

Research Article

Metabolic and inflammatory reprogramming of macrophages by ONC201 translates in a pro-inflammatory environment even in presence of glioblastoma cells

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Tumor-associated macrophages facilitate tumor progression and resistance to therapy. Their capacity for metabolic and inflammatory reprogramming represents an attractive therapeutic target. ONC201/TIC10 is an anticancer molecule that antagonizes the dopamine receptor D2 and affects mitochondria integrity in tumor cells. We examined whether ONC201 induces a metabolic and pro-inflammatory switch in primary human monocyte-derived macrophages that reactivates their antitumor activities, thus enhancing the onco-toxicity of ONC201. Contrary to glioblastoma cells, macrophages exhibited a low ratio of dopamine receptors D2/D5 gene expression and were resistant to ONC201 cytotoxicity. Macrophages responded to ONC201 with a severe loss of mitochondria integrity, a switch to glycolytic ATP production, alterations in glutamate transport, and a shift towards a pro-inflammatory profile. Treatment of macrophages–glioblastoma cells co-cultures with ONC201 induced similar alterations in glutamatergic and inflammatory gene expression profiles of macrophages. It induced as well metabolic changes and a pro-inflammatory switch of the co-culture milieu. However, these changes did not translate into increased onco-toxicity. This study provides the first evidence that ONC201 affects macrophage immunometabolism and leads to a pro-inflammatory tumor environment. This speaks in favor of implementing ONC201 in combinatorial therapies and warrants further investigation of the mechanisms of action of ONC201 in macrophages and other immune cells.

Keywords: glioblastoma · immunometabolism · ONC201 · tumor-associated macrophages · tumor microenvironment



Additional supporting information may be found online in the Supporting Information section at the end of the article.

Introduction

Macrophages are very plastic cells that react to external signals by a fast reprogramming at the genetic and metabolic

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levels, thus facilitating an adequate answer to a changing environment [1]. Macrophages responding to pathogens or pro-inflammatory molecules activate a transcriptional program leading to an increased expression of pro-inflammatory genes and a switch from mitochondrial oxidative phosphorylation (OXPHOS) to glycolysis. Macrophages involved in tissue homeostasis and repair exhibit an anti-inflammatory profile and rely mostly on OXPHOS [2]. Macrophages present in solid tumors undergo reprogramming toward a “homeostatic” profile [3]. As a result, these tumor-associated macrophages (TAM) lose their cytotoxic capacities and gain tumor-supportive functions that largely contribute to tumor growth [4]. Considering their involvement in tumor biology and their capacity to rapidly change their transcriptional and functional profiles, TAMs represent a promising target for cancer therapy.

Glioblastoma, the most aggressive form of brain tumors, host a large number of blood-derived macrophages [5]. Together with microglia, the brain resident macrophages, they constitute a sub-population of cells that actively promote tumor growth [6]. Various *in vitro* and *in vivo* strategies have addressed the feasibility to mitigate the tumor-supportive functions of these cells [7]. We demonstrated that human microglia/macrophages isolated from freshly resected glioblastoma could be reprogrammed by stimulating their Toll-like receptor 3 and consequently exerted anti-proliferative, anti-migratory, and cytotoxic activities, *in vitro*. This reprogramming however was inhibited by co-cultured glioblastoma cells, indicating the difficulty to translate such an approach to therapy [8]. An alternative strategy would consist in targeting the energy metabolism of macrophages. Metabolic adaptation is part of their plasticity and goes together with changes in their inflammatory profiles and functions [4]. TAMs are exposed to metabolic conditions shaped by tumor cells that exhibit strong metabolic alterations [9]. In glioblastoma, such a metabolic condition consists *i.a.* in elevated concentrations of extracellular glutamate [10] resulting from changes in the glutamate metabolism of the tumor cells [11]. We have reported alterations in the expression of glutamatergic genes in human tumor-associated microglia/macrophages and monocyte-derived macrophages exposed to tumor cells [12]. Moreover, recent data, including our own, suggest a connection between the glutamate metabolism and the inflammatory status of macrophages [13, 14].

Considering the potent role of metabolism on the inflammatory and functional status of macrophages and the metabolic changes occurring in tumor cells, metabolic reprogramming represents an extremely attractive therapeutic strategy. Recently, the small molecule ONC201/TIC10 has emerged as a very valuable drug in cancer therapy and has been included in clinical trials for glioblastoma [15–18]. ONC201 works as an antagonist of the dopamine receptor D2 (DRD2) [19] and as a ligand for the mitochondrial ClpP protein [20, 21]. Moreover it induces TRAIL [22] through activation of the transcription factor ATF4 [23]. Recent studies report that ONC201 affects mitochondria integrity [24] and induces a metabolic reprogramming in glioblastoma cells *in vitro*, leading to anti-proliferative and anti-migratory effects [25, 26]. This toxicity seems to be tumor cell-specific since human

astrocytes [25] or monocyte-derived macrophages [27] have been reported to be ONC201 resistant at doses that killed tumor cells. However, ONC201 certainly affects the metabolism of these non-transformed cells present in and around the tumor mass. The impact of these alterations on the efficacy of ONC201 glioblastoma therapy has still to be elucidated.

Based on current data, we hypothesized that ONC201 has the potential to exert its activity in glioblastoma not only by targeting tumor cells, but also by inducing a metabolic switch in TAMs. This would invoke their pro-inflammatory activities, supporting and/or enhancing ONC201 cytotoxicity to tumor cells. To test this hypothesis, we first characterized the response of human monocyte-derived macrophages to ONC201 by analyzing their resistance to the drug, alterations in their glutamate- and energy-metabolism, and their inflammatory status. We then treated co-cultures of monocyte-derived macrophages and tumor cells from three different glioblastoma-derived cell lines with ONC201. We evaluated the degree of ONC201 cytotoxicity on both cell types and monitored inflammatory and metabolic changes in macrophages and the co-culture environment. With this study, we provide, to the best of our knowledge, the first analysis of the effects of ONC201 on the immunometabolism of monocyte-derived macrophages and its relevance to glioblastoma therapy.

Results

Macrophages are resistant to ONC201 and show a low DRD2/DRD5 ratio

The sensitivity of human primary monocyte-derived macrophages (thereafter referred to as macrophages) to ONC201, was tested and compared to the sensitivity of three human glioblastoma (GB) cell lines. Normal human astrocytes were included as nontransformed counterpart of GB cells. We treated cells with increasing concentrations of ONC201 and monitored their viability (Figure 1A; Supporting Information Figure S1A). As expected, ONC201 induced a strong time- and concentration-dependent cytotoxic effect on the three GB cell lines. It had a lower effect on astrocytes, detectable from day 3 and only at the highest doses (50% viable cells at 2.5 μ M and 5.0 μ M). Macrophages were very resistant, with 80% or more viable cells at the latest time point and highest drug concentration.

Macrophage low sensitivity to ONC201 cytotoxicity might be related to their expression of the dopamine receptors D2 (DRD2) and D5 (DRD5). The ratio of DRD2/DRD5 gene expression is reported to be an indicator of tumor cell sensitivity to ONC201 [28]. Therefore, we examined DRD2 and DRD5 gene expression in untreated cells and calculated the corresponding ratio (Figure 1B). Astrocytes expressed very low levels of both receptors and were used for normalization of the data. Each GB cell line expressed higher levels of DRD2 than macrophages and astrocytes which showed comparable levels. Astrocytes (non-normalized data, not shown) and GB cells also expressed a higher level of DRD2 than DRD5, whereas the contrary was observed

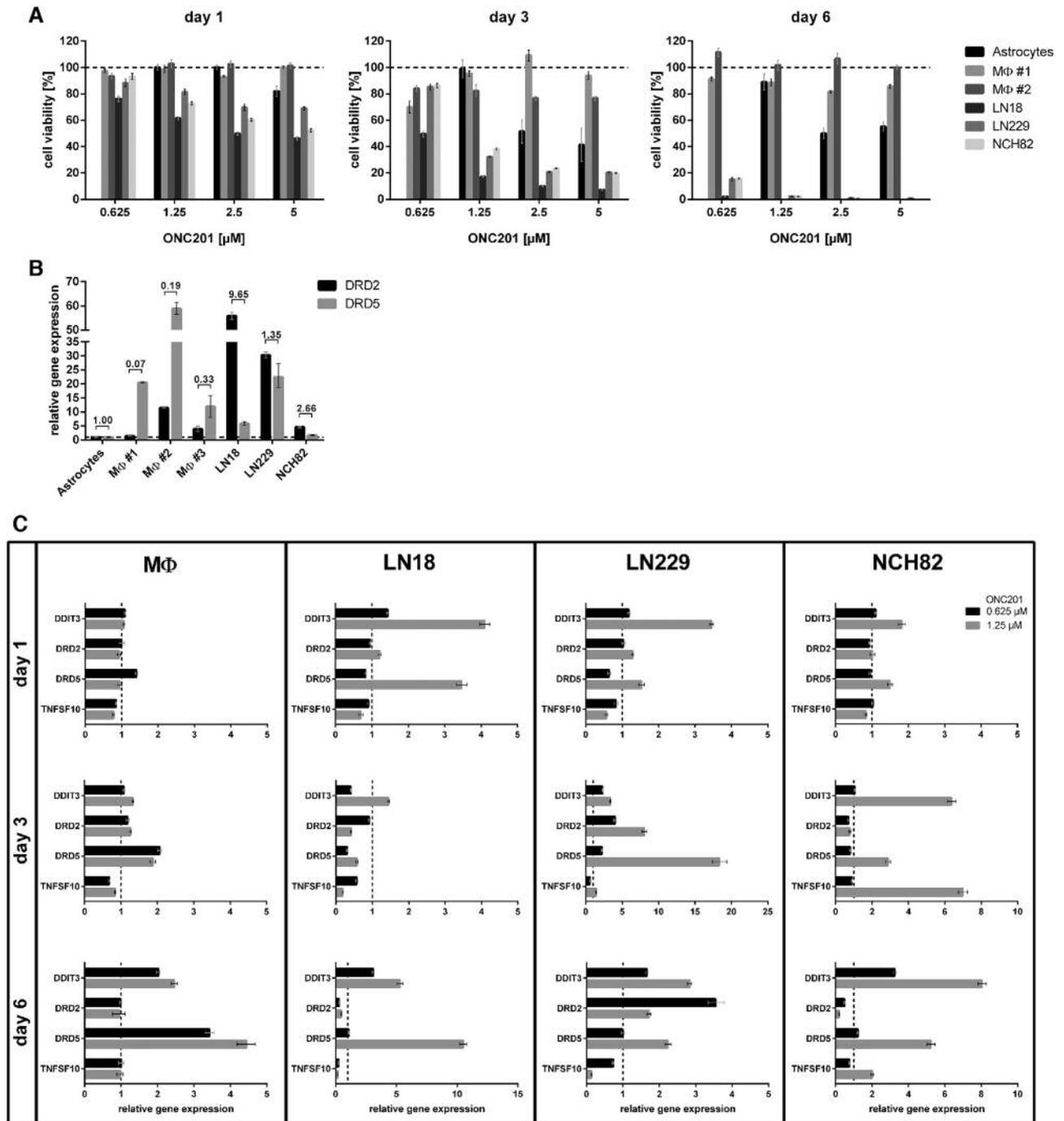


Figure 1. Sensitivity of primary astrocytes, primary macrophages and glioblastoma cell lines to ONC201. (A) Cell viability of the primary cells and GB cell lines LN18, LN229, NCH82 was determined using PrestoBlue reagent after treatment with ONC201. Note that astrocytes were not tested at 0.625 μ M. Values are normalized to the respective DMSO treated controls (dashed line). Two different preparations of primary macrophages (M Φ #1, M Φ #2) were used. Data are given as mean \pm SEM of ten technical replicates from one experiment. (B) Ratio of the relative gene expression levels of DRD2 and DRD5 in primary macrophages and GB cells. Values are normalized to the expression in primary astrocytes (dashed line). Three different preparations of primary macrophages (M Φ #1, M Φ #2, M Φ #3) were used. Data are given as mean \pm SEM of three technical replicates from one experiment. (C) Relative gene expression levels of DDIT-3, DRD2, DRD5, and TNFSF10 in primary macrophages and GB cells after ONC201 treatment. Values are normalized to the gene expression of the respective DMSO-treated controls (dashed line). Data are given as mean \pm SEM of three technical replicates. Macrophages: data are from one experiment representative of three independent experiments (each performed with one macrophage preparation). GB cells, one experiment. Relative gene expression in (B) and (C) was determined by qPCR as described in *Materials and methods*.

for macrophages. In line with published observations, a comparison of the calculated *DRD2/DRD5* ratios with the cells' sensitivity to ONC201 indicated that sensitivity positively correlated with this ratio. Thus, macrophages, the most resistant cells, exhibited the lowest *DRD2/DRD5* ratio whereas the most ONC201-sensitive LN18 cells showed the highest *DRD2/DRD5* ratio. In conclusion, macrophages, as well as astrocytes, are more resistant to ONC201 than GB cells. This resistance correlates with their expression of a low *DRD2/DRD5* ratio.

The titration experiment (Figure 1A) indicated that ONC201 concentrations up to 1.25 μM induced a negligible cytotoxicity in macrophages and astrocytes but were highly toxic to the three GB cell lines. Since higher ONC201 concentrations did not trigger a dramatic increase of GB cell death, further characterization of ONC201 activity on macrophages was performed with concentrations of 0.625 and 1.25 μM ONC201.

ONC201 increases *DDIT-3* and *DRD5* expression in macrophages and GB cells

Macrophage low sensitivity to ONC201 could also reflect a deficiency of ONC201 in triggering signaling pathways in these cells. As an antagonist of *DRD2*, ONC201 induces early activation of the integrated stress response, leading to upregulation of *DDIT-3* (DNA damage-inducible transcript 3 protein—also known as CHOP) and of *TNFSF10* (Tumor necrosis factor ligand superfamily member 10 - also known as TRAIL) [23, 29]. Silencing of *DRD2* is reported to lead to an increase in *DRD5* expression [30], an interesting observation regarding the opposed effects of the signaling cascades activated by both receptors [31]. We examined *DDIT-3*, *DRD2*, *DRD5*, and *TNFSF10* expressions at three time points after ONC201 addition to the cells (Figure 1C). In macrophages, an increased *DRD5* expression was detected at day 3 and enhanced at day 6. Changes in *DDIT-3* expression were weak at day 3 but a clear increase was detectable at day 6. Changes in *DRD2* and *TNFSF10* expression levels were minimal. Contrary to its effect on macrophages, ONC201 affected gene expression levels in each GB cell line at an earlier time point and in a concentration-dependent manner. At day 1, ONC201 increased *DDIT-3* and *DRD5* expression in each GB cell line, with only minor changes in *DRD2* and decreased *TNFSF10* expression in two of the three GB lines. From day 3 on, *DDIT-3* and *DRD5* expression continued to increase whereas *DRD2* expression decreased in LN18 and NCH82 cells and increased in LN229 cells. The expected induction of *TNFSF10* expression was observed only in NCH82 cells. Altogether, this gene expression analysis indicates that macrophages, similar to GB cells, react to ONC201 by activating the integrated stress response (*DDIT-3* increase) and by modulating the expression of *DRD5*.

ONC201 inhibits OXPHOS in macrophages and induces loss of mitochondrial integrity

We next assessed ONC201 effects on macrophage metabolism. In a first step, we determined the total ATP production rate

after treatment with the highest ONC201 concentration (5 μM). ONC201 induced a time-dependent reduction of mitochondrial ATP production and an increase of glycolytic ATP production, starting at day 3 (Figure 2A). We next analyzed the mitochondria status after three days of treatment with increasing ONC201 concentrations. Each tested ONC201 concentration led to increased proton leakage and strong reductions of the maximal and basal respiration, the non-mitochondrial oxygen consumption, the ATP production, and the coupling efficiency (Figure 2B, Supporting Information Figure S2). Reduction of mitochondrial ATP production was dose-dependent whereas the increase in glycolytic ATP production did not significantly change at each ONC201 concentration tested (Figure 2C). In conclusion, ONC201 induces a time- and concentration-dependent switch to glycolysis, concomitant to a severe loss of mitochondrial integrity in macrophages. These results suggest that ONC201 might activate the mitochondrial caseinolytic protease P (ClpP) and its signaling [20, 21] in macrophages.

ONC201 affects glutamate transport in macrophages and GB cells

To further investigate the effects of ONC201 on macrophage energy metabolism, we examined the expression profile of genes coding for transporters of glucose and glutamate. Both are critical metabolites of the TCA cycle and glutamate is specifically relevant in the context of glioblastoma. We analyzed *SLC2A1*, coding for glucose transporter type 1, as an indicator for glucose uptake; *SLC7A11*, coding for the Cystine/glutamate transporter, exporting glutamate in exchange for cystine and known to be upregulated through ATF4 [32]; and *SLC1A2* coding for a major transporter for glutamate uptake (Excitatory amino acid transporter 2) that likely counteracts the effects of *SLC7A11* on glutamate transport. As shown in Figure 3A, expression of *SLC2A1* was not or only slightly affected, suggesting that ONC201 did not impact glucose import in macrophages and GB cells. This lack of effect might reflect a high basal level of the receptor expression as a consequence of culture in high glucose conditions, thus prohibiting further upregulation of *SLC2A1*. On the contrary, ONC201 affected the gene expression of the two glutamate transporters, though to various extents, in both cell types. *SLC1A2* expression increased in LN18 from day 1 on, but decreased in all other cells at day 6. The most striking effect was observed on *SLC7A11*. ONC201 treatment increased its expression in each cell type. This increase, together with *DDIT-3* increase, are clear hints of ATF4 activation by ONC201.

To assess the relevance of altered *SLC1A2* and *SLC7A11* expressions to glutamate transport, we determined the amount of extracellular glutamate in the cell supernatants. Untreated macrophages accumulated much less extracellular glutamate than the three GB cells (Supporting Information Table S1). As shown in Figure 3B, macrophages responded to treatment with a significant ONC201-concentration-dependent increase of extracellular glutamate at day 6. GB cells on the contrary displayed an increased

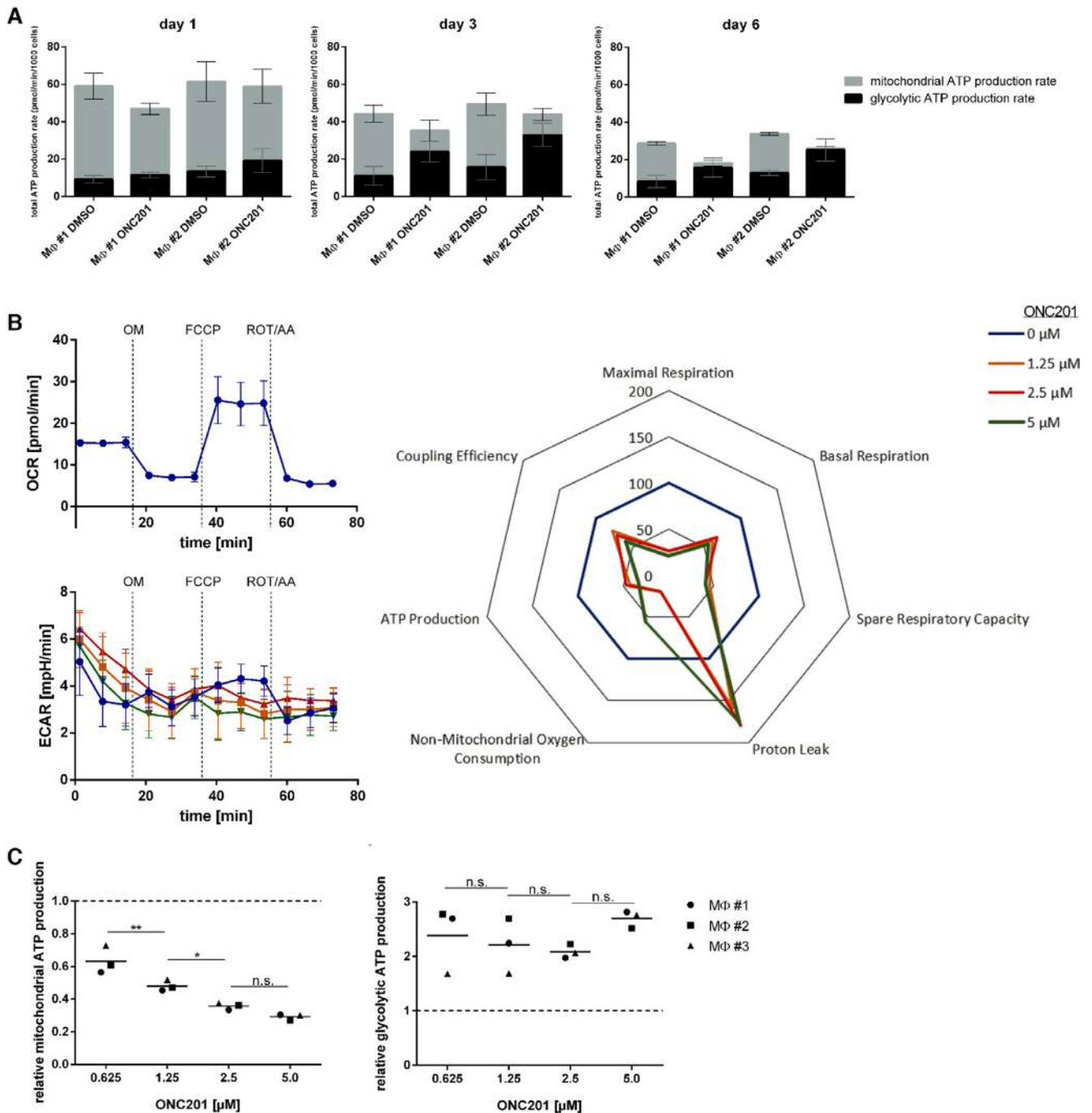


Figure 2. ONC201 reduces mitochondrial integrity and increases glycolytic ATP production rate. ATP production rates were determined by extracellular flux analysis using the Real-Time ATP Rate assay. (A) Mitochondrial and glycolytic ATP production rates of two independent macrophage preparations (MΦ #1, MΦ #2) after treatment with 5 μM ONC201. Data are given as mean ± SEM of four technical replicates from one experiment. (B) Mito Stress Tests: macrophages were treated with indicated doses of ONC201 for three days. Left: OCR and ECAR were calculated based on extracellular flux analysis of these cells sequentially treated with oligomycin (OM), FCCP, and rotenone (ROT) plus antimycin A (AA). Right: Calculated mitochondrial parameters are shown relative to those in DMSO treated macrophages. Data are given as mean ± SEM of four technical replicates and are from one experiment representative of two independent experiments. (C) Relative mitochondrial (left) and glycolytic (right) ATP production by macrophages after three days of treatment with ONC201. Data are from three independent experiments (macrophage preparations MΦ #1, MΦ #2, MΦ #3). Values are normalized to the respective DMSO-treated controls (dashed line) and represent the mean of two independent measurements for each biological sample run in four technical replicates. * $p \leq 0.05$, ** $p \leq 0.01$, n.s. = non-significant (two-way ANOVA corrected for multiple comparisons using Tukey test).

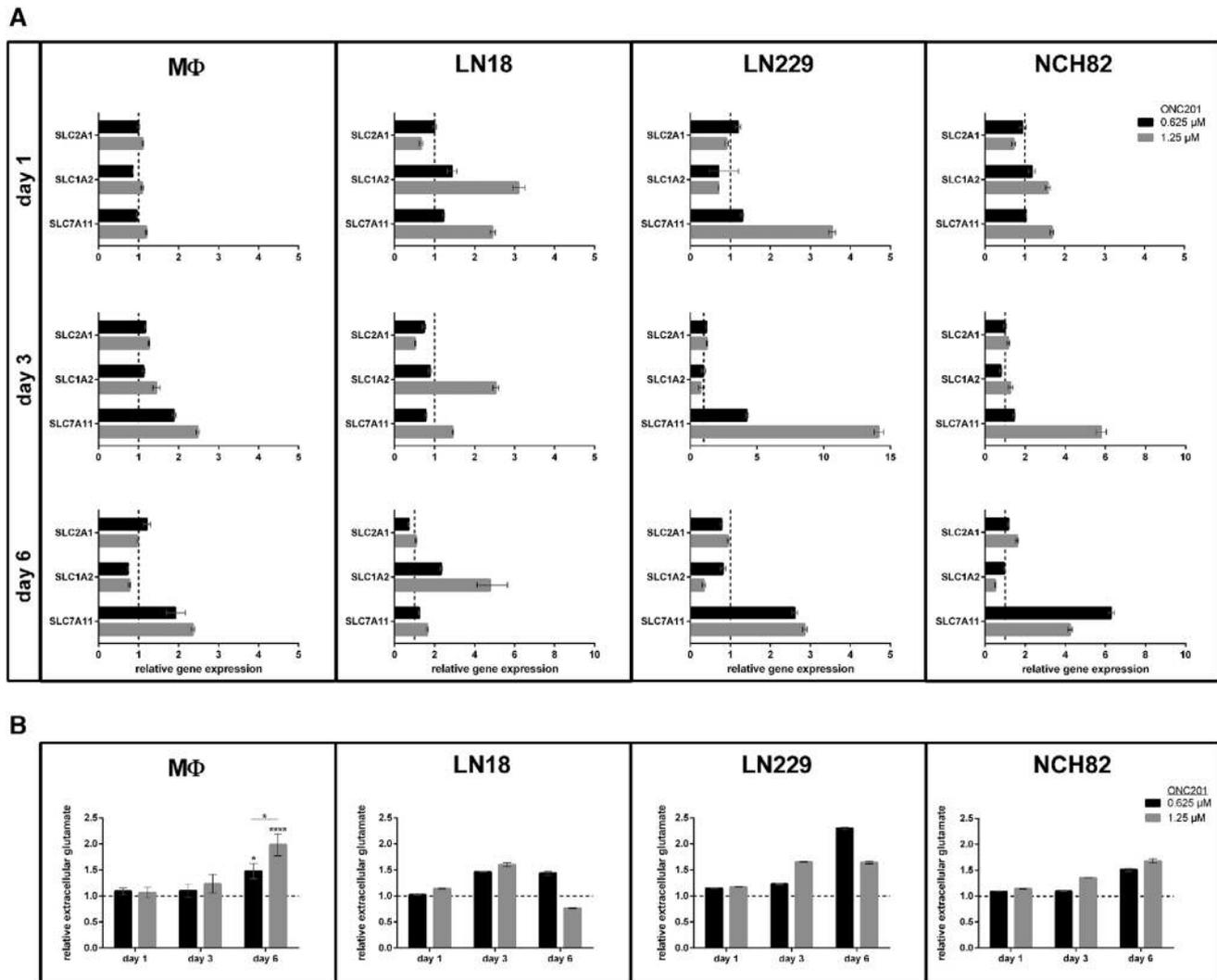


Figure 3. ONC201 induces changes in extracellular glutamate concentrations and expression of metabolic genes. (A) Relative expression levels of *SLC2A1*, *SLC1A2*, and *SLC7A11* were determined by qPCR in ONC201-treated macrophages and GB cells. Values are normalized to the gene expression of the respective DMSO-treated controls (dashed line). Data are given as mean \pm SEM of three technical replicates. Macrophages, two independent experiments; GB cells, one experiment. (B) Extracellular glutamate concentrations of macrophages and GB cells treated with ONC201 were determined with the Glutamate-Glo™ Assay. Data are given as mean \pm SEM of two technical replicates. Macrophages, six independent experiments; GB cells, one experiment * $p \leq 0.05$, **** $p \leq 0.0001$ (two-way ANOVA corrected for multiple comparisons using Tukey test).

amount of glutamate from day 3 of treatment on. The results are in line with the increased expression of *SLC7A11* and the partly decreased expression of *SLC1A2*. Note that the high rate of cytotoxicity in GB cells (Figure 1) possibly explains their lower glutamate concentrations at day 6. Altogether, these data indicate that ONC201 affects glutamate transport in macrophages and enhances its dysregulation in GB cells.

ONC201 induces a pro-inflammatory phenotype in macrophages

Macrophages switching to glycolysis are reported to adopt a pro-inflammatory profile [2]. We characterized the inflammatory sta-

tus of ONC201-treated macrophages by investigating the expression levels of the pro-inflammatory genes *IL1B* (Interleukin-1 β) and *TNF* (Tumor necrosis factor) and of the anti-inflammatory genes *CD163* (Scavenger receptor cysteine-rich type 1 protein M130) and *CD206* (Macrophage mannose receptor 1). As shown in Figure 4A, *IL1B* expression significantly increased after treatment with 1.25 μ M ONC201 for 6 days; the same trend (though not significant, $P = 0.0503$) was observed with 0.625 μ M ONC201. Expression levels of *TNF*, *CD163*, and *CD206* were not significantly affected at any time point tested. However, a dose-dependent trend could be detected at day 6 for these genes. *TNF* on the one hand side and *CD163* and *CD206* on the other hand side were more and less expressed, respectively. Together with alterations in *IL1B* expression, these results suggest the

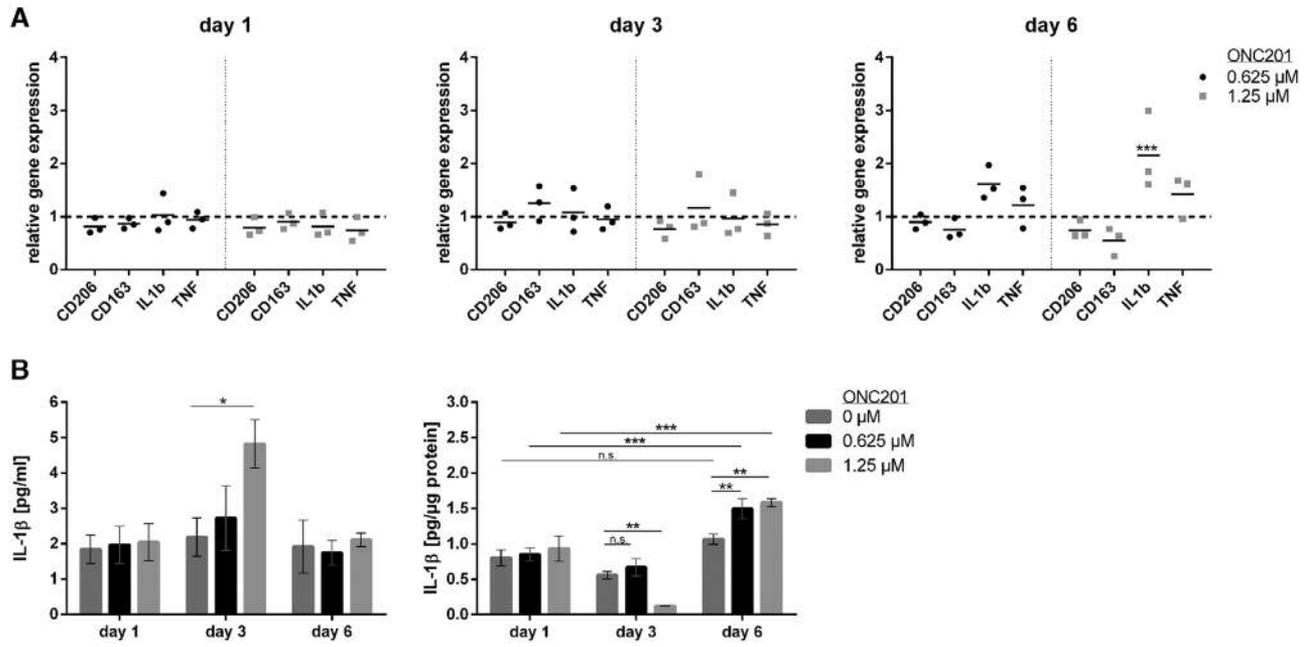


Figure 4. ONC201 induces a pro-inflammatory profile in macrophages. (A) Relative gene expression levels of *CD206*, *CD163*, *IL1B*, and *TNF* in ONC201-treated macrophages were determined by qPCR. Values are normalized to the gene expression of the respective DMSO-treated controls (dashed line). Data are given as mean \pm SEM of three technical replicates and are from three independent experiments. (B) Quantification of IL-1 β in the supernatants (left) and cell homogenates (right) of ONC201-treated macrophages using the IL-1 β Human ProQuantum Immunoassay Kit. Values of intracellular IL-1 β are normalized to the total amount of protein used for analysis. Data are given as mean \pm SEM of three technical replicates and are from three independent experiments. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ (two-way ANOVA corrected for multiple comparisons using Tukey test).

acquisition of a pro-inflammatory profile. It is worth mentioning that treatment of macrophages with ONC201 in the presence of pro- or anti-inflammatory molecules induced a time-dependent shift towards a pro-inflammatory profile (Supporting Information Figure S3).

To confirm the effect of ONC201 on *IL1B* and *TNF* expressions, cells and supernatants were tested for the presence of the respective proteins (Figure 4B). *TNF- α* was detected neither in the supernatants nor in the cells. At day 3, treatment with 1.25 μ M ONC201 led to an increased amount of extracellular IL-1 β that correlated with a decreased intracellular amount, suggesting IL-1 β release from an intracellular pool. At day 6, the intracellular level of IL-1 β increased significantly at both ONC201 concentrations while the extracellular level was back to day 1 levels. Furthermore, a comparison of the intracellular levels at day 1 and day 3 with those at day 6 in treated macrophages, indicates an increase in intracellular IL-1 β . Together with the increased gene expression observed in these conditions (Figure 4A), these data suggest not only a replenishment but also an increase of the intracellular IL-1 β pool in macrophages. To conclude, the gene and protein expression analyses suggest that ONC201 shifts macrophages towards a pro-inflammatory phenotype.

Data reported in Figures 1–4 indicate that ONC201 induces a metabolic and pro-inflammatory shift in macrophages. We next investigated whether this profile could be induced by ONC201 in macrophages co-cultured with tumor cells and thus support or even increase the anti-tumor activities of ONC201.

ONC201 activates the DRD2 signaling cascade in macrophages co-cultured with GB cells

We investigated whether co-cultured macrophages react to ONC201 by activating the integrated stress response and by modulating the expression of *DRD5*. Co-cultures of macrophages with each GB cell line were incubated in the absence or presence of ONC201 for 3 and 6 days. We did not test for day 1 because of the lack of visible change in the monocultures at that time. A comparative analysis of the Δ CT values obtained from qPCR analyses of mono- and co-cultured macrophages indicated that, in the presence of GB cells, macrophages decreased their basal expression of *DDIT-3* and increased *DRD5* and *TNFSF10* expression (data not shown). The addition of ONC201 to the co-cultures induced concentration- and time-dependent alterations in the expression levels of the four analyzed genes in macrophages (Figure 5). Similar tendencies were detected irrespective of the GB cell line used. As shown for macrophages in monoculture (Figure 1C), ONC201 increased *DDIT-3* and *DRD5* expression levels. Increase in *DDIT-3* expression was however detected earlier in co-cultured macrophages, possibly due to their lower basal *DDIT-3* expression. Of note, expression of the down-stream target of ATF4, *SLC7A11*, was consistently increased on days 3 and 6 (Figure 6B). *DRD2* and *TNFSF10* expression was slightly enhanced on day 3 but returned to basal levels on day 6, except for *TNFSF10* expression in macrophages co-cultured with NCH82 cells. These latter alterations were not observed in macrophages in monoculture. Altogether these data indicate that the presence of GB cells

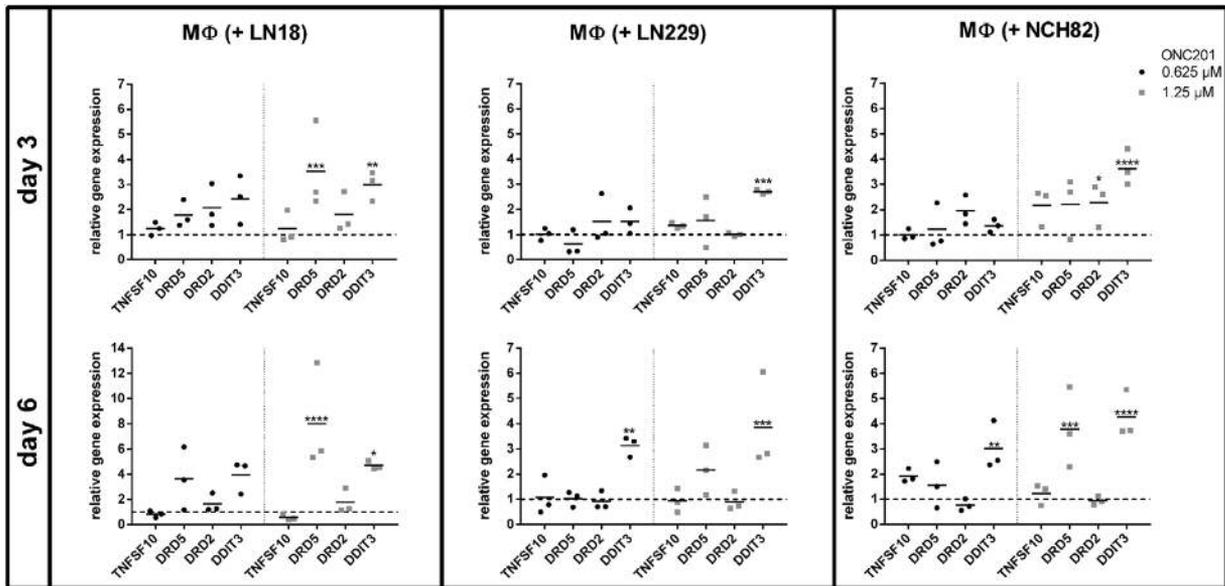


Figure 5. Expression of *TNFSF10*, *DRD5*, *DRD2*, and *DDIT3* in primary macrophages co-cultured with the respective GB cell line and treated with ONC201. Preparations of macrophages from three different donors were used for co-cultures with GB cells in three independent experiments. Gene expression in macrophages was determined by qPCR. Values are normalized to the gene expression of the respective DMSO-treated controls (dashed line). Data are given as mean \pm SEM of the three biological replicates. Each biological replicate was run in three technical replicates. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$ (two-way ANOVA corrected for multiple comparisons using Tukey test).

did not prevent macrophages to activate the *DRD2* signaling cascade in response to ONC201.

ONC201 induces metabolic changes in macrophages/GB cells co-cultures

Technical limitations prevented us to monitor ONC201 effects on energy metabolism at the cell level in the co-culture. We therefore took advantage of the ratio of extracellular lactate and pyruvate that is described as a reliable parameter for estimating the energy state of cells [33]. ONC201 treatment increased this ratio, suggesting that a metabolic switch to glycolysis took place or was enhanced in either macrophages and/or GB cells after treatment (Figure 6A).

We next evaluated changes in glucose and glutamate transport by analyzing *SLC2A1*, *SLC1A2*, and *SLC7A11* expression in macrophages and by measuring the concentration of extracellular glutamate released by the co-cultured cells. As shown in Figure 6B, *SLC2A1* expression was slightly increased at day 3, though non significantly except for the LN229 co-cultures. Similarly, *SLC1A2* expression showed only slight changes at day 3 but not at day 6. As already observed in the monocultures, the clearest effect of ONC201 on co-cultured macrophages was on *SLC7A11* expression which increased in a concentration- and time-dependent manner irrespective of the GB cell line used (Figure 6B). The amount of extracellular glutamate measured at day 3 positively correlated with the increasing concentrations of ONC201 (Figure 6C). At day 6, we observed an ONC201 concentration-dependent decrease of extracellular glutamate in

LN18 and NCH82 co-cultures, whereas glutamate levels continued to increase in LN229 co-cultures. Comparison of glutamate concentrations measured in monocultures and co-cultures suggests that most of the glutamate was contributed by GB cells. As shown in Supporting Information Table S1, untreated monocultured macrophages released up to 50 μM glutamate and monocultured GB cells between 100 and 700 μM whereas concentrations in the co-culture supernatants amounted to approximately 50 to 500 μM depending on the GB cell line. This, moreover, indicates a net decrease in the amount of extracellular glutamate that accumulates in co-cultures.

In conclusion, ONC201 induced a global glycolytic shift and altered glutamate transport also in the co-cultures.

Cytotoxicity of ONC201 and inflammatory status of macrophages/GB cells co-cultures

The results above confirmed that ONC201 activated the integrated stress response and induced transcriptional changes in macrophages as well as metabolic changes in the co-cultures. We next assessed whether these changes support an increased cytotoxicity towards GB cells. As shown in Figure 7A and Supporting Information Figure S1B, we observed a trend for GB cells sensitivity to ONC201 in the presence of macrophages which was very similar to that observed in absence of macrophages (Figure 1A, Supporting Information Figure S1A). LN18 cells were the most sensitive to ONC201 cytotoxicity, followed by NCH82 and LN229 cells that were the most resistant. These two latter were even more resistant to ONC201 in the

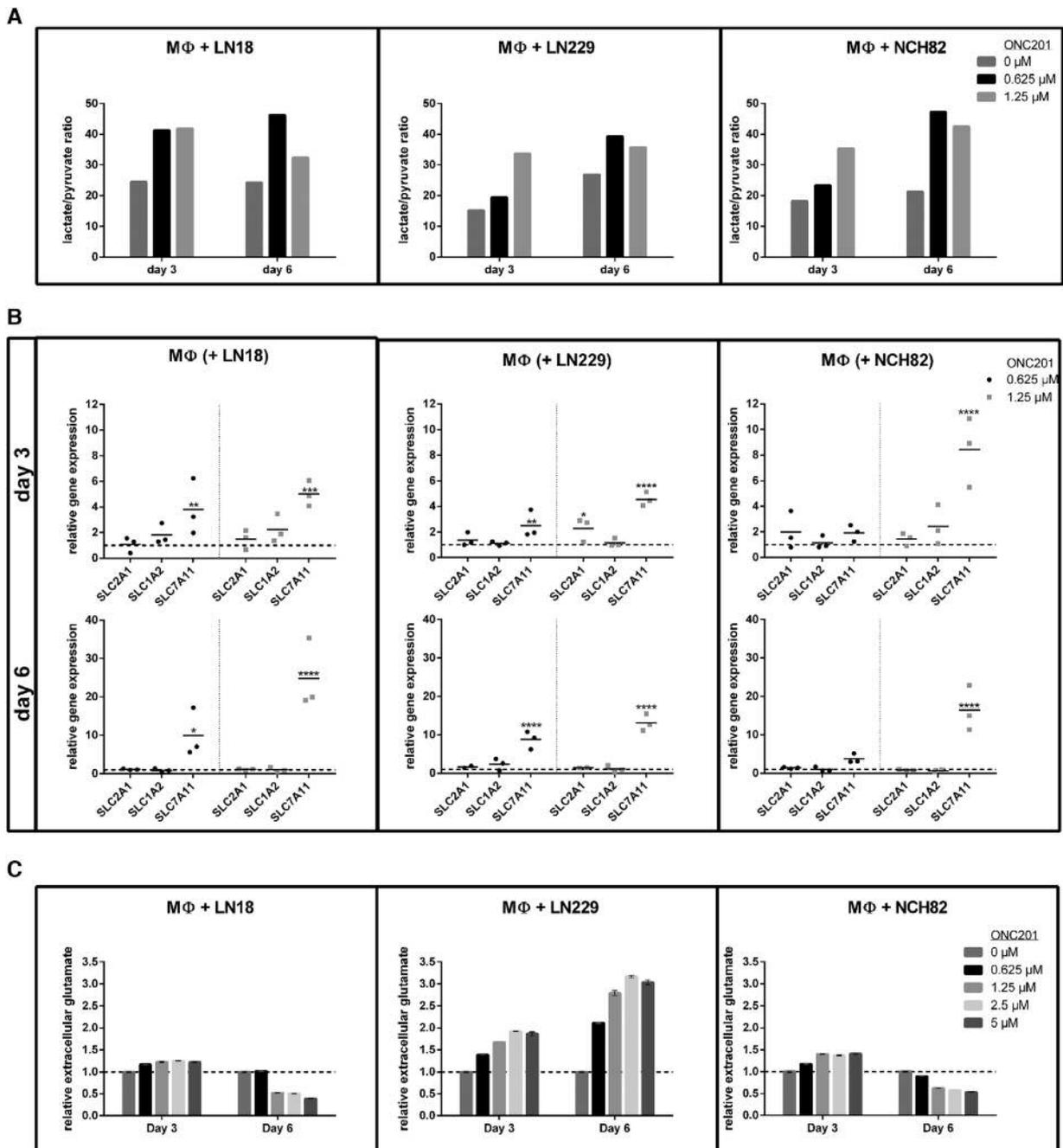


Figure 6. ONC201 induces changes in the energy and glutamate metabolism of co-cultured cells. Supernatants (A, C) and cells (B) harvested from the co-cultures (three independent experiments; same as in Figure 5) were analyzed as follows. (A) The supernatants of the three independent co-cultures were pooled, lactate and pyruvate were measured (technical replicate = 1) and the lactate/pyruvate ratio determined. (B) Relative gene expression levels of SLC2A1, SLC1A2, and SLC7A11 were measured in macrophages by qPCR. Values are normalized to the gene expression of the respective DMSO-treated controls (dashed line). Data are given as mean \pm SEM of three biological replicates. Each biological replicate was run in three technical replicates. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$ (two-way ANOVA corrected for multiple comparisons using Tukey test). (C) Extracellular glutamate concentration was determined in the pooled supernatants (same as in panel A) with the Glutamate-Glo™ Assay. Data are given as mean \pm SEM of two technical replicates; one measurement was performed.

co-culture as indicated by a higher viability, whereas LN18 cells were still efficiently killed, except at the lowest dose (0.625 μ M) of ONC201. Actually, dying LN18 cells most likely triggered the decrease in macrophages viability observed on day 6. Altogether the results reported in Figure 7A indicate that the pres-

ence of macrophages was not increasing the efficacy of ONC201 treatment.

We next assessed the inflammatory profile of the co-cultured macrophages. Gene expression analysis indicated a general trend of reduced levels of the anti-inflammatory markers *CD163* and

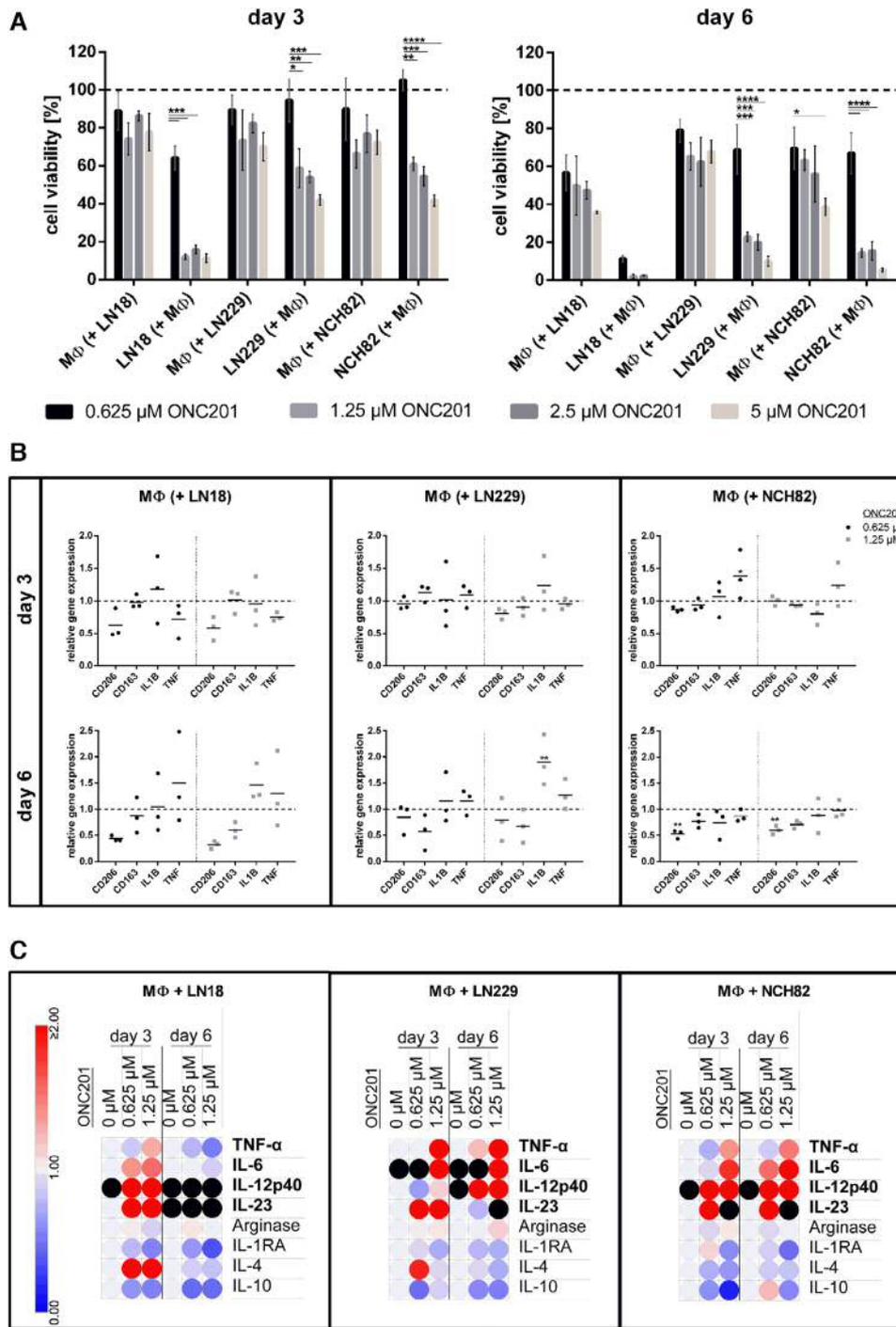


Figure 7. ONC201 induces glioblastoma cell death and a pro-inflammatory profile in the co-culture milieu. Cells and supernatants harvested from the co-cultures (three independent experiments; same as in Figure 5) were analyzed as follows. (A) Cell viability of the co-cultured cells after ONC201 treatment was analyzed with the PrestoBlue reagent. MΦ (+GB cell) = viability of macrophages co-cultured with the respective GB cell line; GB cell (+MΦ) = viability of the respective GB cell line co-cultured with primary macrophages. Values are normalized to the respective DMSO treated controls (dashed line). Data are given as mean ± SEM of the three biological replicates. Each biological replicate was run in three technical replicates. (B) Relative gene expression levels of CD206, CD163, IL1B, and TNF were determined by qPCR in macrophages co-cultured with the respective GB cell line and treated with ONC201. Values are normalized to the gene expression of the respective DMSO-treated controls (dashed line). Data are given as mean ± SEM of the three biological replicates. Each biological replicate was run in three technical replicates. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$ (two-way ANOVA corrected for multiple comparisons using Tukey test). (C) Inflammatory secretome of the co-cultures. The supernatants of the three co-cultures were pooled and analyzed for the indicated molecules with the LEGENDplex™ Human Macrophage/Microglia Panel and the Human ProQuantum Immunoassay Kit. One measurement was performed. Data (mean of two technical replicates ± SEM see Supplementary Table 2 for complete data) are expressed as a heat map (created with [50]). Pro-inflammatory cytokines are written in bold. Color-coding is by mean protein concentration relative to the concentration measured at 0 μM ONC201 at the respective day. In case that molecules were not detectable at 0 μM ONC201, threshold values were used to calculate relative changes. Black dot: not detectable. Measurement was performed once.

CD206 with a significant decrease for this latter in one co-culture. Changes in the expression of the pro-inflammatory markers *TNF* and *IL1B* were minor, with only one significant increase observed for each gene in one co-culture (Figure 7B). This observation suggests that ONC201 mitigates the anti-inflammatory status of macrophages in the presence of GB cells. We further analyzed the secretome of the co-cultures for a set of pro- and anti-inflammatory molecules. As shown in Figure 7C and in Supporting Information Table S2, treatment of each co-culture with ONC201

at 1.25 μM led to an increase in the extracellular concentration of the pro-inflammatory cytokines *TNF-α*, *IL-6*, and *IL-12p40* and a concomitant decrease of the anti-inflammatory cytokines *IL-1RA*, *IL-4*, and *IL-10*. These changes were stable over the period of time analyzed except for the pro-inflammatory cytokine *IL-23* in which production was concentration-dependent. ONC201 treatment did not affect the amount of extracellular arginase, an enzyme that can be produced both by immune and tumor cells and is associated with an anti-inflammatory environment (Supporting

Information Table S2) [34]. IL-1 β , which was secreted by ONC201-treated macrophages (Figure 4B), was not detected in the supernatants of the co-cultures. The consistent shift towards a pro-inflammatory milieu, irrespective of the GB cell line used, was not observed with the lower concentration of ONC201. Note that the cytokine decrease observed at day 6 in the supernatants of co-cultures with the LN18 cells likely results from the decreased number of LN18 cells (see Figure 7A).

Altogether these data suggest that treatment of macrophages/GB cells co-cultures with ONC201, despite inducing a pro-inflammatory shift of the co-culture milieu and decreasing the macrophage anti-inflammatory profile, does not result in an increased onco-toxicity.

Discussion

We hypothesized that ONC201 not only affects GB cells viability but also stimulates the anti-tumor activities of macrophages, by inducing a metabolic and inflammatory switch. We indeed observed that ONC201 reprograms macrophages, as single cells or in co-culture with GB cells. Whereas this reprogramming does not lead to an improved elimination of glioblastoma cells, it contributes to a pro-inflammatory shift of the tumor environment that might provide appropriate conditions for the recruitment and activation of additional immune cells.

Two main modes of action for ONC201-induced cytotoxicity have been described, the most studied being through the antagonism of the DRD2 dopamine receptor leading to TRAIL-dependent or independent cell death [19, 28, 30]. Irrespective of the cell type we analyzed in this study, the cell's sensitivity to ONC201 cytotoxic effects positively correlated with their ratio of *DRD2* and *DRD5* gene expression, not with changes in *TNFSF10* expression. These data are in accordance with reported observations [24, 28] and contribute further support to the use of the *DRD2/DRD5* ratio as an indicator for cell's sensitivity to ONC201 cytotoxicity.

The second mode of action consists in ONC201 binding to and activating the mitochondrial caseinolytic protease P (ClpP) [20, 21], consistent with its targeting of mitochondria [24]. ClpP activation leads to mitochondrial dysfunctions, including inhibition of oxidative phosphorylation. Various facts speak in favor of ClpP activation in ONC201-treated macrophages. A glycolytic switch and severe loss of mitochondria integrity are detected after 3 days of treatment, concomitantly to an increase in *SLC7A11* expression and before an increase in *DDIT-3* expression. These two genes are direct targets of ATF4 of which expression has been reported to increase after ClpP activation [21]. ATF4 thus likely activates *SLC7A11* and *DDIT-3* transcription in macrophages, and might do so in GB cells as well. We hypothesize that macrophages, given their low level of *DRD2* expression, predominantly respond to ONC201 with a mitochondrial stress which, at the ONC201 concentrations used in this study, does not affect -or only minimally- their survival but affects their metabolic and inflammatory status.

Concomitant to the severe loss of mitochondrial integrity, ONC201 induces a time- and concentration-dependent switch to a glycolytic ATP production. This switch is accompanied by alterations in the expression levels of glutamatergic and inflammatory genes. These alterations lead to changes in amounts of extracellular glutamate and cytokines produced by treated macrophages, resulting in a pro-inflammatory profile of macrophages. Whereas the glycolytic switch might be the direct consequence of the integrated stress response induced by ONC201 and inactivation of Akt, a hub for metabolic and inflammatory pathways [35], it might as well be a compensatory mechanism to ensure ATP production in absence of functional mitochondria. Furthermore, it likely triggers the pro-inflammatory profile [2]. Further experiments would be needed to define the cause-to-effect relationship between these events.

The inflammatory reprogramming induced by ONC201 in macrophages might not only rely on the glycolytic switch. CHOP, the product of the *DDIT3* gene is involved in triggering apoptosis and inflammation [36–38]. According to our results, CHOP is more likely to have contributed to inflammation than cell death in ONC201-treated macrophages. It is worth noting that a late increase in *DDIT3* expression was observed in murine macrophages treated with the pro-inflammatory molecule LPS: in those conditions, macrophages did not die but activated the IL-1 β pathway [36]. Dopamine receptors are important players in the regulation of inflammation in the brain levels [39]. Recently, *DRD5* has been shown to inhibit NF- κ B activation and modulate levels of inflammation [40]. The increased *DRD5* expression induced by ONC201 might reflect the contribution of this receptor signaling in returning macrophages to homeostasis after ONC201 treatment.

Considering that glutamate transport, energy metabolism, and oxidative stress are closely linked [41], ONC201-treated macrophages might first react to the mitochondrial stress by increasing their intracellular glutamate pool to sustain ATP production via the TCA cycle and to generate glutathione for scavenging oxidative species. Excess glutamate however must be removed in time to guarantee homeostasis of the cells and this might be accomplished by the glutamate/cystine antiporter which gene (*SLC7A11*) expression is increased 3 days after ONC201 treatment.

Changes induced by ONC201 in glutamate import/export are also observed in GB cells. They occur from the first day of treatment, indicating a fast adjustment of the cell metabolism to ONC201-induced stress. The increased expression of *SLC7A11* and the extracellular accumulation of glutamate might indicate enhancement of their anti-oxidant response. ONC201-treated LN18 cells are the only cells to react with an increased *SLC1A2* expression. The transporter encoded by this gene facilitates uptake of glutamate but also aspartate which might both be used to fuel the TCA cycle. This increased *SLC1A2* expression is intriguing given its very low or even silenced expression in glioblastoma cells [42] and might indicate an unknown aspect of ONC201 activity.

Given the metabolic shift and pro-inflammatory response of macrophages to ONC201, we expected a decreased proliferation and/or increased death of GB cells in a co-culture model. This is not what we observed at the concentrations tested. A simple explanation might rely on the amount of ONC201 available to both cell types in co-culture. On the one hand side, GB cells that express higher levels of *DRD2* than macrophages might capture more ONC201 and thus mitigate the effects of ONC201 on macrophages. On the other hand side, macrophages, as cells endowed with a high endocytic capacity, certainly take up ONC201 and hence lower the dose that would have led to the death of GB cells if they would have been in monoculture. Whatever the explanation may be, the current data suggest that in presence of ONC201, macrophages do not support tumor growth as they do in its absence [6].

A very interesting effect of ONC201 in the co-culture is the dampening of the anti-inflammatory gene expression profile of macrophages and the sustained change of the extracellular milieu in metabolites and inflammatory molecules. It is beyond our capabilities to determine which cell type contributed to the development of the pro-inflammatory milieu that emerged after ONC201 treatment. IL-23, for instance, is reported to be a direct target of CHOP [37] which gene expression was observed to be upregulated in macrophages and GB cells after ONC201 treatment. ONC201 was reported to increase the secretion of IL-12p70 and TNF- α by colorectal carcinoma cell lines [43]. Thus, not only macrophages but also GB cells might secrete TNF- α after ONC201 treatment. These data indicate that ONC201 is a potentially very interesting modulator of the tumor environment as it can turn it into a pro-inflammatory milieu by targeting both tumor cells and macrophages, thus creating conditions for recruiting and activating other immune cells such as NK- and T-cells. Moreover, ONC201 might protect macrophages from being reprogrammed toward an anti-inflammatory status by GB cells. Indeed, loss of mitochondria integrity, which ONC201 triggers in these immune cells, has been reported to prevent reprogramming of pro-inflammatory macrophages towards an anti-inflammatory status [44].

Although these observations speak for the potential of ONC201 to activate macrophage anti-tumor activities, they emphasize its limitations in a multicellular environment and point to the need of combining it with other drugs. GB cells that resisted six days of treatment expressed a high level of *DRD5*, suggesting *DRD5* as a possible target for combinatorial therapy [45, 46]. The glycolytic switch and the correlated glucose uptake represent another interesting target. Pruss et al [45, 46] have demonstrated that combining ONC201 with 2-Deoxyglucose, an analogue of glucose that inhibits its uptake, increased the anti-tumor activities of ONC201. Inhibitors of glucose transporters have recently been shown to kill tumor cells expressing high level of *SLC7A11* [45, 46].

In this study, we have addressed ONC201 activities in an *in vitro* cellular system characterized by a high level of heterogeneity. As expected, primary macrophages exhibit inter-individual variations but mount a similar response to ONC201. This response

is differently affected by each of the three different GB cell lines, suggesting a context-dependent efficacy of ONC201. We provide first evidence for the potential of ONC201 to act on macrophage plasticity. How these changes operate in ONC201-treated macrophages and other immune cells is worth investigating to develop a more efficient therapeutic use of this drug for cancer therapy.

Material and methods

Monocyte isolation and differentiation into macrophages

Buffy coats were purchased from the Transfusion Center of the University Medical Center of the Johannes Gutenberg University (Mainz, Germany) and were obtained from anonymized healthy blood donors. All buffy coats used in this study are residual biological materials made available by the Transfusion Center to scientists on a randomized basis. Blood samples are collected and processed in accordance with the relevant German guidelines and regulations. Personal data is neither collected nor shared for this material.

Peripheral blood mononuclear cells were isolated from buffy coats using a Ficoll gradient. Monocytes were differentiated with 20 ng/ml macrophage-colony stimulating factor [M-CSF (Biolegend, San Diego, CA, USA)] for one week. Monocyte-derived macrophages were further cultured in absence of M-CSF for another week prior to experiment. A detailed description of the protocol is reported in [13]. At the end of these two weeks, cells displayed the typical morphology of macrophages. Flow cytometry indicated that more than 94% of the cells expressed the CD11b protein (data not shown). Average yield was 1×10^7 – 1.5×10^7 macrophages per preparation.

Culture of human primary astrocytes and glioblastoma cell lines

Primary astrocytes purchased from ScienCell (Carlsbad, CA, USA) were cultured at 37°C and 5% CO₂ in astrocyte medium (ScienCell) containing 2% heat-inactivated FCS (Sigma-Aldrich, St. Louis, MO, USA), 1% Astrocyte Growth Supplement (ScienCell) and 50 μ g/ml Gentamicin (Gibco Invitrogen, Carlsbad, California, USA). The glioblastoma cell lines LN18 and LN229 were purchased from American Type Culture Collection. The human primary glioblastoma cell line NCH82 was generated at the Department of Neurosurgery, Heidelberg University Hospital (Heidelberg, Germany) [47]. All glioblastoma cell lines were cultured in cDMEM [DMEM (Sigma-Aldrich), 10% heat-inactivated FCS, 2 mM L-Glutamine (Gibco Invitrogen), 50 μ g/ml Gentamicin] at 37°C and 5% CO₂.

In vitro cell culture and ONC201 treatment

Monoculture experiments: Cells were seeded in cDMEM in tissue culture (TC) vessels (Greiner Bio-One, Frickenhausen, Germany) and incubated for 24 h (37°C, 5% CO₂). At this time point, the medium was removed and replaced by low serum-containing cDMEM (1% FCS) supplemented with ONC201 (dissolved in DMSO; MedChemExpress, Monmouth Junction, NJ, USA) or the respective volume of DMSO (Sigma-Aldrich). Plates were further incubated for one to six days (37°C, 5% CO₂) before analysis. Low serum-containing cDMEM was used to decrease potential side effects of FCS components during treatment. One preparation of macrophages was used for one independent experiment unless otherwise stated.

Co-culture experiments: Glioblastoma cells were seeded in cDMEM onto TC inserts with 0.4 µm membrane (Sarstedt, Nümbrecht, Germany). Macrophages were seeded in cDMEM on 6-well plates. Glioblastoma cells and macrophages were incubated for 24 h (37°C, 5% CO₂). Thereafter, the medium was removed and replaced by low serum-containing cDMEM (1% FCS) supplemented with ONC201 or the respective volume of DMSO. Inserts were placed into the vessels containing macrophages and plates were incubated for three to six days (37°C, 5% CO₂) before analysis. Three independent co-culture experiments each using a macrophage preparation from a different donor, were conducted; each condition was tested in duplicate. Analysis of the cells and supernatants harvested from these co-cultures is presented in Figures 5–7, Supplementary Fig 1B and Supplementary Tables 1 and 2.

Determination of cell viability

Analysis with the Presto Blue reagent: after one to six days of treatment, PrestoBlue™ Cell viability reagent (ThermoFisher Scientific, Waltham, MA, USA) was added directly to the wells in the culture medium, according to manufacturers' instructions. After 30 min of incubation at 37°C (5% CO₂) fluorescence was measured using an Infinite® 200 PRO multiplate reader (Tecan, Männedorf, Switzerland) and cell viability calculated as described in the manufacturers' protocol.

Analysis with the crystal violet staining assay: after one to six days of treatment, cell culture supernatants were removed, cells washed with PBS and stained with crystal violet solution [0.1% crystal violet (Sigma-Aldrich) in 2% Ethanol (Carl Roth, Karlsruhe, Germany)] for 10 min at room temperature. After removal of the staining solution, cells were washed three times with H₂O and plates air-dried upside down. To solubilize the stain, 1% SDS (Carl Roth) was added and plates put on a shaker until the dye was completely dissolved. For quantification, absorbance was measured at 570 nm using an Infinite® 200 PRO multiplate reader (Tecan).

Extracellular flux measurements

Measurements of oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were performed using the XF96e Extracellular Flux analyzer (Agilent Technologies, Santa Clara, CA, USA). Macrophages were plated in cDMEM into XF96 (V3) polystyrene cell culture plates (Agilent Technologies). After 24 h, cells were treated with ONC201 for one to six days and incubated under standard conditions. Prior to performing the assay, the medium was exchanged with the XF assay medium [XF RPMI with 1 mM HEPES (Agilent Technologies) supplemented with 1 mM Pyruvate (Sigma-Aldrich), 2 mM L-Glutamine (Sigma-Aldrich), 10 mM Glucose (Sigma-Aldrich)]. Three baseline measurements were taken prior to addition of any compound and three response measurements were taken after addition of each compound. For Real-Time ATP Rate Assay, sequential injections of ATP Synthase inhibitor Oligomycin (final concentration 1.5 µM), Complex 1 Inhibitor Rotenone and Complex 3 inhibitor Antimycin A (each 0.5 µM) were applied. For Mito Stress Tests, Oligomycin (1.5 µM), Carbonyl cyanide-4 (trifluoromethoxy)phenylhydrazone (FCCP; 1 µM) and Rotenone/Antimycin A (0.5 µM each) were used. ECAR (mpH/min) and OCR (pmoles/min) are reported as absolute rates normalized against cell counts. Cell counts were obtained post flux measurements using the EarlyTox integrity assay (Molecular devices, San Jose, CA, USA) according to manufacturers' instructions. Cells were imaged with a SpectraMax MiniMax 300 Imaging Cytometer (Molecular Devices) using green (541 nm emission) and red (713 nm emission) channels. Cell nuclei were automatically identified by setting size and threshold for object identification in the red channel.

Analysis and quantification of extracellular metabolites and cytokines

Supernatants from the three co-cultures were pooled according to conditions and immediately centrifuged (10 min, 400 rcf, 4°C). Aliquots from each pooled supernatant were prepared. Aliquots saved for the determination of glutamate were diluted (1:40) in ice-cold PBS and stored at -80°C. Aliquots saved for the determination of extracellular lactate and pyruvate, and extracellular cytokines (see below) were stored undiluted at -80°C.

Concentration of extracellular glutamate in cell culture supernatants was determined with the Glutamate-Glo™ Assay (Promega, Madison, WI, USA). The assay was performed according to manufacturers' instructions.

Determination of extracellular lactate and pyruvate was adapted from Uran et al [48]. Supernatants were diluted with ultra-pure water 1:10 (v/v) and mixed with ice-cold methanol 1:4.5 (v/v). 50 µl of these extracts were mixed with 25 µl 140 mM 3-Nitrophenylhydrazine hydrochloride (Sigma-Aldrich), 25 µl methanol, and 100 µl 50 mM Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (Sigma-Aldrich) and incubated for 20 min at 60°C. Separation was carried out using an Acquity

H-class UPLC system coupled to a QDa mass detector (Waters, Milford, MA, USA) using an Acquity HSS T3 column (100 mm × 2.1 mm, 1.8 μm, Waters) heated to 40°C. Separation of derivatives was achieved by increasing the concentration of 0.1 % formic acid in acetonitrile (B) in 0.1% formic acid in water (A) at 0.55 ml/min as follows: 2 min 15% B, 2.01 min 31% B, 5 min 54% B, 5.01 min 90% B, hold for 2 min, and return to 15% B in 2 min. Mass signals for the following compounds were detected in single ion record mode using negative detector polarity and 0.8 kV capillary voltage: Lactate (224.3 *m/z*; 25 V CV) and pyruvate (357.3 *m/z*; 15 V).

The concentration of extracellular cytokines was determined as follows. Supernatants and cells were harvested from monocultures of macrophages after ONC201 treatment. Supernatants were centrifuged (10 min, 400 rcf, 4°C), aliquoted and stored at -80°C. Cells were lysed in Triton-X 100 buffer (150 mM NaCl [Carl Roth], 1.0% [v/v] Triton X-100 [Carl Roth], 50 mM Tris-HCl [Carl Roth], pH 7.4) supplemented with freshly added protease inhibitor [cOmplete, Roche Diagnostics GmbH, Mannheim, Germany] and their protein content measured with the bicinchoninic acid assay (ThermoFisher Scientific). After adjusting the protein concentrations to equal levels, the samples of the three preparations were pooled. IL-1β and TNF-α were measured in supernatants and cell lysates using Human ProQuantum Immunoassay Kits (ThermoFisher Scientific) according to manufacturers' instructions.

Co-culture supernatants were analyzed using the LEGENDplex™ Human Macrophage/Microglia Panel (BioLegend) and an Attune NxT Flow cytometer (ThermoFisher Scientific). The panel of analyzed cytokines is composed of IL-12p70, TNF-α, IL-6, IL-4, IL-10, IL-1β, Arginase, TARC, IL-1RA, IL-12p40, IL-23, IFN-γ, and IP-10. Since the concentrations of IL-4 and IL-10 were below the detection limit, we used the Human ProQuantum Immunoassay Kit (ThermoFisher Scientific) for quantification of these cytokines. The assays were performed according to manufacturers' instructions.

Total RNA isolation, cDNA transcription, and gene expression profiling

After PrestoBlue incubation, cells were washed with PBS and total RNA isolation was performed using RNeasy Mini Kit (Qiagen, Hilden, Germany) according to manufacturers' instructions. RNA concentration and quality were determined using a Nanodrop 2200 (ThermoFisher Scientific). Only samples showing a 260/280 nm ratio between 1.8 and 2.1 were selected for cDNA transcription performed with the FastGene Scriptase II - Ready Mix (NIPPON Genetics Europe, Düren, Germany). Quantitative PCR (qPCR) analysis was done using TaqMan® primers and a StepOnePlus System (Applied Biosystems, Foster City, CA, USA). Briefly, for each well of the 96-well qPCR plate (Sarstedt), 10 μl of qPCRBIO Probe Mix (NIPPON Genetics Europe) were mixed with 5 ng cDNA, 1 μl of the appropriate primer (Table 1), and 4 μl H₂O. Relative quantification (RQ) of gene expression was deter-

Table 1. List of TaqMan® primers used for qPCR

Target gene (associated protein)	Assay ID
CD163 (Scavenger receptor cysteine-rich type 1 protein M130)	Hs00174705_m1
CD206 (Macrophage mannose receptor 1)	Hs00267207_m1
DDIT-3 (DNA damage-inducible transcript 3 protein)	Hs00358796_g1
DRD2 (D(2) dopamine receptor)	Hs00241436_m1
DRD5 (D(1B) dopamine receptor)	Hs00361234_s1
HPRT1 (Hypoxanthine-guanine phosphoribosyltransferase)	Hs02800695_m1
IL1B (Interleukin-1 beta)	Hs01555410_m1
SDHA (Succinate dehydrogenase (ubiquinone) flavoprotein subunit, mitochondrial)	Hs00188166_m1
SLC1A2 (Glutamine synthetase)	Hs01102423_m1
SLC2A1 (Solute carrier family 2, facilitated glucose transporter member 1)	Hs00892681_m1
SLC7A11 (Cystine/glutamate transporter)	Hs00921938_m1
TNF (Tumor necrosis factor)	Hs00174128_m1
TNFSF10 (Tumor necrosis factor ligand superfamily member 10)	Hs00921974_m1

mined using the $2^{-\Delta\Delta Ct}$ method [49]. Reference genes: *SDHA* and *HPRT1* were used in PCR conducted with samples of monocyte-derived macrophages; *SDHA* was used for analyses of astrocytes and tumor cells.

Statistics

Statistical analysis was performed using GraphPad Prism software. The difference between means of unpaired samples was performed using two-way ANOVA with a significance defined by an α of 0.05. The resulting p-values were corrected for multiple comparisons using the Tukey test. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.

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G.P. performed the analysis of data. C.G., C.W., G.P., and A.R.-V. were associated with the interpretation of data. C.G. and A.R.-V. drafted the manuscript. C.G., C.W., G.P., W.R., and A.R.-V. were associated with critical revision for important intellectual content. All authors read and approved the final manuscript.

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Abbreviations: DRD2: dopamine receptor D2 · GB: glioblastoma · OXPHOS: oxidative phosphorylation · TAM: tumor-associated macrophage

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