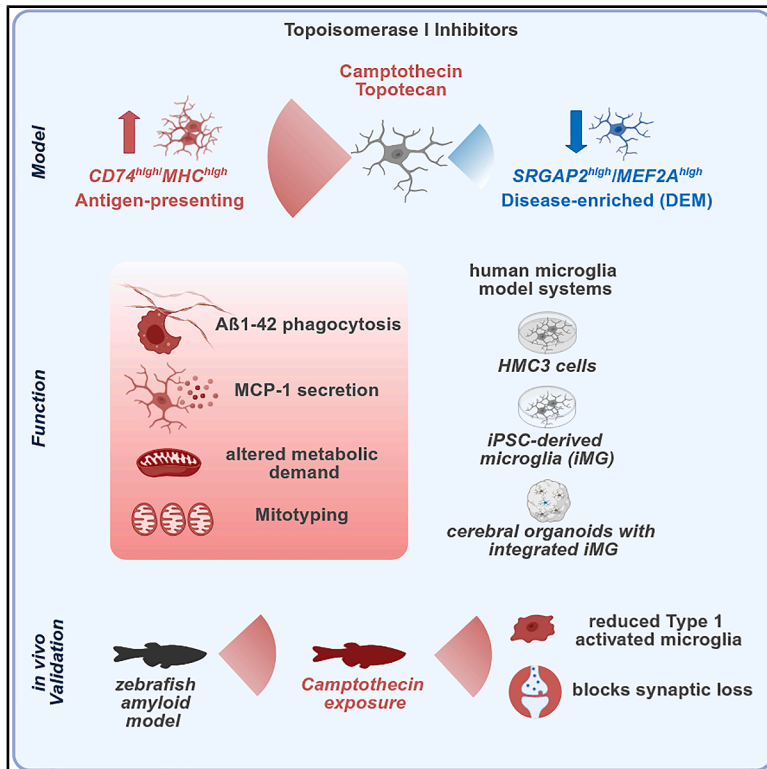


Topoisomerase I inhibition yields a CD74^{high}/MHC^{high} human microglial subtype with enhanced A β uptake

Graphical abstract



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In brief

Camptothecin and its analog Topotecan drive human microglia toward a CD74^{high}/MHC^{high} state with enhanced amyloid- β phagocytosis and altered inflammatory signaling. Using cell, organoid, and zebrafish models, Haage et al. link a transcriptomic microglial subtype to distinct functional properties, uncovering a pharmacological route to model microglial states implicated in neurodegeneration.

Highlights

- Camptothecin induces the transcriptional signature of human CD74^{high}/MHC^{high} microglia
- A pharmacological approach allows the functional analysis of a human microglial subtype
- Specific mitotypes link human microglial subtypes across model systems
- TOP1 inhibition causes functional changes *in vitro/vivo*, fitting a therapeutic role in AD



Article

Topoisomerase I inhibition yields a CD74^{high}/MHC^{high} human microglial subtype with enhanced A β uptake

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SUMMARY

While efforts to identify microglial subtypes have recently accelerated, the relation of transcriptomically defined states to cellular function has been largely limited to *in silico* annotations. Here, we characterize two topoisomerase I inhibitors that polarize human microglia toward a distinct state characterized by increased antigen presentation gene expression. Using HMC3 cells, iPSC-derived microglia, and cerebral organoids, we show that Camptothecin induces a CD74^{high}/MHC^{high} microglia-like cell subtype specialized in amyloid beta phagocytosis and reduced IL-6, IL-8, and MCP-1 secretion in response to TNF- α stimulation. Moreover, Camptothecin suppresses amyloid toxicity and restores microglia to their homeostatic morphology in a zebrafish amyloid proteinopathy model. We also identify Topotecan, a Food and Drug Administration (FDA)-approved Camptothecin analog, as a driver of the CD74^{high}/MHC^{high} signature. This report provides a reproducible approach to recapitulate a human microglial state *in vitro*, enabling its functional characterization and providing a foundation for rational modulation of human microglia *in vivo*.

INTRODUCTION

With the multiplicity of key functions carried out by microglia to maintain homeostasis in the central nervous system (CNS),^{1–3}

the characterization of specialized human microglial subsets with regard to brain region, function, and physiological and pathophysiological context has been a subject of intense interest over the past decade.⁴ Improved single-cell (sc) RNA sequencing



methods have led to the recent emergence of several datasets characterizing human microglia and have transformed our understanding of human microglial heterogeneity.^{4–13}

While currently available datasets propose different human microglial population structures, certain patterns emerge from these transcriptomic data, underscoring the need for a shared nomenclature of human microglia.¹⁴ While there is a range of cellular metabolic states and mitochondrial phenotypes (i.e. mitotypes) that influence cellular behaviors,^{15,16} the greater challenge has been linking transcriptionally defined clusters of human microglia to key microglial functions. This is critical to understand both homeostatic functions and roles in the development and progression of neurodegenerative disease. Moreover, this will be crucial with respect to therapeutic development, to guide the modulation of selected microglial subtypes in different contexts.

Given the range of current human microglial subpopulation models, approaches to build *in vitro* models for specific human microglial subtypes have been limited.^{12,17,18} Here, we deeply characterize one product of our prior *in silico*-driven pharmacologic approach of recapitulating certain human microglial subtypes in model systems to enable their functional evaluation. Specifically, the current report started at the end of our prior effort,¹² which proposed a cross-disease human microglial atlas comprised of 12 subtypes and identified 3 candidate compounds that mimicked selected human microglial subtypes in the Human Microglial Cell 3 (HMC3) cell line *in vitro*.

Here, we further characterize Camptothecin as a foundational compound of a pharmacological toolbox with which to study the function of human microglial subpopulations *in vitro* and *in vivo*. This approach has been previously successfully deployed to model disease associated microglia (DAM).¹⁷ Using several model systems - including the HMC3 microglia-like cell line, iPSC-derived microglia-like cells (iMG) and cerebral organoids with incorporated iMG - we confirm Camptothecin and its Food and Drug Agency (FDA)-approved analog Topotecan as potent drivers of a transcriptional program encoding an immunologically activated microglial subtype with high expression of MHC I and MHC II genes as well as CD74 (MHC II invariant chain and migration inhibitory factor receptor) among the set of 100 genes defining this microglial subset.¹² We therefore refer to them as CD74^{high}/MHC^{high} microglia. These two topoisomerase I inhibitors also suppress the transcriptional program that defines an SRGAP2^{high}/MEF2A^{high} microglial subtype that is enriched in susceptibility genes for Alzheimer's disease (AD) and multiple sclerosis (MS) (Disease Enriched Microglia, DEM).¹² Here, we assess the functional consequences of recapitulating the CD74^{high}/MHC^{high} microglia transcriptional state *in vitro* using our two tool compounds, and we find reproducible evidence that this microglial state could be relevant to the context of AD and future therapeutic efforts to develop immunomodulatory therapies for microglia.

RESULTS

Pharmacologic engagement of a CD74^{high}/MHC^{high} transcriptomic signature in HMC3 microglia-like cells

We previously profiled 215,680 live human microglia from 74 donors across diverse neurological diseases and CNS

regions. We used an *in silico* approach to first identify and then validate compounds recapitulating this subtype *in vitro* using HMC3 cells. Camptothecin had the most robust effect, downregulating the signature of the DEM (SRGAP2^{high}/MEF2A^{high}) subtype (101 genes) and inducing the CD74^{high}/MHC^{high} signature (100 genes), previously associated with AD¹² (Figure 1A; Table S1).

Single-cell level characterization of the tool compound

To determine the extent of heterogeneity among HMC3 cells at baseline and after exposure to Camptothecin, we replicated our previously established pharmacologic modulation paradigm¹² by exposing HMC3 cells to Camptothecin (1 μ M) compared to untreated and dimethyl sulfoxide (DMSO) treated control conditions. After 24 h of exposure, cells were labeled with cell hashing antibodies, pooled, and underwent single-cell RNA sequencing (scRNA-seq; untreated $n = 4729$ cells; DMSO $n = 3434$ cells; Camptothecin $n = 2790$ cells) (Figure 1B). Camptothecin polarized HMC3 cells into a distinct transcriptional space (Figure 1B) and distinct clusters were identified in association with the different treatment conditions (Figures S1A and S1B). Interestingly, as illustrated by the UMAP, untreated and DMSO-treated cells clustered into at least three distinct groups highlighting that (1) HMC3 cultures are heterogeneous at baseline, exhibiting transcriptionally distinct subtypes and (2) DMSO does not induce a transcriptionally distinct state (Figure 1B; Figure S1C).

To assess whether Camptothecin induced the respective target transcriptional signature, we assessed the predefined list of the top 100 genes that define the CD74^{high}/MHC^{high} signature. We illustrated the analysis by coloring each cell by the level of expression of this signature (Figures S1D and S1F; Table S1). We also assessed the distinct SRGAP2^{high}/MEF2A^{high} signature (Figures S2A and S2G; Table S1). Figures 1C and 1D depicts a quantitative analysis of our data, showing that there is a statistically significant increase of the CD74^{high}/MHC^{high} signature with Camptothecin treatment (untreated vs. Camptothecin - $p \leq 5E-324$; DMSO vs. Camptothecin - $p \leq 5E-324$) (Figure 1C), while the SRGAP2^{high}/MEF2A^{high} signature is significantly reduced by Camptothecin (untreated vs. Camptothecin - $p = 2.81E-279$; DMSO vs. Camptothecin - $p = 4.72E-275$) (Figure 1D; Table S2). These results are consistent with our *in silico* predictions: Camptothecin clearly induced the CD74^{high}/MHC^{high} signature and noticeably depleted the SRGAP2^{high}/MEF2A^{high} DEM signature (Figures S1D and S2A–S2C). We note that these p values are extreme both because of the large number of cells that yield individual transcriptomes and the challenge that, while the cDNA library of each cell is generated in an independent reaction, all cells are growing in the same culture environment, which will tend to enhance the correlation of gene expression across the profiled cells and hence will tend to inflate p values. However, we note that our single-cell data uncovers substantial heterogeneity in HMC3 cell states, highlighting that the culture harbors variation in gene expression despite the shared cell culture environment.

To assess the impact of these results beyond our predefined CD74^{high}/MHC^{high} signature, we assessed whether signatures defined in other studies for human microglial subsets

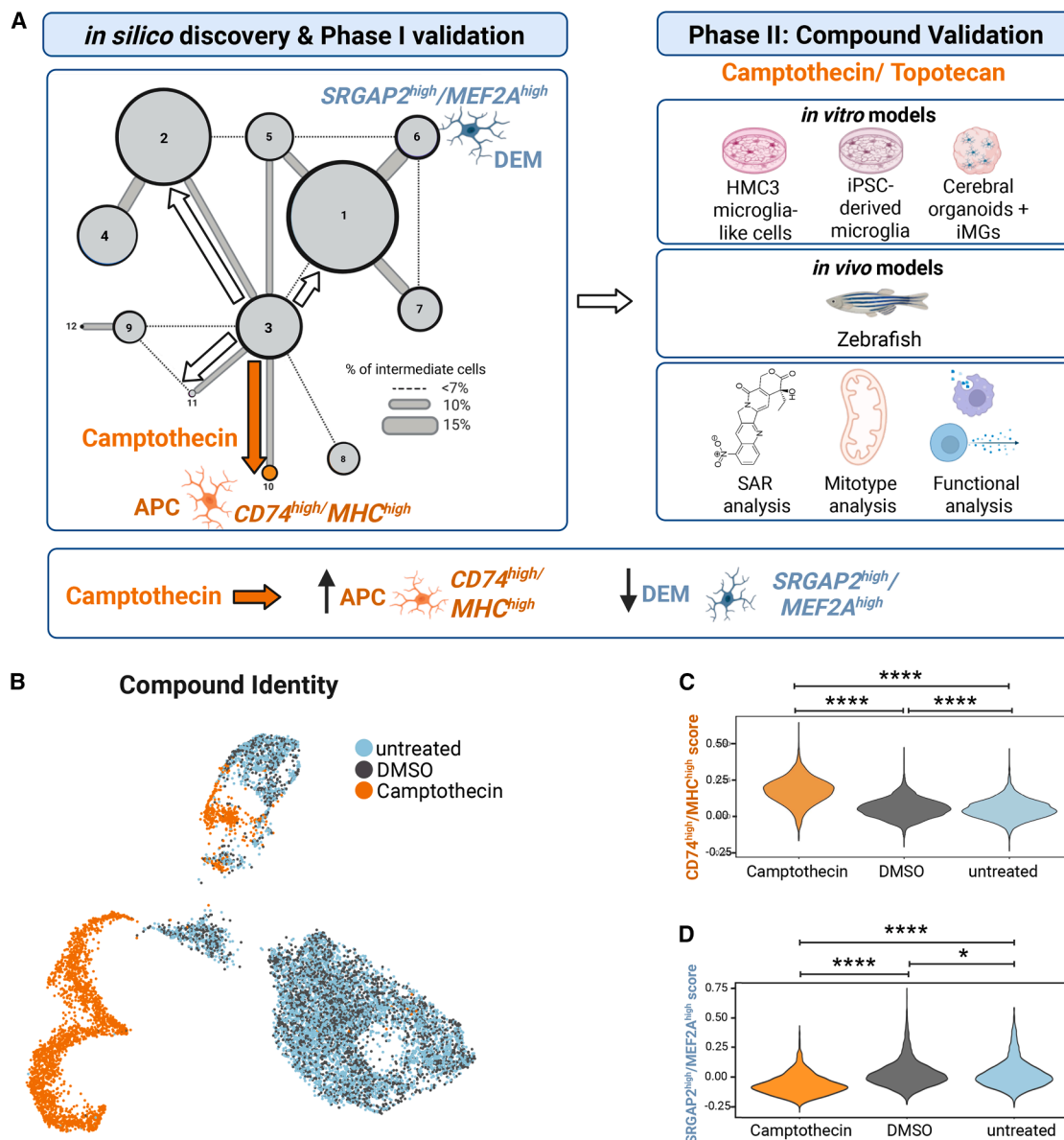


Figure 1. Camptothecin drives human microglia toward a CD74^{high}/MHC^{high} phenotype: study design and single-cell validation in HMC3 cells
(A) Overview of the study design and sc-RNA sequencing analysis of compound-treated HMC3 cells. Schematic diagram depicts the *in silico* discovery phase, phase I of compound validation via RT-qPCR expression analysis, and phase II of compound validation for Camptothecin in various *in vitro* microglial model systems and in an *in vivo* zebrafish model. In validation phase II structure-activity relationship analysis (SAR), mitochondrial phenotyping (mitotype), and functional assays were performed. Camptothecin was previously identified as a compound to polarize human microglia toward a phenotype characterized by high expression of CD74 and MHC genes.¹²

(B) UMAP of compound-treated and control HMC3 cells. Each dot is a single cell, colored by the treatment condition - untreated (light blue, $n = 1,4729$ cells), DMSO control (dark gray; $n = 1,3434$ cells), Camptothecin (1 μ M; orange; $n = 1,2790$ cells).

(C) Violin plots depict the per-cell CD74^{high}/MHC^{high} module score grouped by treatment condition. For statistical analyses, pairwise Wilcoxon rank-sum tests were performed, ns > 0.05, *p.adj $\leq$ 0.05; **p.adj $\leq$ 0.01; ***p.adj $\leq$ 0.001; and ****p.adj $\leq$ 0.0001.

(D) Violin plots show SRGAP2^{high}/MEF2A^{high} module score/cell grouped by treatment condition. For statistical analyses, pairwise Wilcoxon sum-rank tests were performed, ns > 0.05, *p.adj $\leq$ 0.05; **p.adj $\leq$ 0.01; ***p.adj $\leq$ 0.001; and ****p.adj $\leq$ 0.0001. Detailed statistical results are presented in Table S2.

characterized by high expression of MHC genes were induced by Camptothecin treatment. Specifically, we extracted four other versions of the MHC^{high}-expressing human microglial signature^{18–21} (listed in Tables S3A and S3B), and assessed their

expression in untreated, DMSO treated and Camptothecin-treated HMC3 cells (Figure S2D). Interestingly, we found all four signatures to be increased in HMC3 cells treated with Camptothecin when compared to DMSO-treated or untreated

Table 1. Overview of statistical results for four different versions of the MHC^{high} microglia signature tested in our dataset

Dataset	Comparison	Adjusted <i>p</i> value (rank biserial correlation)
Dolan et al. 2023 ³	DMSO vs. untreated	$p = 0.136$ ($r_{rb} = 0.019$)
	DMSO vs. Camptothecin	$p = 1.08E-220$ ($r_{rb} = 0.467$)
	untreated vs. Camptothecin	$p = 8.04E-269$ ($r_{rb} = 0.483$)
Sun et al. 2023 ⁴	DMSO vs. untreated	$p = 0.122$ ($r_{rb} = 0.020$)
	DMSO vs. Camptothecin	$p = 3.36E-07$ ($r_{rb} = 0.077$)
	untreated vs. Camptothecin	$p = 1.4E-12$ ($r_{rb} = 0.098$)
Gazestani et al. 2023 ⁵	DMSO vs. untreated	$p = 0.016$ ($r_{rb} = 0.031$)
	DMSO vs. Camptothecin	$p = 1.13E-236$ ($r_{rb} = 0.484$)
	untreated vs. Camptothecin	$p = 5.82E-292$ ($r_{rb} = 0.504$)
Mancuso et al. 2024 ⁶	DMSO vs. untreated	$p = 0.073$ ($r_{rb} = 0.023$)
	DMSO vs. Camptothecin	$p = 6.56E-60$ ($r_{rb} = 0.241$)
	untreated vs. Camptothecin	$p = 1.21E-77$ ($r_{rb} = 0.258$)

For statistical analyses, pairwise Wilcoxon rank-sum tests were performed. R_{rb} refers to the rank biserial correlation reporting effect sizes for Wilcoxon signed-rank tests.

HMC3 cells, highlighting that our result is robust to the exact composition of the gene set (Table 1).

Compound-treated iMG and CO-iMG confirm Camptothecin as a potent inducer of the CD74^{high}/MHC^{high} signature

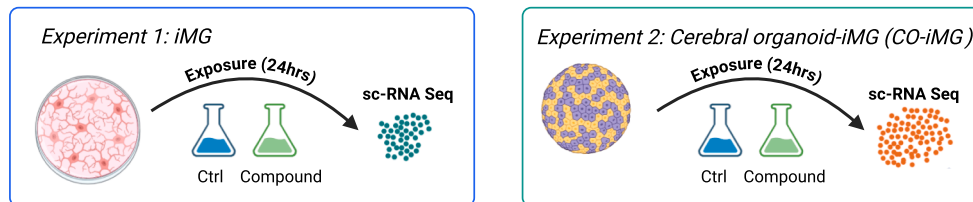
To address the robustness of our observed Camptothecin-induced differences in gene expression, we tested Camptothecin in a complementary microglial model system: iMG. In addition to assessing the effect of Camptothecin, we also considered the Camptothecin derivative Topotecan. The latter compound emerged from structure-activity relationship experiments in which we selected and tested three analogs for the lead compound Camptothecin and assessed the RNA expression of two marker genes (*SRGN*, *CXCR4*) as proxies for the CD74^{high}/MHC^{high} microglial subtype, as we previously did in our compound screen.¹² Results of the assessment of the analog compounds are presented in detail in Figure S3. We note that the topoisomerase I inhibitor Topotecan is a clear driver of the CD74^{high}/MHC^{high} signature (Figures S3A and S3B). Topotecan is of particular interest as it has fewer adverse events than its parent compound, Camptothecin, and is FDA-approved for the treatment of small-cell lung cancer.²² Thus, we exposed iMG differentiated from the FA0000010 hiPSC line on day 28–29 of differentiation from iPSC²³ to Camptothecin. Lower doses were used – Camptothecin (0.1 μ M) and Topotecan (0.1 μ M) – given the higher susceptibility of iMG to toxicity (relative to HMC3 cells). After 24 h of compound exposure, cells were collected, and scRNA-seq data were generated (Figure 2A, Experiment 1).

In parallel with these experiments, we performed a second line of experiments in a more complex model system: cerebral organoids (CO) co-cultured with isogenic iMG (CO-iMG), to evaluate microglia in a 3-dimensional microenvironment (see STAR Methods).²⁵ In short, CO were first differentiated from iPSC (25–35 days old) derived from two independent iPSC lines, WTC11 and MSN38. Hematopoietic progenitor cells (HPC, 12 days old) differentiated from iPSC (WTC11) were then seeded onto the CO. The CO and HPC were then co-cultured for 7 days, allowing the HPC to differentiate into microglia (iMG) and integrate into the cerebral organoids (CO-iMG). On day 7 after co-culture, CO-iMG were treated with Camptothecin (0.1 μ M, 0.5 μ M) or DMSO for 24 h (for dose-titration on iMG, see Figure S4A) followed by processing for scRNA-seq data generation (Figure 2A, Experiment 2; $n = 2$ organoids per condition, derived from $n = 1$ differentiation of WTC11 iPSC and $n = 1$ differentiation of MSN38 iPSC). For our analyses, the microglia-like cells were first isolated, *in silico*, from the other CO-iMG cells using microglial markers (*AIF1*, *C1QA*, *C1QB*, *CSF1R*, *CTSS*, *CX3CR1*, *GPR34*, *HEXB*, *ITGAM*, *MERTK*, *P2RX7*, *P2RY12*, *PTPRC*, *SLC2A5*, *TGFB1*, *TMEM119*; see STAR Methods) and then pre-processed together with the iMG-derived data (Figure 2A, Experiment 2). From the DMSO-treated cerebral organoids, we identified $n = 131$ CO-iMG cells, and, from the Camptothecin-treated organoids, we identified $n = 130$ CO-iMG cells. We then interrogated the effect of the selected compound on the transcriptional states of iMG and CO-iMG in a single framework to assess the extent to which the expected drug effects are recapitulated in these two model systems. Specifically, we first projected both datasets onto an integrated space in an unsupervised manner²⁶ using the iMG data ($n = 29,177$ cells total) as reference and the CO-iMG data as query, such that cells from both systems could be jointly analyzed. Figure S4B shows the Uniform Manifold Approximation and Projection (UMAP) embeddings of the iMG and the CO-iMG projected onto the same space. Most CO-iMG cells were able to be mapped within the iMG space, suggesting transcriptional similarity between the two model systems.

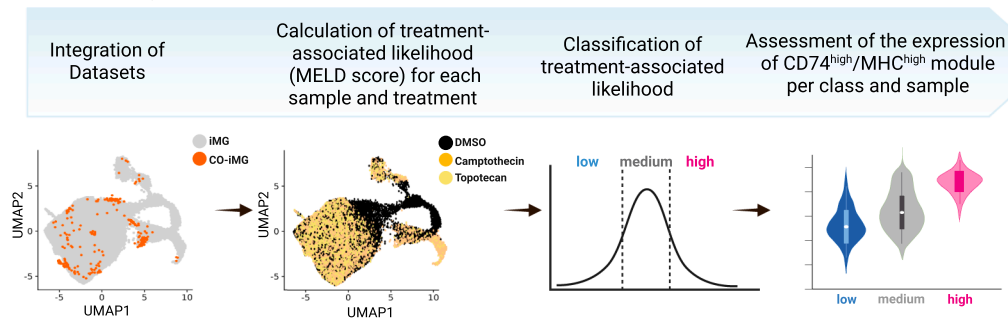
To quantify the effect of compound treatment across conditions and model systems at the single-cell level, we applied the MELD algorithm²⁴ to the integrated iMG and CO-iMG dataset. MELD captures the continuous and heterogeneous nature of cell populations in response to perturbations, and it models the treatment-associated relative likelihood for each cell from a given condition compared to the control condition. The cells are binned into three groups based on their likelihood of being in the Camptothecin-treated condition: low, medium, and high likelihood. Thus, instead of a binary control/treatment comparison, MELD allows for capturing a continuum of response states (as individual cells treated with Camptothecin exist on a continuum of response), providing more statistical power in downstream analyses. Figure 2B shows the treatment-associated likelihood for individual cells in the iMG and CO-iMG integrated dataset.

The iMG cells with the highest likelihood of being derived from the treatment condition are enriched on the left side of the UMAP (Figure 2B). The CO-iMG cells showed a similar distribution. We then statistically evaluated these data using the three classes of

A Experimental Models



Data analysis pipeline



B CD74^{high}/MHC^{high} score in iMG and CO-iMG across established classes of treatment-associated likelihood

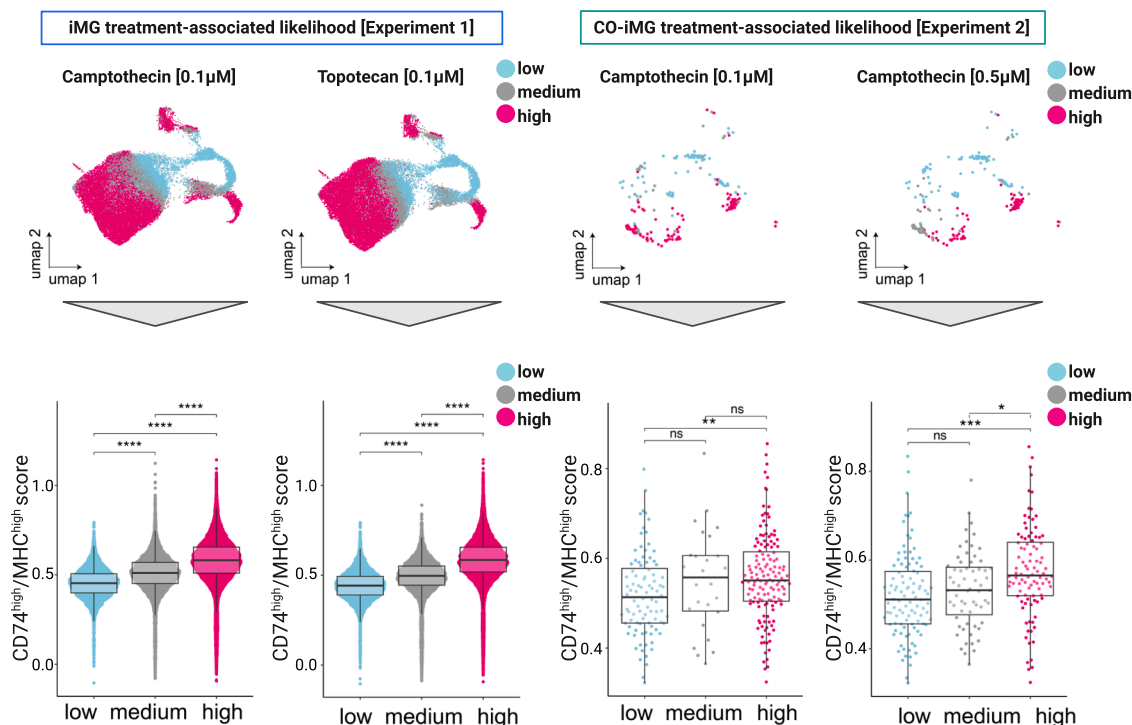


Figure 2. sc-RNA sequencing of compound-treated iMG and CO-iMG reveals upregulation of the CD74^{high}/MHC^{high} module following Camptothecin and Topotecan treatment

(A) Overview of experimental design. iPSC-derived microglia (iMG), treated with compounds (Camptothecin ($n = 1$), Topotecan ($n = 1$); Experiment 1) and cerebral organoids containing co-cultured iMG (CO-iMG), treated with compounds (Camptothecin 0.1 μ M and 0.5 μ M) or DMSO as control (24 h; $n = 1$) were subjected to sc-RNA sequencing. CO-iMG were projected onto the iMG data, and MELD²⁴ was used to quantify the relative treatment-associated likelihood for individual (legend continued on next page)

cells (low, medium, or high likelihood of being affected by treatment) (Figure 2B, see STAR Methods), assessing the expression of the CD74^{high}/MHC^{high} signature in the three groups. iMG show a statistically significant increase in the signature with increasing likelihood of belonging to the group of cells that responded to Camptothecin (0.1 μ M) (Figure 2B; Table S4): for example, compared to the low likelihood group, the high likelihood has a significantly higher expression of the CD74^{high}/MHC^{high} signature ($p \leq 0.0001$, $r = -0.551$). Results with Topotecan (0.1 μ M) also showed significance, validating the role of topoisomerase I inhibition in eliciting a microglial CD74^{high}/MHC^{high} phenotype in iMG with a different, mechanistically related compound (Figure 2B; Figure S4B): low vs. high likelihood $p \leq 0.0001$, $r = -0.625$.

In addition, we applied a simpler, complementary approach for analysis by performing a comparison between all cells belonging to each of the treatment groups (Figure S4C); these results are similar but have smaller effect sizes, illustrating the value added by the MELD analysis (Table S4). MELD also highlights a progressive, graded increase in CD74^{high}/MHC^{high} scores across the low/medium/high likelihood groups. Finally, we performed a third analytical approach in which we used a factor-based approach and a new primary microglial resource which delineates 23 transcriptional programs (factors) across primary human microglia.²⁷ We tested all 23 factors, and reanalyzed our compound-treated iMG data by scoring each cell based on their expression of the top 50 genes of each factor.²⁷ In this analysis, Camptothecin significantly increases the expression of two factors: factor 15 (the MHC^{high} transcriptional program) and factor 17 (the C1Q^{high} factor, Figure S4D) in comparison to DMSO. This approach, using an unbiased analysis following data dimensionality reduction, further underlines our findings of Camptothecin as a potent inducer of the CD74^{high}/MHC^{high} signature.

The CO-iMG data consist of a small number of cells (261 CO-iMG cells vs. 29,177 cells analyzed for the iMG experiment); therefore, our data are somewhat sparse for the CO-iMG model system. Nonetheless, we see a significantly higher CD74^{high}/MHC^{high} signature in the cells of the high likelihood of treatment effect class when compared to the low likelihood cell class at both doses of Camptothecin (Figure 2B; Table S4). Thus, the CO-iMG results are consistent with the iMG results.

In addition, we assessed the effect of treatment on other cell types present in the cerebral organoids. We identified cortical neurons, neural progenitor cells (NPCs) and ciliate mesenchymal cells as additional cell types present in the CO's using predetermined gene sets²⁸ (Figures S5 and S6). Differential gene expression analysis in these cell types showed that they were also affected by Camptothecin treatment, underscoring the need for future studies to dissect the direct and indirect effects of Camptothecin treatment as studies evaluating the therapeutic

potential of topoisomerase I inhibition proceed. (Figures S5 and S6). A summary of the differentially regulated pathways across the analyzed cell types is presented in Figure S7.

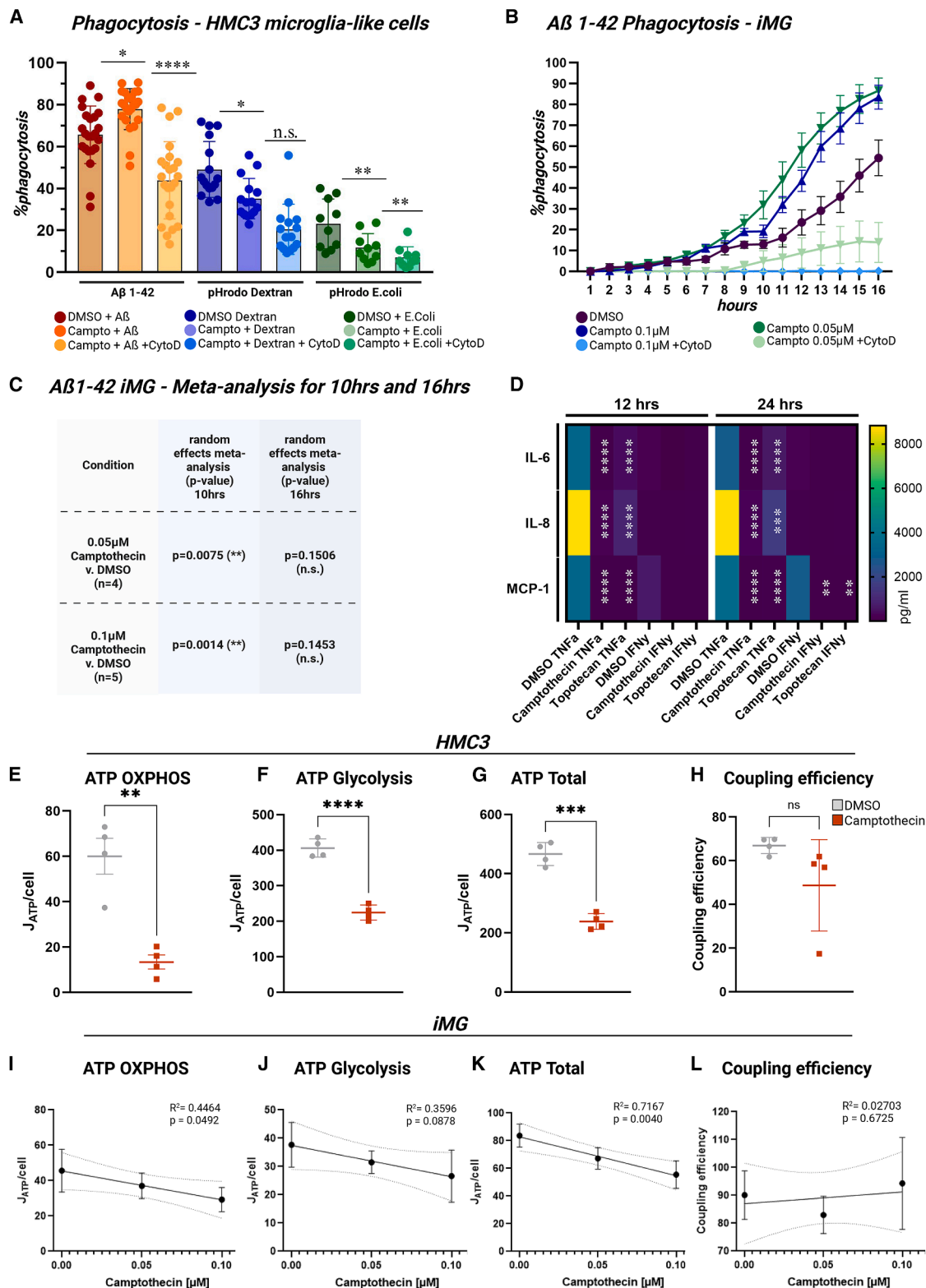
Camptothecin and Topotecan therefore have consistent effects across human microglia-like model systems: they upregulate the CD74^{high}/MHC^{high} signature. To address the p value inflation of these single-cell analyses, we also calculated Cohen's d , a standardized effect size measure defined as the difference between two groups' means normalized by the pooled within-group standard deviation. We did this separately in each model system and then combined the results across the cell types (HMC3, iMG and CO-iMG) since they represent independent assessments of different microglia-like model systems. Namely, we performed a one-sided one-sample test and found that effect sizes were significantly positive across the four samples ($n = 4$ scRNA-seq conditions, $t = 2.71$, $p = 0.037$; Figure S8). This effect remained significant when the analysis was restricted to the iMG and CO-iMG comparisons ($n = 3$ conditions, $t = 3.84$, $p = 0.031$), indicating that the association was not driven solely by the HMC3 treatment comparison. Topotecan also showed increased CD74^{high}/MHC^{high} signature in the single comparison ($d = 1.15$; Figure S8). Thus, in aggregate, our four single-cell datasets produce a clear and consistent result.

Functional characterization: Endocytic and phagocytic properties

One of the key functions of microglia involves uptake of a variety of substrates to maintain brain homeostasis. We chose to use HMC3 cells and a series of substrates to assess different uptake phenotypes of the Camptothecin-polarized CD74^{high}/MHC^{high} microglia-like cell subtype: (1) pHrodo Dextran was used to assess endocytosis and macropinocytosis, (2) fluorescently labeled A β 1–42 assessed uptake in an AD context, and (3) pHrodo-labeled heat-inactivated *E. coli* was used as a readout for phagocytosis in an inflammatory context²⁹ (Figure 3). Cytochalasin D, an inhibitor of actin polymerization, was used as a control condition for the experiments and administered jointly with A β for one hour prior to flow cytometry-based readout and following 24 h pre-treatment of the cells with Camptothecin or DMSO as a control (Figure S9A). Interestingly, we observed a specialization of uptake phenotypes (Figure 3A): in HMC3 microglia-like cells, Camptothecin (Figure 3) led to a reduction in endocytic activity of Dextran (DMSO vs. Camptothecin – $p = 0.0057$; $R^2 = 0.514$) as well as a downregulation of phagocytosis of *E. coli* (DMSO vs. Camptothecin – $p = 0.0141$; $R^2 = 0.417$) while the uptake of monomeric A β was upregulated (DMSO vs. Camptothecin – $p = 0.0142$; $R^2 = 0.497$). This enhanced A β uptake of the CD74^{high}/MHC^{high} microglia-like cells may be relevant to the context of A β clearance in the trajectory to AD.

cells. Cells were then classified based on their relative treatment-associated likelihood (low; medium; high) and expression of CD74^{high}/MHC^{high} signature genes was assessed across these three groups of cells.

(B) CD74^{high}/MHC^{high} score in iMG and CO-iMG cells. Top row - UMAP of integrated CO-iMG and iMG, with cells colored by three different levels of treatment-associated likelihood (low, medium, high) for each drug-treatment condition. Bottom row - single-cell distribution of the CD74^{high}/MHC^{high} module scores across the three levels of treatment-associated likelihood for each of the given samples. Each dot represents a single cell; statistical analysis was performed using an unpaired t test; ns > 0.05, * p .adj $\leq$ 0.05; ** p .adj $\leq$ 0.01; *** p .adj $\leq$ 0.001; **** p .adj $\leq$ 0.0001. Detailed statistics are presented in Table S4. Each boxplot highlights the median, lower, and upper quartiles. Whiskers indicate 1.5 times the interquartile range.



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To validate our findings on a specialization of CD74^{high}/MHC^{high} microglia for enhanced uptake of amyloid beta, we conducted further experiments in iMG ($n = 4$ –5 independent differentiations). To confirm the uptake of A β into the lysosome, we conducted this set of phagocytosis assays using pHrodo-conjugated A β 1-42 and assessed phagocytosis longitudinally over a period of 16 h (Figures 3B and 3C), using 0.05 μ M Camptothecin ($n = 4$) and 0.1 μ M Camptothecin ($n = 5$) as previously determined by titration experiments (Figure S9B). We also included Cytochalasin D treatment as a control. A representative image of longitudinal phagocytosis is depicted in Figure 3B. For our primary analysis, we analyzed the percentage of A β -positive iMG at 10 h in control and treated cells of each experiment. We then summarized the statistical analyses of each experiment using a random-effects meta-analysis approach (Figure 3C; for detailed results see Table S5). For completeness, we also performed a less conservative fixed-effects meta-analysis; these results are presented in Table S5 and are consistent with the results of the primary analysis. Namely, we observed that after 10 h, both concentrations, 0.05 μ M Camptothecin ($n = 4$; $p = 0.0075$) and 0.1 μ M Camptothecin ($n = 5$; $p = 0.0014$), significantly increased A β phagocytosis in iPSC-derived microglia, confirming the HMC3 results. In addition, we analyzed red fluorescence protein (RFP) mean intensity for all cells above the DMSO mean +2 SD (standard deviations) across conditions (Figures S9C and S9D). Using this approach, we could show that Camptothecin, at both selected concentrations, increased the amount of A β 1-42 phagocytosis per iMG cell after 10 h, thereby supporting our prior findings in HMC3 microglia-like cells (Figure 3A).

Thus, the transcriptionally distinct state of CD74^{high}/MHC^{high} microglia displays distinct, context-specific functional differences in uptake. These experiments help to prioritize Camptothecin as a lead compound for further investigation in the realm of AD.

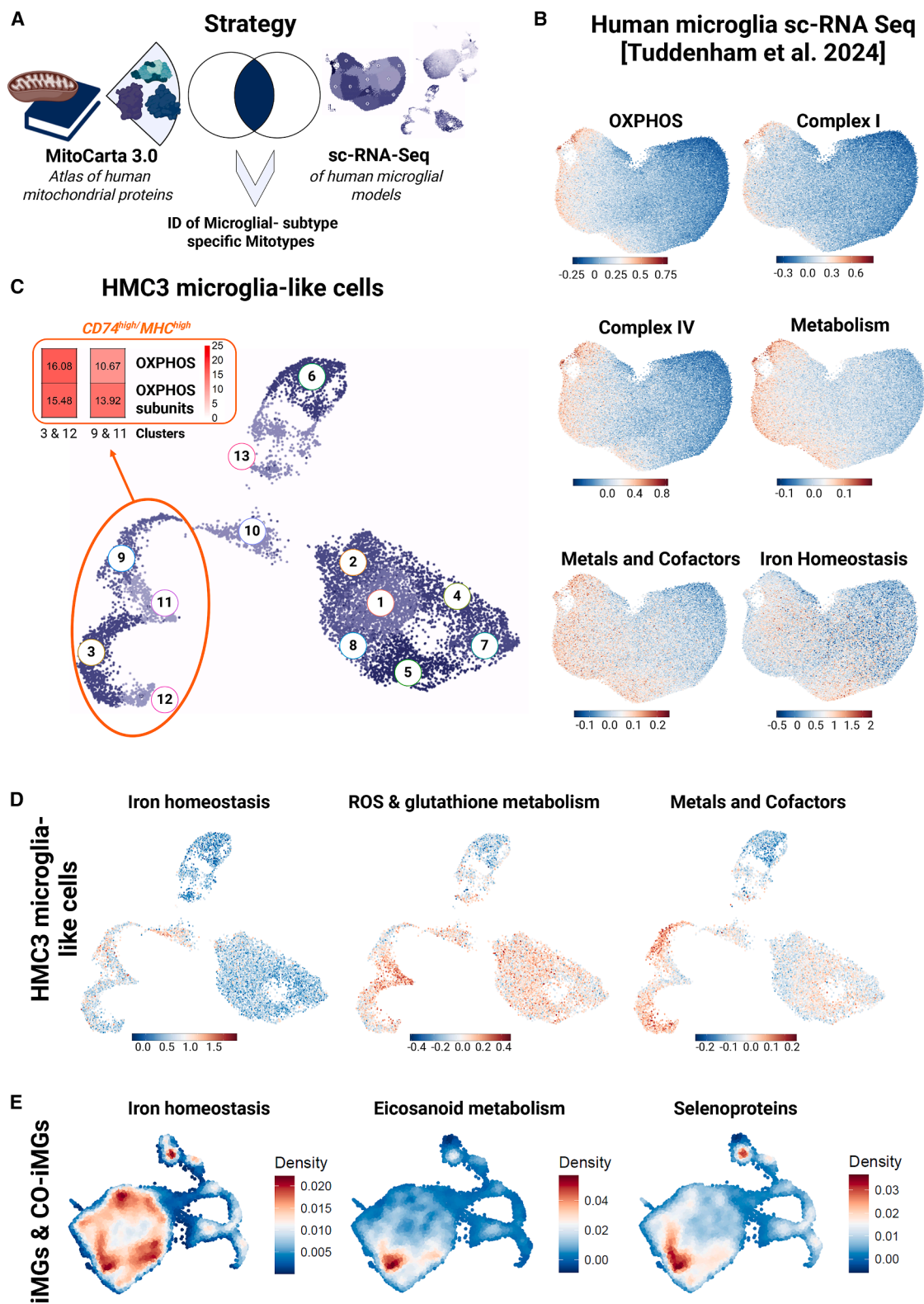
Functional characterization: Secretion of pro-inflammatory chemokines and cytokines

Activating immune responses is another important function of microglia, so we evaluated the effect of Camptothecin and Topotecan in modulating responses to two cytokines that induce important systemic immune responses: TNF- α and IFN- γ (Figure 3D; Figures S10A and S10B). A panel of 15 pro-inflammatory cytokines was measured in culture supernatants of DMSO-, Camptothecin- or Topotecan-pretreated HMC3 microglia following 12 h and 24 h of stimulation with TNF- α or IFN- γ ($n = 3$ per condition; see STAR Methods). In general, HMC3 cells responded more strongly to TNF- α than to IFN- γ , and cytokine secretion responses to these two stimuli were changed by Camptothecin (Tables S5 and S6). Polarization toward the CD74^{high}/MHC^{high} microglial phenotype using Camptothecin treatment significantly reduced IL-6, IL-8, as well as MCP-1 secretion at 12 h following stimulation with TNF- α , and the effect persisted at 24 h (Figure 3D; Tables S5 and S6). These results are consistent with reports of Camptothecin (1) suppressing inflammatory gene expression, including IL-8, IL-6 as well as MCP-1 secretion in different model systems,^{30,31} (2) inhibiting the activation of NF- κ B,³² and (3) polarizing microglia toward an anti-inflammatory phenotype.³²

Consistent with this shared mechanism of action, Topotecan treatment fully recapitulated the cytokine-release pattern observed with Camptothecin (Figure 3D; Tables S5 and S6), thereby functionally validating the observation that polarization of microglia-like cells by topoisomerase I inhibition induces a functionally distinct state, with a diminished response to systemic immune activation with TNF- α (Figures S10A and S10B). This further positions Topotecan as an interesting candidate for inducing the CD74^{high}/MHC^{high} human microglial signature *in vitro* in future studies and for its potential as a lead compound for modulating immune responses in the CNS.

Figure 3. Compound-treated HMC3 cells exhibit substrate-specific endocytic and phagocytic phenotypes as well as differences in secretion of pro-inflammatory cytokines and metabolic activity

(A–D) Phagocytic phenotypes. (A) HMC3 microglia-like cells, pretreated with Camptothecin (1 μ M) or DMSO as control (24 h), were exposed to AlexaFluor 647-labeled A β 1-42 (left; orange shading), pHrodo-labeled Dextran (center; blue shading), or pHrodo-labelled *E. coli* particles (right; green shading) for 1 h, and uptake was assessed via flow cytometry. Each dot represents one independent experiment (mean $\pm$ SD; A β – $n = 23$; pHrodo Dextran – $n = 14$; pHrodo *E. coli* – $n = 10$). For statistical analysis, one-way ANOVA followed by Tukey's multiple comparison test was performed. ns > 0.05, *p.adj $\leq$ 0.05; **p.adj $\leq$ 0.01; ***p.adj $\leq$ 0.001; and ****p.adj $\leq$ 0.0001. (B) Representative graph showing iMG phagocytosis of pHrodo-labelled amyloid beta over a period of 16 h. iPSC-derived human microglia-like cells were pretreated with Camptothecin (0.05 μ M (4n), 0.1 μ M (5n) or 0.5 μ M (4n)) or DMSO as a control for 24 h, exposed to pHrodo-labeled amyloid beta, and the uptake of pHrodo-labeled amyloid beta was assessed hourly over a period of 16 h using BioTek BioSpa Live Cell Analysis System (Agilent), capturing two-four images/well and 3 wells/conditions. Individual measurements were analyzed with a Cell Profiler pipeline to extract the percentage of RFP-positive cells and are summarized for each condition and each time point, depicting mean $\pm$ SEM. Graphs depict results for a single experiment for representation. (C) Results of the random-effects and fixed-effects meta-analyses summarizing phagocytosis assays performed on $n = 4$ –5 independent preparations of human iPSC-derived microglia ($n = 4$ –0.05 μ M Camptothecin; $n = 5$ –0.1 μ M Camptothecin). ns > 0.05, *p.adj $\leq$ 0.05; **p.adj $\leq$ 0.01; ***p.adj $\leq$ 0.001; and ****p.adj $\leq$ 0.0001. (D) Heatmap summarizing results of cytokine secretion of compound- or DMSO-pretreated HMC3 cells (24 h) followed by stimulation with either TNF- α (0.3 μ g/mL), IFN- γ (0.3 μ g/mL), or H₂O as a control for 12 or 24 h. Pro-inflammatory cytokine secretion was assessed using a human pro-inflammatory cytokine discovery assay. Different colors depict the measured amounts of cytokines IL-6, IL-8, and MCP-1 (mean $\pm$ SEM) in pg/mL for DMSO control-treated samples, Camptothecin- (1 μ M) or Topotecan- (1 μ M) treated samples ($n = 3$ biological replicates per condition). For statistical analysis, one-way ANOVA followed by Tukey's multiple comparisons test with a single pooled variance was performed. ns > 0.05, *p.adj $\leq$ 0.05; **p.adj $\leq$ 0.01; ***p.adj $\leq$ 0.001; and ****p.adj $\leq$ 0.0001. (E–H) MitoStress test on HMC3 cells treated with Camptothecin (1 μ M) and DMSO as control ($n = 4$ biological replicates), depicting ATP generated through OxPhos (E), ATP Glycolysis (F), ATP total/cell (G), and coupling efficiency (H). Data points show experimental replicates, including mean $\pm$ SEM. For statistical analysis, Welch's *t* test was performed. ns > 0.05, *p.adj $\leq$ 0.05; **p.adj $\leq$ 0.01; ***p.adj $\leq$ 0.001; and ****p.adj $\leq$ 0.0001. (I–L) MitoStress test on iMG cells treated with Camptothecin (0.05 μ M, 0.01 μ M) and DMSO as control ($n = 3$ biological replicates), depicting ATP generated through OxPhos (I), ATP Glycolysis (J), ATP total/cell (K), and coupling efficiency (L). Graphs show linear regression analysis for Seahorse parameters assessed in iMG. Datapoints indicate mean $\pm$ SD (Standard Deviation) for each treatment condition (DMSO, 0.05 μ M Camptothecin, 0.1 μ M Camptothecin). Simple linear regression was performed, and dotted lines indicate 95% confidence bands of the best-fit line (continuous line); goodness of fit is indicated by the R^2 value, and the *p* value indicates whether the slope is significantly non-zero. Table S5 summarizes all statistical results from this figure.



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Functional characterization: Mitochondrial phenotypes

Our recent report describing human microglial subpopulations (illustrated in Figure 1) also suggested a central metabolic divide between the two major poles of our human microglial population structure model,¹² with microglial subtypes on the left side of the population structure (Clusters 2/4/9, Figure 1A) being enriched in genes related to oxidative phosphorylation (OxPhos) and the right side of the population structure corresponding to the SRGAP2^{high}/MEF2A^{high} subtypes (Clusters 1/6), being enriched for genes involved in alternative metabolic pathways and heterocyclic metabolism.¹² Metabolic phenotypes have been previously linked to distinct microglial activation states with homeostatic microglia being most reliant on OxPhos; following activation, reactive microglia switch their metabolism to glycolysis, a faster but less efficient way to generate ATP.³³

We therefore assessed respiratory states of HMC3 cells with a MitoStressTest (STAR Methods) 24 h following treatment with Camptothecin (1 μ M) or DMSO as control ($n = 4$ biological replicates) and derived interpretable ATP production rates for OxPhos and glycolysis. Camptothecin induced a hypometabolic state where the total ATP production rate ($J_{ATP-Total}$) relative to DMSO control was decreased by 49% (Figure 3G; $p = 0.0001$; $R^2 = 0.947$). Additionally, Camptothecin had an effect on both, ATP production rates derived from glycolysis ($J_{ATP-Glycolysis}$) and ATP production rates derived from oxidative phosphorylation ($J_{ATP-OxPhos}$), suggesting that this compound induces a general hypometabolic state that reflects lower energy demand (Figures 3E–3G; Figures S10C–S10E; $J_{ATP-Glycolysis} - p = < 0.0001$, $R^2 = 0.954$; $J_{ATP-OxPhos} - p = 0.0059$, $R^2 = 0.885$). Thus, the functional changes in uptake and response to TNF- α described above are occurring in the context of altered metabolic demand (Figures 3A–3D).

In addition, we repeated these experiments in iPSC-derived microglia-like cells, using 0.05 μ M and 0.1 μ M Camptothecin determined by titration using Alamar Blue Assay (Figure S4A). Following FCCP (carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone) titration (Figure S11A), we performed three independent experiments using three independent iMG differentiations. Using linear regression, we find that an increasing dose of Camptothecin leads to reduced basal Total ATP levels ($p =$

0.004; $R^2 = 0.717$, $\beta = -281.1$; Figure 3K), consistent with the results in HMC3 cells. Its two components— $J_{ATP-Glycolysis}$ and $J_{ATP-OxPhos}$ —show the same direction of effect, but only $J_{ATP-OxPhos}$ is significant ($p = 0.049$; $R^2 = 0.446$; $\beta = -163$; Figures 3I and 3J).

We also analyzed coupling efficiency, the proportion of oxygen consumption used for ATP synthesis in comparison with the oxygen consumption rate (OCR) driving the proton leak (ATP-linked OCR/basal OCR)³⁴; proton leak is defined as the remaining basal respiration not coupled to ATP production.³⁵ However, we found no difference in this measure. Overall, Camptothecin-induced CD74^{high}/MHC^{high} microglial-like cells display alterations in empirically measured metabolic function, reflected in a hypometabolic state.

Human microglial subtypes exhibit enrichment of specific mitotypes

Given these differences in empirically measured mitochondrial function in an *in vitro* reconstituted microglia-like cell subtype, we repurposed our sc-RNA Seq data generated from living human microglia¹² and implemented a higher-resolution computational approach to mitochondrial phenotyping (i.e., mitotyping) by deploying mitochondrial pathway annotations from the human MitoCarta3.0 resource, a catalog comprised of 1136 human mitochondrial genes and 149 pathway annotations.³⁶ Specifically, we assessed the enrichment of these 149 MitoPathways (Table S7) in the 12 human microglial subtypes that we previously defined¹² (Figures 1A, 4A, and 4B) and in our compound-driven *in vitro* reconstitution of the CD74^{high}/MHC^{high} (microglial cluster 10) subtype (HMC3 cells: Figures 4C and 4D; iMG & CO-iMG; Figure 4E).

Interestingly, we observed an enrichment of genes related to OxPhos on the left side of the human microglial population structure, which contains homeostatic cells¹² (Figure 1A and 4B), and OxPhos-related MitoPathways, including Complexes I, III, and IV were strongly downregulated on the right side of the population structure, consistent with prior observations using GO analyses of this dataset,¹² suggesting that cells at opposite extremes of our diagram exhibit distinct metabolic states.

The CD74^{high}/MHC^{high} signature induced by Camptothecin, is not at the extreme end of this major metabolic axis of our

Figure 4. Mitochondrial phenotyping identifies association between distinct human microglial subtypes and specific mitochondrial phenotypes

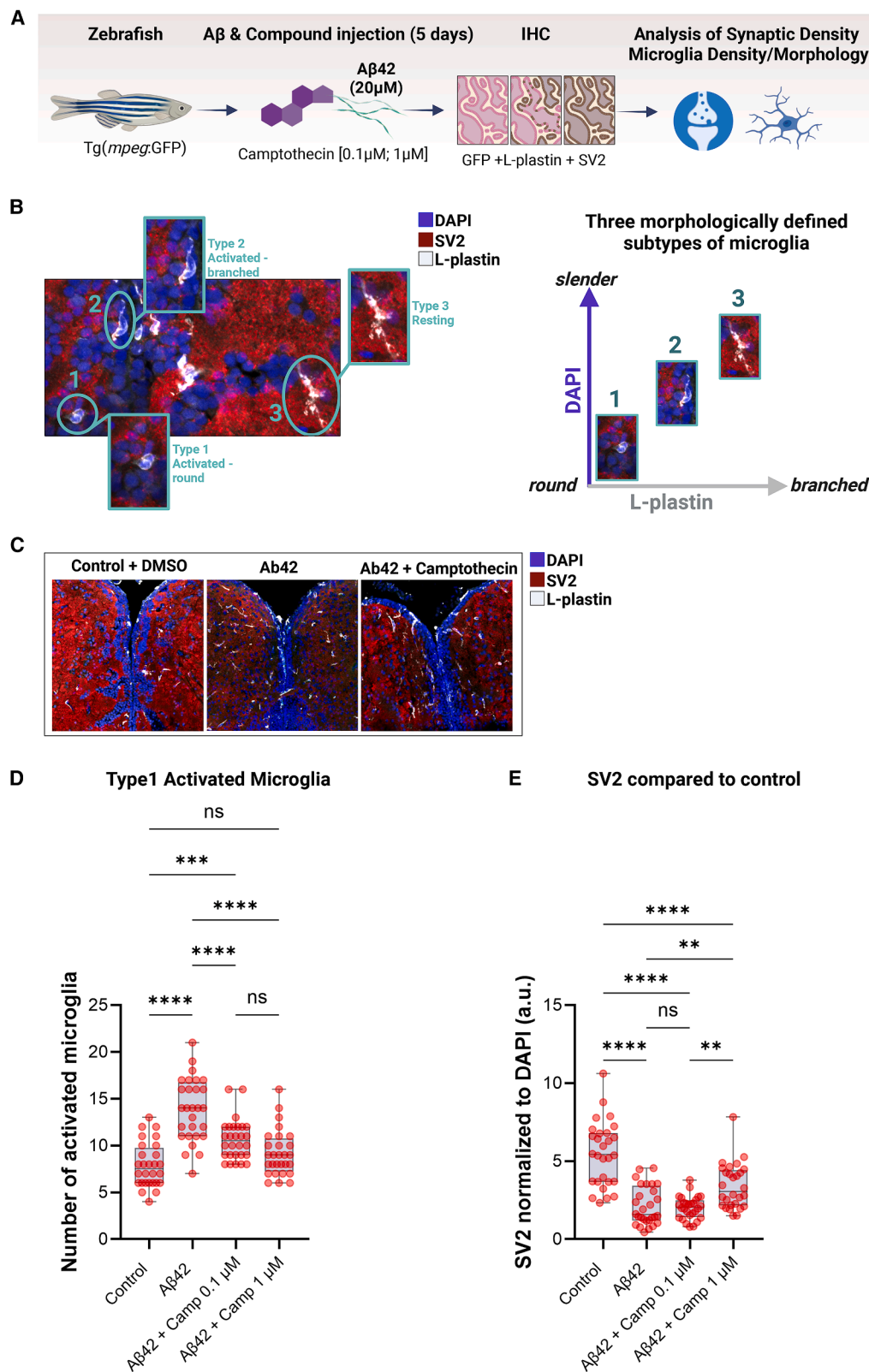
(A) Strategy for mitochondrial phenotyping analysis. 149 mitochondrial pathways defined from the MitoCarta 3.0,³⁶ were used to perform Gene Set Enrichment Analysis (GSEA) in the human microglial sc-RNA-Seq dataset.¹² Detailed results are presented in Tables S8 and S9.

(B) Selected results of GSEA for specific mitotypes in human microglial subtypes are shown. Legend depicts module scores, showing average expression for module genes vs. a background of similarly expressed control genes. The legend depicts module scores, showing average expression for module genes versus a background of similarly expressed control genes.

(C) Mitotype analysis results in Camptothecin-treated HMC3 microglia-like cells following GSEA for the 149 mitochondrial MitoCarta pathways.³⁶ Genes from treated HMC3- scRNA-seq data were ordered based on average log-fold change between cells from the respective treatment condition vs. control. Enrichment of gene modules associated with different aspects of mitochondrial activity was performed in upregulated gene lists associated with each cluster using a hypergeometric test with Benjamini-Hochberg correction. Detailed results are presented in Tables S8 and S9.

(D) Selected results of mitotype GSEA projected into UMAP of compound-treated HMC3 cells. Plots illustrate some of the most distinct GSEA results for mitochondrial phenotypes in compound-treated HMC3 cell clusters. The legend depicts module score, showing average expression for module genes versus a background of similarly expressed control genes.

(E) Selected results of mitotype GSEA projected into joint UMAP of compound-treated iMG and CO-iMG. Plots illustrate some of the GSEA results for mitochondrial phenotypes in compound-treated iMG and CO-iMG microglial clusters. Legend depicts module scores, showing average expression for module genes vs. a background of similarly expressed control genes.



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microglial population structure model (Figure 1); this specialized set of microglial cells appears to be a minor microglial subset following a different vector of differentiation which is distinct from the main axis of differentiation.¹² Using MitoPathway annotations, we detected an upregulation of OxPhos and Complex I subunit pathways in the cells induced by Camptothecin (Figure 4C, clusters 3,9,11,12 in Figure S1A), relative to DMSO-treated control HMC3 microglia-like cells (Figure 4C, clusters 1–2, 4–10 in Figure S1A). Thus, while our functional characterization of Camptothecin-treated HMC3 cells uncovered a hypometabolic state (Figures 3E–3H), the transcriptional profiles suggest that cells might be preparing to re-enter a state that is driven by OxPhos.

Repurposing the purified living primary human microglia data, the CD74^{high}/MHC^{high} subtype (labeled as cluster 8/10 in the model in Figure 1A) displays enrichment of MitoPathways related to lipid metabolism, reactive oxygen species, and glutathione metabolism, as well as metals and cofactors. When performing the same type of analysis in our compound-based models, including HMC3 cells, iMG, and CO-iMG, we could recapitulate these findings in all of our *in vitro* models: mitotype analysis of Camptothecin-treated HMC3 as well as Camptothecin- or Topotecan-treated iMG and CO-iMG showed a higher degree of OxPhos and iron homeostasis mitotypes (Figures 4D and 4E). A summary of all results can be found in Tables S8 and S9.

Overall, CD74^{high}/MHC^{high} human microglia-like cells therefore represent a state enriched for certain functions: (1) enhanced RNA expression of antigen presentation machinery genes, (2) selective enhancement of A β uptake, (3) diminished secretion of selected cytokines in response to TNF- α stimulation, and (4) changes in OxPhos metabolism, reactive oxygen species (ROS) generation and glutathione metabolism as well as higher levels of metals and cofactors, indicating biological catalysis³⁷ (Figure 4D; Figure S12).

Camptothecin dose-dependently restores synaptic density and modulates microglial activation in zebrafish

To assess the effects of Camptothecin on amyloid-induced neuroinflammation and synaptic loss, we next used an established zebrafish amyloid proteinopathy model,³⁸ which shows cellular transcriptomic changes and neurodegenerative phenotypes that are reminiscent of human disease.^{38–40} We injected the zebrafish with A β 1-42 and treated them with either 0.1 μ M or 1 μ M Camptothecin (Figure 5A); we then performed immunohisto-

chemistry with L-plastin to label microglia and SV2 as a presynaptic marker. Data were captured using confocal imaging, which led to quantitative outcome measures used in downstream morphometric analyses (Figures 5B–5E).

Following an established schema,⁴¹ we distinguished three morphologically distinct microglial subtypes based on the soma shape visualized with L-plastin staining: Type 1 (amoeboid, activated-round), Type 2 (activated-branched), and Type 3 (resting, highly branched) (Figure 5B). Morphologically, the Type 1 amoeboid microglia observed in zebrafish correspond to disease-associated microglia, as reported previously in zebrafish amyloid and interferon-response models.⁴⁰ A β 1-42 injection led to a pronounced shift toward Type 1 microglia, consistent with a reactive phenotype (Figure 5D). Quantification across four independent animals ($n = 4$) per condition showed a significant increase in the number of Type 1 microglia in A β 1-42-injected fish compared with controls (two-way ANOVA, $p < 0.0001$, Mean diff. = -5.96 , 95% CI of diff. = -7.87 to -4.06). Camptothecin treatment reduced this cell population in a dose-dependent manner, with both concentrations significantly lowering Type 1 microglial numbers compared to A β 1-42 alone (Figure 5D; Figure S13; 0.1 μ M Camptothecin: $p < 0.0001$, Mean diff. = 3.25 , 95% CI of diff. = 1.35 to 5.15 ; 1 μ M Camptothecin: $p < 0.0001$, Mean diff. = 4.68 , 95% CI of diff. = 2.78 to 6.58).

To evaluate whether these microglial changes correlated with synaptic preservation, we quantified SV2 immunoreactivity and normalized it to DAPI signal intensity (Figure 5E). A β 1-42 injection markedly decreased SV2 signal compared to control zebrafish ($p < 0.0001$, Mean diff. = 3.37 , 95% CI of diff. = 2.38 to 4.35). Low-dose Camptothecin (0.1 μ M) did not change synaptic density. In contrast, high-dose Camptothecin (1 μ M) led to significantly greater SV2 levels relative to the A β 1-42 condition ($p < 0.01$, Mean diff. = -1.28 , 95% CI of diff. = -2.27 to -0.30) (Figure 5E). These effects were consistent across all biological replicates, as shown in the nested individual-animal plots (Figure 5E; Figure S13). These findings demonstrate that Camptothecin counteracts A β 1-42-induced microglial activation and synaptic loss in zebrafish in a dose-dependent manner.

DISCUSSION

Leveraging reference data identifying transcriptionally distinct microglial subtypes,¹² we have developed a pipeline to prioritize,

Figure 5. Dose-dependent effects of Camptothecin on microglial activation and synaptic density in a zebrafish amyloidosis model

(A) Schematic of experimental design. Adult animals from a zebrafish reporter line for microglia were injected with A β 1-42 (20 μ M) and treated for 5 days with Camptothecin (0.1 μ M or 1 μ M). Brains were analyzed by immunohistochemistry for L-plastin (microglia) and SV2 (presynaptic marker).

(B) Representative confocal images showing the three morphological subtypes of microglia: Type 1 (activated, round), Type 2 (activated, branched), and Type 3 (resting, ramified).

(C) Representative confocal images of microglia (L-plastin, white), synapse (SV2, red), and nuclei (DAPI, blue) staining in the different treatment conditions control + DMSO, A β 42, A β 42 + Camptothecin.

(D) Quantification of Type 1 activated microglia per animal ($n = 4$ per group) presented as a grouped boxplot. All data represent mean $\pm$ standard error of the mean (SEM). $n = 4$ independent animals per condition with 7 images analyzed per animal. Two-way ANOVA followed by Tukey's post hoc test was used for statistical comparisons. Significance is indicated as: ns > 0.05, *p.adj $\leq$ 0.05; **p.adj $\leq$ 0.01; ***p.adj $\leq$ 0.001; ****p.adj $\leq$ 0.0001. Detailed statistical results are presented in Table S10.

(E) Quantification of SV2 immunoreactivity normalized to DAPI, presented as a grouped boxplot. All data represent mean $\pm$ SEM. $n = 4$ independent animals per condition with 7 images analyzed per animal. Two-way ANOVA followed by Tukey's post hoc test was used for statistical comparisons. Significance is indicated as: ns > 0.05, *p.adj $\leq$ 0.05; **p.adj $\leq$ 0.01; ***p.adj $\leq$ 0.001; and ****p.adj $\leq$ 0.0001. Detailed statistical results are presented in Table S10.

Table 2. Summary of the structured functional evaluation of CD74^{high}/MHC^{high} microglia in multiple *in vitro* and *in vivo* model systems

Assay	Model System(s)	Effect of Camptothecin
scRNA-Seq	HMC3/iMG/CO-iMG	RNA signature ↑
Proteomics	HMC3	Protein signature ↑
Phagocytosis - Dextran	HMC3	uptake ↓
Phagocytosis - Aβ	HMC3/iMG	uptake ↑
Phagocytosis - <i>E. coli</i>	HMC3	uptake ↓
Cytokine secretion	HMC3	↓ IL-6, IL-8, MCP-1
Mitochondrial fitness (Seahorse)	HMC3/iMG	OXPPOS ↓ (HMC3 & iMG) Glycolysis ↓ (HMC3)
Immunohistochemistry	Zebrafish	↓ Type 1 activated microglia; ↑ SV2 (synaptic) levels

validate and then functionally evaluate candidate compounds that are predicted to engage selected microglial signatures. Camptothecin engages the RNA signature of the CD74^{high}/MHC^{high} microglia subtype, enabling us to link this microglial subtype to empirically measured cellular function using multiple *in vitro* and *in vivo* model systems (summarized in Table 2); this provides a critical link between single-cell RNA-sequencing profiles and behavior of microglial subtypes. One of the most interesting functional results may be the specialization of the CD74^{high}/MHC^{high} subtype for enhanced phagocytosis of Aβ, especially in view of the fact that this subtype also suppresses the SRGAP2^{high}/MEF2A^{high} subtype signature,¹² which is enriched with susceptibility genes for AD, multiple sclerosis, and Parkinson's Disease.

Small molecules and their effect on the modulation of cell fate or cell states have been previously used in the stem cell and other fields with the goal of regulating self-renewal, differentiation, *trans*-differentiation, cell reprogramming or modulation for desired therapeutic applications.⁴² It has also been used for direct reprogramming from one cell type to another in transplantation therapy (chemical-compound induced cells - ciCells⁴³). Our approach of modulating subtypes of terminally differentiated CNS-resident cells in a rationally designed manner highlights that this approach may be more generally applicable to the brain. The identification of pharmacological compounds that recapitulate established cell-subtype specific transcriptional states allows the validation of *in silico* predictions along with the characterization of the functional properties of human microglial subtypes.

Recently, another approach to model human microglial subtypes *in vitro* has been reported,¹⁸ exposing human iPSC-derived microglia (iMG) to selected CNS-substrates, perturbing their transcriptome. The authors subsequently correlated the induced subtypes with previously described human microglial subtypes derived from snRNA-Seq datasets.⁴⁴ While this approach yielded intriguing insights, it is limited by the variability of the preparation of some of the stimulants (e.g., synaptic preparations) and the lack of direct translation to therapeutic development. Our structured pharmacological approach arguably bears higher potential to yield reproducible results when applied to model systems and, in our case, resulted in both translation to an *in vivo* system and repurposing of a Food and Drug Administration (FDA) approved compound, Topotecan. Additionally, by showing that Camptothecin treatment induces not only our predefined CD74^{high}/MHC^{high} signature¹² *in vitro*

but also the different versions of this signature defined in four different publications,^{18,19,21,44} we establish the generalization of the results.

We also relate transcriptome-derived mitochondrial signatures to direct bioenergetic measurements across complementary human microglial *in vitro* model systems. In freshly isolated human microglia, the CD74^{high}/MHC^{high} state shows enrichment of mitochondrial modules linked to metals and cofactors and iron homeostasis (Figure 4B). In Camptothecin-treated HMC3 cells, we observed enrichment of the same modules, together with ROS/glutathione-related modules. Importantly, this transcriptomic signature is accompanied by a reduction in ATP-linked mitochondrial respiration measured by the Seahorse platform in HMC3 cells. These findings extend to Camptothecin-treated iMG, where scRNA-seq and Seahorse measures point in the same direction, linking the induced CD74^{high}/MHC^{high} state to lower-ATP-generating respiration alongside enrichment of redox stress and cofactor-related pathways. Thus, both *in vitro* model systems converge on a consistent mitochondrial phenotype associated with the CD74^{high}/MHC^{high} program, aligning with the mitotype observed in freshly isolated human microglia.

Multiple studies by colleagues^{45,46} and our laboratory^{11,12} have repeatedly identified a neuroimmunologically active human microglial subpopulation characterized by high expression of CD74 and MHC molecules, which we can now mimic using low doses of the topoisomerase I inhibitors Camptothecin or Topotecan. Characterization of the functional features of our CD74^{high}/MHC^{high} model system are consistent with previously reported Camptothecin-mediated suppression of microglial activation³² and cytokine signaling. Future work might further refine the relationship between distinct microglial subsets and amyloid load to directly assess the connection between transcriptional and morphological findings and functionally relevant outcomes in the context of AD.

Topoisomerases influence DNA topology associated with DNA replication, transcription, recombination and chromatin remodeling to maintain chromosome superstructure and integrity.⁴⁷ The inhibition of topoisomerases has previously been linked to altered expression of cell surface receptors including MHC I in colon cancer⁴⁸ and melanoma.⁴⁹ Moreover, Topotecan, a topoisomerase I inhibitor used in our study, previously induced phenotypic and activation status changes in monocyte-derived dendritic cells (moDCs), resulting in the stimulation of CD8⁺ T cell proliferation.⁵⁰ While we cannot comment on the exact mechanism by which topoisomerase inhibition drives a

specific human microglial transcriptional program, our study adds to existing literature showing that topoisomerase inhibition can skew phenotypes of monocytes, another type of myeloid cell.⁵⁰

Topoisomerase I is upregulated in mouse microglia following LPS stimulation,⁵¹ and Camptothecin has previously been shown to inhibit nucleotide-binding oligomerization domain-like receptor 3 (NLRP3) inflammasome activation and pyroptosis, attenuating proinflammatory cytokine levels and improving spinal cord injury recovery in mice.⁵² Camptothecin also appears to impact mitochondrial function and copy number which can lead to cell death.⁵³ Thus, we extend this literature by confirming that topoisomerase I inhibition leads to the polarization of microglia-like cells toward a phenotype with enhanced MHC I and II expression, an altered inflammatory response, and an Aβ42-specialized phagocytic phenotype. Further, we identified Topotecan as a structural and functional analog of Camptothecin that robustly induces the CD74^{high}/MHC^{high} signature and functionally mimics Camptothecin's downregulation of pro-inflammatory cytokine secretion in HMC3 cells (Figure 3B). These results are of particular interest as Topotecan is an FDA-approved drug that is clinically more effective and safer than Camptothecin.⁵⁴ Considering our findings, Topotecan might represent an entry point for therapeutic development of a microglia-targeted AD therapy. It may help to boost the effects of current approved anti-amyloid antibodies^{55,56} since the mechanisms are different. It could also be an alternative for those who do not respond to or have adverse events from antibody treatments. Side effects of current clinical doses in cancer therapy using Topotecan, e.g., hematopoietic toxicity, can be serious due to its function as a topoisomerase I inhibitor. Its anti-inflammatory effects on microglia, however, are found at lower doses both in preclinical studies of Parkinson's disease (PD)³² and in our own dataset. Thus, as with repurposing of chemotherapy agents such as cyclophosphamide and cladribine at low doses in multiple sclerosis,^{57,58} initial clinical research in AD could start off with low Topotecan doses followed by TSPO PET imaging and other markers of microglial activation in CSF. However, further studies are needed to elucidate the functions, temporal dynamics, and disease associations of the human microglial subsets described here before considering them as potential therapeutic targets.

Finally, development of a new generation of Camptothecin analogs with minimal topoisomerase I inhibitory activity and maximal Camptothecin-based structural efficacy is currently under way.⁵⁴ This new generation of Camptothecin analogs might also bear great potential for our proposed microglia-targeting approaches in AD therapy and potentially other neurodegenerative diseases, as the goal of therapy probably involves subtle shifts in the frequency of certain microglial subtypes and not wholesale elimination of microglia.

Limitations of the study

Our current functional analyses were initially conducted using a human embryonic microglia cell line (HMC3), which has the usual limitations of an immortalized cell line but recapitulates many features of myeloid and microglial cell behavior. We address this limitation by validating our work in iMG, iMG-containing cerebral organoids, and an *in vivo* model system.

While all of these systems have their own limitations, ranging from the number of iMG differentiations for each experiment to the number of sequenced iMG extracted from human cerebral organoids, these results lay the groundwork for further functional characterization of our compounds in a variety of complementary model systems. A limitation concerning our functional readouts of phagocytosis using pHrodo-conjugated substrates is that pHrodo fluorescence, which is only detectable in acidic compartments, cannot definitively distinguish between increased phagocytic uptake and reduced lysosomal degradation of internalized substrate. As both mechanisms could theoretically contribute to the observed intracellular signal at the analyzed timepoint, future studies employing pulse-chase designs or lysosomal inhibitor controls would be required to formally resolve this ambiguity. An additional limitation regarding our functional experiments is that the cytokine secretion data were not normalized to the number of cells present at the moment of collection.

This deep characterization of two putative tool compounds for microglial modulation has further validated our approach to identify chemical matter that influences microglia in a specific, reproducible manner. Its foundation rests on a large dataset of single-cell transcriptomes from live human microglia.¹² Camptothecin and Topotecan expand the list of translational reagents with which to manipulate human microglia in a manner that could enhance therapeutic intervention in the early amyloid phase of AD to reduce amyloid accumulation and synaptic loss. Since the therapeutic goal is a moderate shift in the proportion of certain microglial states, these tool compounds can be further optimized to achieve this modulation at low doses.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Philip L. De Jager (pld2115@cumc.columbia.edu).

Materials availability

This study did not generate new unique reagents.

Data and code availability

- All new deposited data from this study, including sc-RNA Sequencing of compound-treated HMC3 cells, sc-RNA Sequencing of compound-treated iMG cells, sc-RNA Sequencing of compound-treated CO-iMG, seahorse analysis of compound-treated HMC3 cells and iMG, and proteomics of compound-treated HMC3 cells, can be found and accessed from the AD knowledge portal using the following accession link (see also [key resources table](#)): <https://www.synapse.org/Synapse:syn53124932>.
- All code generated for this study was deposited in the GitHub repository and is accessible through the following link (see also [key resources table](#)): https://github.com/masashi-CU/CUMC_Compounds_Polarizing_Human_Microglial_Subtypes.
- Any additional information required to reanalyze the data reported in this paper is available from the [lead contact](#) upon request.

CONSENT FOR PUBLICATION

Not applicable.

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DECLARATION OF INTERESTS

The authors declare that they have no competing interests.

STAR★METHODS

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SUPPLEMENTAL INFORMATION

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
TotalSeq™-B0256 anti-human hashtag 6 antibody	BioLegend	Cat#: 394641; RRID:AB_2814352
TotalSeq™-B0257 anti-human hashtag 7 antibody	BioLegend	Cat#: 394643; RRID:AB_2814353
TotalSeq™-B0258 anti-human hashtag 8 antibody	BioLegend	Cat#: 394645; RRID:AB_2814354
TotalSeq™-B0259 anti-human hashtag 9 antibody	BioLegend	Cat#: 394647; RRID:AB_2814355
Chicken anti-GFP (zebrafish)	Invitrogen	Cat#: PA1-9533; RRID:AB_1074893
Mouse anti-SV2	DSHB	Cat#: SV2, RRID:AB_2315387
Rabbit anti-L-Plastin	Gift from Michael Redd; (Redd et al. ⁵⁹)	–
Chemicals, peptides, and recombinant proteins		
Camptothecin	EMD Millipore	Cat #:390238
Luotonin A	Sigma-Aldrich	Cat #: L4045-10MG
Rubitecan	Sigma-Aldrich	Cat#: R3655-10MG
Topotecan	APEX BIO	Cat#: B4982
Cytochalasin D	Sigma-Aldrich	Cat#: C8273
pHrdo Green Dextran	life technologies	Cat#: P10361
human beta-Amyloid (1–42), HiLyte™ Fluor 647-labeled	Anaspec Inc.	Cat#: AS-64161
pHrodo™ Green <i>E. coli</i> BioParticles™	ThermoFisher	Cat#: P35366
Oligomycin	Sigma-Aldrich	Cat#:75351
FCCP	Selleckchem	Cat #: S8276
Rotenone	Sigma-Aldrich	Cat #: R8875
Antimycin A	Sigma-Aldrich	Cat #: A8674
XF Media	Agilent Technologies	Cat #: 103334-100
Pyruvate	Sigma-Aldrich	Cat #: Sigma-Aldrich #P9767
Glutamine	fisher scientific	Cat #: G7513
Uridine	Sigma-Aldrich	Cat#: U6381
Palmitate	Sigma-Aldrich	Cat#: P9767
Hoechst	ThermoFisher	Cat#: 62249
TNF-α	Peprtech	Cat#: 300-02
IFN-γ	Peprtech	Cat#: 300-01A
DMSO	Sigma-Aldrich	Cat #:472301
Trypsin	Gen Clone	Cat #:25-510F
PBS	Corning	Cat#:21-040-CV
BSA	pluriSelect	Cat#: 60-00020-10BSA
Fc Block	BioLegend	Cat#: 422301
SYTOX Blue	ThermoFisher	Cat#: S34857
StemFlex media	ThermoFisher	Cat# A3349401
Cultrex BME	Biotechne	Cat# 3434-010-02
ReLesSR™	Stem Cell Technologies	Cat# 100-0483
knockout serum replacement	ThermoFisher	Cat# 10828010
human insulin	Sigma-Aldrich	Cat.# 12585014

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
M-CSF	R&D	Cat.# 216-MC-010/CF
EMEM	ATCC	Cat#: 30-2003
DMEM/F12	Thermo Fisher	Cat.# 11320033
2X insulin-transferrin-selenite	Thermo Fisher	Cat.# 41400045
B27	Thermo Fisher	Cat.# 17504044
N2	Thermo Fisher	Cat.# 17502048
1X glutamax	Thermo Fisher	Cat.# 35050061
1X non-essential amino acids	Thermo Fisher	Cat.# 11140050
Monothioglycerol	Sigma-Aldrich	Cat.# M-6145
IL-34	R&D	Cat.# 5265-IL-010/CF
TGFβ1	STEMCELL Technologies	Cat.# 100-0856
CD200	Bon Opus Biosciences	Cat#: 50-203-1792
CX3CL1	R&D Systems	Cat#: 365-FR-025/CF
paraformaldehyde	ThermoFisher	Cat#: J61899-AP
20% Sucrose	Invitrogen	Cat#: 15503-022
ethylenediaminetetraacetic acid (EDTA)	Sigma	Cat#: ED4SS
Triton X-100	Millipore Sigma,	Cat#: 648466
aquamount	Polysciences Inc.	Cat#: 18606
RLT buffer	Qiagen	Cat#: 74136
2-Mercaptoethanol	ThermoFisher	Cat#:63689
P/S	Gibco	Cat#: 15140-122
Cytochalasin D	Sigma-Aldrich	Cat#: C8273
Trypsin	Gen Clone	Cat#:25-510F
FCS	Fisher Scientific	Cat#: 10-438-026
Cell Staining buffer	BioLegend	Cat#: 420201
NaOH	ThermoFisher	Cat#: 63736.AK

Critical commercial assays

10x Chromium Next GEM Single Cell 5' Reagent Kits v2 (Dual Index)	10x Genomics, Pleasanton, CA	Cat#: PN-1000263
TapeStation D5000 screening tapes and reagents	Agilent, Santa Clara, CA	Cat#: 5067-5588; 5067-5589
Qubit HS DNA quantification kit	ThermoFisher	Cat#: Q32851
STEMdiff Hematopoietic kit	STEMCELL Technologies	Cat#: 05310
STEMdiff™ Cerebral Organoid Kit	STEMCELL Technologies	Cat#: 08570, Cat#: 08571
MTT assay	Invitrogen	Cat#: M6494
Alamar Blue HS	Thermo Fisher	Cat#: DAL1100
Qiagen RNEasy mini plus kit	Qiagen	Cat#: 74136
BioRad iScript cDNA Synthesis kit	BioRad	Cat#:1708891
Qubit HS DNA quantification kit	ThermoFisher	Cat#: Q32851
Human Cytokine Proinflammatory Focused 15-Plex Discovery Assay® Array (HDF15)	Eve Technologies	N/A
Alamar Blue Assay	ThermoFisher	Cat#: DAL1025
Beta Amyloid (1–42), Aggregation Kit	rPeptide	A-1170-02

Deposited data

AD Knowledge Portal:	sc-RNA Sequencing HMC3 compound-treated	https://www.synapse.org/Synapse:syn53124932
AD Knowledge Portal:	sc-RNA Sequencing iMG compound-treated	https://www.synapse.org/Synapse:syn53124932

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
AD Knowledge Portal:	sc-RNA Sequencing CO-IMG compound-treated	https://www.synapse.org/Synapse:syn53124932
AD Knowledge Portal:	Seahorse data for HMC3 cells and iMG	https://www.synapse.org/Synapse:syn53124932
AD Knowledge Portal:	Proteomics HMC3 compound-treated	https://www.synapse.org/Synapse:syn53124932
GitHub:	Code	https://github.com/masashi-CU/CUMC_Compounds_Polarizing_Human_Microglial_Subtypes

Experimental models: Cell lines

HMC3	ATCC	Cat#: CRL-3304
hiPSC line WTC11	Allen Institute for Cell Science; https://www.allencell.org/genomics.html	–
hiPSC line MSN38	Mount Sinai Normal, Icahn School of Medicine at Mount Sinai https://icahn.mssm.edu/research/portal/resources/deans-cores/stem-cell-engineering/services	–
FA000010 iPSCs	RUCDR/BiologyX	Cat#: 3434-010-02
FA000011	CUIMC002-A (Patel et al. ¹⁰)	–

Experimental models: Organisms/strains

Tg (mpeg1:EGFP) zebrafish line	Zebrafish International Resource Center (ZIRC); Ellett et al. ⁶⁰	–
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Oligonucleotides

CXCR4 - fw: ACGCCACCAACAGTCAGAG	IDT Custom DNA Oligos	–
CXCR4 - rev: AGTCGGGAATAGTCAGCAGGA	IDT Custom DNA Oligos	–
SRGN - fw: GGACTACTCTGGATCAGGCTT	IDT Custom DNA Oligos	–
SRGN - rev: CAAGAGACCTAAGTTGTCATGG	IDT Custom DNA Oligos	–
HPRT1 - fw: CCTGGCGTCGTGATTAGTGAT	IDT Custom DNA Oligos	–
HPRT1 - rev: AGACGTTAGTCCTGTCCATAA	IDT Custom DNA Oligos	–

Software and algorithms

Cell Ranger (Version 6.0.1)	10x Genomics	–
DoubletFinder (2.0.3)	McGinnis et al. ⁶¹	–
ChooseR	Patterson-Cross et al. ⁶²	–
clusterProfiler	Wu et al. ⁶³	–
freemuxlet software	https://github.com/statgen/popsicle	–
GraphPad Prism (Version 9.5.1; Version 9.2.0.)	Dotmatics	–
ImageJ	Abramoff et al. ⁶⁴	–
FlowJo (Version 10.8.1)	BD Biosciences	–
MELD (1.0.0)	Burkhardt et al. ²⁴	–
scvi-tools (0.20.3)	Gayoso et al. ⁶⁵	–
scikit-learn (1.2.2)	Pedregosa ⁶⁶	–
Seurat (4.3.0)	Hao et al. ⁶⁷	https://github.com/satijalab/seurat/releases/tag/v4.0.4
R (4.2.2; 4.4.1)	https://www.R-project.org/	–

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Python (3.8)	https://www.python.org/downloads/release/python-380/	–
Demuxmix	Klein ⁶⁸	–
MAST (v1.14.0)	Finak et al. ⁶⁹	https://github.com/RGLab/MAST
DESeq2 (1.32.0)	Love et al. ⁷⁰	https://bioconductor.org/packages/release/bioc/html/DESeq2.html
Picard (v1.83)	http://broadinstitute.github.io/picard/	http://broadinstitute.github.io/picard/
RSeQC (v2.6.1)	Wang et al. ⁷¹	http://rseqc.sourceforge.net/#download
STAR (v2.5.2a)	Dobin et al. ⁷²	https://github.com/alexdobin/STAR
Bowtie2 (v2.1.0)	Langmead et al. ⁷³	https://github.com/BenLangmead/bowtie2
featureCounts (v1.4.3-p1)	Liao et al. ⁷⁴	http://subread.sourceforge.net/
GSEA	Subramanian et al. ⁷⁵	–
MitoCarta3.0	Rath et al. ³⁶	–
Other		
NovaSeq 6000 with S4 flow cell	Illumina, San Diego, CA	–
Zeiss confocal LSM800 microscope	Zeiss	–
Tecan Infinite@200 PRO plate reader	Tecan	Cat#:30050303
Nanodrop	ThermoFisher	–
Aurora 3L analyzer	Cytek Bio	–

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Cell culture

HMC3 microglia-like cells

HMC3 microglia-like cells were purchased from ATCC (Cat#: CRL-3304) and cultured in complete EMEM media (ATCC, Cat#: 30-2003, supplemented with 10% FCS (Fisher Scientific, Cat#: 10-438-026), 1% penicillin/streptomycin (Gibco, Cat#: 15140-122)). Prior to experiments, HMC3 microglia-like cells were tested for mycoplasma contamination.

Differentiation and culture of microglia-like cells (iMG)

hiPSCs were maintained in StemFlex media (ThermoFisher) on reduced growth factor Cultrex BME (Biotechne, Cat.# 3434-010-02) and routinely split 1–2 times a week with ReLesSR (Stem Cell Technologies) without ROCKi as described previously.⁷⁶ hiPSCs were differentiated into iMGLs as described previously with minor adaptations.^{23,77} In brief, FA000010 (RUCDR/BiologyX) iPSCs were differentiated into hematopoietic precursor cells (HPCs) using the STEMdiff Hematopoietic kit (STEMCELL Technologies) largely by manufacturer's instructions. In brief, on day –1 iPSCs were detached with ReLesSR and passaged to achieve a density of 1–2 aggregates/cm² of 100–150 cells. Multiple densities were plated in parallel. On day 0, colonies of appropriate density were switched to Medium A from the STEMdiff Hematopoietic Kit to initiate HPC differentiation. On day 3, cells were switched to Medium B with a full media change and fed again with a full media change on day 5. Cells remained in Medium B for the rest of the HPC differentiation period with Medium B overlay feeds every other day. HPCs were collected 3 independent times by gently removing the floating population with a serological pipette on days 11, 13 and 15 (or days 12, 14 and 16). HPCs were either cryobanked in 45% Medium B, 45% knockout serum replacement (ThermoFisher) and 10% DMSO and stored in liquid nitrogen or directly plated for iMGL induction. HPCs were terminally differentiated at 28,000–35,000 cells/cm² in microglia medium (DMEM/F12, 2X insulin-transferrin-selenite, 2X B27, 0.5X N2, 1X glutamax, 1X non-essential amino acids, 400 mM monothioglycerol, and 5 mg/mL human insulin (ThermoFisher)) freshly supplemented with 100 ng/mL IL-34, 25 ng/mL M-CSF (R&D System) and 50 ng/mL TGFβ1 (STEMCELL Technologies) for every other day until day 24. On day 25, 100 ng/mL CD200 (Bon Opus Biosciences) and 100 ng/mL CX3CL1 (R&D Systems) were added to Microglia medium to mimic a brain-like environment. For all experiments in this study, iMGLs were treated between day 28–29.

Cerebral organoids co-cultured with iPSC-derived human microglia

Cortical brain organoids (CO) were cultured using the STEMdiff™ Cerebral Organoid Kit (STEMCELL Technologies, Cat #08570, #08571). This four-stage protocol is based on the original Lancaster et al. protocol.⁷⁸ hiPSC lines used were MSN38 (Mount Sinai Normal, Icahn School of Medicine at Mount Sinai) and WTC-11 (Allen Institute for Cell Science). At mature age the COs were co-cultured with microglial progenitors (derived from WTC-11) to obtain microglia-containing organoids (CO-iMG), as previously described.²⁵ For more details on the co-culture protocol, please refer to Buonfiglioli et al.²⁸ On day 6/7

of co-culturing iPSC-derived microglia (iMG) with the respective cerebral organoids derived from the same lines (CO-iMG) were treated with compounds for subsequent single-cell RNA-Seq experiments.

Animals

Tg(mpeg1:EGFP) zebrafish,⁶⁰ obtained from the Zebrafish International Resource Center (ZIRC), of both sexes and 14 months of age, were used for the experiments described here. Animals were maintained under a temperature and humidity-controlled environment at Columbia University Medical Center on a 14/10-h light/dark cycle, with regular scheduled feeding. Animals from the same clutch were randomly distributed across experimental groups. All zebrafish experiments were performed in accordance with the applicable regulations and approved by the Institutional Animal Care and Use Committee (IACUC) at Columbia University (protocol number AC-AACB9700).

METHOD DETAILS

In vitro sc-RNA seq datasets

Single-cell (sc-) RNA seq of compound-treated HMC3s

0.5×10^6 HMC3 microglia-like cells per condition were seeded into a 6-well plate and incubated o.n. in complete EMEM media (ATCC, Cat#: 30-2003, containing 10% FCS (Fisher Scientific, Cat#: 10-438-026), 1% P/S (Gibco, Cat#: 15140-122)). The next day, HMC3 cells were left untreated or treated ($n = 1$) with Camptothecin (1 μ M; EMD Millipore; Cat #:390238; $n = 1$) or DMSO (Sigma-Aldrich, Cat #:472301; 1:1000–0.1% (v/v); $n = 1$) as control and incubated for 24 h before harvest. Cells were trypsinized (Gen Clone; Cat #:25-510F) and each treated HMC3 sample was subsequently barcoded using “hashing antibodies” consisting of antibodies targeting beta-2-microglobulin and CD298 and conjugated to “hash-tag” oligonucleotides (HTOs). In order to do so, cells were washed with PBS (Corning; Cat#:21-040-CV), centrifuged at 300g, 4 °C, 5 min and filtered through a 70 μ m filter (fisher scientific; Cat#: 08-771-23). Following centrifugation, cells were resuspended in 25 μ L PBS+0.04% BSA (pluriSelect; Cat#: 60-00020-10BSA), 1 μ L of FcBlock (BioLegend, Cat#: 422301) was added and cells were incubated for 10 min on ice. 2 μ L of each hashtag antibody (TotalSeq-B0256 anti-human Hashtag 6 Antibody, Cat#: 394641; TotalSeq-B0257 anti-human Hashtag 7 Antibody, Cat#: 394643; TotalSeq-B0258 anti-human Hashtag 8 Antibody, Cat#: 394645; TotalSeq-B0259 anti-human Hashtag 9 Antibody, Cat#: 394647; 0.5 mg/mL) was mixed with 25 μ L PBS+0.04% BSA, passed through 0.1 μ m filter at 12,000g for 2 min, 4 °C and antibody mix for each hashtag was added to the respective tube containing cells in FcBlock. Cells were incubated for 30 min on ice and subsequently washed 4 times with staining buffer. Cells for each sample were then counted, mixed 1:1:1:1, filtered, then counted and subsequently subjected to single-cell library preparation.

The single-cell library preparation was constructed using 10x Chromium Next GEM Single Cell 5' Reagent Kits v2 (Dual Index) with Feature Barcode technology for Cell Surface Protein (10x Genomics, Pleasanton, CA) according to the manufacturer's protocol. Briefly, a total of 30,000–40,000 cells were loaded on the 10x Genomics chromium controller single-cell instrument. Reverse transcription reagents, barcoded gel beads, and partitioning oil were mixed with the cells for generating single-cell gel beads in emulsions (GEMs). After reverse transcription reaction, the GEMs were broken. Feature Barcode cDNA amplification was performed. The amplified cDNA was then separated by SPRI size selection into cDNA fractions containing mRNA derived cDNA (>400 bp) and HTO-derived cDNAs (<180 bp), which were further purified by additional rounds of SPRI selection. Independent sequencing libraries were generated from the mRNA and HTO cDNA fractions, which were analyzed and quantified using TapeStation D5000 screening tapes (Agilent, Santa Clara, CA) and Qubit HS DNA quantification kit (Thermo Fisher Scientific). Libraries were pooled and sequenced together on a NovaSeq 6000 with S4 flow cell (Illumina, San Diego, CA) using paired-end, dual-index sequencing with 28 cycles for read 1, 10 cycles for i7 index, 10 cycles for i5 index, and 90 cycles for read 2.

Analysis of sc-RNA seq of compound-treated HMC3s

FASTQ files of single-cell sequencing libraries were processed using the “count” command of Cell Ranger (10x Genomics, version 6.0.1). Gene expression library and hashtag oligo library were processed together, and the human transcriptome (GRCh38-2020-A) was used as the reference for alignment. As HMC3s have higher median feature number as compared to live human microglia, our filtering thresholds were adjusted, and more aggressive doublet removal approaches were applied. We did not set a top bound for UMI-based filtering, only discarding low-quality cells below 500 UMIs. Hashtags were deconvoluted with demuxmix. Subsequently, we checked for doublets by using a recently published tool, DoubletFinder,⁶¹ that was the top performer in a recent benchmark of computational doublet detection approaches.⁷⁹ We followed the standard workflow for DoubletFinder analysis, setting our expected rate to 0.2 based on extrapolation of expected multiplet rates based on loading densities from the 10x Genomics website. The parameters we used for DoubletFinder were: 15 PCs and pN of 0.25. pK was calculated from our dataset. Substantial overlap was found between the doublets identified by demuxlet and DoubletFinder, and DoubletFinder-identified doublets generally had higher counts than identified singlets. As such, all doublets from demuxmix and DoubletFinder were excluded from further analysis. Clustering was conducted with the standard single-sample SCTransform workflow. Briefly, SCTransform was run with default settings, followed by PCA calculation with RunPCA. We chose to retain 20 PCs for downstream analysis by inspecting an elbow plot. To optimize clustering resolution, the recently reported method ChooseR,⁶² which compares silhouette scores across separate resolutions of Louvain clustering, was used. The final choice of resolution was 0.6, and this was used for downstream analyses. Differentially expressed genes per cluster were calculated as detailed in.¹² Annotation of the genes

associated with different clusters was performed by way of Reactome pathway annotation using the clusterprofiler package, as described in.⁶³ To calculate module enrichment per cell, the Seurat AddModuleScore function was used. In brief, this function takes an input list of genes, and for each of these genes, assigns a “background” of 100 genes expressed at similar levels, then calculates the overall enrichment of input genes versus control gene sets on a per-cell level.⁸⁰ This approach was applied for the top 100 genes per cluster to derive a cluster enrichment score on a per-cell level. Testing for significant differences between clusters in module scores was performed with pairwise Wilcoxon testing. Assessment of enrichment of cluster signatures in association with compound treatment, in contrast, was performed with GSEA.⁷⁵ Here, log fold change was calculated for all genes in the dataset between control (both, DMSO and untreated) and Camptothecin treatment, and genes were ranked based on average log fold change across all cells in each condition. Enrichment of cluster signatures in this rank-ordered list was then performed with GSEA.

Analysis of additional MHC^{high} signatures in HMC3 scRNA-Seq

To evaluate other signatures in our dataset, we applied a similar process of assessing signature scores in the HMC3 scRNA-seq dataset. We used the same approach as in [Analysis of single-cell RNA Sequencing of compound-treated HMC3s](#), electing to use the top 50 genes per signature to compute a module score. For Dolan et al.,¹⁸ we used the top 50 genes that were common across the three signatures that overlapped with our HLA signature, clusters 3, 4, and 7. For Sun et al.,¹⁹ we first filtered the “MG3” signature to remove all mitochondrial and ribosomal genes, as our pre-processing approach involved removing those, then chose the top 50 remaining genes. For Gazestani et al.,²⁰ we selected the top 50 genes associated with the “GBNMB_NACA” signature. Finally, for Mancuso et al., we filtered the “HLA” signature to remove all mitochondrial and ribosomal genes for reasons noted above, then selected the top 50 remaining genes. Results were displayed in violin plots, details of construction and statistical analysis for which are described in [Analysis of single-cell RNA Sequencing of compound-treated HMC3s](#).

Evaluating mitotype enrichment in RNA-Seq data

Using mitochondrial pathways defined from the MitoCarta3.0 gene sets for murine and human datasets,³⁶ we evaluated enrichment of different sets in the rank-ordered log-fold-change lists for compound-treated versus control/DMSO conditions for either bulk RNA-seq or for aggregated cells in scRNA-seq. GSEA^{75,81} was used as previously described to calculate enrichment of genes associated with different mitochondrial pathways in sets of genes associated with different compound treatment conditions.

Mitochondrial pathway analysis

To delve more into the metabolic differences between compound treatment conditions in the human single-cell data, we leveraged recent annotated gene sets from MitoCarta3.0³⁶ as mentioned in [Evaluating mitotype enrichment in transcriptomic data](#). Using gene sets tied to distinct mitochondrial properties, we calculated enrichment of different sets in upregulated cluster signatures using a hypergeometric test with an FDR-corrected threshold for significance at $q = 0.01$.⁸² The results of this analysis were visualized in heatmaps where the color intensity corresponds to the $-\log_{10} p$ -value of the FDR q -value for enrichment of each given gene set in the differentially expressed genes associated with a given cluster.

Compound-treatment of iPSC-derived human microglia (iMG)

On day 28/29 of iMG differentiation, iMG in 12-wells were treated for 24 h with the following compounds and the following concentrations: Camptothecin (EMD Millipore, Cat#: 390238) – 0.1 μ M and Topotecan (APEX BIO, Cat#: B4982) – 0.1 μ M (summarized in [Table S11](#)). Differentiated microglia were cultured in 500 μ L microglia medium (DMEM/F12, 2X insulin-transferrin-selenite, 2X B27, 0.5X N2, 1X glutamax, 1X non-essential amino acids, 400 mM monothioglycerol, and 5 mg/mL human insulin (ThermoFisher)). For compound treatment, 2x solutions were prepared for each compound and subsequently 500 μ L of 2x solution was added to each well. After 24 h, treated iMG were harvested in low-protein binding tubes (fisher scientific; Cat#: 13-864-407) using media and PBS (Corning; Cat#:21-040-CV) to wash off iMG from cell culture wells. Subsequently, cells were washed 1x with PBS, centrifuged at 300g, 4 $^{\circ}$ C, 5 min, filtered through a blue lid filter (FACS tube), resuspended in 100 μ L PBS+0.04% BSA for counting using counting chambers (Bulldog Bio, Portsmouth, NH) and subsequently subjected to single-cell library preparation.

The single-cell library preparation was constructed using 10X Chromium Next GEM Single Cell 3' Reagent Kits v3.1 (Dual Index) according to the manufacturer's protocol. Briefly, a total of $\sim$ 10,000 cells were loaded on the 10X genomics chromium controller single-cell instrument. Reverse transcription reagents, barcoded gel beads, and partitioning oil were mixed with the cells for generating single-cell gel beads in emulsions (GEM). After reverse transcription reaction, the GEMs were broken. cDNA amplification was performed. The amplified cDNA was then separated by SPRI size selection into cDNA fractions containing mRNA derived cDNA (>300 bp) which were further purified by additional rounds of SPRI selection. Sequencing libraries were generated from the mRNA cDNA fractions, which were analyzed and quantified using TapeStation D5000 screening tapes (Agilent, Santa Clara, CA) and Qubit HS DNA quantification kit (Thermo Fisher Scientific).

Pre-processing of compound-treated iMG sc-RNA seq

The h5 file output by Cell Ranger for each sample was read in R (4.2.2) using the Seurat package (4.3.0). For quality control, we removed cells with a mitochondrial RNA percentage above a statistical threshold of either two absolute deviations above the median mitochondrial reads within the sample, or 1.5 times the interquartile range above the third quartile, whichever

is a lower threshold. If this threshold is lower than 10%, we set the threshold to 10%. All ribosomal genes, mitochondrial genes and pseudogenes were removed. Cells with more than 500 UMI count were kept for downstream analysis. To predict and remove potential doublets, we applied DoubletFinder (2.0.3)⁶¹ on the first 15 principal components of the iMG data, using the function *doubletFinder_v3* (with $pN = 0.25$, $nExp = 0.075 * (\# \text{ cells in library}) * (1 - \text{homotypic proportion})$, $sct = F$). The homotypic proportion was estimated using the *modelHomotypic* function, whereas the pK parameter was optimized for each sample using *paramSweep_v3*. To correct for batch effect in the iMG data, we used the scVI model from the scvi-tools package in python (3.8). scVI generates non-negative corrected counts and has recently been benchmarked to perform well in data integration.⁸³ We trained the scVI model using raw counts as input, with two hidden layers ($n_layers = 2$), 30 dimensions of the latent space ($n_latent = 30$) and assuming a negative binomial gene count distribution. The batch corrected counts were then log normalized using the *log1p* function from the numpy package (1.21.6). The scVI generated latent space were then used for dimension reduction using Uniform Manifold Approximation and Projection (UMAP). The batch corrected counts results from scVI were used for query mapping and MELD analysis described later in discussion.

Single-cell RNA seq of CO-iMG

On day 6/7 of co-culturing iPSC-derived microglia (iMG) with the respective cerebral organoids derived from the same lines (CO-iMG) were treated in 500 μ L CO-iMG-specific media containing Camptothecin (EMD Millipore; Cat #:390238; 0.1 μ M and 0.5 μ M) or DMSO as control (Sigma-Aldrich, Cat #:472301), with $n = 2$ organoids per hiPSC line (WTC11, MSN38) and per condition (DMSO, 0.1 μ M Camptothecin, 0.5 μ M Camptothecin). 2x compound-containing media was prepared and for treatment, 250 μ L were removed from the 500 μ L-containing 24-well plate and subsequently 250 μ L of the 2x solution were added to achieve 1x solution for compound treatment. CO-iMG were incubated for 24 h at 37 °C, 5%CO₂. Subsequently, cerebral organoids were collected, transferred into a tissue homogenizer (5 mL) containing 2 mL CO-iMG media, and dissociated on ice using the loose pestle. Cell solution was subsequently filtered through a 35 μ m strainer (5 mL, fisher scientific, Cat#: 08-771-23) into a low protein binding tube (fisher scientific; Cat#: 13-864-407). Cell solution was centrifuged at 300g, 4 °C, 5 min, twice washed with 1x with PBS (Corning; Cat#:21-040-CV), filtered, centrifuged at 300g, 4 °C, 5 min and resuspended in 60 μ L of PBS (Corning; Cat#:21-040-CV) containing 0.04% BSA (pluriSelect; Cat#: 60-00020-10) for counting using disposable counting chambers (Bulldog Bio, Portsmouth, NH). Subsequently, volumes corresponding to the same cell number of corresponding samples (DMSO, Camptothecin) from each genetic line (MSN38 or WTC11) were mixed 1:1 to achieve equal cell loading for each sample within one single-cell sequencing experiment.

For 10x Genomics, 30,000 cells were targeted for cell loading. The single-cell library preparation was constructed using 10X Chromium Next GEM Single Cell 3' Reagent Kits v3.1 (Dual Index) according to the manufacturer's protocol. Briefly, a total of ~30,000 cells were loaded on the 10X genomics chromium controller single-cell instrument. Reverse transcription reagents, bar-coded gel beads, and partitioning oil were mixed with the cells for generating single-cell gel beads in emulsions (GEMs). After reverse transcription reaction, the GEMs were broken. cDNA amplification was performed. The amplified cDNA was then separated by SPRI size selection into cDNA fractions containing mRNA derived cDNA (>300 bp) which were further purified by additional rounds of SPRI selection. Sequencing libraries were generated from the mRNA cDNA fractions, which were analyzed and quantified using TapeStation D5000 screening tapes (Agilent, Santa Clara, CA) and Qubit HS DNA quantification kit (Thermo Fisher Scientific).

Preprocessing of CO-iMG sc-RNA seq

To demultiplex the samples, cells were grouped into two clusters based on SNPs in RNA-seq reads using freemuxlet software (<https://github.com/statgen/popsicle>). Positions of SNPs were obtained from a VCF file of the 1000 Genomes Project phase 3 after excluding SNPs with minor allele frequency <10%. Average expression of *XIST* RNA was computed in each cluster. A cluster with higher *XIST* expression should be female origin and thus was annotated as MSN38. Conversely, a cluster with lower *XIST* expression was annotated as WTC11. Quality control (i.e., low-quality cell removal, gene filtering and doublet removal) was performed as in the iPSC-derived microglia preprocessing. To identify microglia from the organoid data, we performed the following standard Seurat pipeline to cluster cells: *SCTransform*, *RunPCA*, *RunUMAP* (using the first 30 principal components with default parameters), *FindNeighbors*, and *FindCluster* using a resolution of 0.5. Cells of the cluster with the highest co-expression of microglial markers (*AIF1*, *C1QA*, *C1QB*, *CSF1R*, *CTSS*, *CX3CR1*, *GPR34*, *HEXB*, *ITGAM*, *MERTK*, *P2RX7*, *P2RY12*, *PTPRC*, *SLC2A5*, *TGFBF1*, *TMEM119*) were annotated as microglia. This process resulted in 492 cells annotated as microglia. Here we focused on DMSO- ($n = 131$) and Camptothecin-treated cells ($n = 130$) for further analysis, since these two conditions contained a sufficient number of cells for analysis.

To understand the similarity and differences between *in vitro* iMG and organoid-iMG in drug response, we mapped the CO-iMG treated with DMSO and Camptothecin as query onto the iMG, of which the batch-corrected count matrix was used as reference data. The reference-query mapping was performed using the Seurat query mapping pipeline.²⁶ Briefly, the query data was first normalized using the function *NormalizeData* (with *normalization.method* = "LogNormalize" and *scale.factor* = 10000). We then identified the top 1000 variable genes from the log-normalized query data and from the log-transformed scVI batch-corrected counts of the reference data, using the *FindVariableFeatures* (with *nfeatures* = 1000). A set of mapping anchors (pairs of cells that encode the relationships between the two datasets) between the reference and query was identified and scored using the *FindTransferAnchors* function (using the first 15 principal components with default parameters). The anchors were then used as input

for the *MapQuery* function to calculate the transformation vectors that transform query gene expression to be combined with the reference for downstream analyses, including UMAP dimension reduction and MELD analysis.

Software and algorithms for analysis of sc-RNA seq data

Additional software and algorithms used for the analysis of the diverse single-cell RNA-Seq datasets generated for this study include Seurat (4.3.0),⁶⁷ demuxmix,⁶⁸ MAST (v1.14.0),⁶⁹ DESeq2 (1.32.0),⁷⁰ STAR (v2.5.2a),⁷² Bowtie2 (v2.1.0),⁷³ featureCounts (v1.4.3-p1),⁷⁴ scvi-tools (0.20.3),⁶⁵ scikit-learn (1.2.2)⁶⁶ and RSeQC.⁷¹ For further reference, refer to the [key resources table](#).

Treatment of an adult amyloidosis zebrafish model

To administer the amyloid beta 42 and drugs, cerebroventricular microinjections (CVMI) into adult zebrafish brain were performed as described.^{38,41} For treatment, 14 months old transgenic animals with fluorescently labeled microglia (*Tg (mpeg1:EGFP)*⁶⁰; Zebrafish International Resource Center (ZIRC)) of both sexes were injected with the following: human amyloid-beta 42 (A β 42, 20 μ M) and Camptothecin (0.1 μ M and 1 μ M) or DMSO as control ([Table S12](#)). Total injection volume was 0.5–1 μ L.

Immunohistochemistry (IHC) of treated adult amyloidosis zebrafish

Zebrafish were euthanized at 5 days post injection; the heads were dissected and fixed overnight at 4 °C using 4% paraformaldehyde (ThermoFisher, Cat#: J61899-AP). After several washes the heads were incubated in 20% Sucrose (Invitrogen, Cat#: 15503-022) with 20% ethylenediaminetetraacetic acid (EDTA) (Sigma, Cat#: ED4SS) solution at 4 °C for cryoprotection and decalcification, embedded in cryoprotectant sectioning resin and cryosectioned into 12 μ m thick sections. For immunofluorescence, the sections were dried at room temperature, followed by washing steps in PBS (Millipore Sigma, Cat#: P3813) with 0.03% Triton X-100 (Millipore Sigma, Cat#: 648466) (PBSTx). The slides were then incubated overnight with primary antibodies at 4 °C. On the next day, the slides were washed 3 times with PBSTx and incubated for 2 h in appropriate secondary antibodies at room temperature. The slides were then washed several times before mounting using aquamount (Polysciences Inc., Cat#: 18606). The following antibodies were used: chicken anti-GFP (1:1000, Invitrogen Cat#: PA1-9533), mouse anti-SV2 (1:500, DSHB Cat#: SV2, RRID:AB_2315387) and rabbit anti-L-Plastin (1:3000,⁵⁹ gift from Michael Redd).

Images were acquired using a Zeiss confocal LSM800 microscope in a 20X and 40X objectives with tiles/z stack function wherever necessary. At least 7 images of telencephalon sections between the caudal end of the olfactory bulb and anterior commissure from each animal were acquired and used for analysis. The quantification of SV2-positive synapses was performed using 3D object counter module of ImageJ software⁶⁴ with a same standard cut-off threshold for every image within the brain parenchyma. Based on cellular morphology with respect to sphericity of DAPI+ nuclei (slender or round) and L-plastin+ microglial cell body (branched or round), three different microglial states were classified to analyze the microglial dynamics. The statistical evaluation was performed using GraphPad Prism (Version 9.5.1) for two-way ANOVA followed by a Tukey's multiple comparison test. Error bars shown are the SEM and asterisks indicate significance according to: *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$. $p > 0.05$ is considered not significant (n.s.).

Structure activity relationship (SAR) analysis

Compounds of interest were obtained from a wide range of reputable vendors (see [Table S13](#)) and resuspended in DMSO (Sigma-Aldrich, Cat #:472301). To keep the design of our experiment as similar as possible to the CMAP study, the target stock concentration was 10 mM. Dose titration with doses ranging from 0.01 μ M to 0.1 mM was conducted to determine the highest tolerable dose for each compound. Each concentration of drug was plated in triplicate with early-passage HMC3 cells (ATCC; Cat #: CRL-3304), and cell viability was readout using MTT assay, by incubating the cells in 0.25 mg/mL MTT (Invitrogen; Catalog #: M6494) in PBS (Corning, Cat #:21-040-CV) for 1 hr at 37 °C, following removal of MTT solution and addition of 200 μ L DMSO (Sigma-Aldrich, Cat #:472301) and further incubation of the cells for 15 min at 37 °C before measuring the absorbance at 570 nm with a Tecan Infinite 200 PRO plate reader (Tecan; Cat#: 30050303). An optimal dose of each drug was then chosen based on cell morphology and viability. Subsequently, 0.35×10^6 HMC3 microglial cells were seeded into a 6-well plate and incubated overnight. The next day, microglia were treated with the respective concentrations of selected drugs (see [Table S13](#)) or DMSO (Sigma-Aldrich, Cat #:472301) as control and incubated for 6 h and 24 h before harvest for RNA extraction. Lysis was performed in-well with buffer RLT (Qiagen; Cat #: 74136) containing 2-Mercaptoethanol (Thermo Fisher Scientific, Cat #:63689), and RNA extraction was performed with the Qiagen RNeasy mini plus kit (Qiagen; Cat #: 74136) following the manufacturer's instructions. gDNA eliminator columns were used to remove contaminating genomic DNA. Initial RNA quality and quantity was assessed using Nanodrop (ThermoFisher Scientific) followed by cDNA preparation using the BioRad iScript cDNA Synthesis kit (BioRad; Cat #:1708891). cDNA was subsequently purified with AMPure XP beads (Thermo Fisher Scientific; Cat #: A63880) using a 1:1.8 ratio of cDNA: beads, concentration determined using Qubit HS DNA quantification kit (Thermo Fisher Scientific, Cat#: Q32851) and subsequently adjusted to 1 ng/ μ L. RT-qPCR analysis was subsequently performed as described in.¹² For cluster 8_10 primers, the following primers were used: *CXCR4* – fw: ACGCCACCAACAGTCAGAG, rev: AGTCGGAATAGTCAGCAGGA; *SRGN* – fw: GGACTACTCTGGATCAGGCTT, rev: CAAGAGACCTAAGGTTGTCATGG. *HPRT1* – fw: CCTGGCGTCGTGATTAGTGAT, rev: AGACGTTTCAGTCCTGTCCATAA) was used as a housekeeping gene. For statistical analysis, log-fold change values in comparison to DMSO-treated control samples were analyzed using one-way ANOVA followed by Dunnett's multiple comparison test. Data was subsequently plotted in GraphPad Prism 9.2.0.

Phagocytosis assays in HMC3 microglia-like cells

A β phagocytosis

0.1 mg human beta-Amyloid (1–42), HiLyte Fluor 647-labeled (Anaspec Inc., Cat#: AS-64161) was resuspended to a 4 μ M stock in 50 μ L 1% NaOH and 3950 μ L Corning Eagle's Minimum Essential Medium (MEM) (Corning, Cat#: 10-009-CV) and aliquots stored at -80°C until usage. For phagocytosis assay, 75K HMC3 cells were seeded in a 24-well plate and incubated at 37°C , 5% CO_2 o.n. The next day, cells were treated with Camptothecin (1 μ M; EMD Millipore; Cat #:390238) or DMSO (Sigma-Aldrich, Cat #:472301) as control and incubated for another 24 h. Media was removed and cells were subsequently incubated in 50 nM A β -containing complete DMEM media (10% FCS (Fisher Scientific, Cat#: 10-438-026), 1% P/S (Gibco, Cat#: 15140-122) or DMSO as a control, additionally containing either 5 μ M Cytochalasin D (Sigma-Aldrich, Cat#: C8273) as a negative control for phagocytosis, or DMSO as control for Cytochalasin D treatment. Cells were incubated for 1 hr at 37°C , 5% CO_2 and subsequently washed twice with pre-warmed PBS (Corning, Cat #:21-040-CV) before harvest using Trypsin (Gen Clone; Cat #:25-510F). Cells were subsequently harvested in Flow tubes (MTC Bio, Cat#: T9005), centrifuged at 1500 rpm for 5 min at 4°C and resuspended in 500 μ L Cell Staining buffer (BioLegend, Cat#: 420201) containing 1:4000 dilution of SYTOX Blue (ThermoFisher, Cat#: S34857) for labeling of live/dead cells. To assess phagocytosis, samples were subsequently assessed using Aurora 3L analyzer (Cytek Bio), followed by analysis using FlowJo_v10.8.1. Cells were gated for single cells, live cells and then Alexa 647-positive cells and analyzed percentages subsequently normalized to DMSO control for each experiment to allow comparison across experiments performed on different days. For statistical analysis, log-fold change values in comparison to DMSO-treated control samples were analyzed using one-way ANOVA followed by Dunnett's multiple comparison test. Data was subsequently plotted in GraphPad Prism 9.2.0.

pH-rhodo Dextran phagocytosis

pHrhodo Green Dextran (life technologies, Cat #: P10361) was resuspended in 0.5 mL deionized water (Sigma-Aldrich, Cat#: 95284) to 1 mg/mL stock solution and aliquots stored at -20°C . For phagocytosis assay, 75K HMC3 cells were seeded in a 24-well plate and incubated at 37°C , 5% CO_2 o.n. The next day, cells were treated with Camptothecin (1 μ M; EMD Millipore; Cat #:390238) or DMSO (Sigma-Aldrich, Cat #:472301) as control and incubated for another 24 h. Media was removed and cells were subsequently incubated in 100 μ g/mL pHrhodo Green Dextran-containing complete DMEM media (10% FCS (Fisher Scientific, Cat#: 10-438-026), 1% P/S (Gibco, Cat#: 15140-122)) or DMSO as a control, additionally containing either 5 μ M Cytochalasin D (Sigma-Aldrich, Cat#: C8273) as a negative control for phagocytosis, or DMSO as control for Cytochalasin D treatment. Cells were incubated for 1 hr at 37°C , 5% CO_2 and subsequently washed twice with pre-warmed PBS (Corning, Cat #:21-040-CV) before harvest using Trypsin (Gen Clone; Cat #:25-510F). Cells were subsequently harvested in Flow tubes (MTC Bio, Cat#: T9005), centrifuged at 1500 rpm for 5 min at 4°C and resuspended in 500 μ L Cell Staining buffer (BioLegend, Cat#: 420201) containing 1:4000 dilution of SYTOX Blue (ThermoFisher, Cat#: S34857) for labeling of live/dead cells. To assess phagocytosis, samples were subsequently assessed using Aurora 3L analyzer (Cytek Bio), followed by analysis using FlowJo_v10.8.1. Cells were gated for single cells, live cells and then pHrhodo-positive cells and analyzed percentages subsequently normalized to DMSO control for each experiment to allow comparison across experiments performed on different days. For statistical analysis, log-fold change values in comparison to DMSO-treated control samples were analyzed using one-way ANOVA followed by Dunnett's multiple comparison test. Data was subsequently plotted in GraphPad Prism 9.2.0.

pH-rhodo E. coli phagocytosis

pHrodo Green E. coli BioParticles Conjugate for Phagocytosis (ThermoFisher Scientific, Cat#: P35366) were resuspended in 2 mL PBS (Corning, Cat #:21-040-CV) and incubated for 45 min in a sonicator bath to generate 1 mg/mL stock suspension. For phagocytosis assay, 75K HMC3 cells were seeded in a 24-well plate and incubated at 37°C , 5% CO_2 o.n. The next day, cells were treated with Camptothecin (1 μ M; EMD Millipore; Cat #:390238) or DMSO (Sigma-Aldrich, Cat #:472301) as control and incubated for another 24 h. Media was removed and cells were subsequently incubated in 160 μ L E. coli solution containing either 5 μ M Cytochalasin D (Sigma-Aldrich, Cat#: C8273) as a negative control for phagocytosis, or DMSO as control for Cytochalasin D treatment. Cells were incubated for 1 hr at 37°C , 5% CO_2 and subsequently washed twice with pre-warmed PBS (Corning, Cat #:21-040-CV) before harvest using Trypsin (Gen Clone; Cat #:25-510F). Cells were subsequently harvested in Flow tubes (MTC Bio, Cat#: T9005), centrifuged at 1500 rpm for 5 min at 4°C and resuspended in 500 μ L Cell Staining buffer (BioLegend, Cat#: 420201) containing 1:4000 dilution of SYTOX Blue (ThermoFisher, Cat#: S34857) for labeling of live/dead cells. To assess phagocytosis, samples were subsequently assessed using Aurora 3L analyzer (Cytek Bio), followed by analysis using FlowJo_v10.8.1. Cells were gated for single cells, live cells and then pHrodo-positive cells and analyzed percentages subsequently normalized to DMSO control for each experiment to allow comparison across experiments performed on different days. For statistical analysis, log-fold change values in comparison to DMSO-treated control samples were analyzed using one-way ANOVA followed by Dunnett's multiple comparison test. Data was subsequently plotted in GraphPad Prism 9.2.0.

A β phagocytosis assay in iMG

Labeling of A β with pHrodo-red

Human iPSC-derived microglia were differentiated as described above. For labeling of the substrate amyloid beta with pHrodo Red, we followed the iCell Microglia application protocol from Fujifilm and Cellular Dynamics using the Beta Amyloid (1–42), Aggregation Kit (rPeptide; A-1170-02). In short, Beta Amyloid (1–42), Aggregation Kit (rPeptide; A-1170-02) was equilibrated to room temperature, 250 μ L of 10 mM NaOH (ThermoScientific, Cat#: 63736.AK) was added to the A β vial and swirled. Rehydrated A β was transferred to a

1.5 mL Eppendorf tube and 250 μ L of HPLC water was added. Then, 56 μ L of 10X TBS pH 7.4 was added and mixed well to neutralize the solution. A β solution was then incubated at 37 $^{\circ}$ C overnight to allow for aggregation. The next day, A β solution at 16,000 $\times$ g for 1 min to collect the aggregates. Supernatant was aspirated and aggregates were washed with 1 mL of HBSS followed by centrifugation of 16,000 $\times$ g for 1 min to collect the aggregates. Supernatant was removed and 200 μ L of 0.1 M sodium bicarbonate added to the aggregates and mixed by pipetting. 10 mg/mL pHrodo Red dye was prepared by diluting 1 mg of pHrodo Red in 100 μ L of DMSO and mixed by vortexing. Then, 36 μ L of the 10 mg/mL pHrodo Red dye was added to the A β aggregate suspension to initiate the labeling reaction and incubated for 1 h at room temperature in the dark followed by centrifugation at 16,000 $\times$ g for 1 min. Supernatant was then aspirated, 1 mL methanol was added and vortexed for 5–10 s followed by centrifugation at 16,000 $\times$ g for 1 min. Supernatant was then aspirated and 200 μ L of HBSS was added to the reaction tube and mixed. 800 μ L of HBSS was additionally added, vortexed and tube was centrifuged at 16,000 $\times$ g for 1 min. Supernatant was aspirated and washing step repeated an additional three times by adding 1 mL HBSS, vortexing, centrifugation at 16,000 $\times$ g for 1 min and aspirating the supernatant. 200 μ L HBSS was then added, mixed and pHrodo Red-labeled A β was stored at -80° C until further usage.

Phagocytosis assay in iMG

For the phagocytosis assay, we treated FA000011 (CUIMCi002-A)⁸⁴-derived iMG between days 28–35 of differentiation with 0.05 μ M, 0.1 μ M or 0.5 μ M Camptothecin (EMD Millipore; Cat #: 390238) or DMSO as vehicle control for 24 h. The same day, wells of 96-well plates were coated with 1:100 Cultrex (R&D Systems, Cat #: 3434-005-02) (in ddH₂O) overnight. The next day, iMG were lifted of the plate using resuspension, cell viability was assessed and 20,000–35,000 iMG were subsequently plated in 50 μ L media (DMEM/F12, 2X insulin-transferrin-selenite, 2X B27, 0.5X N2, 1X Glutamax, 1X non-essential amino acids, 400 mM monothioglycerol, and 5 mg/mL human insulin (ThermoFisher)) in triplicates on a 96-well Cultrex-coated plate. pHrodo red-labeled A β was thawed and prepared as 2X (0.8 μ M) in media (DMEM/F12, 2X insulin-transferrin-selenite, 2X B27, 0.5X N2, 1X Glutamax, 1X non-essential amino acids, 400 mM monothioglycerol, and 5 mg/mL human insulin (ThermoFisher)). Once the BioTek Cytation 5 Cell Imaging Multimode Reader (Agilent) was setup, 50 μ L of 2X cargo was added to each well, mixing gently by pipetting up and down. As negative controls, no substrate and substrate + Cytochalasin D (Sigma-Aldrich, Cat#: C8273) was added to triplicate wells for each of the conditions (DMSO, 0.05 μ M Camptothecin, 0.1 μ M Camptothecin, 0.5 μ M Camptothecin). For imaging of phagocytosis, using 20 $\times$ magnification, we captured 2–4 positions/well with 3 wells/condition (DMSO; 0.05 μ M Camptothecin, 0.1 μ M Camptothecin; 0.5 μ M Camptothecin; all either without substrate, with substrate or with substrate containing Cytochalasin D (Sigma-Aldrich, Cat#: C8273) over a period of 16 h from $n = 4$ –5 independent iMG differentiations.

Analysis of phagocytosis assay in iMG

For the analysis, we established a Cell Profiler pipeline to extract absolute mean intensity for each segmented and identified microglia cell. In order to do so, we set up the following sequence of modules: Identify primary objects (input: OrigPhaseContrast, Threshold: adaptive and robust background, Threshold smoothing scale: 1.3488), followed by SplitOrMergeObjects to create new objects called merged_iMG if the maximum distance between objects is 0; then we implemented the module MeasureObjectIntensity with selecting the OrigRFP images to measure and selection of merge_iMG objects to measure, resulting in measurement of the mean fluorescent intensity of RFP per identified microglia cell. The here extracted values were then used to plot the distribution of RFP mean intensity at the timepoints of 10 h for all cells and all wells pooled per condition as a histogram. For statistical analysis, we then set a threshold of 2 standard deviations (SD) above the mean and compared all cells in this category with regards to their RFP mean intensity across conditions. To test for statistical significance, we performed one-way ANOVA followed by Dunnett's multiple comparison test. In order further assess results per condition across the three experiments, we performed two sets of meta-analyses – fixed effect meta-analysis as well as random-effect meta-analysis to account for heterogeneity of effect and standard error (SE). Data from independent experiments were analyzed separately at 10 and 16 h. Within each experiment, treatment effects were estimated using a one-way analysis of variance, with experimental condition included as a categorical factor. Each Camptothecin condition was compared with the DMSO control using Dunnett contrasts implemented with the `glht()` function in the R package `multcomp`. The resulting mean differences between Camptothecin and DMSO and their standard errors were subsequently combined across experiments. Separate meta-analyses were conducted for 0.05 μ M and 0.1 μ M Camptothecin at each time point. Random-effects models were fitted using restricted maximum likelihood to estimate between-experiment heterogeneity, as implemented by the `rma()` function in the R (Version 4.4.1) package `metafor`.

In order to assess the percentage of iMG phagocytosing pHrodo Red-labeled A β over time for each condition, we implemented additional modules in the CellProfiler pipeline: we filtered AbetaPosiMG from Merge_iMG based on RFP mean intensity measured in OrigRFP and a minimum value of 0.15–0.32 followed by the ClassifyObjects module, classifying Merge_iMG based on mean RFP intensity measurements from image OrigRFP and the custom threshold 0.3. With the CalculateMath module we then selected to count all AbetaPosiMG from all measured Merge_iMG followed by multiplication of the result by 100 in order to get percentage as a readout. We then plotted for each condition the percentage of AbetaPosiMG (RFP+ iMG) over the time course of 16 h.

Alamar Blue Assay with iMG

iMG cells were seeded at $\sim$ 5,000 cells per well in a 96-well plate pre-coated with Cultrex and allowed to adhere for 24 h. Cells were then treated with Camptothecin across a 7-point concentration series spanning 5 μ M–0.001 μ M (5 μ M, 1 μ M, 0.5 μ M, 0.1 μ M, 0.05 μ M, 0.01 μ M, 0.001 μ M), prepared from a 10 mM stock, and incubated for 24 h. Following treatment, viability was quantified using Alamar Blue HS (Thermo Fisher; Cat #: DAL1100) as a metabolic viability assay: the reagent was equilibrated to room temperature, added

directly to wells at 1:10 of the culture volume, and plates were incubated at 37 °C protected from light for four hours. After incubating, the fluorescence is measured using the excitation/emission settings within 540–570 nm and 580–610 nm respectively, and absorbance was measured at a wavelength of 570 nm with 600 nm as a reference. Viability signals for each concentration were quantified to generate a dose-response curve, selecting final Camptothecin working concentrations from the cells with the highest dose that have the least reduction in metabolic activity.

Seahorse assay to assess mitochondrial fitness

Oxidative capacity and extracellular pH

Oxygen consumption rate (OCR) and extracellular acidification rate (ECAR; pH change) were measured over a confluent cell monolayer of HMC3 cells using the XF96 Seahorse extracellular flux analyzer (Agilent Technologies). First, 10K HMC3 cells or 20K iPSC-derived human microglia per well were seeded in 80 μ L of a Agilent Seahorse XF96 Cell Culture Microplate (Agilent Technologies; Cat #: 101085-004) and incubated overnight. The next day, cells were treated with Camptothecin (1 μ M; EMD Millipore; Cat #:390238) or DMSO (Sigma-Aldrich, Cat #:472301) as control in a total volume of 80 μ L/well and incubated for 24 h. Subsequently, OCR and ECAR were measured as follows: cell culture media was removed and 80 μ L of XF Media (Agilent Technologies; Cat #: 103334-100) containing no pH buffers and supplemented with 5.5 mM glucose, 1 mM pyruvate (Sigma-Aldrich; Cat #: Sigma-Aldrich #P9767), 1 mM glutamine (fisher scientific; Cat #: G7513), 50 μ g/mL Uridine (Sigma-Aldrich, Cat#: U6381), 10 μ M palmitate (Sigma-Aldrich, Cat#: P9767) conjugated to 1.7 μ M BSA (pluriSelect; Cat#: 60-00020-10) was added to the cells. The plate was then incubated in a non-CO₂ incubator for one hour to equilibrate temperature and atmospheric gases. The Seahorse instrument was programmed to assess various respiratory states using the manufacturer's protocol of sequential substrate addition and measurements (4x, 3 measurements over 18 min). Basal respiration, ATP turnover, proton leak, coupling efficiency, maximum respiration rate, respiratory control ratio, spare respiratory capacity and non-mitochondrial respiration were all determined by the sequential additions of the ATP synthase inhibitor oligomycin (final concentration: 1 μ M; Sigma-Aldrich Cat#:75351), the protonophoric uncoupler FCCP (final concentration: 0.45 μ M; Selleckchem, Cat #: S8276), and the electron transport chain Complex I and III inhibitors, rotenone (final concentration: 1 μ M; Sigma-Aldrich, Cat #: R8875) and antimycin A (final concentration: 1 μ M; Sigma-Aldrich, Cat #: A8674) respectively as well as the staining dye Hoechst (1:200 dilution; ThermoFisher Scientific #62249). The optimal concentration for the uncoupler FCCP yielding maximal uncoupled respiration was previously determined based on a titration performed on HMC3 microglia-like cells and iMG (see [Figure S11](#)).

After the seahorse run, nuclei were counted using the cytoation1 Cell Imager (BioTek). Both OCR and ECAR were normalized relative cell counts on a per-well basis. Bioenergetic metrics were calculated using the P/O ratios of OxPhos and glycolysis as described in.⁸⁵ These calculations assume that the pyruvate was derived entirely from glucose via glycolysis. The raw and processed data are available as detailed in the Data Availability statement.

Cytokine multiplex assay

10K HMC3 cells from three different passages (P12, P19, P21) were seeded in 96-well plates containing 200 μ L of complete Eagle's Minimum Essential Medium (EMEM; ATCC, Cat#: 30-2003) and incubated o.n. at 37 °C, 5% CO₂. The next day, cells were treated with DMSO (1:1000), Camptothecin (1 μ M) or Topotecan (1 μ M) and incubated for 24 h. The next day, 2 wells/condition were stimulated with TNF- α (0.3 μ g/mL, Peprotech, Cat#: 300-02), 2 wells/condition were stimulated with IFN- γ (0.3 μ g/mL, Peprotech, Cat#: 300-01A) and as a control 2 wells/condition were stimulated with H₂O as control, each dissolved in a fresh dilution of the respective drug treatment and in a final volume of 200 μ L. For subsequent cytokine expression analysis, cell culture supernatant was harvested after 12 h and 24 h and stored at –20 °C until further analysis.

For cytokine multiplex assay, 100 μ L/sample were sent to Eve Technologies (Calgary, Alberta) and analyzed using Human Cytokine Proinflammatory Focused 15-Plex Discovery Assay Array (HDF15). Data was subsequently plotted in GraphPad Prism 9.2.0. For statistical analysis, one-way ANOVA followed by Tukey's multiple comparisons test with a single pooled variance was performed using GraphPad Prism 9.2.0 software.

QUANTIFICATION AND STATISTICAL ANALYSIS

All data were statistically analyzed by GraphPad Prism (Version 9.5.1; Version 9.2.0), R (4.2.2) or Python (3.8) and displayed with mean $\pm$ SEM ([Figures 3B](#) and [3E–3H](#); [Figures 5D](#) and [5E](#)), mean $\pm$ SD ([Figures 3A](#) and [3I–3L](#)), as violin plots ([Figures 1C](#) and [1D](#)), boxplots (highlight the median, lower, and upper quartiles. Whiskers indicate 1.5 times interquartile ranges; [Figure 2B](#)) or UMAPs ([Figure 1B](#); [Figure 4](#)). The sample sizes (n) for each experiment are reported in the figure legends. For statistical analysis, pairwise Wilcoxon rank-sum tests ([Figures 1C](#) and [1D](#)), unpaired *t* test ([Figure 2B](#)), one-way ANOVA followed by Tukey's multiple comparison test ([Figures 3A](#) and [3D](#); [Figures 5D](#) and [5E](#)), one-way ANOVA followed by Dunnett's test ([Figure 3C](#)) and a random- and fixed-effects meta-analysis ([Figure 3C](#)), Welch's *t* test ([Figures 3E–3H](#)), linear regression analysis ([Figures 3I–3L](#)) and GSEA ([Figure 4](#)) were performed.

Statistical results are reported in the figures as well as detailed in [Tables S2–S10](#). Asterisks denote corresponding statistical significance: ns – not significant - > 0.05, **p*.adj $\leq$ 0.05; ***p*.adj $\leq$ 0.01.; ****p*.adj $\leq$ 0.001; *****p*.adj $\leq$ 0.0001.