

Glioma-associated microglia and macrophages as a potential target for mTOR inhibition in glioblastoma

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Abstract. Glioma-associated microglia/macrophages (GAM) constitute the predominant immune cell population in glioblastoma (GB). Both GB cells and GAM exhibit upregulated mTOR signaling. The present study aimed to investigate the effects of pharmacological mTOR inhibition (mTORi) specifically on GAM. The effects of mTORi on signal transduction, cell growth and viability were analyzed in immortalized microglia cell lines. Additionally, a comprehensive analysis of the GAM phenotype was conducted, including whole transcriptome analyses and cytokine profiling. Effects were investigated in a tumor cell/GAM co-culture model under mTORi with rapamycin or torin2 or treatment with temozolomide, the standard chemotherapy agent for patients with GB. In the *in vitro* model, mTORi had significant effects on central biological functions of GAM, resulting in reduced proliferation and oxygen consumption. Additionally, treatment with mTORi induced a pro-inflammatory phenotype in microglia cell lines. These findings demonstrate the relevance of mTOR signaling on GAM biology. Moreover, they provide rationales

for therapeutic interventions targeting mTOR signaling specifically in GAM as a potential novel treatment strategy.

Introduction

In patients with glioblastoma (GB), overall survival (OS) remains poor despite advances in diagnostic precision (1) and targeted therapies (2,3). Standard therapy for patients in good clinical condition consists of tumor biopsy/resection followed by radiotherapy with temozolomide chemotherapy yielding a median overall survival slightly below 15 months after diagnosis (4). The predominant immune cell population in GB comprises glioma-associated microglia and macrophages (GAM), which can originate from brain-resident microglia (GAM-MG) or monocyte-derived macrophages (GAM-MDM) from the periphery (5-7). The immune polarization of GAM is complex (8-13) and the specific role of different microglial subtypes in GB pathogenesis still only poorly understood with conflicting prognostic data on the role of the innate immune system (12,14-17). Moreover, the effects of GB therapy with standard alkylating or novel targeted treatment approaches on GAM have not been investigated in detail, while in preclinical brain metastases models profound effects of tumor-associated innate immune cells on therapy efficacy have been reported (18,19).

Activation of the mammalian target of rapamycin (mTOR) pathway is frequently found in GB (20). Enhanced signaling occurs through amplified and/or mutated receptor tyrosine kinases including the epidermal growth factor receptor (EGFR), deletion of the tumor suppressor PTEN and other alterations in the upstream signaling cascade. The mTOR pathway engages a central role in the regulation of proliferation, metabolism and

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other biological processes important for cell growth proliferation and the adaptation to the tumor microenvironment (21,22) (Fig. 1A). mTOR exists as part of 2 different multiprotein complexes termed mTOR complex 1 and 2 (mTORC1 and 2) that integrate upstream signals from diverse stimuli including growth factor receptors but also nutrient and oxygen availability (23). Important downstream effects of mTORC1 are mediated through targets that regulate mRNA translation. For instance, the activation of the mTORC1 pathway triggers phosphorylation of the eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1) at the phosphorylation sites Thr37/Thr46, as well as phosphorylation of the S6 ribosomal protein (S6RP) at Ser240/244 via S6-kinase (S6K). mTORC2 signaling is less well understood and plays important roles in the reorganization of the cytoskeleton as well as regulation of proliferation (23). mTORC2 targets include the glucocorticoid-regulated kinase 1 (SGK1), which in turn activates N-myc downstream-regulated gene 1 (NDRG1) through phosphorylation of Thr346 (24). 4E-BP1 phosphorylation is to some degree resistant to first-generation mTORC1 inhibitors such as rapamycin and its derivatives, while second-generation mTOR inhibitors including torin2 effectively induce dephosphorylation of 4E-BP1 (Fig. 1A) (25). Taken together, EGFR and its downstream target mTOR are plausible targets for therapeutic intervention that have been extensively studied in various cancers, including GB (3,21,22,26).

However, despite the biological rationale and consistent activation of this pathway, clinical trials with mTOR inhibitors in GB have so far only produced only weak and sometimes even antagonistic signals in unselected GB patient cohorts (3,27). Therefore, the results of biomarker selected GB cohorts treated with mTOR inhibition (mTORi) in multi-arm trials are eagerly awaited. The NCT Neuro Master Match trial (N2M2, NOA-20) investigated temsirolimus-based mTORi in newly diagnosed GB with upregulated mTOR signaling in bulk tumor tissue (28), while the GBM AGILE trial is testing paxalisib-based PI3K/mTORi in newly diagnosed or recurrent tumors (29). Additionally, several resistance mechanisms to mTORi in the complex GB microenvironment, such as protection of tumor cells from hypoxia-induced cell death or from temozolomide chemotherapy have been reported (21,22,30,31). Furthermore, biomarkers of mTOR activity could indicate a subpopulation of GB patients more prone to benefit from mTOR inhibitor treatment (26,32).

Interestingly, mTORC1 not only drives GB cell growth and proliferation (21), but also shows significant activity in GAM *in vivo*, as reflected by the analysis of phosphorylation status of the mTOR target proteins 4E-BP1 and S6RP (33). The regulation of peripheral innate immune cell functions by mTOR has been extensively studied (34,35). For example, the mTORC1 inhibitor rapamycin can enhance pro-inflammatory cytokine production in macrophages (35). Also, various effects of mTORi on tumor-associated macrophages in peripheral cancers have been identified (36). In contrast, the role of mTOR signaling for innate immune functions in the central nervous system (CNS) and specifically MG and GAM has not been understood in detail, yet. In certain preclinical models, mTOR inhibition has been shown to exert anti-inflammatory effects on MG during stroke (37,38) or aging (39) and to suppress LPS-induced pro-inflammatory cytokine production (40). In contrast, in the context

of glioma, mTOR inhibition showed a pro-inflammatory GAM phenotype in a rat glioma model (41) as well as a reduced expression of anti-inflammatory markers in cell culture (42). Additionally, the landmark paper of Dumas *et al* (43) identified mTOR regulation in GAM-MG (as opposed to GAM-MDM) as a central mechanism for glioma progression with a correlation of mTOR activity and effector immune evasion in the GB microenvironment. Genetic inactivation of mTORC1 in GAM-MG had anti-tumor effects and reduced glioma growth, suggesting a prognostic relevance of mTOR signaling specifically in microglia (43). Furthermore, we previously reported that GB patients with higher levels of GAM had improved OS when EGFR/mTOR signaling was targeted (within the OSAG 101-BSA-05 trial) (26). These findings support the hypothesis that GAM could be a promising target for a precision mTORi approach in GB. The aim of our study was to further investigate the properties of GAM under pharmacological mTORi compared to standard chemotherapy in GB.

Materials and methods

Reagents, cell lines and culture conditions. A split of passage three of the human C20 MG cell line was kindly provided in 2017 by Dr. David Alvarez-Carbonell from the Department of Molecular Biology and Microbiology at the Case Western Reserve University (10900 Euclid Ave., SOM WRT 205, Cleveland, Ohio 44106, USA). The C20 cell line has the advantage that it was generated from primary human MG obtained from fresh CNS cortical tissue of adult patients by magnetic cell sorting (44). Negativity for human viruses (including HIV, HCV, HBV) in C20 cells was confirmed. The resulting classification as biosafety level 1 of the C20 by the department for biological safety of the University Hospital Frankfurt was approved by the local authority (Regierungspräsidium Gießen, Central regional council of Hesse). The human microglial clone 3 cell line HMC3 was purchased from the ATCC (45) and originally derives from human embryonic MG (46). LNT-229 cells were a kind gift of Dr. Nicolas de Tribolet from the Department of Neurosurgery and Laboratory of Brain Tumor Biology and Genetics (Centre Hospitalier Universitaire Vaudois and University of Lausanne, Lausanne, Switzerland) (47). No passage number was available for LN-229 cells that are amongst the most widely spread glioblastoma cell lines. LNT-229 glioma cells differ from LN-229 cells in having retained wild-type p53 status (48). LNT-229 cells were authenticated using short tandem repeat analysis (Multiplexion, Heidelberg, Germany) and the STR profile of LNT-229 cells matched with the known profile for LN-229 (49). Cells were cultured in Dulbecco's modified eagle medium (DMEM) containing 100 IU/ml penicillin and 100 mg/ml streptomycin (Life Technologies, Darmstadt Germany) and 10% fetal calf serum (FCS) (Biochrom KG, Berlin, Germany). All reagents not specified were purchased from Sigma/Merck.

Transwell co-cultivation of microglia and glioma cells as an *in vitro* GAM model. Transwell co-cultivation of the human MG cell lines C20 or HMC3 with the glioma cell line LNT-229 was performed using 6- or respective 24-well cell culture inserts (1 μ m pore; Greiner bio-one, Frickenhausen, Germany).

6-well inserts were used for experiments with protein and RNA analyses and 24-well inserts for oxygen consumption analyses. MG were seeded into the lower chamber (6-well: 500,000/well, 24-well: 120,000/well) and glioma cells into the inserts (6-well insert: 400,000/well, 24-well insert: 30,000/well) separately in DMEM with 10% FCS 24 h prior to co-cultivation. To provide a MG monoculture control condition, C20 or HMC3 MG were seeded into the inserts and lower chambers. Cultivation was performed in serum-free DMEM with a total volume of 3 ml/500 μ l or 2 ml/200 μ l added to the well and insert (6-/24-well plate, respectively). Cultured cells were treated with the mTOR inhibitors 100 nM rapamycin and 100 nM torin2 as well as 400 μ M temozolomide or corresponding vehicle controls for 24 h. Rapamycin and torin2 concentrations were selected based on previous studies in glioblastoma cells demonstrating effective target inhibition without cytotoxicity (21,25). Treatment durations were designed to allow for steady-state cellular adaptation and to model prolonged drug exposure, approximating clinical administration schedules of mTOR inhibitors and temozolomide over a continuous period. The temozolomide condition was used as a control to enable comparison between the effects of standard GB chemotherapy and mTORi treatment. After the incubation period, C20 or HMC3 cells in the lower chamber were harvested for further analyses (RNA sequencing, immunoblot, human cytokine array or oxygen consumption).

Cell density and viability assays. Cell density was assessed by crystal violet staining as previously described (50) after treatment of C20 or HMC3 with 100 nM rapamycin and 100 nM torin2 as well as vehicle controls for 72 h in serum-free medium or DMEM with 10% FCS. Cell viability measurement using propidium iodide (PI) uptake and flow cytometry was performed as previously described (21).

Oxygen consumption. After 24 h Transwell cultivation in mono- or co-culture condition (see above), oxygen consumption was assessed during 24 h treatment of C20 or HMC3 with 100 nM rapamycin, 100 nM torin2 or vehicle control. Plates with oxygen sensors (Oxo Dish OD24, PreSens, Regensburg, Germany) were used to quantify oxygen consumption. Airtight conditions were achieved by covering with sterile paraffin oil as previously described (22). Fluorescence-based measurement of oxygen consumption was performed in triplicates.

Immunocytochemistry. Immunocytochemistry (ICC) was performed in C20 or HMC3 cells after treatment with 100 nM rapamycin and 100 nM torin2 as well as a vehicle control. Paraffin embedded cell pellets were prepared as previously described (33). Pellets were cut in slices of 3 μ m thickness using a microtome (Leica Microsystems, Nussloch GmbH, Nussloch, Germany) and placed onto SuperFrost slides (Thermo Scientific, Dreieich, Germany). mTORi was investigated according to the phosphorylation status of the mTORC1 target protein 4E-BP1 using the phospho-4E-BP1 (Thr37/46) antibody diluted 1:1,000 (236B4; Cell Signaling). ICC was performed according to standardized protocols of the Leica BOND-III automated stainer (Leica, Wetzlar, Germany) and stainings were analyzed using a light microscope (BX41, Olympus, Hamburg, Germany).

Protein isolation and immunoblot analysis. Protein lysates were prepared using lysis buffer P followed by a determination of protein concentration by Bradford-Assay to analyze equal amounts of protein loading of each sample as previously described (51). Electrophoretic separation of the denatured proteins was performed on 12% SDS-polyacrylamide gels followed by a blotting process as described previously (12). Membranes were blocked in 1x Roti-Block blocking buffer (Roth, Karlsruhe, Germany) and then incubated with antibodies against 4E-BP1 (Cell Signaling), phospho-4E-BP1 (Thr37/46) (236B4; Cell Signaling), S6RP (5G10; Cell Signaling), phospho-S6RP (Ser240/244) (D68F8; Cell Signaling), NDRG1 (D8G9; Cell Signaling), phospho-NDRG1 (Thr346) (D98G11; Cell Signaling) or beta-actin (Santa Cruz Biotechnology) as a loading control. The secondary antibodies were purchased from Jackson ImmunoResearch, USA. Immunodetection was performed by HRP enzyme-coupled secondary antibodies, which oxidize luminol (AppliChem GmbH, Darmstadt, Germany) resulting in a chemiluminescent reaction on X-ray films (Super RX, Fujifilm Europe GmbH, Düsseldorf, Germany).

Human cytokine array. After 24 h Transwell cultivation of the MG cell lines C20 or HMC3 with the glioma cell line LNT-229 in serum-free DMEM, protein lysates of HMC3 or C20 cells were prepared. Experiments were performed in biological triplicates and pooled for a sufficient protein concentration to perform the human cytokine array according to the manufacturer's protocol (ARY005B, R&D Systems). The array enables a simultaneous detection of 36 different cytokines, chemokines and acute-phase proteins per sample (CCL1/I-309, CCL2/MCP-1, MIP-1 α /MIP-1 β , CCL5/RANTES, CD40 Ligand/TNFSF5, Complement Component C5/C5a, CXCL1/GRO α , CXCL10/IP-10, CXCL11/I-TAC, CXCL12/SDF-1, G-CSF, GM-CSF, ICAM-1/CD54, IFN- γ , IL-1 α /IL-1F1, IL-1 β /IL-1F2, IL-1 α /IL-1F3, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12 p70, IL-13, IL-16, IL-17A, IL-17E, IL-18/IL-1F4, IL-21, IL-27, IL-32 α , MIF, Serpin E1/PAI-1, TNF- α and TREM-1). The coordinates of the array are available in the protocol (<https://resources.rndsystems.com/pdfs/datasheets/ary005b.pdf?v=20250615>). Immunodetection was performed as described in the section immunoblot analysis. Quantification was performed by measuring the pixel density of scanned films using ImageJ software (NIH).

Whole transcriptome analysis. Extraction of total RNA using TRIzol and the ExtractMe Total RNA Kit (Blirt, Danzig, Poland) was performed as described previously (22). Paired end, 150 base pair, sequencing was performed with an Illumina HiSeq2500 by Genewiz (New Jersey, USA). RNA-sequencing libraries were generated with the SMART-Seq preparation kit (CloneTech) and fragmented with the Nextera XT kit (Illumina). The following steps were performed as previously described (52,53). Pre-processing of fastq-files of bulk sequenced samples including filtering for quality scores, poly-A trimming, removal of N containing reads, artifact removal and clearing of rRNA contamination was achieved using a pipeline in the HUSAR platform, provided by DKFZ (Heidelberg, Germany). Transcriptomes were mapped to the human genome using the genecode annotations (release v. 32; genome

assembly version GRCh38.p13) and TopHat2 (v. 2.0.14) (54). The number of reads per gene was determined by HTSeq count. Overlaps were considered as unique. Further analysis was performed within R (v. 4.1.2), operating in RStudio (v. 2022.02.0) with BioMart package (v. 2.50.3) and DESeq2 (v. 1.34.0) (55).

Gene expression analyses. Gene annotation and species conversion was done using the biomaRt R package (v. 2.38.0). For principal component analysis (PCA) and unsupervised clustering, read counts were normalized using variance-stabilized transformed (vst) data ('vst' function of DESeq2 package which equals log2 transformation), respecting a base mean >20 and an adjusted P-value of 0.05 (= FDR 5 %). PCAs were plotted using the 'autoplot' function of ggplot2 package (v. 3.3.5). Heatmaps of the top 2,000 genes were created with the pheatmap package (v. 1.0.12). Hierarchical clustering of the top 100 gene loadings of the PCAs was performed in JMP 16.2.0 by use of the complete linkage variance method. The first PC obtained by the PCA was used as an ordering column sorting the clusters by these values.

Gene set enrichment analysis (GSEA). GSEA was performed using fgsea R package (v. 1.18.0) as reported before (53). Genes with base mean <20 were removed and the remaining genes were ranked ascendingly based on the log2 fold change. The ranked gene list was compared against the MSigDB database (v. 7.4) using 'fgseaMultilevel' function with the default settings. Normalized enrichment scores (NES) were used for subsequent heatmaps of the top 50 Hallmark signatures graphed with JMP 16.2.0.

Statistical analysis and data visualization. Statistical analyses were conducted using JMP 16.2.0 (SAS, Cary, USA), GraphPad Prism V9 or R (v. 3.5 or v. 4.2.1). Detailed information on the respective analyses was indicated in the corresponding figure legend or specific methods section. Quantitative data were depicted including mean and standard deviation (SD). Statistically significant P-values were indicated (*P<0.05; **P<0.01; ***P<0.001). BioRender was used for illustrations.

Results

Pharmacological inhibition of mTOR signaling in microglia cell lines. To determine the sensitivity of human MG cell lines to pharmacological mTORi, the C20 cell line (with the advantage of having been generated by a cell sorting approach) (44) and the established HMC3 MG cell line (45,46) were treated with the allosteric mTORC1 inhibitor rapamycin or torin2 that inhibits both mTORC1 and mTORC2 in an ATP-competitive manner. Both MG cell lines showed a strong expression of mTOR signaling per se and were sensitive to treatment with rapamycin and torin2. Treatment with torin2 resulted in a profound reduction of the phosphorylation of all investigated target proteins, whereas rapamycin treatment mainly reduced the signal of S6RP phosphorylation (Fig. 1B). These results are in line with the known effects of the first generation mTORC1 inhibitors rapamycin and its derivatives in contrast to second generation mTOR inhibitors like torin2 in glioma cells (25,33). The assessment of basal MG cellular functions

commonly affected by mTORi with rapamycin and torin2 showed a reduced proliferation (Fig. 1C) without displaying major cytotoxicity (Fig. 1D) and a reduced oxygen consumption in the MG cell lines (C20 in Fig. 1E, HMC3 in Fig. S1) similar as previously reported from glioma cell lines with stronger effects of torin2 (22,25).

Effects of pharmacological mTOR inhibition on the GAM transcriptome. To examine effects of mTORi on the GAM phenotype we employed a defined co-culture model (Fig. 2A). The C20 or the HMC3 cell line was cultured either in co-cultivation with the GB cell line LNT-229 (GAM co-culture) or in a monoculture condition with the MG cell line in both compartments of the well (MG mono-culture). The PCAs of the entire transcriptome dataset as well as the unsupervised clustering of the top regulated genes across all conditions exhibited a discernible C20 or HMC3 signature induced by rapamycin, which triggered marked differences compared to temozolomide or the untreated condition (Figs. 2B, S2, S3A, B). For example, in C20 cells, PC 1 of the PCA primarily differentiated the samples based on temozolomide treatment, while PC 2 distinguished the samples based on rapamycin treatment and the culture condition (Fig. 2B). Notably, the pharmacological treatment with rapamycin or temozolomide had more influence on the transcriptional phenotype than the presence or absence of glioma cells (GAM co-culture vs. MG mono-culture condition). These effects were observed in both the C20 and HMC3 cell lines, although they were clearer in the C20 cell line (C20 in Figs. 2B, S2; HMC3 in Fig. S3A, B). Effects of co-culturing in the unsupervised transcriptome-based clustering analysis were most apparent in the rapamycin treatment condition in the C20 as compared to the HMC3 cell system (Figs. S2, S3B). Upon analyzing the most significantly regulated genes, we found that treatment with rapamycin resulted in increased expression levels of genes associated with inflammatory processes. These included pro-inflammatory cytokines, components of the complement system, and signaling molecules involved in migration and invasion (such as *ADAMTS5*, *C4A*, *CCL2*, *CEBPD*, *CEMIP*, *CXCL12*, *IL7R*, *KRT7*, *SOCS3*, *TNFRSF11B* or *TRIB3*). Notably, this effect was more explicit in the GAM co-culture condition compared to the MG mono-culture condition. In contrast to rapamycin, temozolomide treatment had a significant impact on genes related to cell death (such as *AEN*, *BBC3*, *BTG2* or *FAS*) (Fig. 3). Also, there was a substantial overlap in the top regulated genes of C20 and HMC3 cells (Fig. S4A, B). To gain a broader understanding of the signaling pathways and gene programs involved, we conducted GSEA, which corroborated that rapamycin upregulated key immunogenic hallmark gene sets in both MG and GAM, whereas the standard alkylating chemotherapy agent temozolomide appeared to be associated with cell cycle related hallmark gene sets (Fig. 4). As a proof of concept, the GSEA indicated that the rapamycin-induced phenotype in both MG and GAM showed reduced mTORC1 signaling (Fig. 4).

Effects of pharmacological mTOR inhibition on the GAM phenotype. In line with the transcriptome analysis, the human cytokine array revealed elevated protein levels of pro-inflammatory cytokines following mTORi by rapamycin or torin2 especially under co-culture with GB cells (GAM

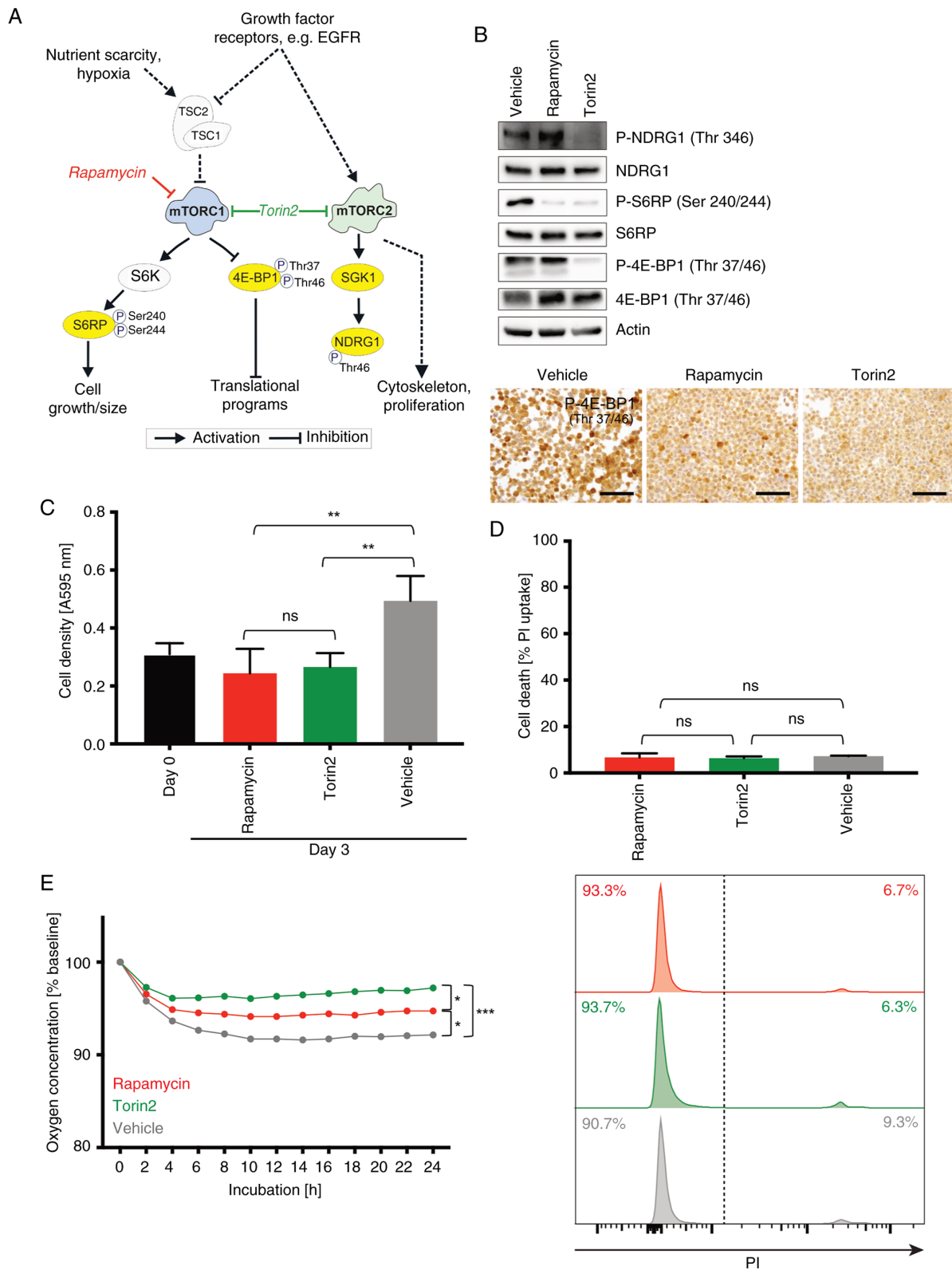


Figure 1. Impact of pharmacological mTOR inhibition on basal functions of human microglia cell lines. (A) Overview of the mTOR pathway and targets of the mTOR inhibitors investigated in this study. Scheme adapted from (33). (B) C20 cells were incubated with 100 nM rapamycin, 100 nM torin2 and vehicle control for 24 h. Protein lysates were analyzed by immunoblotting with antibodies for NDRG1, P-NDRG1 (Thr346), S6RP, P-S6RP (Ser240/244), 4E-BP1, P-4E-BP1 (Thr37/46) and actin as well as by immunocytochemistry stainings of P-4E-BP1 (scale bar, 100 μ m) of C20 cell pellets incubated with 100 nM rapamycin, 100 nM torin2 and vehicle control for 24 h. (C-E) C20 microglia cells were treated with 100 nM rapamycin (red), 100 nM, torin2 (green) or vehicle control (grey). (C) Crystal violet staining was used to quantify cell density at baseline (day 0, after a 24 h attachment period prior to any treatment intervention, black) and after 72 h of exposure to the respective treatment conditions. Data are presented as mean $\pm$ SD (n=4; one-way ANOVA, Tukey's multiple comparisons test). (D) Cell death was quantified by PI FACS after 72 h. Data represent mean $\pm$ SD (n=3; one-way ANOVA, Tukey's multiple comparisons test). Histograms were depicted. (E) Quantification of oxygen by a fluorescence-based assay was performed in C20 cells during 24 h treatment. Oxygen consumption is shown relative to the start of the experiment as mean (n=3) every hour; treatment groups were then compared at timepoint 24 h (n=3; one-way ANOVA, Tukey's multiple comparisons test). *P<0.05, **P<0.01 and ***P<0.001. ns, not significant; PI, propidium iodide; SD, standard deviation; NDRG1, N-myc downstream-regulated gene 1; P-, phosphorylated; S6RP, S6 ribosomal protein; 4E-BP1, 4E-binding protein 1.

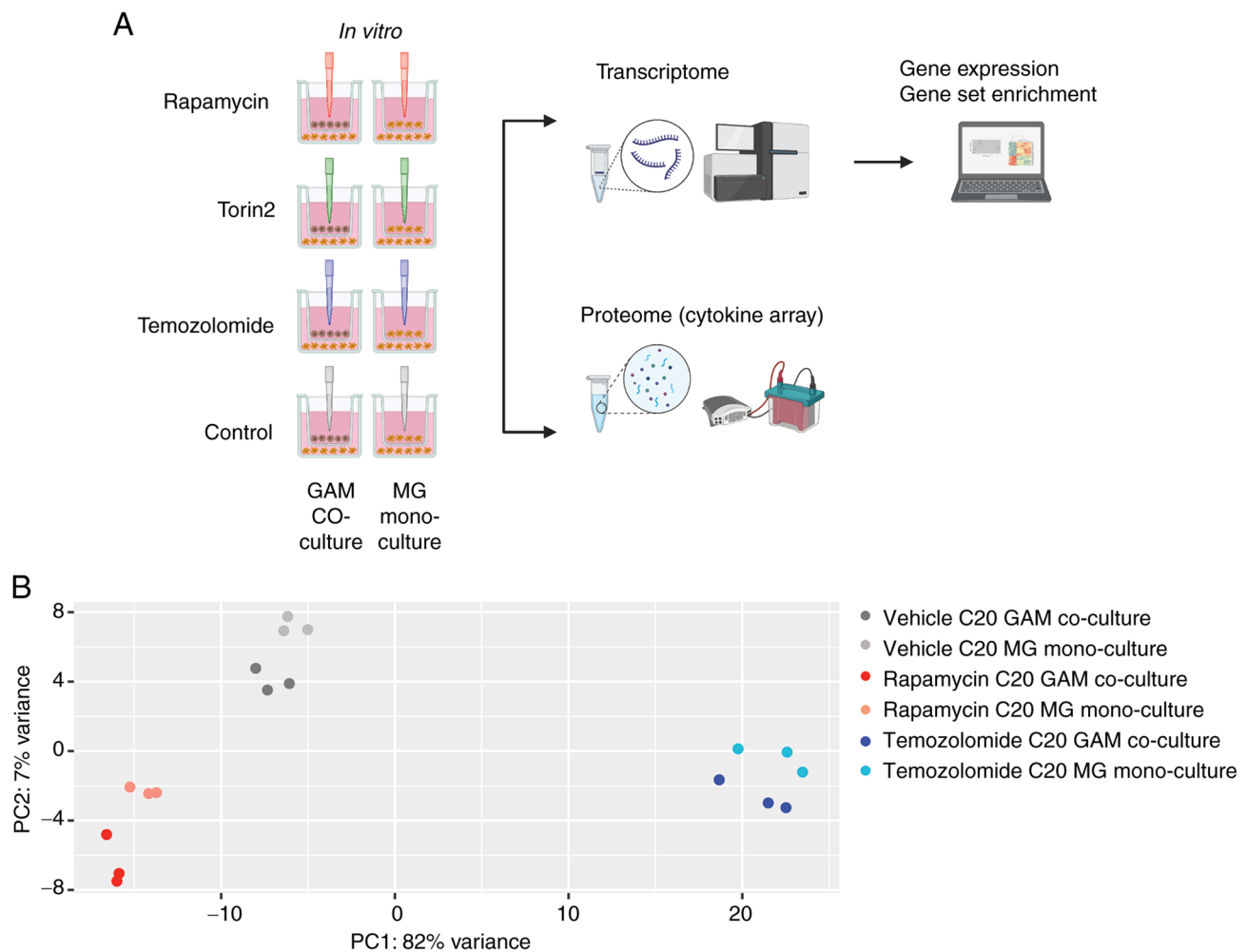


Figure 2. Impact of rapamycin compared with temozolomide on the GAM transcriptome. (A) Overview on all treatment conditions used throughout the experimental setup (created in BioRender, Strecker, M. (2025); <https://BioRender.com/wgg6ezp>). Temozolomide effects were only analyzed by RNA sequencing (transcriptome), torin2 co-culture experiments were only analyzed in the cytokine assay. (B) Gene expression was analyzed in the whole transcriptome dataset by PCA across all C20 samples including triplicates of the different treatments (rapamycin, temozolomide and vehicle control) in the co-culture (C20 microglia with LNT-229 glioma cells) or the C20 mono-culture condition (bright colors, GAM co-culture of C20 with LNT-229; light colors, MG mono-culture control condition; red, treatment with 100 nM rapamycin; blue, 400 μ M temozolomide; grey, vehicle control) (biological replicates were labelled 1 to 3). PCA, principal component analysis; GAM, glioma-associated microglia/macrophages; MG, microglia.

model). Strikingly, torin2 induced 28 whereas rapamycin only 2 out of 36 proteins of the cytokine array in C20 GAM (Fig. 5). Comparing the transcriptome (C20: Figs. 3, 4, HMC3: Figs. S5, S6) and cytokine array protein data (C20: Fig. 5, HMC3: Fig. S7), rapamycin-induced enrichment for certain gene sets including Hallmark_IL6_JAK_STAT3_signaling (Fig. 4) could be confirmed by an increased IL-6 cytokine protein level in the GAM condition in C20 cells (Fig. 5B, C). Comparatively minor effects were observed in the HMC3 model (Fig. S7). Rapamycin-induced enrichment of other inflammatory gene sets or immunogenic pathways in GAM in C20 or HMC3 was not traceable in the human cytokine array except for an upregulation of IL-8 in C20 (Fig. 5B, C). However, cytokines corresponding to a portion of the gene sets regulated by rapamycin on the transcriptional level (Fig. 4) could be detected in GAM and/or MG following treatment with torin2, e.g. TNF- α or IFN- γ signaling (Fig. 5B, C). When comparing effects in a microglia mono-culture (MG model) rapamycin and torin2 induced the same cytokines. Both in C20 as well as HMC3

cells these included macrophage migration inhibitory factor (MIF) as well as Serpin E1/PAI-1 (Figs. 5C; S7B).

Discussion

Our study demonstrated a regulation of key immunogenic pathways in GAM by pharmacological mTORi with rapamycin and torin2 in a human cell line co-culture model. These findings are in line with the study by Dumas *et al* (43) and other pre-clinical glioma models (41,42), which showed that mTORi in GAM-microglia reshaped the GB microenvironment towards a pro-inflammatory and anti-tumorigenic state with effects on survival in glioma tumor models. Mechanistically, mTORi interfered with a GB cell-mediated activation of mTOR in GAM microglia which otherwise promoted an immunosuppressive phenotype that hampered the anti-tumor adaptive immune response (43). Of note, mTOR inhibition has also been reported to exert anti-inflammatory effects on MG, e.g. in the context of stroke or aging (37-39). Therefore, our observed pro-inflammatory effects in GAM-microglia might

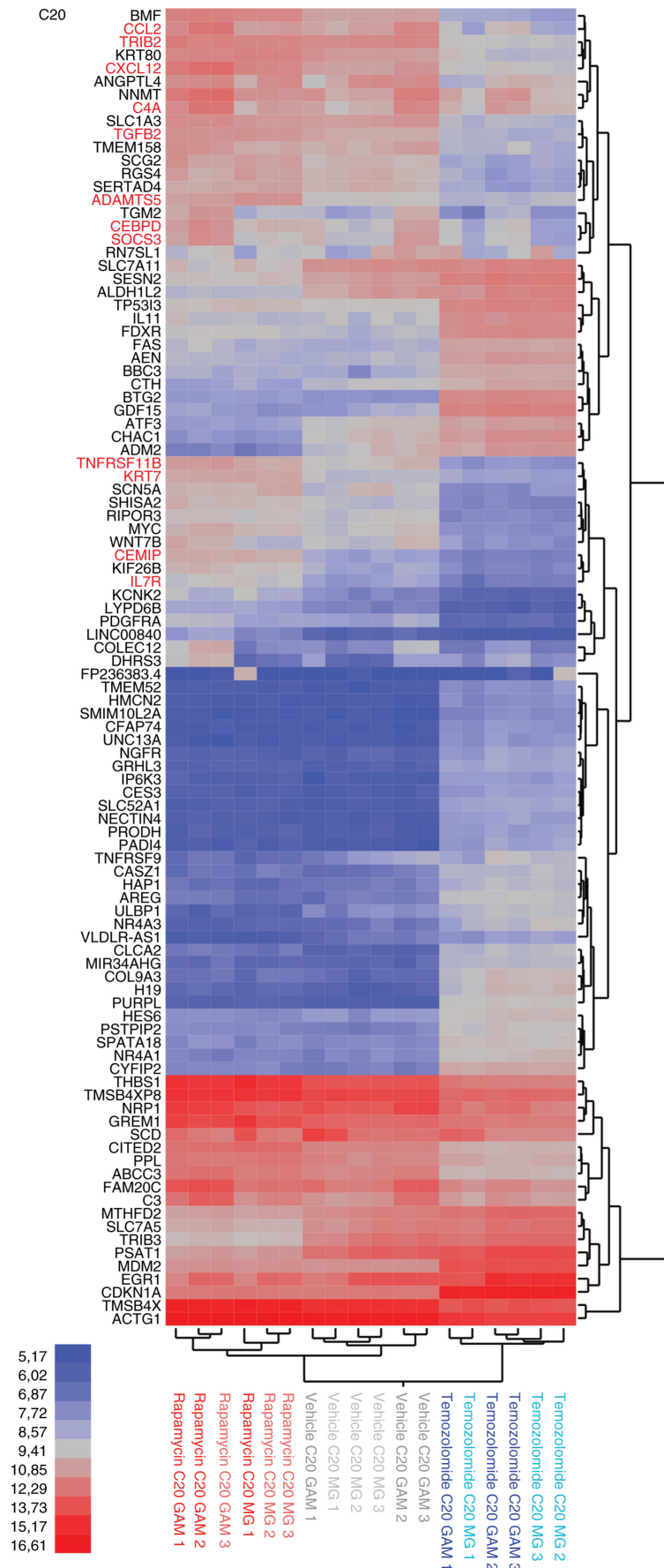


Figure 3. Differential expression of genes in GAM following rapamycin and temozolomide treatment. Heatmap depicts hierarchical clustering of the top 100 PCA gene loadings in C20 GAM (co-cultivated) or C20 MG (C20 monoculture control condition) following pharmacological mTORi with 100 nM rapamycin versus vehicle control or treatment with 400 μ M temozolomide versus vehicle control. Variance-stabilized transformed data values are shown. Genes involved in inflammatory processes were highlighted in red. GAM, glioma-associated microglia/macrophages; PCA, principal component analysis; MG, microglia; mTORi, mTOR inhibition.

C20

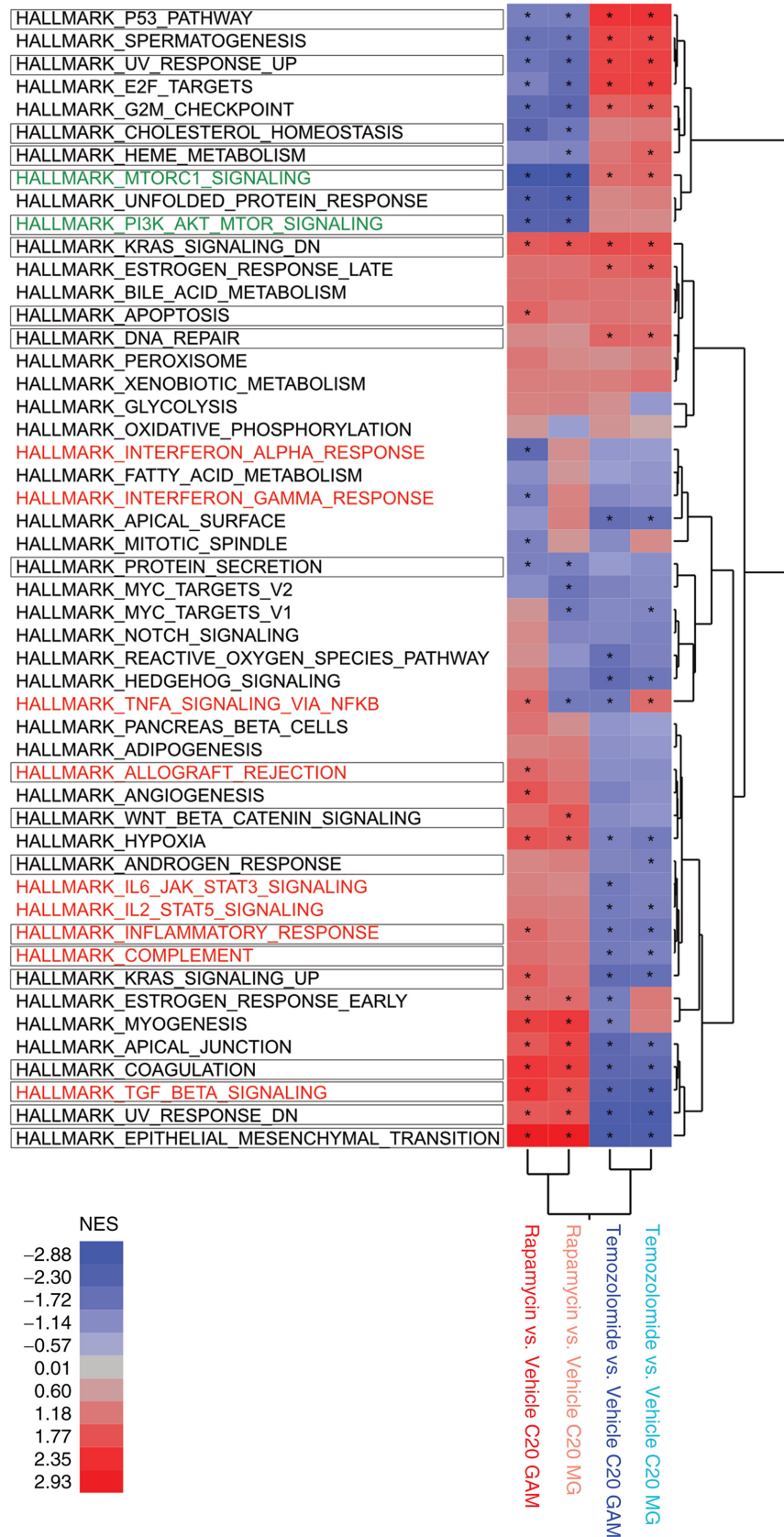


Figure 4. Impact of rapamycin and temozolomide on key gene programs in GAM. Gene set enrichment analysis was performed to decipher gene programs most relevant to the respective culture and treatment conditions. Heatmap of normalized enrichment score of the hallmark gene sets (v7.4) in C20 GAM (co-cultivated) or C20 MG (C20 monoculture control condition) following pharmacological mTORi with 100 nM rapamycin versus vehicle control or treatment with 400 μ M temozolomide versus vehicle control. Normalized enrichment scores are shown. A significance level of $*P < 0.05$ was depicted. Hallmark gene sets involved in key immunogenic pathways were highlighted in red and for mTORC1 signaling in green. Hallmark gene sets displaying the same regulation pattern under rapamycin in C20 and HMC3 (Fig. S6) were highlighted with squares. GAM, glioma-associated microglia/macrophages; MG, microglia; mTORC1, mTOR complex 1; mTORi, mTOR inhibition.

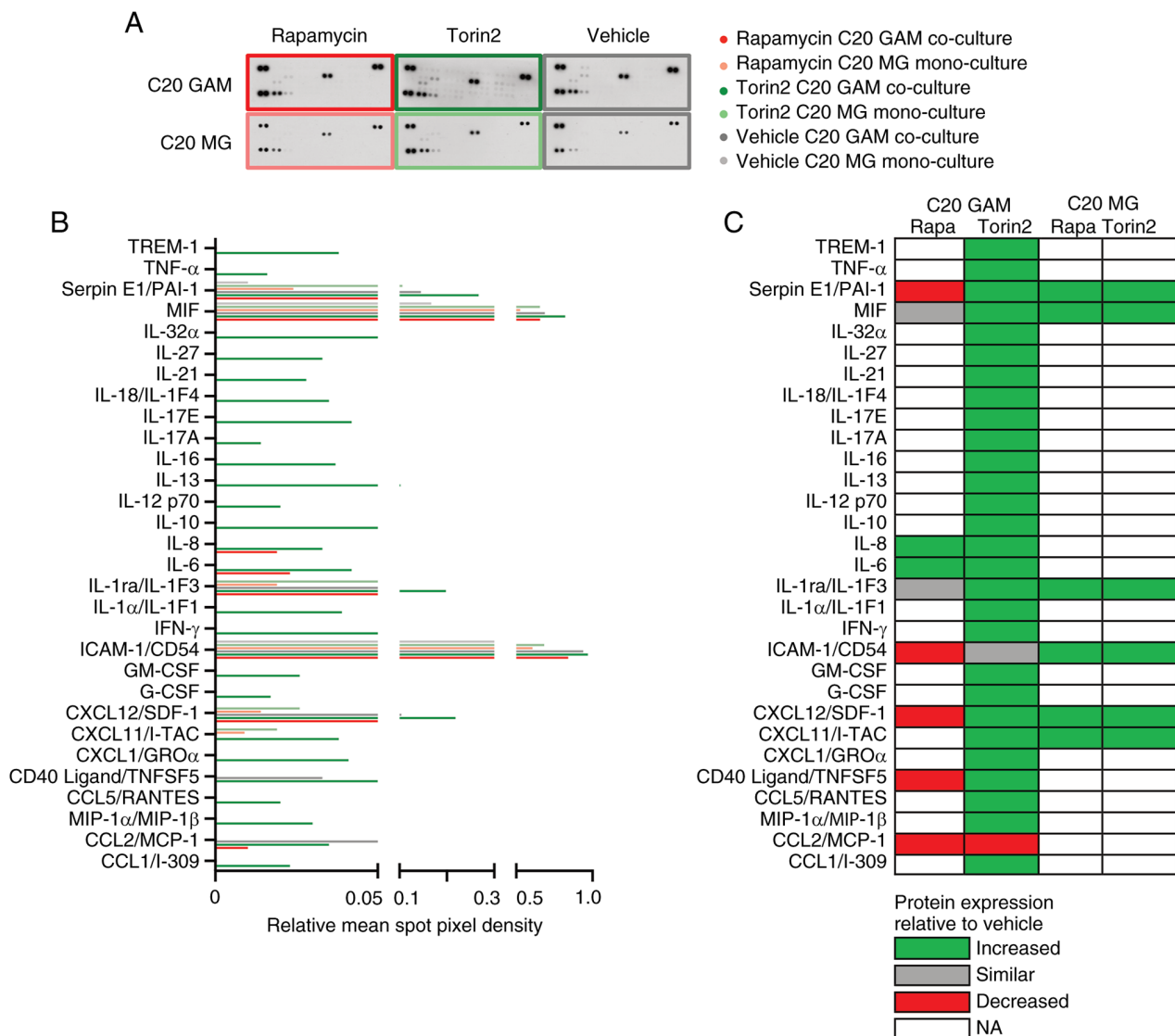


Figure 5. Impact of pharmacological mTOR inhibition on cytokine expression profiles of GAM. (A) Immunodetection of 36 different cytokines (each in duplicate) per pooled sample (pool of n=3) incubated with rapamycin, torin2 or vehicle. (B) Quantification by relative mean spot pixel density for each cytokine was depicted. (A and B) Bright colors, GAM co-culture of C20 with LNT-229; light colors, MG mono-culture control condition; red, treatment with 100 nM rapamycin; green, 100 nM torin2; grey, vehicle control. (C) Overview of protein expression in the treatment conditions rapamycin and torin2 compared to vehicle control in C20 GAM and C20 MG, respectively. Green, increased; grey, similar; red, decreased; no color, not analyzable/detectable. GAM, glioma-associated microglia/macrophages; MG, microglia.

depend on the cellular microenvironment or disease model which needs to be considered when evaluating mTORi as a therapeutic. Our previous study identified the GB immune microenvironment as a potential factor relevant for the response to therapies targeting EGFR/mTOR signaling in human GB patients (26). Analyses of the GB patient cohort of the OSAG 101-BSA-05 trial revealed that higher levels of GAM in the initial treatment-naïve tissue were associated with improved OS in patients treated with the anti-EGFR antibody nimotuzumab (26), despite the overall negative outcome of the study (of a non-biomarker selected study population) (56). These findings (26,43), together with our present study, support the hypothesis that GAM could pose a co-target for therapeutic mTORi in GB.

In our study, we investigated the effects of pharmacological mTORi on GAM and observed overall largely similar outcomes with both the first generation mTORC1 inhibitor rapamycin and

the more potent second-generation ATP-competitive mTOR inhibitor torin2, which targets both mTORC1 and mTORC2. Because mTOR besides protein translation affects various other cellular processes including autophagy and metabolism a longer incubation period was chosen to allow cellular processes to reach their new equilibrium. Both inhibitors had similar effects on basal cellular functions, such as reduced proliferation and oxygen consumption, also comparable to previous results from mTORi treatment in glioma cells (22,25), but divergent from effects on immune cells, like T cells that have been shown to increase oxidative phosphorylation under rapamycin treatment (57). Our transcriptome analysis revealed a distinct GAM phenotype triggered by rapamycin-mediated mTORi, which differed from the profile of temozolomide that-expectably for a chemotherapeutic agent-mainly triggered cell death and cell cycle related alterations. Interestingly, at the transcriptional level rapamycin treatment led to an

upregulation of pro-inflammatory genes and key immunogenic pathways in the GAM co-culture setting (for example in C20 and HMC3 Hallmark_TNFA_SIGNALING_VIA_NFKB) as well as more general in the GAM and MG setting (for example in C20 the Hallmark_IL6_JAK_STAT3, summarizing the transcriptional response driven by IL-6 signaling via the JAK/STAT3 pathway), but also exclusively in the C20 MG mono-culture setting (for example Hallmark_INTERFERON response genesets). Of note, there was a substantial overlap in the top regulated genes of C20 and HMC3 cells as well as in the differential gene expression and GSEA analyses of both cell lines. The overall pro-inflammatory effect of mTOR inhibition on the GAM and MG profile was supported by protein level data, which revealed an increase in pro-inflammatory cytokines following mTORi treatment with rapamycin and torin2 treatment. On protein level, the strongest and most complex effects were observed in the C20 GAM setting treated with torin2, where many pro-inflammatory proteins were upregulated. Taken together, the torin2-induced microglial profile in especially C20 GAM, and less pronounced C20 MG, indicates an activated, mostly pro-inflammatory microglial state important for immune cell recruitment (via chemokines) and modulation of the immune microenvironment (via for example IL-1 β , TNF- α , IFN- γ , but also IL-10, IL-1ra, Serpin E1, MIF). C20 GAM were characterized by the expression of chemotactic chemokines (CCL1/I-309, MIP-1 α (CCL3), CCL5/RANTES, CXCL1/GRO α , CXCL11/I-TAC or CXCL12/SDF-1), co-stimulatory/immune regulation ligands (CD40 Ligand/TNFSF5, TREM-1), colony-stimulating growth factors (G-CSF and GM-CSF), type II cytokines/interferon (IFN- γ), pro-inflammatory interleukins (IL-1 α , IL-6, IL-8, IL-12p70, IL-17A, IL-17E, IL-18, IL-21, IL-27, IL-32 α), interleukins linked to T cell differentiation (IL-13, IL-16), regulatory or mixed interleukins (IL-1ra (IL-1F3), IL-10), pro-inflammatory mediators linked to invasion and migration (MIF, Serpin E1 (PAI-1) as well as TNF- α as a central mediator of inflammation. Beyond this strong torin2-induced upregulation of pro-inflammatory cytokines in the C20 GAM co-culture setting, torin2 also induced several, though not all, pro-inflammatory cytokines in the C20 MG mono-culture. In contrast, rapamycin showed a partially divergent effect in the C20 GAM co-culture setting compared to the C20 MG mono-culture setting: Despite a generally pro-inflammatory signature (e.g., IL-6 and IL-8 upregulation), rapamycin-treated C20 GAM also displayed a reduced expression of several cytokines compared to the vehicle control, suggesting a dampened expression of some cytokines under tumor cell influence. This divergence between C20 MG mono- and GAM co-cultures was also apparent in the transcriptomic data, with interferon α/γ response genes upregulated only in mono-cultures. Notably, cytokines reduced by rapamycin in C20 GAM, such as ICAM-1 and CD40L, are interferon-associated, therefore supporting concordant transcriptomic and proteomic changes. Also, the rapamycin-induced activation of the Hallmark_IL6_JAK_STAT3_signaling pathway in C20 GAM and MG was supported by increased IL-6 and IL-8 protein levels. However, some immunogenic pathways enriched at the transcriptomic level did not show matching protein changes under rapamycin, while related cytokines such as TNF- α and IFN- γ were detected in GAM and/or MG after torin2 treatment.

Interestingly, in our *in vitro* model, MIF exhibited high baseline levels in both MG and GAM of C20 and HMC3, even under untreated (vehicle) controls and mTOR inhibition further increased MIF expression in the HMC3 GAM co-culture setting as well as the C20 microglia mono-culture and GAM co-culture setting. In HMC3 GAM, rapamycin appeared to exert even stronger effects than torin2 on the cytokines MIF and Serpin E1/PAI-1. In contrast, in C20 GAM, torin2 led to a marked upregulation of both cytokines compared to control conditions. However, rapamycin treatment in C20 GAM showed lower levels of Serpin E1 and relatively unchanged MIF expression. Notably, elevated levels of GAM subtype expressing the MIF receptor CD74 were associated with prolonged OS in patients with IDH-wildtype GB (12). In this regard, it is important to mention that the dichotomic categorization of macrophages as either pro-tumor M1 or anti-tumor M2 (58) has been challenged in microglia and especially in the brain tumor immune microenvironment. Over the past decade, complex GAM phenotypes with simultaneous expression of pro- and anti-inflammatory cytokines have been identified and extensively studied (8-13). Overall, our data suggest that torin2 more effectively modulates the inflammatory GAM and MG phenotype than rapamycin, which may have implications for the use of its derivatives, such as temsirolimus, currently under investigation in the N2M2 trial (28).

Pro-inflammatory effects of mTOR inhibition in GAM could be highly relevant, considering the increasing importance of immunotherapeutic approaches in GB treatment (59-62) that may be more effective in a pro-inflammatory and anti-tumorigenic state of the innate GB microenvironment. However, despite the intriguing findings in our GAM model that provided a consistent and standardized experimental framework, our study is limited by its *in vitro* nature and the use of immortalized microglia cell lines. Both the C20 and the HMC3 cell line display overall high similarities but also show distinct context-dependent differences and therefore offer distinct advantages and limitations for studying various aspects of microglial biology that researchers should be aware of. For example, a recent comparative study analyzing morphology, proteome, and secretome of HMC3 and C20 revealed both shared and distinct responses to inflammatory stimuli (LPS or IFN- γ). While the baseline proteome of HMC3 was described as more transcriptionally and metabolically active, the C20 proteome was interpreted as displaying a higher phagocytic capacity. Upon inflammatory stimulation, HMC3 activated immune and metabolic pathways, whereas C20 activated mitochondrial besides immune pathways. Both cell lines secreted IL-6 under LPS treatment (63). For our experimental setting in the context of GAM, we considered the newer C20 line, derived from adult human cortical microglia via magnetic cell sorting, a more physiologically suitable model than the HMC3 cell line, derived from human embryonic microglial cells and modified via SV40-dependent immortalization. Therefore, the HMC3 cell line was primarily used as a secondary validation model to support the reproducibility of key findings. The C20 microglia cell line showed a broader and more robust response to the mTOR inhibitors, whereas the effects observed in HMC3 cells were less pronounced but remained largely consistent with those seen in C20 cells. For example, in the cytokine expression analysis torin2 treatment of C20 cells (co-cultured

with GB cells) yielded 30 different cytokines while in HMC3 cells only two of the 36 covered cytokines of the assay were regulated. Although other genetic models (43) support our findings, potential off-target effects of mTOR inhibitors cannot be fully excluded and represent a limitation of our experimental setting. In this regard, rapamycin is considered an uncommonly specific inhibitor due to its association with the intracellular adaptor FKBP12 which then allosterically inhibits mTORC1. Torin2 was developed as an ATP-competitive inhibitor and off-target effects are more likely. Torin2 has been shown to potentially interfere also with signaling from phosphoinositide 3-kinase (PI3K) however with an approximately 800-fold lesser selectivity than for cellular mTOR. Additionally, torin2 can interfere with signaling from the kinases ATM, ATR and DNA-PK, however, again with reduced selectivity in comparison to mTOR (64). Thus, while the major effects of torin2 observed in our study are most likely due to on-target mTOR inhibition, some minor contribution of other kinases cannot be ruled out. Also, treatment durations in our study were chosen to approximate steady-state cellular adaptation and mimic continuous exposure to the substances (21,25). Nonetheless, shorter or extended treatment periods, not assessed here, may provide further mechanistic insights. Therefore, future studies should incorporate genetic models and rescue experiments as well as a broader variety of treatment durations to confirm the specificity of the observed effects. Dumas *et al* (43) supported their preclinical models employing an *in silico* analysis in which they could demonstrate a correlation of enhanced mTOR signaling in GAM-microglia with reduced effector immune cell infiltration. Unfortunately, a similar approach was not feasible in our experimental setting due to a lack of GAM-microglia specific transcriptome data in publicly available datasets of human GB patients as well as no *in vivo* datasets of human GB samples under mTORi treatment.

Given the recent report on a potential therapeutic activity of temsirolimus in mTOR-activated GB in the N2M2 trial (65), human GB tissue samples of responders vs. non-responders as well as of recurrent tumors after mTORi treatment might become available in the near future. The analysis of such samples with a focus on tumor-intrinsic and microenvironmental features, particularly GAM, would represent an ideal setting to validate both our *in vitro* findings and the effects observed in animal models (43). Ideally, such analyses could be incorporated into future prospective studies.

In conclusion, our findings support targeting of mTOR signaling in GAM to promote a favorable state within the GB microenvironment as a therapeutic approach. Notably, innovative techniques for even a cell-type specific delivery of therapeutic agents are currently under development (66). Furthermore, our results stress the importance of a comprehensive understanding of therapy effects in the GB microenvironment which is essential for optimizing the design of treatment combinations and identifying specific patient subgroups that are more likely to benefit from both established and novel targeted therapies. Given that mTORC1 activation in bulk tumor tissue is estimated in approximately 35% of cases (28,32), a significant proportion of GB patients might be candidates for mTOR inhibitor therapy. Eagerly awaited initial results of the N2M2 trial reported a benefit from treatment with mTOR inhibition in mTOR-activated GBs (65)

emphasizing the clinical need for a deeper understanding of mTOR-mediated effects in the GB immune microenvironment.

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Availability of data and materials

Bulk RNA sequencing data have been deposited to the NCBI Gene Expression Omnibus and can be accessed via accession no. GSE242829 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE242829>). The rest of the data generated in the present study may be requested from the corresponding author

Authors' contributions

PSZ, JPS, PNH, MWR conceived and designed the study. PSZ, MS, JS, JBW, NIL, BS, BR, KJW, ALL, AB, KHP, LS, JPS, MHM, PNH and MWR acquired and analyzed the data. PSZ and MS confirm the authenticity of all the raw data. PSZ, MS, JPS, MHM, PNH and MWR drafted the manuscript. All authors read, reviewed and edited the manuscript and approved the final version.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

JPS has received honoraria for lectures, advisory board participation, consulting or travel grants from Abbvie, Roche, Boehringer, Bristol-Myers Squibb, Medac, Mundipharma, Servier and UCB. MHM is meanwhile an employee of Sanofi. MWR has received a research grant from UCB as well as honoraria for advisory board participation from Alexion and Servier. PSZ, MS, JS, NIL, JBW, BS, BR, KJW, ALL, AB, KHP, LS and PNH report no disclosures relevant to the manuscript.

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