Scatter-plot showing correlation of initial ADP-induced Ca^{2+} response (store-release) and cytoplasmic Ca^{2+} after 5 minutes (SOCE) in WT (grey) and KO (green) cells ($N= 866-935$ cells from multiple imaging runs with a range of ADP doses). (C) Correlation between ADP-induced store-release and SOCE using the same data-set in B but with each data-point representing average Ca^{2+} response to the following doses of ADP in 1 mM Ca^{2+} (in μM : 0.001, 0.1, 0.5, 1, 2, 2.5, 5, 10). WT and TREM2 KO data are overlaid in the same scatter-plot. (Comparison of slopes between WT and TREM2 KO: p-value = 0.7631; Extra sum of squares F test).

Figure 3-figure supplement 1: ADP-mediated process extension in WT iPSC-microglia. (A)

Representative images of a cell (Cell 1) from a time-lapse experiment showing increased branching and extension of processes in GFP-expressing WT iPSC-microglia, at times indicated following addition of 2.5 μ M ADP. Bright field DIC images (top row) and GFP images (bottom row) are shown. **(B)** Another example of a cell (Cell 2) showing process extension in the same imaging field. **(C)** A motile cell (Cell 3) in the same imaging field is shown for comparison. Note the lack of displacement in cells that extend their process, and lack of significant process extension in a highly motile cell. Scale bar: 15 μ M.

Figure 4-figure supplement 1: Motility analysis in WT and TREM2 KO iPSC-microglia. (A)

Scratch Wound performed via IncuCyte S3 WoundMaker shows no difference in migration into the scratch wound area. Two-way ANOVA with multiple comparisons, n=2 images per well over 3 independent wells. **(B)** Summary of microglial mean speeds, displacement over 10 min, and track straightness in open-field migration (student's t-test). **(C)** Flower plots show similar displacement from origin for WT (left) and TREM2 KO (right) cells. **(D, E)** Directional Autocorrelation calculated via DiPer excel macro. Due to lack of directional gradient, directional autocorrelation of motility vectors is expected to drop quickly. Time constants for best-fit single exponential curves are indicated, consistent with increased straightness for TREM2 KO cells treated with ADP. **(E)** Directional autocorrelation of WT (grey) and TREM2 KO (green) iPSC-microglia at baseline (open circles) or after ADP addition (filled circles). Mean autocorrelation values in the first 5 minutes (one-way ANOVA) and time (min) until autocorrelation reaches zero.

Figure 4-figure supplement 2: Comparison of process extension in WT and TREM2 KO Microglia.

Branching and process extension in WT and TREM2 KO iPSC-microglia 30 min after addition of ADP in 1 mM **(A, B)** or 0 mM extracellular Ca^{2+} buffer **(C, D)**. **(A)** Data displayed as paired-plots showing average branch number per cell in an imaging field (top row) and normalized to pre-ADP values for each imaging field (middle row). Bottom row shows fold change in branching after ADP treatment for WT (grey) and KO (green) iPSC-microglia. **(B)** Changes in process length in the same dataset as **A**. n=151-158 cells, WT; 133-167 cells, KO; 9-10 imaging fields, 3-4 experiments. **(C, D)**

Same analysis as **A, B** but with ADP in Ca^{2+} free buffer. n=137-143 cells, 8 imaging fields, 2-3 experiments. (**A-D**) p-values calculated by two-tailed paired Students t-test for the paired-plots, and by unpaired t-test when comparing fold-change in WT and KO cells.

Figure 5-figure supplement 1: Generation of Salsa6f iPSC line. Construct design for the Salsa6f knock-in template (top) Primers for sequence validation marked with blue arrows. Sanger sequencing to confirm proper integration of Salsa6f cassette into AAVS1 Safe Harbor Locus (bottom).

Figure 6-figure supplement 1: Motility analysis with Ca^{2+} “clamp”. (**A**) Ca^{2+} ratios and microglia motility in WT (top) and KO (bottom) microglia. Ca^{2+} clamp experiments with ADP added: yellow (0.2 mM Ca^{2+} , TG + ADP), green (2 mM Ca^{2+} , TG + ADP). Students t-test **** $p < 0.0001$; *** $p = 0.0001$. n=164-393 cells. (**B, C**) Ca^{2+} tuning of mean speed and track displacement length in 0.2 mM “ Ca^{2+} clamp”: correlation between instantaneous Ca^{2+} and cumulative displacement. Each dot represents an individual cell for an individual frame. Dotted red line represents displacement of 200 μm^2 . WT cells (**B**) displace less than KO cells (**C**). For each cell type, larger displacements are correlated with lower G/R Ca^{2+} ratios. Cells which maintain elevated cytoplasmic Ca^{2+} do not displace as far. For WT: $p < 0.0001$; $r = -0.4778$; number pairs = 5973. For KO: $p < 0.0001$; $r = -0.3699$; number pairs = 5761 (Spearman’s correlation).

578 Materials and Methods

579 Key Resources Table

Reagent type (species) or resource	Designation	Source or reference	Identifiers	Additional Information
Cell line (Human)	WT iPSC-microglia	UCI ADRC iPSC Core	ADRC5	iPSC-derived microglial line
Cell line (Human)	TREM2 KO iPSC microglia	Blurton-Jones lab	ADRC5 Clone 28-18	CRISPR-mediated knockout of TREM2 on the WT iPSC line
Cell line (Human)	WT GFP- expressing iPSC-microglia	Coriell	AICS-0036	iPSC-line with GFP tagged to α tubulin. Originally developed by Dr. Bruce Conklin
Cell line (Human)	TREM2 KO GFP-expressing iPSC-microglia	Blurton-Jones lab	GFP Clone 1	CRISPR-mediated knockout of TREM2 on the WT GFP ⁺ iPSC line
Cell line (Human)	WT RFP-expressing iPSC-microglia	Coriell	AICS-0031-035	iPSC-line with RFP tagged to α tubulin. Originally developed by Dr. Bruce Conklin
Cell line (Human)	TREM2 KO RFP-expressing iPSC-microglia	Blurton-Jones lab	RFP Clone 6	CRISPR-mediated knockout of TREM2 on the WT RFP ⁺ iPSC line
Cell line (Human)	WT Salsa6f-expressing iPSC-microglia	UCI ADRC iPSC Core	ADRC76 Clone 8	iPSC-line expressing a GCaMP6f-tdTomato fusion construct (Salsa6f)
Cell line (Human)	TREM2 KO Salsa6f-expressing iPSC microglia	Blurton-Jones lab	ADRC76 Clone 8 Clone 98	CRISPR-mediated knockout of TREM2 on the WT Salsa6f ⁺ iPSC line
Plasmid Construct (transgene)	Salsa6f	Addgene	Plasmid# 140188	A genetically encoded Calcium Indicator with tdTomato linked to GCaMP6f by a V5 epitope tag.
Other	DMEM/F12, HEPES, no Phenol red	Thermo Fisher Scientific	11038021	Microglia differentiation cell culture medium
Other	TeSR™-E8™	STEMCELL Technologies	05990	Stem cell culture medium
Other	StemDiff Hematopoietic kit	STEMCELL Technologies	05310	
Peptide/ Recombinant protein	Non-essential amino acids	Gibco	11140035	

Peptide/ Recombinant protein	Glutamax	Gibco	35050061	
Peptide/ Recombinant protein (human)	Insulin	Sigma	I2643	
Peptide/ Recombinant protein	B27	Gibco	17504044	
Peptide/ Recombinant protein	N2	Gibco	A1370701	
Peptide/ Recombinant protein	Insulin-transferrin- selenite	Gibco	41400045	
Peptide/ Recombinant protein (human)	IL-34	Peprtech	200-34	
Peptide/ Recombinant protein (human)	TGFβ1	Peprtech	100-21	
Peptide/ Recombinant protein (human)	M-CSF	Peprtech	300-25	
Peptide/ Recombinant protein	CX3CL1	Peprtech	300-31	
Peptide/ Recombinant protein	CD200	Novoprotein	C311	
Peptide/ Recombinant protein	Fibronectin	STEMCELL Technologies	07159	
Other	Matrigel	Corning	356231	
Other	ReLeSR	STEMCELL Technologies	5872	Human pluripotent stem cell selection and passing reagent
Other	Goat Serum	Thermo Fisher Scientific	10000C	
Other	fluorescent beta- amyloid 1-42 (647)	Anaspec	AS64161	
Other	pHrodo tagged zymosan A beads	Thermo Fisher Scientific	P35364	
Other	pHrodo tagged S. Aureus	Thermo Fisher Scientific	A10010	
Other	Human Stem Cell Nucleofector kit 2	Lonza	VPH-5022	

Other	Alt-R CRISPR-Cas9 tracrRNA	IDTDNA	107253	
Other	Alt-R HiFi Cas9 Nuclease	IDTDNA	1081061	
Antibody	Anti-human P2RY12	Sigma	HPA014518	
Antibody	Anti-human P2RY13	Alomone	APR-017	
Antibody	Anti-human Orai1	Alomone	ALM-025	
Antibody	Anti-human IBA1	Wako	019-19741	
Antibody	Goat anti-rabbit 488	Thermo Fisher Scientific	A11008	Secondary antibody
Antibody	Goat anti-mouse 555	Thermo Fisher Scientific	A21424	Secondary antibody
Antibody	Goat anti-rabbit 555	Thermo Fisher Scientific	A21429	Secondary antibody
Chemical compound, drug	Fluo-4 AM	Thermo Fisher Scientific	F14201	
Chemical compound, drug	Fura-red AM	Thermo Fisher Scientific	F3021	
Chemical compound, drug	Pluronic F-127	Thermo Fisher Scientific	P3000MP	
Chemical compound, drug	Cal-520 AM	AAT Bioquest	21130	
Chemical compound, drug	ci-IP3/PM	SiChem	6210	Caged-inositol triphosphate analog
Chemical compound, drug	Hoeschst	Thermo Fisher Scientific	R37165	
Chemical compound, drug	ADP	Sigma-Aldrich	A2754	
Chemical compound, drug	ATP	Sigma-Aldrich	A9187	
Chemical compound, drug	UTP	Sigma-Aldrich	U1006	
Chemical compound, drug	PSB 0739	Tocris	3983	
Chemical compound, drug	MRS 2211	Tocris	2402	
Chemical compound, drug	2-APB	Sigma-Aldrich	D9754	
Chemical compound, drug	Gadolinium	Sigma-Aldrich	G7532	
Chemical compound, drug	EGTA	Sigma-Aldrich	E8145	
Chemical compound, drug	1-thioglycerol	Sigma-Aldrich	M6145	
Chemical compound, drug	CloneR	STEMCELL Technologies	05888	Defined supplement for single-cell cloning of human iPS cells

Chemical compound, drug	Thiazovivin	STEMCELL Technologies	72252	ROCK inhibitor
Other	35mm glass-bottom dish	MatTek	P35G-1.5-14-C	1.5 coverslip, 14mm glass diameter
Other	Incubation perfusion Lid for 35mm dishes	Tokai Hit	LV200-D35FME	Perfusion lid with inlet and outlet
Other	Laser Scanning Confocal Microscope	Olympus	FV3000	Equipped with Resonant Scanner, IX3-ZDC2 Z-drift compensator, 40x silicone oil objective, 20x air objective
Other	Stage Top Incubation System	Tokai Hit	STXG	Temperature and humidity control for FV3000 microscope stage
Other	Nikon Eclipse Ti microscope system	Nikon		Equipped with a 40x oil immersion objective (N.A.: 1.3; Nikon) and an Orca Flash 4.0LT CMOS camera (Hamamatsu)
Other	Chemotaxis Assay Chamber	Hansang Cho Lab		
Other	Incucyte S3 Live-Cell Analysis System	Sartorius		
Other	Essen Incucyte WoundMaker	Sartorius	4493	
Software, algorithm	GraphPad Prism 9.1.0			Data analysis, statistical analysis
Software, algorithm	Fiji (Image J)			Image analysis
Software, algorithm	Incucyte 2020C			Image acquisition and analysis
Software, algorithm	IMARIS 9.7.0			Cell tracking and image analysis
Software, algorithm	Flika			Image analysis
Software, algorithm	DiPer Excel Macros		PMID: 25033209	Data analysis, directional persistence

580

581 **Generation of iPSCs from human fibroblasts:** Human induced pluripotent stem cell lines were

582 generated by the University of California, Irvine Alzheimer's Disease Research Center (UCI ADRC)

583 Induced Pluripotent Stem Cell Core from subject fibroblasts under approved Institutional Review

Boards (IRB) and human Stem Cell Research Oversight (hSCRO) committee protocols. Informed consent was received from all participants who donated fibroblasts. Reprogramming was performed with non-integrating sendai virus in order to avoid integration effects. To validate new iPSC lines, cells were karyotyped by G-banding and tested for sterility. Pluripotency was verified by Pluritest Array Analysis and trilineage in vitro differentiation. Additional GFP- and RFP- α tubulin expressing iPSC lines (AICS-0036 and AICS-0031-035) were purchased from Coriell and originally generated by Dr. Bruce Conklin. iPSCs were grown antibiotic free on Matrigel (Corning) in complete mTeSR1 or TeSR-E8 medium (STEMCELL Technologies) in a humidified incubator (5% CO₂, 37° C). All lines will be available upon request to the corresponding author.

CRISPR-mediated knockout of TREM2: Genome editing was performed as in McQuade et al. 2020³⁹. Briefly, iPSCs were nucleofected with RNP complex targeting the second exon of TREM2 and allowed to recover overnight. Transfected cells were dissociated with pre-warmed Accutase then mechanically plated to 96-well plates for clonal expansion. Genomic DNA from each colony was amplified and sequenced at the cut site. The amplification from promising clones was transformed via TOPO cloning for allelic sequencing. Knockout of TREM2 was validated by western blotting (AF1828, R&D) and HTRF (Cisbio) ³⁹.

iPSC-microglia differentiation: iPSC-microglia were generated as described in ⁴⁶. Briefly, iPSCs were directed down a hematopoietic lineage using the STEMdiff Hematopoiesis kit (STEMCELL Technologies). After 10-12 days in culture, CD43+ hematopoietic progenitor cells are transferred into a microglia differentiation medium containing DMEM/F12, 2 $\times$ insulin-transferrin-selenite, 2 $\times$ B27, 0.5 $\times$ N2, 1 $\times$ Glutamax, 1 $\times$ non-essential amino acids, 400 μ M monothioglycerol, and 5 μ g/mL human insulin. Media was added to cultures every other day and supplemented with 100 ng/mL

IL-34, 50 ng/mL TGF- β 1, and 25 ng/mL M-CSF (Peprotech) for 28 days. In the final 3 days of differentiation 100 ng/mL CD200 (Novoprotein) and 100 ng/mL CX3CL1 (Peprotech) were added to culture.

Confocal Laser Scanning Microscopy: Unless otherwise stated, cells were imaged on an Olympus FV3000 confocal laser scanning inverted microscope equipped with high-speed resonance scanner, IX3-ZDC2 Z-drift compensator, 40x silicone oil objective (NA 1.25) and a Tokai-HIT stage top incubation chamber (STXG) to maintain cells at 37°C. To visualize Salsa6f, 488 nm and 561 nm diode lasers were used for sequential excitation of GCaMP6f (0.3% laser power, 450V channel voltage, 494-544nm detector width) and TdTomato (0.05% laser power, 450V channel voltage, 580-680nm detector width), respectively. Fluo-4 and Fura-red were both excited using a 488 nm diode laser (0.07% laser power, 500V channel voltage, 494-544nm detector width for Fluo-4; 0.07% laser power, 550V channel voltage, 580-680nm detector for Fura-Red). Two high-sensitivity cooled GaAsP PMTs were used for detection in the green and red channels respectively. GFP was excited using the same settings as GCaMP6f. Other image acquisition parameters unique to Ca²⁺ imaging, microglia process and cell motility analysis are indicated in the respective sections.

Measurement of intracellular Ca²⁺:

Cell preparation: iPSC-microglia were plated on fibronectin-coated (5 μ g/mL) glass-bottom 35 mm dishes (MatTek, P35G-1.5-14-C) overnight at 60 % confluence. Ratiometric Ca²⁺ imaging was done using Fluo-4 AM and Fura-Red AM dyes as described previously³⁹. Briefly, cells were loaded in microglia differentiation medium with 3 μ M Fluo-4 AM and 3 μ M Fura-Red AM (Molecular Probes) in the presence of Pluronic Acid F-127 (Molecular Probes) for 30 min at room

temperature (RT). Cells were washed with medium to remove excess dye and 1 mM Ca^{2+} Ringer's solution was added to the 35 mm dish before being mounted on the microscope for live cell imaging. We note that iPSC-microglia are sensitive to shear forces and produce brief Ca^{2+} signals in response to solution exchange that are dependent on extracellular Ca^{2+} , and that these are more prominent at 37° C. To minimize these confounding effects, cells were imaged at RT and perfusion was performed gently. Salsa6f-expressing iPSC-microglia were prepared for Ca^{2+} imaging in the same way as conventional microglia, but without the dye loading steps. The following buffers were used for Ca^{2+} imaging: (1) 1 or 2 mM Ca^{2+} Ringer solution comprising 155 mM NaCl, 4.5 mM KCl, 1 mM CaCl_2 , 0.5 mM MgCl_2 , 10 mM glucose, and 10 mM HEPES (pH adjusted to 7.4 with NaOH), (2) Ca^{2+} -free Ringer solution containing: 155 mM NaCl, 4.5 mM KCl, 1.5 mM MgCl_2 , 10 mM glucose, 1 mM EGTA, 10 mM HEPES, pH 7.4. Live cell imaging was performed as described earlier. Cells were treated with ADP as indicated in the results section.

Data acquisition: Time-lapse images were acquired in a single Z-plane at 512 x 512 pixels (X = 318.2 μm and Y = 318.2 μm) and at 2-3 sec time intervals using Olympus FV3000 software. Images were time averaged over 3 frames to generate a rolling average and saved as .OIR files.

Data analysis: Time-lapse videos were exported to Fiji- ImageJ (<https://imagej.net/Fiji>), converted to tiff files (16-bit) and background subtracted. Single-cell analysis was performed by drawing ROIs around individual cells in the field and average pixel intensities in the green and red channels were calculated for each ROI at each time-point. GCaMP6f/ TdTomato (G/R Ratio) and Fluo-4/Fura-Red ratio was then obtained to further generate traces showing single-cell and average changes in cytosolic Ca^{2+} over time. Single-cell ratio values was used to calculate Peak Ca^{2+} signal and responses at specific time points after agonist application. Peak Ca^{2+} signal for

each cell was baseline subtracted, which was calculated as an average of 10 minimum ratio values before application of agonist.

Microglia process extension analysis:

Data acquisition: GFP-expressing iPSC-microglia were plated overnight on 35 mm glass bottom dishes at 40-50% confluence. Cells were imaged by excitation of GFP on the confocal microscope at 37° C as described earlier. To study process extension in response to ADP, two sets of GFP images were obtained for each field of view across multiple dishes: before addition of ADP (baseline) and 30 min after application of ADP. Images were acquired as a Z-stack using the Galvo scanner at Nyquist sampling. Adjacent fields of view were combined using the Stitching function of the Olympus FV3000 Software and saved as .OIR files.

Process Analysis: The basic workflow for microglia process analysis was adapted from Morrison et al, Sci. Rep, 2017⁶⁶. Image stacks (.OIR files) were exported to Fiji- Image J and converted into 16-bit Tiff files using the Olympus Viewer Plugin (<https://imagej.net/OlympusImageJPlugin>). Maximum intensity projection (MIP) image from each Z-stack was used for further processing and analysis. MIP images were converted to 8-bit grey scale images, to which a threshold was applied to obtain 8-bit binary images. The same threshold was used for all sets of images, both before and after ADP application. Noise reduction was performed on the binary images using the Process -> Noise -> Unspeckle function. Outlier pixels were eliminated using Process -> Noise -> Outliers function. The binary images were then skeletonized using the Skeletonize2D/3D Plugin for Image J (<https://imagej.net/plugins/skeletonize3d>). Sparingly, manual segmentation was used to separate a single skeleton that was part of two cells touching each other. The Analyze Skeleton Plugin (<https://imagej.net/plugins/analyze-skeleton/>) was then applied to the

skeletonized images to obtain parameters related to process length and number of branches for each cell in the imaging field. Processes were considered to be skeletons > 8 μm . The data was summarized as average process length and number of branches, before and after ADP application for a specific imaging field, normalized to the number of cells in the field which allowed for pairwise comparison. Additionally, single cell data across all experiments were also compared in some instances.

IP₃ uncaging: Whole-field uncaging of i-IP₃, a poorly metabolized IP₃ analog, was performed as previously described⁶⁷ with minor modifications. Briefly, iPSC-microglia were loaded for 20 min at 37° C with either Cal520 AM or Cal590 AM (5 μM , AAT Bioquest), and the cell permeable, caged i-IP₃ analog ci-IP₃/PM (1 μM , SiChem) plus 0.1% Pluronic F-127 in Microglia Basal Medium. Cells were washed and incubated in the dark for further 30 min in a HEPES-buffered salt solution (HBSS) whose composition was (in mM): 135 NaCl, 5.4 KCl, 1.0 MgCl₂, 10 HEPES, 10 glucose, 2.0 CaCl₂, and pH 7.4. Intracellular Ca²⁺ ([Ca²⁺]_i) changes were imaged by employing a Nikon Eclipse Ti microscope system (Nikon) equipped with a 40x oil immersion objective (N.A.: 1.3; Nikon) and an Orca Flash 4.0LT CMOS camera (Hamamatsu). Cal520 or Cal590 were excited by a 488 or a 560 nm laser light source (Vortran Laser Technologies), respectively. i-IP₃ uncaging was achieved by uniformly exposing the imaged cells to a single flash of ultraviolet (UV) light (350-400 nm) from a Xenon arc lamp. UV flash duration, and thus the amount of released i-IP₃, was set by an electronically controlled shutter.

Image acquisition was performed by using Nikon NIS (Nikon) software. After conversion to stack tiff files, image sequences were analyzed with Flika, a custom-written Python-based imaging analysis software (<https://flika-org.github.io/>;⁶⁸). After background subtraction, either

Cal520 and Cal590 fluorescence changes of each cell were expressed as $\Delta F/F_0$, where F_0 is the basal fluorescence intensity and ΔF the relative fluorescence change ($F_x - F_0$). Data are reported as superplots⁶⁹ of at least three independent replicates. Experiments were reproduced with two independent lines. Comparisons were performed by unpaired non-parametric t-test.

Immunocytochemistry: Cells were fixed with 4 % paraformaldehyde for 7 min and washed 3x with 1X DPBS. For P2RY12 staining, plates were pre-treated with citrate buffer (pH 6.0) for 20 min 95° C. Blocking was performed at room temp for 1 hr in 5 % Goat Serum, 0.1 % Triton X-100. Primary antibodies were added at 1:200 overnight 4° C (P2RY12; HPA014518 Sigma) (P2RY13; APR-017, Alomone). Plates were washed 3x before addition of secondary antibodies (Goat anti-Rabbit 488/555, Thermo Fisher Scientific) and Hoeschst (Thermo Fisher Scientific). Images were captured on an Olympus FV3000RS confocal microscope with identical laser and detection settings. Images were analyzed with IMARIS 9.7.0 software.

Scratch wound assay: Nondirectional motility was analyzed using Essen Incucyte WoundMaker. iPSC-microglia were plated on fibronectin (STEMCELL Technologies) at 90% confluence. Scratches were repeated 4x to remove all cells from the wound area. Scratch wound confluency was imaged every hour until scratch wound was closed (15 hrs). Confluence of cells within the original wound ROI was calculated using IncuCyte 2020C software.

IMARIS Cell Tracking: For motility assays, iPSC-microglia were tracked using IMARIS 9.7.0 software. For videos of GFP lines, cells were tracked using spot identification. For videos of Salsa6f lines, surface tracking was used to determine ratiometric Ca^{2+} fluorescence and motility per cell. In both conditions, tracks were defined by Brownian motion with the maximum distance jump of 4 microns and 10 frame disturbance with no gap filling. Tracks shorter than 3 minutes in

length were eliminated from analysis. After automated track formation, tracks underwent manual quality control to eliminate extraneous tracks, merge falsely distinct tracks, and add missed tracks. After export, data was plotted in Prism 9.1.0 or analyzed in excel using DiPer macros for Plot_At_Origin and mean squared distance ⁵⁷.

Generation of Salsa6f-expressing iPSC lines: iPSCs were collected following Accutase enzymatic digestion for 3 min at 37° C. 20,000 cells were resuspended in 100 µL nucleofection buffer from Human Stem Cell Nucleofector™ Kit 2 (Lonza). Salsa6f-AAVS1 SHL plasmid Template (2 µg; Vector Builder) and RNP complex formed by incubating Alt-R® S.p. HiFi Cas9 Nuclease V3 (50 µg; IDTDNA) was fused with crRNA:tracrRNA (IDTDNA) duplex for 15 min at 23° C. This complex was combined with the cellular suspension and nucleofected using the Amaxa Nucleofector program B-016. To recover, cells were plated in TeSR™-E8™ (STEMCELL Technologies) media with 0.25 µM Thiazovivin (STEMCELL Technologies) and Cloner™ (STEMCELL Technologies) overnight. The following day, cells were mechanically replated to 96-well plates in TeSR™-E8™ media with 0.25 µM Thiazovivin and Cloner™ supplement for clonal isolation and expansion. Plates were screened visually with a fluorescence microscope to identify TdTomato⁺ clones. Genomic DNA was extracted from positive clones using Extracta DNA prep for PCR (Quantabio) and amplified using Taq PCR Master Mix (Thermo Fisher Scientific) to confirm diallelic integration of the Salsa6f cassette. A clone confirmed with diallelic Salsa6f integration in the AAVS1 SHL was then retargeted as previously described³⁹ to knock-out Trem2.

Phagocytosis assay: Phagocytosis of transgenic iPSC-microglia was validated using IncuCyte S3 Live-Cell Analysis System (Sartorius) as in McQuade et al. 2020³⁹. Microglia were plated at 50% confluency 24 hours before substrates were added. Cells were treated with 50 µg/mL pHrodo

tagged human AD synaptosomes (isolated as described in McQuade et al. 2020), 100 ng/mL pHrodo tagged zymosan A beads (Thermo Fisher Scientific), 100 ng/mL pHrodo tagged *S. Aureus* (Thermo Fisher Scientific), or 2 µg/mL fluorescent beta-amyloid (Anaspec). Image masks for fluorescence area and phase were generated using IncuCyte 2020C software.

Chemotaxis assay: iPSC-microglia were loaded into the angular chamber (2-5K cells/device) to test activation and chemotaxis towards the central chamber containing either ADP (100 ng/mL) or vehicle. When noted, PSB 0739 (10 µM) was added to both the central and angular chamber to inhibit P2RY12. To characterize motility, we monitored the number of recruited microglia in the central chamber for 4 days under the fully automated Nikon TiE microscope (10× magnification; Micro Device Instruments, Avon, MA, USA).

Acknowledgments

The authors would like to thank Dr. Andy Yeromin for the development of Excel macros to analyze IMARIS cell tracking. The authors would also like to thank Morgan Coburn for sharing python scripts that aided in the organization of IMARIS output files. This work was supported by T32 NS082174 and ARCS foundation (A.M.); NIH R01 NS14609 and AI121945 (M.D.C.); NRF 2020R1A2C2010285, 2020M3C7A1023941, and NIH AG059236-01A1 (H.C.); NIH AG048099, AG056303, and AG055524 (M.B.J.); RF1DA048813 (M.B.J. and S.G.); UCI Sue & Bill Gross Stem Cell Research Center Seed Grant (S.G.); and a generous gift from the Susan Scott Foundation (M.B.J.). iPSC lines were generated by the UCI-ADRC iPS cell core funded by NIH AG066519. Experiments using the GFP-expressing iPSC line AICS-0036 were made possible through the Allen Cell Collection, available from Coriell Institute for Medical Research.

760
761
762
763
764
765
766
767
768
769
770
771
772

Declaration of interest: M.B.J. is a co-inventor of patent application WO/2018/160496, related to the differentiation of pluripotent stem cells into microglia. M.B.J and S.P.G. are co-founders of NovoGlia Inc.

Ethics: Human iPSC lines were generated by the University of California Alzheimer’s Disease Research Center (UCI ADRC) stem cell core. Subject fibroblasts were collected under approved Institutional Review Boards (IRB) and human Stem Cell Research Oversight (hSCRO) committee protocols. Informed consent was received for all participants.

Data Availability: RNA sequencing data referenced in Figure 1- figure supplement 2 is available through Gene Expression Omnibus: GSE157652.

References

1. Andersen, M. S. *et al.* Heritability Enrichment Implicates Microglia in Parkinson's Disease Pathogenesis. *Annals of Neurology* **89**, 942–951 (2021).
2. Crotti, A. *et al.* Mutant Huntingtin promotes autonomous microglia activation via myeloid lineage-determining factors. *Nat Neurosci* **17**, 513–521 (2014).
3. Fahira, A., Li, Z., Liu, N. & Shi, Y. Prediction of causal genes and gene expression analysis of attention-deficit hyperactivity disorder in the different brain region, a comprehensive integrative analysis of ADHD. *Behav Brain Res* **364**, 183–192 (2019).
4. Jansen, I. E. *et al.* Genome-wide meta-analysis identifies new loci and functional pathways influencing Alzheimer's disease risk. *Nat. Genet.* (2019) doi:10.1038/s41588-018-0311-9.
5. McQuade, A. & Blurton-Jones, M. Microglia in Alzheimer's disease: Exploring how genetics and phenotype influence risk. *J Mol Biol* **431**, 1805–1817 (2019).
6. Pimenova, A. A. *et al.* Alzheimer's-associated PU.1 expression levels regulate microglial inflammatory response. *Neurobiol Dis* 105217 (2020) doi:10.1016/j.nbd.2020.105217.
7. Tan, L. *et al.* Association of GWAS-linked loci with late-onset Alzheimer's disease in a northern Han Chinese population. *Alzheimers Dement* **9**, 546–553 (2013).
8. Davalos, D. *et al.* ATP mediates rapid microglial response to local brain injury in vivo. *Nat. Neurosci.* **8**, 752–758 (2005).
9. De Simone, R. *et al.* TGF- β and LPS modulate ADP-induced migration of microglial cells through P2Y1 and P2Y12 receptor expression. *J. Neurochem.* **115**, 450–459 (2010).
10. Honda, S. *et al.* Extracellular ATP or ADP Induce Chemotaxis of Cultured Microglia through Gi/o-Coupled P2Y Receptors. *J. Neurosci.* **21**, 1975–1982 (2001).

- 795 11. Koizumi, S. *et al.* UDP acting at P2Y₆ receptors is a mediator of microglial phagocytosis.
796 *Nature* **446**, 1091–1095 (2007).
- 797 12. Dissing-Olesen, L. *et al.* Activation of neuronal NMDA receptors triggers transient ATP-
798 mediated microglial process outgrowth. *J Neurosci* **34**, 10511–10527 (2014).
- 799 13. Madry, C. & Attwell, D. Receptors, ion channels, and signaling mechanisms underlying
800 microglial dynamics. *J Biol Chem* **290**, 12443–12450 (2015).
- 801 14. Zhang, Y. *et al.* An RNA-Sequencing Transcriptome and Splicing Database of Glia,
802 Neurons, and Vascular Cells of the Cerebral Cortex. *J. Neurosci.* **34**, 11929–11947 (2014).
- 803 15. Prakriya, M. & Lewis, R. S. Store-Operated Calcium Channels. *Physiol Rev* **95**, 1383–1436
804 (2015).
- 805 16. McLarnon, J. G. Microglial Store-operated Calcium Signaling in Health and in Alzheimer’s
806 Disease. *Curr Alzheimer Res* **17**, 1057–1064 (2020).
- 807 17. Mizuma, A. *et al.* Microglial Calcium Release-Activated Calcium Channel Inhibition
808 Improves Outcome from Experimental Traumatic Brain Injury and Microglia-Induced
809 Neuronal Death. *J Neurotrauma* **36**, 996–1007 (2019).
- 810 18. Gilbert, D. F. *et al.* Store-Operated Ca²⁺ Entry (SOCE) and Purinergic Receptor-Mediated
811 Ca²⁺ Homeostasis in Murine bv2 Microglia Cells: Early Cellular Responses to ATP-
812 Mediated Microglia Activation. *Front Mol Neurosci* **9**, 111 (2016).
- 813 19. Hasselmann, J. *et al.* Development of a Chimeric Model to Study and Manipulate Human
814 Microglia In Vivo. *Neuron* (2019) doi:10.1016/j.neuron.2019.07.002.
- 815 20. Keren-Shaul, H. *et al.* A Unique Microglia Type Associated with Restricting Development
816 of Alzheimer’s Disease. *Cell* **0**, (2017).

21. Krasemann, S. *et al.* The TREM2-APOE Pathway Drives the Transcriptional Phenotype of Dysfunctional Microglia in Neurodegenerative Diseases. *Immunity* **47**, 566-581.e9 (2017).
22. Olah, M. *et al.* Single cell RNA sequencing of human microglia uncovers a subset associated with Alzheimer's disease. *Nature Communications* **11**, 6129 (2020).
23. Sala Frigerio, C. *et al.* The Major Risk Factors for Alzheimer's Disease: Age, Sex, and Genes Modulate the Microglia Response to A β Plaques. *Cell Rep* **27**, 1293-1306.e6 (2019).
24. Weisman, G. A., Woods, L. T., Erb, L. & Seye, C. I. P2Y receptors in the mammalian nervous system: pharmacology, ligands and therapeutic potential. *CNS Neurol Disord Drug Targets* **11**, 722–738 (2012).
25. Cserép, C. *et al.* Microglia monitor and protect neuronal function through specialized somatic purinergic junctions. *Science* **367**, 528–537 (2020).
26. Haynes, S. E. *et al.* The P2Y₁₂ receptor regulates microglial activation by extracellular nucleotides. *Nat Neurosci* **9**, 1512–1519 (2006).
27. Suzuki, T. *et al.* Extracellular ADP augments microglial inflammasome and NF- κ B activation via the P2Y₁₂ receptor. *European Journal of Immunology* **50**, 205–219 (2020).
28. Wu, Y. *et al.* Aberrant expression of long noncoding RNAs in the serum and myocardium of spontaneous hypertensive rats. *Mol Biol Rep* **46**, 6399–6404 (2019).
29. Eyo, U. B. *et al.* Neuronal hyperactivity recruits microglial processes via neuronal NMDA receptors and microglial P2Y₁₂ receptors after status epilepticus. *J Neurosci* **34**, 10528–10540 (2014).
30. Lou, N. *et al.* Purinergic receptor P2RY₁₂-dependent microglial closure of the injured blood–brain barrier. *PNAS* **113**, 1074–1079 (2016).

31. Leissring, M. A. *et al.* Capacitative calcium entry deficits and elevated luminal calcium content in mutant presenilin-1 knockin mice. *J Cell Biol* **149**, 793–798 (2000).
32. Brawek, B. *et al.* Impairment of in vivo calcium signaling in amyloid plaque-associated microglia. *Acta Neuropathol* **127**, 495–505 (2014).
33. Demuro, A., Parker, I. & Stutzmann, G. E. Calcium Signaling and Amyloid Toxicity in Alzheimer Disease. *J Biol Chem* **285**, 12463–12468 (2010).
34. Mustaly, S., Littlefield, A. & Stutzmann, G. E. Calcium Signaling Deficits in Glia and Autophagic Pathways Contributing to Neurodegenerative Disease. *Antioxid. Redox Signal.* (2018) doi:10.1089/ars.2017.7266.
35. Eichhoff, G., Brawek, B. & Garaschuk, O. Microglial calcium signal acts as a rapid sensor of single neuron damage in vivo. *Biochim Biophys Acta* **1813**, 1014–1024 (2011).
36. Irino, Y., Nakamura, Y., Inoue, K., Kohsaka, S. & Ohsawa, K. Akt activation is involved in P2Y₁₂ receptor-mediated chemotaxis of microglia. *J Neurosci Res* **86**, 1511–1519 (2008).
37. Milior, G. *et al.* Distinct P2Y Receptors Mediate Extension and Retraction of Microglial Processes in Epileptic and Peritumoral Human Tissue. *J Neurosci* **40**, 1373–1388 (2020).
38. Cheng-Hathaway, P. J. *et al.* The Trem2 R47H variant confers loss-of-function-like phenotypes in Alzheimer’s disease. *Mol Neurodegener* **13**, 29 (2018).
39. McQuade, A. *et al.* Gene expression and functional deficits underlie TREM2-knockout microglia responses in human models of Alzheimer’s disease. *Nature Communications* **11**, 5370 (2020).
40. Ulrich, J. D. *et al.* Altered microglial response to A β plaques in APPPS1-21 mice heterozygous for TREM2. *Mol Neurodegener* **9**, 20 (2014).

41. Andrews, S. J., Fulton-Howard, B. & Goate, A. Interpretation of risk loci from genome-wide association studies of Alzheimer's disease. *Lancet Neurol* (2020) doi:10.1016/S1474-4422(19)30435-1.
42. Karch, C. M. *et al.* Expression of Novel Alzheimer's Disease Risk Genes in Control and Alzheimer's Disease Brains. *PLOS ONE* **7**, e50976 (2012).
43. Cheng, Q. *et al.* TREM2-activating antibodies abrogate the negative pleiotropic effects of the Alzheimer's disease variant Trem2R47H on murine myeloid cell function. *J. Biol. Chem.* **293**, 12620–12633 (2018).
44. Gratuze, M. *et al.* Impact of TREM2^{R47H} variant on tau pathology–induced gliosis and neurodegeneration. *J Clin Invest* **130**, 4954–4968 (2020).
45. Abud, E. M. *et al.* iPSC-Derived Human Microglia-like Cells to Study Neurological Diseases. *Neuron* **94**, 278-293.e9 (2017).
46. McQuade, A. *et al.* Development and validation of a simplified method to generate human microglia from pluripotent stem cells. *Molecular Neurodegeneration* **13**, 67 (2018).
47. Andreone, B. J. *et al.* Alzheimer's-associated PLCγ2 is a signaling node required for both TREM2 function and the inflammatory response in human microglia. *Nature Neuroscience* 1–12 (2020) doi:10.1038/s41593-020-0650-6.
48. Cosker, K. *et al.* Microglial signalling pathway deficits associated with the patient derived R47H TREM2 variants linked to AD indicate inability to activate inflammasome. *Sci Rep* **11**, 13316 (2021).
49. Kontinen, H. *et al.* PSEN1ΔE9, APP^{swe}, and APOE4 Confer Disparate Phenotypes in Human iPSC-Derived Microglia. *Stem Cell Reports* (2019) doi:10.1016/j.stemcr.2019.08.004.

50. Piers, T. M. *et al.* A locked immunometabolic switch underlies TREM2 R47H loss of function in human iPSC-derived microglia. *FASEB J.* (2019) doi:10.1096/fj.201902447R.
51. Reich, M. *et al.* Alzheimer's Risk Gene TREM2 Determines Functional Properties of New Type of Human iPSC-Derived Microglia. *Front. Immunol.* **11**, (2021).
52. Dong, T. X. *et al.* T-cell calcium dynamics visualized in a ratiometric tdTomato-GCaMP6f transgenic reporter mouse. *eLife* **6**, e32417 (2017).
53. Michaelis, M., Nieswandt, B., Stegner, D., Eilers, J. & Kraft, R. STIM1, STIM2, and Orai1 regulate store-operated calcium entry and purinergic activation of microglia. *Glia* **63**, 652–663 (2015).
54. Visentin, S., Nuccio, C. D. & Bellenchi, G. C. Different patterns of Ca²⁺ signals are induced by low compared to high concentrations of P2Y agonists in microglia. *Purinergic Signal* **2**, 605–617 (2006).
55. Borges, P. A. *et al.* Adenosine Diphosphate Improves Wound Healing in Diabetic Mice Through P2Y₁₂ Receptor Activation. *Front. Immunol.* **12**, (2021).
56. Hall-Roberts, H. *et al.* TREM2 Alzheimer's variant R47H causes similar transcriptional dysregulation to knockout, yet only subtle functional phenotypes in human iPSC-derived macrophages. *Alzheimers Res Ther* **12**, 151 (2020).
57. Gorelik, R. & Gautreau, A. Quantitative and unbiased analysis of directional persistence in cell migration. *Nature Protocols* **9**, 1931–1943 (2014).
58. Das, R. & Chinnathambi, S. Actin-mediated Microglial Chemotaxis via G-Protein Coupled Purinergic Receptor in Alzheimer's Disease. *Neuroscience* **448**, 325–336 (2020).
59. Negulescu, P. A., Krasieva, T. B., Khan, A., Kerschbaum, H. H. & Cahalan, M. D. Polarity of T cell shape, motility, and sensitivity to antigen. *Immunity* **4**, 421–430 (1996).

60. Park, J. *et al.* A 3D human triculture system modeling neurodegeneration and neuroinflammation in Alzheimer's disease. *Nat. Neurosci.* **21**, 941–951 (2018).
61. Meilandt, W. J. *et al.* Trem2 deletion reduces late-stage amyloid plaque accumulation, elevates the A β 42:A β 40 ratio, and exacerbates axonal dystrophy and dendritic spine loss in the PS2APP Alzheimer's mouse model. *J. Neurosci.* (2020) doi:10.1523/JNEUROSCI.1871-19.2019.
62. Lim, H. M. *et al.* UDP-Induced Phagocytosis and ATP-Stimulated Chemotactic Migration Are Impaired in STIM1-/- Microglia In Vitro and In Vivo. *Mediators Inflamm* **2017**, 8158514 (2017).
63. S, P. *et al.* Loss of TREM2 Function Increases Amyloid Seeding but Reduces Plaque-Associated ApoE. *Nature neuroscience* vol. 22 https://pubmed.ncbi.nlm.nih.gov/30617257/?from_single_result=30617257&expanded_search_query=30617257 (2019).
64. Alector Inc. *A Phase 2 Randomized, Double-Blind, Placebo-Controlled, Multicenter Study to Evaluate the Efficacy and Safety of AL002 in Participants With Early Alzheimer's Disease.* <https://clinicaltrials.gov/ct2/show/NCT04592874> (2021).
65. Wang, S. *et al.* Anti-human TREM2 induces microglia proliferation and reduces pathology in an Alzheimer's disease model. *J Exp Med* **217**, (2020).
66. Morrison, H., Young, K., Qureshi, M., Rowe, R. K. & Lifshitz, J. Quantitative microglia analyses reveal diverse morphologic responses in the rat cortex after diffuse brain injury. *Sci Rep* **7**, 13211 (2017).

- 928 67. Lock, J. T., Parker, I. & Smith, I. F. Communication of Ca(2+) signals via tunneling
929 membrane nanotubes is mediated by transmission of inositol trisphosphate through gap
930 junctions. *Cell Calcium* **60**, 266–272 (2016).
- 931 68. Ellefsen, K. L., Settle, B., Parker, I. & Smith, I. F. An algorithm for automated detection,
932 localization and measurement of local calcium signals from camera-based imaging. *Cell*
933 *Calcium* **56**, 147–156 (2014).
- 934 69. Lord, S. J., Velle, K. B., Mullins, R. D. & Fritz-Laylin, L. K. SuperPlots: Communicating
935 reproducibility and variability in cell biology. *J Cell Biol* **219**, (2020).
- 936
- 937

Figure 1: Microglia lacking TREM2 show exaggerated Ca^{2+} responses to purinergic stimulation.

Figure 1-figure supplement 1: Differential engagement of SOCE by purinergic agonists.

bioRxiv preprint doi: <https://doi.org/10.1101/2021.08.24.457491>; this version posted August 24, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

Figure 1-figure supplement 2: Expression of STIM1, Orai1, and IP3R are not altered in TREM2 KO iPSC-microglia.

Figure 1-figure supplement 3: SOCE engagement is not altered in TREM2 KO.

bioRxiv preprint doi: <https://doi.org/10.1101/2021.08.24.457491>; this version posted August 24, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

Figure 2: Higher sensitivity of TREM2 KO microglia to ADP is driven by increased purinergic receptor expression

Figure 3: Nondirectional ADP exposure increases WT microglial speed and process extension.

Figure 3-figure supplement 1: ADP-mediated process extension in WT iPSC-microglia.

Post-ADP

Figure 4: ADP-driven process extension and cell displacement are increased in TREM2 KO iPSC-microglia.

Figure 4-figure supplement 1: Motility analysis in WT and TREM2 KO iPSC-microglia.

Figure 4-figure supplement 2: Comparison of process extension in WT and TREM2 KO Microglia.

Figure 5: Validation of Salsa6f transgenic iPSC-microglia.

Figure 5-figure supplement 1: Generation of Salsa6f iPSC line.

Figure 6: Cytosolic Ca²⁺ levels tune microglial motility in TREM2 KO cells.

Figure 6-figure supplement 1: Motility analysis with “Ca²⁺ clamp”.

Figure 7: Migration deficits in TREM2 KO microglia are rescued by inhibition of purinergic signaling.

Figure 8: A Model for Ca²⁺ regulation of ADP-mediated chemotaxis in microglia lacking TREM2.