Role of mitochondrial dynamics in microglial activation and metabolic switch

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Research

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Abstract

Background

Microglia are the endogenous immune cells of the central nervous system (CNS) and act as sensors of injury in the brain, favouring its homeostasis. Their activation and polarization towards a pro-inflammatory phenotype are associated to injury and disease. These processes are linked to a metabolic reprogramming of the cells, characterized by high rates of glycolytic function and suppressed levels of oxidative phosphorylation. This metabolic switch can be reproduced in vitro by stimulation with lipopolysaccharide (LPS) plus Interferon-γ (IFNγ). In an attempt to understand the mechanisms regulating mitochondrial respiration abolishment, we examined potential alterations in mitochondrial features during the metabolic switch. In addition, we studied the possible implication of mitochondrial dynamics in the metabolic switch using the mitochondrial division inhibitor-1 (Mdivi-1), which blocks Drp1-dependent mitochondrial fission.

Methods

Cultured microglia was treated with LPS + IFNγ to reproduce the metabolic switch under pro-inflammatory stimuli in the absence or in the presence of Mdivi-1 to block mitochondrial fission. Mitochondrial membrane potential and mitochondrial calcium were measured with living cell imaging, and microglial polarization was assessed by immunofluorescence and qRT-PCR. The metabolic profile of the cells was measured using the Seahorse XFe96 Extracellular Flux Analyzer.

Results

Under conditions of mitochondrial respiration abolishment, microglia did not show any change in mitochondria morphology, nor in mitochondrial membrane potential, indicative of a limited impact in its viability. We provided evidence that reverse operation of F_0F_1-ATP synthase contributes to mitochondrial membrane potential. On the other hand. mitochondrial fission blockage significantly reduced the expression of pro-inflammatory markers in LPS + IFNγ-treated microglia, such as the inducible nitric oxide synthase (iNOS). However, this inhibition did not lead to a recovery of the oxidative phosphorylation ablation by LPS + IFNγ or to a microglia repolarization.

Conclusions

Altogether, these results suggest that Drp1-dependent mitochondrial fission, although potentially involved in microglial activation, does not play an essential role in metabolic reprogramming and repolarization of microglia.

Background

Microglial cells are the resident macrophages of the central nervous system (CNS). They contribute to the tissue development, integrity and homeostasis through an active surveillance process [1, 2] that can lead to different states of activation. Microglia display an enormous plasticity of responses to injury, ranging from effects that may contribute to neuroinflammation and eventually tissue damage, to essential responses for regenerative processes [3]. These mechanisms are associated to a broad spectrum of activation states, which are in turn related to molecular changes and adaptations [4]. One of these adaptive mechanisms is the metabolic reprogramming of microglia and other immune cells in response to immune activation [5–7]. Similar to macrophages [6, 7], microglia exposed to pro-inflammatory stimulus shift their metabolism from oxidative phosphorylation (OXPHOS) to aerobic glycolysis [8, 9], in an event similar to the Warburg effect suffered by cancer cells [7]. This metabolic reprogramming is essential to activate cellular defense mechanisms and to manage various microenvironments in inflamed tissue [7, 10, 11]. Moreover, these cells overexpress inflammatory markers such as interleukin-1β (IL-1β), tumour necrotic factor α (TNFα), or the inducible nitric oxide synthase (iNOS), which takes part in the metabolism of arginine. In contrast, macrophages exposed to anti-inflammatory stimulus are primarily characterized by oxidative phosphorylation and increased fatty acid oxidation for ATP synthesis [6, 11], a fact that it has not been corroborated in microglia cells. This is commonly accompanied by the expression of anti-inflammatory markers, such as arginase 1 (Arg1), which competes with iNOS to hydrolyse arginine to ornithine [12], or the mannose receptor (MNR).

Mitochondria are dynamic organelles that are crucial for energy metabolism of the cells. Mitochondria constantly undergo fusion and fission processes that contribute to the homeostasis of the cell and the maintenance of mitochondrial functions[13]. Basal mitochondrial fission is required for mitochondrial trafficking to synapses, mitochondrial quality control, and brain development [14]. Misbalances in these dynamic mechanisms are even thought to contribute to the development of various neurological disorders [15–17]. Moreover, these processes are associated to the production of reactive oxygen species (ROS). Mitochondrial fission is a mechanism involved in the maintenance of the organelle, and is triggered by the dynamin-related protein 1 (Drp-1) recruitment from the cytosol to the outer mitochondrial membrane, which is mediated by different adaptor proteins [18]. Lately, the pharmacological inhibition of Drp1 with the mitochondrial division inhibitor 1 (Mdivi-1), a quinazonilone derivative, has become a promising strategy to analyse the physiological and pathological role of mitochondrial fission. However, Mdivi-1 can also interact directly with mitochondria [19, 20], a fact that should be taken into consideration in Mdivi's effects interpretations.

To elucidate the mechanisms underlying the metabolic reprogramming of primary microglia and whether mitochondrial dynamics are involved in this process or not, we have used Mdivi-1 to block Drp1-dependent mitochondrial fission *in vitro*. We found that whereas this inhibition leads to a modulation of the expression of inflammatory markers in microglial cells, this is not sufficient to alter the metabolic switch suffered by the cells, in sharp contrast with the results reported in a previous study [21].

Furthermore, as Mdivi-1 treatment generated a decrease in iNOS expression, we have assessed whether this outcome was capable to favour a repolarization process in microglia.

Materials And Methods

Animals

All experiments were performed according to the procedures approved by the Ethics Committee of the University of the Basque Country (UPV/EHU). Animals were handled in accordance with the European Communities Council Directive, and were kept under conventional housing conditions (22 ± 2 °C, 55 ± 10% humidity, and 12-h day/night cycle) at the UPV/EHU animal facilities. All possible efforts were made to minimize animal suffering and the number of animals used.

Microglial culture

Primary mixed glial cultures were prepared from the cerebral cortex of neonatal rats (P0-P2). After 10–15 days in culture, microglia were isolated by mechanical shaking (400 rpm, 1 h) and purified by plating them on non-coated bacterial grade Petri dishes (Sterilin; Thermo Fisher) as previously described [22]. Microglial cells obtained with this procedure were cultured in Dulbecco's Modified Eagle Medium (DMEM; Gibco) supplemented with 10% Fetal Bovine Serum (FBS; Gibco).

Microglial cells were polarized with specific stimuli. To generate an anti-inflammatory phenotype in these cells, they were treated for 24 hours with IL-4 (20 ng/ml; Peprotech) and IL-13 (50 ng/ml; Peprotech). On the other hand, pro-inflammatory microglia were generated with a treatment of both LPS (10 ng/mL) and IFNγ (20 ng/ml; Peprotech) for 24 hours. The repolarization protocol implied a 24 h pro-inflammatory activation followed by the treatment with the anti-inflammatory stimuli for the same amount of time.

Immunofluorescence analysis

Several immunocytochemistry (ICC) assays were performed on the cells in different conditions. Cells in culture were fixed in 4% p-formaldehyde (PFA) in PBS and processed for ICC as previously described [23]. Primary antibodies were used as follows to: iNOS (1:500, BD Bioscience), a pro-inflammatory marker, and mannose receptor C type 1 (MRC1; 1:1000, Abcam), MRC1 (1:1000, Abcam), specific for anti-inflammatory cells. As secondary antibodies, we used goat anti-rabbit Alexa Fluor 488 (1:250 Invitrogen) and goat anti-mouse Alexa Fluor 594 (1:250, Invitrogen). The morphology of microglial cells was measured using MRC1 as an specific marker, as the whole soma was stained by it. All the image analysis was performed with the ImageJ software (NIH). Immunoreactivity of the different markers was calculated as the fluorescence intensity and normalized to the number of cells in the selected field of view.

Cell viability assays

Microglial viability was assessed using both the calcein-AM dye (Invitrogen) and the Cytotox 96® Non-Radioactive Cytotoxicity Assay (Promega).

For the performance of the calcein assay, cells were incubated with 0.5 μM of the dye for 30 minutes at 37º C. The wells were then washed and the number of viable cells were calculated with a Synergy HT fluorimeter/spectrophotometer reader (Bio-tek) with 485 nm excitation and at 528 nm of emission wavelengths. A well with PBS was also measured in each experiment to subtract its values as a background control. The results are expressed as the relative percentage of cellular death with respect to non-treated microglial cells.

For the Cytotox 96® Non-Radioactive Cytotoxicity Assay, culture supernatants were harvested from the plate, mixed with the specific Assay Buffer and incubated for 30 minutes at 37º C. The reaction was then stopped and the absorbance at 490 nm was immediately recorded in the fluorimeter. The levels of absorbance are associated with the amount of lactate dehydrogenase in the medium, a stable cytosolic enzyme that is released upon cellular death and its consequent lysis. All the data obtained through both assays was analysed using Gen5 software (Bio-tek).

Quantitative RT-PCR

Total RNA of microglial cells was extracted using Trizol (Invitrogen) according to the manufacturer's instructions. Afterwards, 1 μg of this RNA was used to perform a retrotranscription protocol, using SuperScript III retrotranscriptase (200 U/μl; Invitrogen) and random hexamers as primers (Promega).

Real-time quantitative PCR reactions (qPCRs) were conducted in a Bio-Rad CFX96 real-time PCR detection system, as previously described [24]. The reactions were performed using SYBR-Green as a DNA-binding dye, and specific primers for anti-inflammatory and pro-inflammatory markers which were currently available in the laboratory (Table 1). The amount of cDNA was quantified using a standard curve from a pool of cDNA obtained from the different conditions of each experiment. Subsequently, the results were normalized with a factor, obtained for each condition using GeNorm v3.5 software, based on the expression levels of housekeeping genes (Table 1).

Mitochondrial membrane potential and mitochondrial calcium measurements

For quantification of mitochondrial membrane potential, microglial cells were loaded with quenching concentrations of Rhodamine 123 (Rh123; 10 μM) for 20 min in medium without phenol red. After this, microglia were washed for 10 minutes in the same medium. Living cell imaging was performed with a 63x objective in an inverted Leica LCS SP2-AOBS confocal microscope at an acquisition rate of 1 frame every 15 seconds for 5 min. After obtaining some basal images, FCCP (1 μM), an uncoupler of the mitochondrial oxidative phosphorylation, was added to the plates and the increase in the Rh123 fluorescence level was measured to determine the membrane potential. For each plate, a homogenous population of approximately 20 cells was selected in the field of view, and the background fluorescence signal was subtracted from the individual values.

For the measurement of the mitochondrial calcium content time-lapse images were acquired using a 63X objective in a Leica TCS STED CW SP8 confocal microscope, at an acquisition rate of 1 frame every 15

seconds for 5 min. Cells were loaded with 1 μM Fluo-4 (Thermo Fisher) in HBSS (Ca^{2+}- and Mg^{2+}-free) with 0.5 μM EGTA for 30 minutes at 37ºC. Subsequently, microglial cells were washed and after obtaining some basal images, 1 μM FCCP was added to depolarize the mitochondrial membrane and force the release of Ca^{2+} from mitochondrial matrix to the cytosol. The analysis was made as above.

Oxygen consumption rate analysis

Real time measurements of oxygen consumption rate (OCR) were performed using a Seahorse XFe96 Extracellular Flux Analyzer (Agilent), following manufacturer's instructions to carry out the XF Cell Mito Stress Test Kit (Agilent). Microglia cells were seeded as a monolayer in the XF96 microplate. Before the assay, cells were washed and equilibrated in the XF Assay modified DMEM medium for 30 minutes at 37ºC.

The real levels of oxygen consumption rate were determined in response to the sequential addition of oligomycin (2 μM), FCCP (1 μM) and rotenone/antimycin A (0.5 μM). Specifically, basal mitochondrial respiration was calculated subtracting OCR rate after rotenone/antimycin A addition from basal OCR. as the last measurement before addition of oligomycin – non-mitochondrial respiration (minimum rate measurement after the treatment with rot/antA). The spare respiratory capacity was obtained by subtracting the basal respiration level from the maximum rate measurement after addition of FCCP. ATP-linked OCR was determined by subtracting oligomycin-induced OCR from basal OCR. All these parameters were obtained using the specific Agilent Report Generator. For each single experiment, eight replicates were performed.

Data analysis

All the data shown in the figures are presented as the mean ± SEM. Every comparison between two groups was analysed using unpaired Student's two-tailed t-test. Comparison among multiple groups, such as in those in the viability experiment or the oligomycin-induced ECAR increase paradigm, were analysed by one-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparison tests for post hoc analysis. Statistical significance was considered at $p < 0.05$.

Results

Metabolic reprogramming in microglia is not associated to mitochondrial damage

It is not entirely known whether microglia metabolic reprogramming from OXPHOS to glycolysis upon pro-inflammatory stimulation is associated to mitochondrial damage. In order to test this idea, we set up an *in vitro* protocol to study the metabolic switch and we measured oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) in real time as indicative of mitochondrial respiration and glycolysis respectively. Cells treated with classical pro-inflammatory stimuli (LPS and IFN-γ) showed a flat profile regarding OXPHOS, and all the parameters (basal OCR, ATP-linked respiration and spare respiratory

capacity) related to this molecular process were practically abolished (Fig. 1A), as previously described [25]. This suggests a metabolism shift from OXPHOS to glycolysis in order to rapidly obtain energy. Conversely, microglia treated with anti-inflammatory factors showed a significant increase in basal OCR, spare respiratory capacity and ATP-linked respiration, thus, indicating that mitochondrial OXPHOS was boosted. In contrast, ECAR measurement showed an increase in basal glycolysis in both pro-inflammatory and anti-inflammatory microglia (Fig. 1B), suggesting that activated microglia have higher energetic demands. Moreover, inhibition of mitochondrial F_0F_1-ATP synthase with oligomycin, thus blocking OXPHOS, increased glycolytic rate in control and anti-inflammatory microglia but not in pro-inflammatory microglia (Fig. 1B). These results further support the idea that pro-inflammatory microglia metabolism relies exclusively on glycolysis.

In other immune cells, such as dendritic cells, the metabolic switch to glycolysis occurs within minutes after TLR activation with LPS [26]. However, microglia acute treatment with LPS did not induce any change in OCR (Fig. 1C). Time course analysis demonstrated that the metabolic switch began to occur at 2 h, was clear at 6 h and completed at 24 h (Fig. 1D).

Since OXPHOS takes place in the mitochondrial inner membrane and it is determinant for H^+ movement and mitochondrial membrane potential ($\Delta\Psi m$) maintenance, the complete arrest of mitochondrial OXPHOS by pro-inflammatory stimuli could induce a collapse in $\Delta\Psi m$. So, we checked whether mitochondrial membrane potential was altered in microglia treated with pro-inflammatory stimuli (LPS plus IFN-γ). To do that, we performed live cell imaging of Rhodamine 123 (Rh123) fluorescent dye in microglia under “quenching” conditions [27]. Addition of FCCP to dequench Rh123 increased cytoplasmic fluorescence two fold over baseline (100%) in control microglia as well as in pro-inflammatory microglia (Fig. 2A), indicating that basal mitochondrial potential is maintained in pro-inflammatory microglia despite the arrest of OXPHOS. To further assess mitochondrial integrity we measured resting $[Ca^{2+}]_{mit}$ by recording $[Ca^{2+}]_{cyt}$ upon addition of FCCP in the absence of extracellular Ca^{2+}, which is indicative for $[Ca^{2+}]_{mit}$ [28]. Again, we observed no significant differences in the release of mitochondrial calcium to the cytosol after the addition of FCCP (Fig. 2B). Moreover, pro-inflammatory stimulation of microglia induced a significant increase in ROS production, as revealed with DCFDA dye (Fig. 2C), despite the blockage of OXPHOS. All these results suggest that pro-inflammatory activation and its subsequent lack of OXPHOS activity in microglia is not related to and it does induce any mitochondrial dysfunction or alteration in mitochondrial membrane potential.

Maintaining mitochondrial membrane potential is essential to prevent the release of pro-apoptotic factors into the cytosol and subsequent cell death. Indeed, despite the blockage of OXPHOS, LPS plus IFNγ stimulation did not induce any significant microglia cell death (Fig. 2D). $\Delta\Psi m$ could be maintained in cells with respiration inhibited by the reverse operation of F_0F_1-ATP synthase and the adenine nucleotide translocase (ANT) that pump H^+ out of the matrix [29]. To test this hypothesis, we analysed the impact of the F_0F_1-ATP synthase and ANT in cell viability and $\Delta\Psi m$. Indeed, treatment of LPS + IFN-γ stimulated microglia with oligomycin, a F_0F_1-ATP synthase inhibitor, induced a significant increase in microglia cell

death (Fig. 2D). In contrast, bongkrekic acid, an inhibitor of ANT, did not induce microglia cell death (Fig. 2D). Accordingly, a role of F_0F_1-ATP synthase in mitochondrial ΔΨm maintenance in pro-inflammatory microglia was further corroborated by live cell imaging of TMRE. Addition of oligomycin induced a significant decrease in ΔΨm in pro-inflammatory microglia but not in control microglia (Fig. 2E).

Mitochondrial fission inhibition does not reverse mitochondrial metabolic reprogramming

Previous data have showed that activation of microglia with LPS induced a shortening of mitochondria by fission [21]. However, this effect was transient (for about 2 h) and mitochondrial length was elongated and recovered at 6–12 h after stimulation, the time when we observed the metabolic switch in microglia (Fig. 1D). Nevertheless, since microglia metabolic switch has been associated previously to mitochondrial fission [8], we further analysed this hypothesis in our cultures. As the GTPase dynamin related protein (Drp-1) is one of the main mediators of the mitochondrial fission [18, 30], we used the mitochondrial fission inhibitor Mdivi-1 [31] to examine the impact of mitochondrial fission in microglia and their metabolic switch. A 24 h-treatment with Mdivi-1 (50 μM) provoked an increased in microglia cytoplasm circularity (as described in the ImageJ software), indicating that Mdivi-1 treatment induces the amoeboid morphology (Fig. 3A). Although this characteristic is generally associated with cell activation, we found no differences in the basal expression of pro-inflammatory (iNOS) or anti-inflammatory markers (mannose receptor, MNR) (Fig. 3B), nor in cell viability (Fig. 3C). However, Mdivi-1 reduced significantly the increased expression of iNOS in response to LPS and IFN-γ stimulation (Fig. 3D). We further analysed by qPCR the expression of different pro-inflammatory markers. Treating microglia with LPS + IFN-γ induced an increase in the expression of all the pro-inflammatory markers (*Ccl2, Il1b, iNOS)* assessed with respect to control cells and also the expression of arginase-1 (*Arg1*), an anti-inflammatory marker with opposite function to iNOS regarding cellular metabolism. The treatment with mdivi-1 significantly reduced pro-inflammatory markers overexpression as well as that of Arg1 (Fig. 3E). These results suggest that Drp1-dependent mitochondrial fission is potentially involved in microglial activation, as suggested earlier [32].

Next, we checked whether the impact of mdivi-1 on microglial activation could affect microglia metabolic reprogramming. In addition to the blockage of drp-1 mitochondrial fission, mdivi-1 could inhibit acutely and reversibly mitochondrial complex I in neurons [19]. To determine the direct impact of Mdivi-1 on microglial bioenergetics, we treated microglia with Mdivi-1 for 1 h and 24 h and we measured microglial OCR. We also observed a reduction in mitochondrial respiration at 1 h, however the effect was transient and disappeared at 24 h (Fig. 4A, B). Next, we analysed the impact of mdivi-1 on the metabolic switch of pro-inflammatory microglia. Treatment with Mdivi-1 did not increase OCR levels in cells stimulated with LPS and IFN-γ, to recover the normal bioenergetics profile (Fig. 4B). These results indicate that, although Drp1-mediated mitochondrial fission could modulate or contribute to microglia activation, it does not play an active role in the metabolic switch produced in inflammatory paradigms.

Mdivi-1 treatment does not improve microglial repolarization

Redirection of microglia from a detrimental to a regenerative phenotype is a major concept to develop new therapies targeting these cells [33]. Nitric oxide (NO), the product of iNOS activity, blunts mitochondrial respiration of pro-inflammatory macrophages and this dysfunction prevents editing macrophage towards an anti-inflammatory phenotype. Thus, inhibiting NO production improves mitochondrial metabolic impairment and macrophages reprogramming towards an anti-inflammatory phenotype [34]. Given that mdivi-1 reduced iNOS expression in pro-inflammatory microglia, we assessed the capacity of Mdivi-1 to promote repolarization in microglia. To that end, microglia cells were primed for 24 hours with LPS + INFγ (with or without Mdivi-1, to reduce NO production), and then treated 24 h with anti-inflammatory cytokines (see scheme on Fig. 5A). We first compared the IL-4 + IL-13-induced response in cells previously primed with LPS + INFγ with the response of cells exposed to anti-inflammatory stimulus only. After exposure to IL-4 + IL-13, microglia primed with LPS + INFγ exhibited efficient upregulation in the expression of the anti-inflammatory marker mannose receptor and downregulation in the expression of iNOS (Fig. 5B). The expression of iNOS and MNR were further reduced or increased respectively in cells treated with Mdivi-1 during LPS + INFγ priming (Fig. 5B). When the metabolic profile of the cells was assessed using Seahorse XFe96 Analyzer, we observed that microglia primed with LPS + INFγ recovered partially mitochondrial respiration after exposure to IL-4 + IL-13. However, Mdivi-1 treatment during LPS + INFγ priming did not further enhance the final OCR and therefore the OXPHOS activity (Fig. 5C).

Discussion

Lately, immunometabolism has emerged as an important focus of research, as it opens a novel therapeutic approach for inflammatory and autoimmune diseases. To conduct some responses, effector immune cells such as microglia/macrophages undergo a metabolic reprogramming process [5, 7]. Here, we have monitored this effect in primary microglia and its consequences in mitochondrial integrity; moreover, we have checked whether this metabolic switch is associated to mitochondrial dynamics or not. It is noteworthy to mention that the ablation of oxidative phosphorylation in active cells is not due to a challenge to mitochondrial integrity. Moreover, we have shown that Drp1-dependent mitochondrial fission, although potentially involved in microglial activation, does not play an essential role in metabolic reprogramming of microglia.

Upon pro-inflammatory stimulation, cells are able to redirect their entire metabolic processes to the glycolytic pathway, in order to rapidly obtain energy. This process is an outcome of different molecular pathways; nevertheless, the precise mechanisms involved are yet to be defined. Here, we have observed that even though the oxidative phosphorylation machinery is completely halted, this is not associated to mitochondrial damage or dysfunction nor with microglia cell death. This is opposed to what was observed when the Warburg effect was firstly described; he hypothesized that dysfunctional mitochondria

would be the reason underlying the switch in metabolism and eventually, the development of cancer cells. [35]. Indeed, recent studies have highlighted the importance of mitochondria regarding the production of ROS as agents to support not only the development of cancer cells but also the pro-inflammatory state of macrophages [36, 37]. Thus, toll like receptor activation in macrophages induced mitochondrial ROS generation, an essential step for efficient intracellular bacteria killing [37]. We observed that ΔΨm was maintained in pro-inflammatory microglia through the reverse operation of F_0F_1-ATP synthase and that this protects microglia from cell death. Indeed, the blockage of complex I, III and IV abolishes H^+ translocation and it would lead to a transient drop in ΔΨm. However, the F1 subunit of F_0F_1-ATP synthase can hydrolyse mitochondrial ATP under these circumstances and drives the F0-rotor to pump H^+ out of the matrix to be able to maintain the ΔΨm [38]. Thus, the mitochondria of pro-inflammatory microglia would become consumers, rather than ATP generators, further increasing the energetic demand of these cells [39]. We have not found any essential role in the ANT reversal activity, which has also been described as key in the ΔΨm maintenance process in similar paradigms [29].Signalling events mediated by extracellular signals can regulate the metabolic pathways in immune cells, such as macrophages or microglia [40]. Accordingly, diverse cellular functions have been associated to metabolic reprogramming, including those related to mitochondrial function in general. Previous data suggested that mitochondrial dynamics contribute to this mechanism [8]. Our results demonstrated that Mdivi-1, a mitochondrial fission inhibitor a putative division inhibitor, reduced the enhancement of markers associated to microglial activation after LPS and IFN-γ exposure. This effect on microglial activation is in agreement with other studies, even in other paradigms of treatment [8, 21]. Drp1-mediated mitochondrial fission has been associated to enhanced activation of both p38 and NF-κβ, both mediators of signalling cascades leading to the expression of pro-inflammatory genes, in a paradigm of diabetic nephropathy [41]. Moreover, blocking Drp1-dephosphorylation with oleuropein reduced the production of pro-inflammatory factors in microglia as well [42]. In contrast, blockage of mitochondrial fission with Mdivi-1 did not avoid the microglial metabolism switch to glycolysis upon LPS + IFN-γ exposure, nor did it provoke any effect in the control cells. We concluded that mitochondria fission does not contribute to the metabolic switch in microglia. This result is apparently at odd with previous results [8]. The contradiction may be explained in the basis of the different paradigm used; in this study, microglia is exposed to Mdivi-1 as a pre-treatment, before the stimulation with LPS.

Distinct arginine metabolism plays a key role in the metabolic plasticity of immune cells. Pro-inflammatory microglia convert arginine into NO trough iNOS activity, increased in this phenotype [12, 43]. It has been described that the upregulation of iNOS and the resulting generation of NO contributes to the impairment of mitochondrial respiration both in immune cells as well as in astrocytes [44, 45]. Moreover, editing macrophage and microglia (re)polarization is emerging as a new therapeutic approach and iNOS have been described as a target. Thus, iNOS inhibition improve metabolic and phenotypic reprograming to anti-inflammatory macrophages [34]. Despite that Mdivi-1 treatment consistently reduced iNOS expression in pro-inflammatory microglia as well as in M1 to M2 reprogramed microglia, we did not detect any significant improvement on mitochondrial respiration. There are two possible interpretations. The complete blockage of iNOS activity and total abolishment of NO production, as observed with the

iNOS inhibitor 1400W, could be required to prevent the metabolic switch [46]. In this sense, Mdivi-1 only partially reduced iNOS expression in pro-inflammatory microglia. Alternatively, signalling pathways controlling metabolic switch could differ from those regulating phenotypic and inflammatory expression. Indeed, iNOS inhibition does not affect phenotypic polarization of cells, nor the inflammatory cytokine secretion of macrophages [31]. Accordingly, the effect of Mdivi-1 on pro-inflammatory gene expression does not produce any change on metabolism. For instance, Mdivi-1 rapidly and reversible attenuated complex I-dependent reverse electron transfer-mediated reactive oxygen species (ROS) production by brain mitochondria oxidizing succinate [18].

Conclusions

In summary, the present study sheds some light into the role of mitochondria in the metabolic reprogramming process in microglia. Pro-inflammatory stimuli dampen mitochondrial function without compromising their integrity and our results point to F_0F_1-ATP synthase as a key regulator of mitochondrial potential and cell viability maintenance in these conditions. However, we concluded that mitochondrial dynamics, fusion-fission, although potentially involved in pro-inflammatory gene expression, do not contribute to microglial glycolytic switch after pro-inflammatory stimulation.

Abbreviations

ANT
adenine nucleotide translocase
CNS
Central nervous system
DMEM
Dulbecco's Modified Eagle Medium
Drp1
Dynamin-related protein 1
ECAR
Extracellular acidification rate
EGTA
Egtazic acid
FBS
Fetal bovine serum
FCCP
Carbonyl cyanide p-trifluoromethoxyphenylhydrazone
IFNγ
Interferon gamma
IL
Interleukin

iNOS
Inducible nitric oxide synthase
LPS
Lipopolysaccharide
Mdivi-1
Mitochondrial division inhibitor 1
NO
Nitric oxide
OCR
Oxygen consumption rate
OXPHOS
Oxidative phosphorylation
Rh123
Rhodamine 123
TLR
Toll-like receptor
TNF
Tumor necrosis factor

Declarations

Ethics approval and consent to participate

All experiments were performed according to the procedures approved by the Ethics Committee of the University of the Basque Country (UPV/EHU).

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare no competing interests.

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Authors' contributions

MD contributed to the conception and design of the study, data interpretation and manuscript writing. AM contributed to the data acquisition and analysis, data interpretation and manuscript writing. AR assisted to the data acquisition and interpretation. CM substantially contributed to the revision of the work. All authors contributed to the manuscript revision, read, and approved the submitted version.

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Authors' information (optional)

Not applicable.

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Tables

Table 1. List of primers for quantitative real-time PCR.

Target gene	Forward sequence (5'->3')	Reverse sequence (5'->3')
Arg1	GGATTGGCAAGGTGATGGAA	CGACATCAAAGCTCAGGTGAA
Ccl2	AGCAGCAGGTGTCCCAAA	TTCTTGGGGTCAGCACAGAC
Il1b	TGGCAACTGTTCCTGAACTCA	GGGTCCGTCAACTTCAAAGAAC
Nos2	GAGGAGCAGGTGGAAGACTA	GGAAAAGACTGCACCGAAGATA
Housekeeping gene	Forward sequence (5'->3')	Reverse sequence (5'->3')
Hprt2	CAGTACAGCCCCAAAATGGTTA	AGTCTGGCCTGTATCCAACA
Ppia	AGGGTTCCTCCTTTCACAGAA	TGCCGCCAGTGCCATTA

Figures

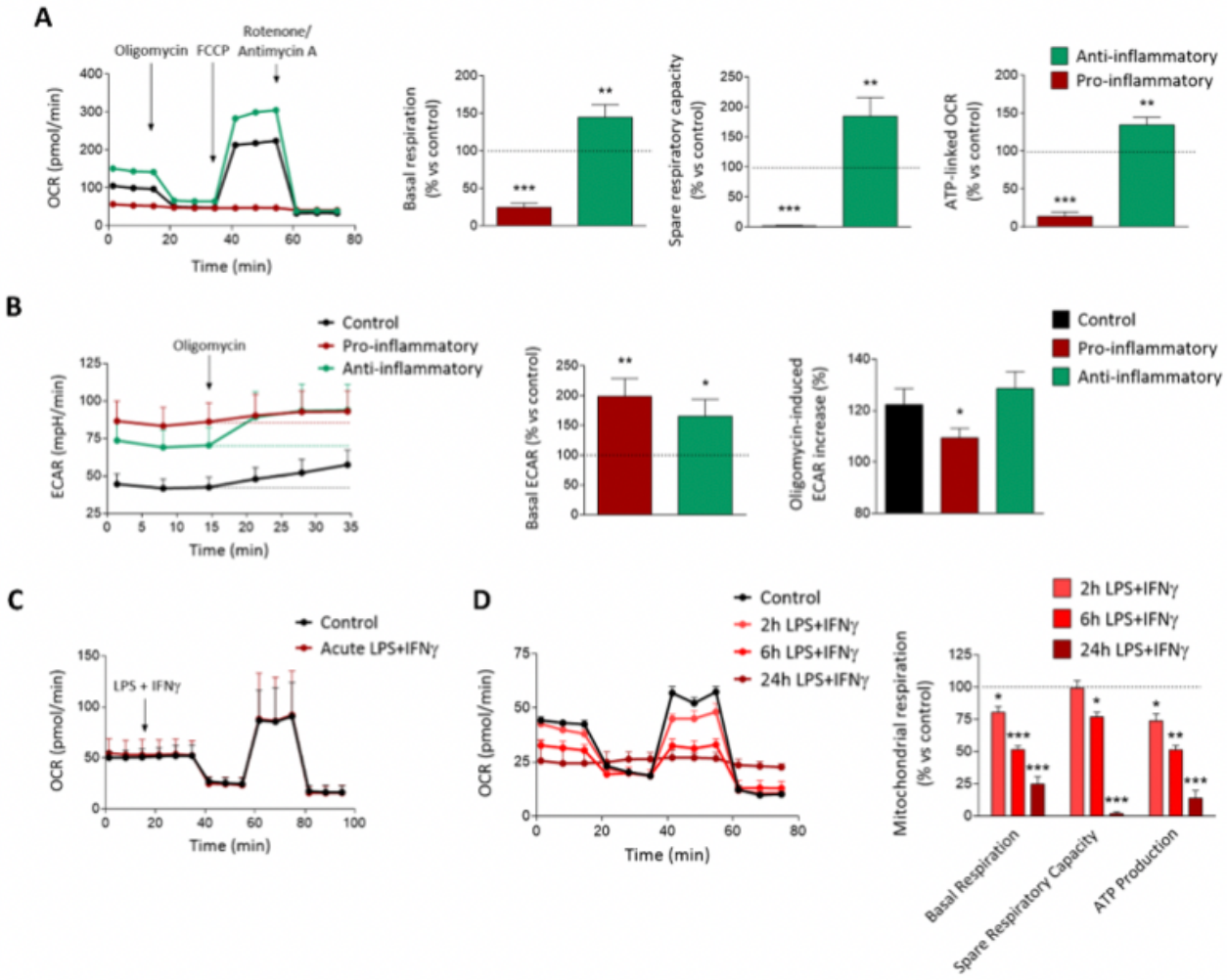

Figure 1

Robust metabolic reprogramming to glycolytic pathway after 24 hours of pro-inflammatory stimulation in microglia. (A) Representative experiment of OCR measurements in control microglia and in microglia after 24 hours of pro- and anti-inflammatory stimulations. Histograms show metabolic parameters obtained by the analysis of this metabolic profile compared to control cells (n = 7). (B) ECAR measurement obtained by the secretion of lactate of control microglia and microglia after 24-hour incubation with pro- and anti-inflammatory factors (n = 7). Histograms show the basal level of this parameter as well as the increase provoked by the acute treatment with oligomycin, relative to the control cells. Statistical analysis was performed by one-way ANOVA followed by Bonferroni post-hoc test. (C) Metabolic profile of microglia after the acute treatment with LPS and IFN-γ (n = 3) (D) Metabolic profile of microglia treated during different time lapses with LPS and IFN- γ. Histograms shows the metabolic parameters compared to control cells (n = 3). Data are presented as means ± SEM. *p < 0.05, **p < 0.005, ***p < 0.001. Unless otherwise stated,Student's t-test was used to analyse the data.

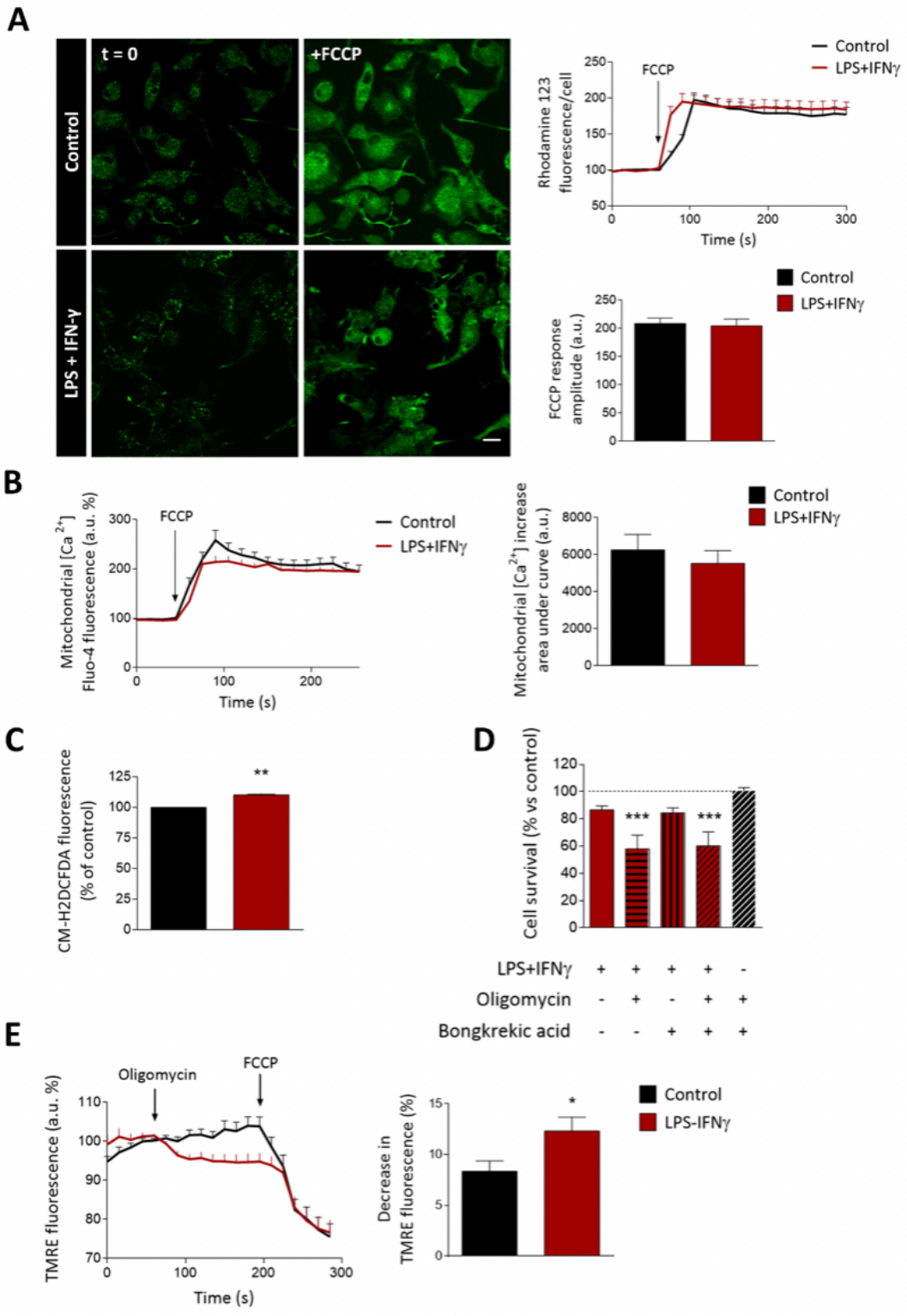

Figure 2

Pro-inflammatory microglia maintain mitochondrial integrity upon ATPase activity blockade. (A) Measurement of mitochondrial potential, represented as the increase in cytoplasmic Rh123 fluorescence measured after acute exposure to FCCP in control and LPS+IFN-γ treated microglia (n = 50-75 cells from three independent experiments). (B) Measurement of mitochondrial-specific calcium represented as the increase in cytoplasmic Fluo-4 fluorescence in a Ca2+-free medium, after exposure to FCCP, in control and

LPS+IFN-γ treated cells (n = 50-75 cells from three independent experiments).. (C) Reactive oxygen species quantification in control and pro-inflammatory cells (n = 3 experiments performed in triplicate). (D) Microglial viability after 24-hour treatment with LPS+IFNγ, oligomycin and/or bongkrekic acid, compared to control cells. One-way ANOVA followed by Bonferroni post-hoc analysis (n = 3 experiments performed in triplicate). (E) Effect of ATPase inhibitor oligomycin in mitochondrial potential of control and LPS+IFN-γ treated cells (n = 70-80 cells from three independent experiments). Data are presented as means ± SEM. *p < 0.05, **p < 0.005, ***p < 0.001. Unless otherwise stated, Student's t-test was used to analyze the data.

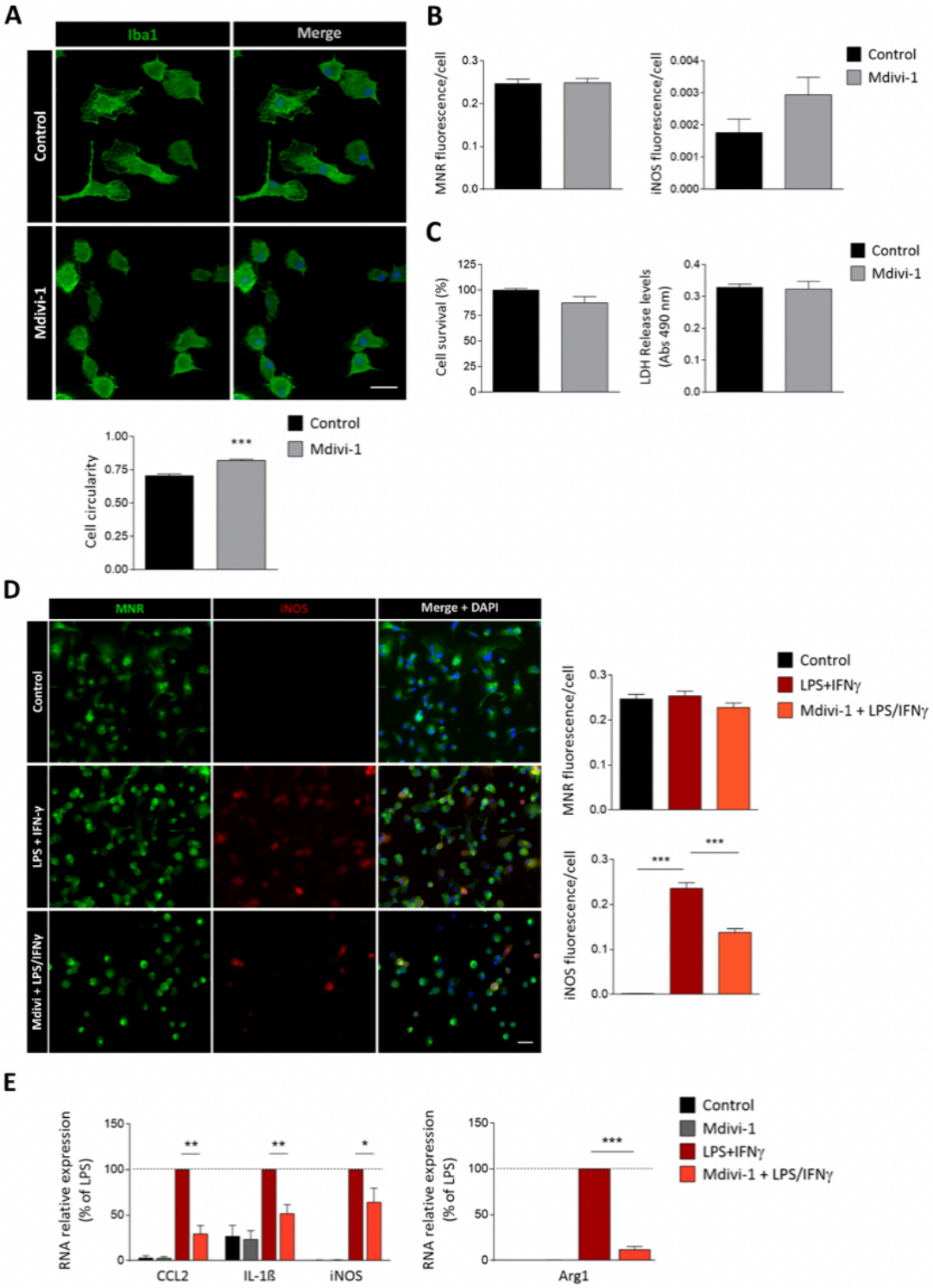

Figure 3

Mitochondrial fission inhibition reduces the microglial activation markers. (A) Representative images of Iba1+ control and Mdivi-1 treated microglia. Right column shows Iba1+ and DAPI staining (merge). Scale bar = 25 µm. Histogram represent the circularity of the cells (n = 200 cells from three independent experiments). (B) Expression of anti-inflammatory (MRC1) and pro-inflammatory markers (iNOS) in control and Mdivi-1 treated microglia (n = 4 experiments performed in duplicate). (C) Microglial viability in

control and Mdivi-1 cells, measured by both calcein assay and the quantification of LDH release to the medium, indicative of cell death (n = 3 experiments performed in triplicate). (D) Representative immunostaining of MRC1 and iNOS in control microglia, as well as in cells treated with LPS+IFN-γ or Mdivi-1 and LPS+IFN-γ. Scale bar = 40 μm. Histograms represent the mean fluorescence of the staining per cell (n = 4 experiments performed in duplicate). (E) Quantitative real time PCR (qRT-PCR) of pro-inflammatory (left) and an anti-inflammatory marker (right) in control microglia, as well as in microglia treated with Mdivi-1, LPS+IFN-γ, and Mdivi-1 + LPS+IFN-γ (n = 4 experiments performed in duplicate). Data are expressed relative to the expression in LPS+IFN-γ. Data are presented as means ± SEM. *p < 0.05, **p < 0.005, ***p < 0.001. Student's t-test.

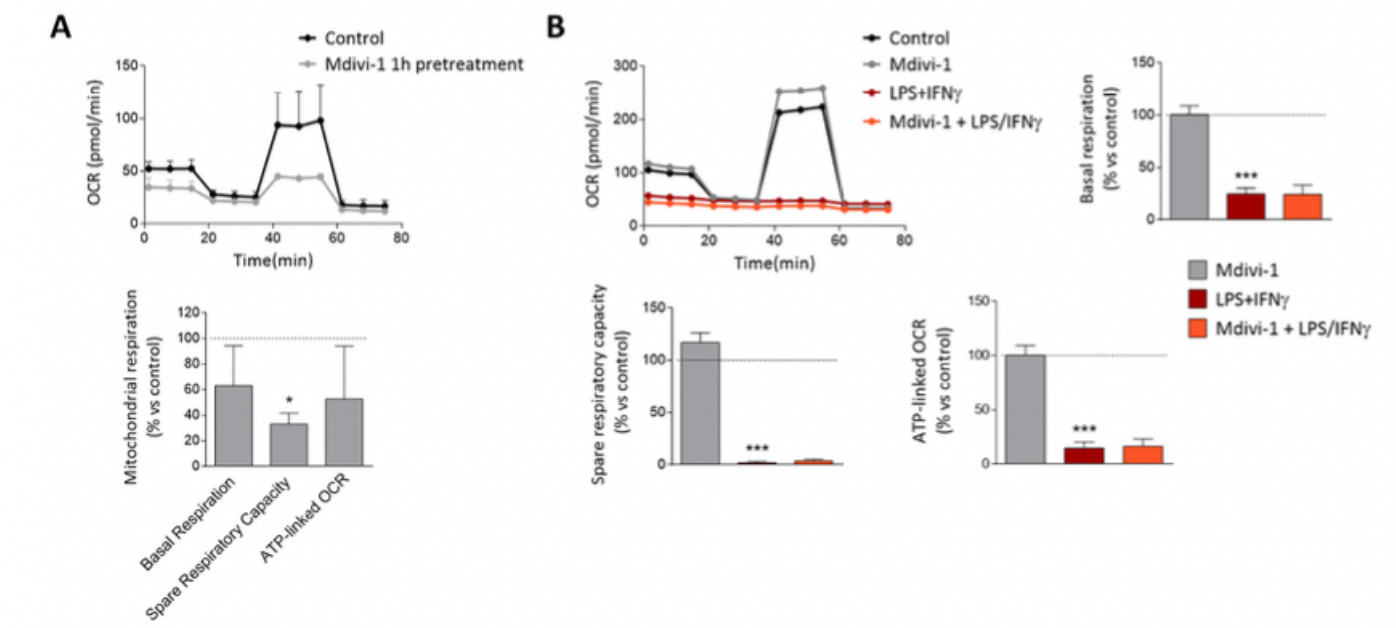

Figure 4

Mitochondrial fission inhibition does not reverse mitochondrial metabolic reprogramming. (A) Metabolic profile of control and microglia treated for 1 hour with Mdivi-1 (above). Histogram shows the metabolic parameters related to OCR of Mdivi-1 treated cells, compared to the control ones (n = 3). (B) Metabolic profile of control microglia, as well as microglia treated for 24 hours with Mdivi-1, LPS+IFN-γ and Mdivi-1 + LPS+IFN-γ. Histograms show the metabolic parameters related to OCR compared to control microglia (n = 3). Data are presented as means ± SEM. *p < 0.05, ***p < 0.001. Student's t-test

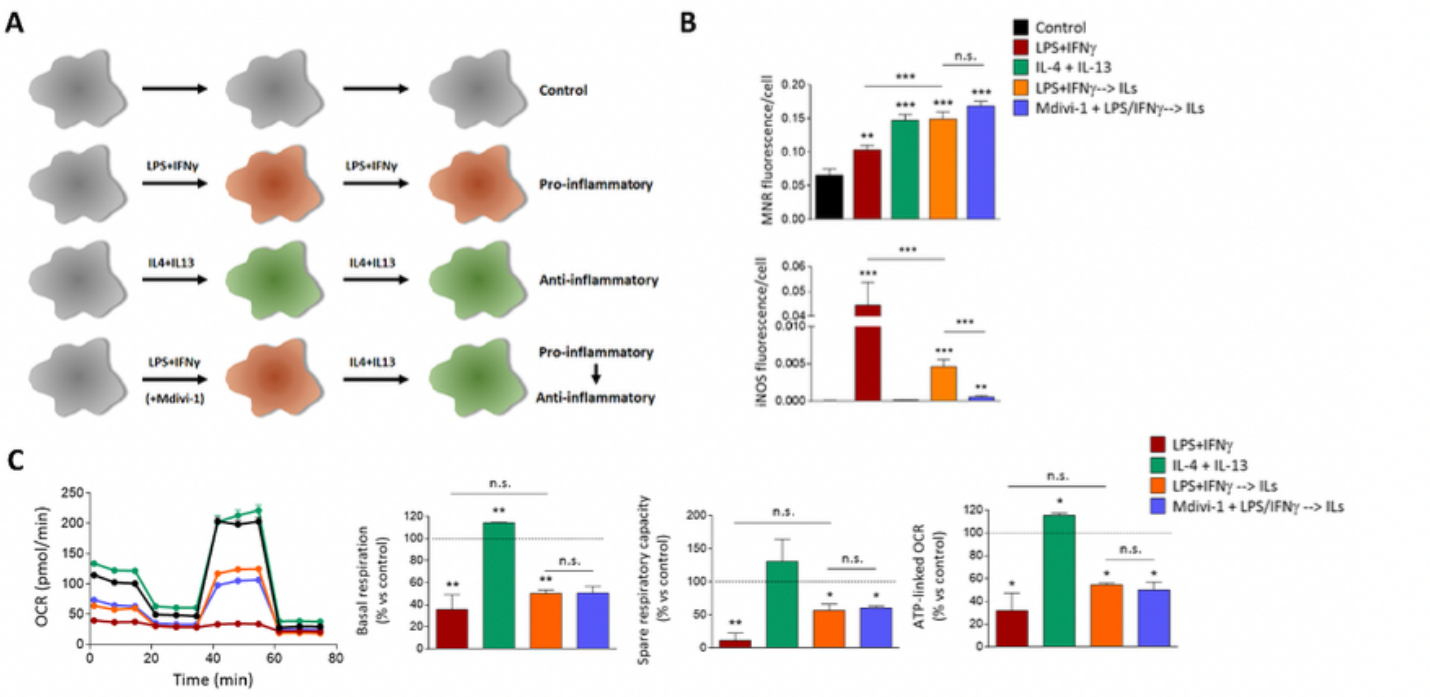

Figure 5

Mdivi-1 treatment does not promote microglial repolarization. (A) Scheme of the conditions used to study the role of Mdivi-1 in the repolarization capacity of microglia. Each treatment indicated lasts 24 hours. Anti-inflammatory microglia (ILs- treated) were compared to microglia that were primed for 24 hr with LPS + IFNγ before 24 hr treatment with ILs. (B) Expression of anti-inflammatory (MRC1) and pro-inflammatory (iNOS) markers in every condition of the repolarization experiment. Histograms represent the mean ± s.e.m of the marker fluorescence intensity per cell (n =3 experiments performed in triplicate). (C) Metabolic profile (left) of microglia treated as schematized in subfigure A. Histograms (right) show metabolic parameters regarding OCR of the cells (n =3). Data was expressed as fold change versus control non-treated microglia. Data are presented as means ± SEM. *p < 0.05, **p < 0.005, ***p < 0.001. Student's t-test.