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L-glutamine is a key parameter in the immunosuppression phenomenon

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ABSTRACT

Suppression of tumour-specific T-cell functions by myeloid-derived suppressor cells (MDSCs) is a dominant mechanism of tumour escape. MDSCs express two enzymes, i.e. inducible nitric oxide synthase (iNOS) and arginase (ARG1), which metabolize the semi-essential amino acid L-arginine (L-Arg) whose bioavailability is crucial for T-cell proliferation and functions. Recently, we showed that glutaminolysis supports MDSC maturation process by ensuring the supply of intermediates and energy. In this work, we used an immortalized cell line derived from mouse MDSCs (MSC-1 cell line) to further investigate the role of L-glutamine (L-Gln) in the maintenance of MDSC immunosuppressive activity. Culturing MSC-1 cells in L-Gln-limited medium inhibited iNOS activity, while ARG1 was not affected. MSC-1 cells inhibited Jukat cell growth without any noticeable effect on their viability. The characterization of MSC-1 cell metabolic profile revealed that L-Gln is an important precursor of lactate production via the $NADP^+$-dependent malic enzyme, which co-produces NADPH. Moreover, the TCA cycle activity was down-regulated in the absence of L-Gln and the cell bioenergetic status was deteriorated accordingly. This strongly suggests that iNOS activity, but not that of ARG1, is related to an enhanced central carbon metabolism and a high bioenergetic status. Taken altogether, our results suggest that the control of glutaminolysis fluxes may represent a valuable target for immunotherapy.

1. Introduction

Complex interactions between tumours and the immune system are known to significantly affect the efficiency of anti-cancer treatments. While the adaptive immune system has the potential to recognize and prevent tumour outgrowth, the accumulation of myeloid-derived suppressor cells (MDSCs) suppresses the anti-tumour specific T-cell functions by metabolizing the semi-essential amino acid L-arginine (L-Arg) [1]. The latter is the substrate for two principal enzymes expressed by MDSCs: (i) iNOS that oxidizes L-Arg to generate nitric oxide (NO) and L-citrulline; and (ii) ARG1 that converts L-Arg into urea and L-ornithine. Both enzymes compete for L-Arg at low substrate concentration [2]. The sparse concentration of L-Arg and the accumulation of NO derivatives in the blood result in the down-regulation of T-cell proliferation, the loss of their immunomodulatory functions and ultimately induce their death [3,4].

Despite considerable progresses accomplished in the field of immune escape in the past years, the metabolic events that favor the maturation of MDSCs and the activation of their functions are still misunderstood. We have recently shown that MDSC maturation process is dependent on an enhanced central carbon metabolism and a high bioenergetic status [5]. Interestingly, L-glutamine (L-Gln) was shown to play an important role in maintaining high TCA cycle activity. Indeed, glutaminolysis is known as a key metabolic pathway supporting cell proliferation and energy supply in tumours and immune system [6,7].

Therefore, in order to further understand the role of L-Gln on MDSCs immunosuppressive functions, we have characterized the effect of a limitation in L-Gln on immortalized MSC-1 cells. These are phenotypically similar to primary MDSCs and constitutively express iNOS and ARG1 *in vitro* [8,9]. MSC-1 cells inhibit antigen-specific proliferative and functional cytotoxic T-lymphocyte response without any additional treatment by specific cytokines or endotoxins [8,10].

2. Methods

2.1. Cell culture

MSC-1 cells were grown in 75 cm^2 T-flasks (VWR, Ontario, Canada) in RPMI1640 medium (Sigma) supplemented with 10% (v/v) irradiated FBS (Cedarlane, Burlington, Ontario, Canada), 1 mM Sodium Pyruvate (Sigma), 100 U mL^{-1} Penicillin, 150 U mL^{-1} Streptomycin (Cedarlane) and 2 mM L-glutamine (Cedarlane) when required, in a 5% CO_2 and 37 °C incubator. Cultures were inoculated

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at a cell density of 0.2×10^6 cells mL^{-1} and cells were passaged when they reached 80% of confluence.

2.2. Assays

Glucose, lactate, glutamate and glutamine concentrations in supernatants were measured using a 2700 SELECT biochemistry analyser (YSI Inc, Ohio). Ammonia and nitric oxide concentrations in supernatants were respectively assayed by the following enzymatic kits with respect to manufacturer technical instructions: Ammonia Assay Kit (Sigma) and Nitrate/Nitrite Colorimetric Assay Kit (Cedarlane).

2.3. Determination of Arginase 1 activity

Total MSC-1 cells were lysed with 50 μL of a lysis buffer containing 0.1% Triton X-100 (Sigma) and 100 μg mL^{-1} of pepstatin, antipain and aprotinin (all from EMD BioSciences, San Diego, CA). After 30 min in a thermomixer at 37 °C, cells debris were removed by centrifugation at 15000*g* for 20 s and cells lysates were kept in −80 °C prior to analysis. The determination of ARG1 activity was performed as previously described by Munder and colleagues [11].

2.4. Cytotoxicity assay

The immunosuppressive activity of MSC-1 cells was assessed as their ability to inhibit Jurkat cell (leukemic T-cells, clone E6-1, Cedarlane) growth and induce their death. Experiments were performed in 24-well tissue culture plates (VWR) in a final volume of 1 mL. First, MSC-1 cells were cultured in the absence of L-Gln for 12 h. Jurkat cells were then inoculated (500 μL at 0.2×10^6 cells mL^{-1}) in MILLICELL®PC 0.4 μm culture plate inserts (Millipore) and added to wells containing 500 μL of the conditioned medium. L-Gln was added to a final concentration of 2 mM to avoid down-regulation of Jurkat cell proliferation due to the absence of L-Gln. For the control culture, Jurkat cells in culture plate inserts were added to wells containing 500 μL of complemented culture media at 0.2×10^6 MSC-1 cells mL^{-1}.

Mixed cultures were kept in a 5% CO_2 and 37 °C incubator for 32 h only to prevent the arrest of cell growth or the induction of cell death associated to nutrient limitation or toxic metabolite accumulation. Jurkat cells were then counted using a hemocytometer and viability was determined by the Trypan Blue exclusion method.

2.5. Respirometry test

Respirometry assays were performed as described by Lamboursain and colleagues [12]. Briefly, MSC-1 cells were cultured for 48 h in the absence of L-Gln. 3 mL of a 5×10^6 cells mL^{-1} suspension were then inoculated in a 10-mL borosilicate glass syringe (Sigma) in which the plunger was substituted by a Ingold pO_2 probe (Mettler Toledo, Quebec, Canada). The respirometer was kept at 37 °C and magnetically agitated (60 RPM) to ensure the homogeneity of cell suspension. Dissolved oxygen was recorded by an acquisition system (Virgo, Longueuil, Canada).

2.6. Metabolite extraction

The extraction protocol was based on the method developed by Kimball and colleagues [13]. Briefly, for each sample, 5×10^6 cells were washed with cold PBS and extracted with 400 μL of 80% cold methanol in the presence of 0.2 g of Sand (Sigma). After 10 min on dry ice, the mixture was vortexed and then sonicated in ice and water for 5 min. The samples were then centrifuged for 7 min at 21,000*g* and 4 °C to collect supernatants. The pellets were extracted a second and third time as described above with 200 μL of 50% cold methanol and 200 μl of cold water. Supernatants were mixed and stored in −80 °C prior to analysis.

2.7. Nucleotide concentrations

Extracts were filtered through 0.2 μm filters (Millipore, Ontario, Canada) before analysis. Nucleotide concentrations were determined by ion-pairing liquid chromatography–electrospray ionization mass spectrometry (positive mode) using an HPLC–MS system (Waters, Milford, MA) equipped with a Symmetry C18 column (150 × 2.1 mm, 3.5 μm) (Waters) and a Security C18 guard-column (Waters, 10 × 2.1 mm, 3.5 μm).

DMHA was used as ion-pair reagent to improve the signal-to-noise ratio with positive ionization mode. Mobile phase consisted in Buffer A: 10 mM ammonium acetate, 15 mM DMHA at pH 7.0, and Buffer B: 40% (v/v) acetonitrile in water. Flow rate was set at 0.3 mL min^{-1} using the following gradient: 0–10 min at 15% B, 10–12 min at linear gradient from 15% to 40% B, 12–30 min at linear gradient from 40 to 70% B, 30–35 min at 70% B, 35–37 min at linear gradient from 70% to 15% B and 37–45 min at 15% B.

2.8. Organic acid concentrations

Extracts were filtered through 0.2 μm filters (Millipore, Ontario, Canada) before UPLC–MS/MS (Agilent, Quebec, Canada) analysis equipped with a Hypercarb column (100 × 2.1 mm, 5 μm) and a Hypercarb pre-column (2.1 × 10, 5 μm) (Thermo Fisher, Ontario, Canada).

Mobile phase consisted in Buffer A: 20 mM ammonium acetate at pH 7.5, and Buffer B: 10% (v/v) methanol in water. Flow rate was set at 0.3 mL min^{-1} using the following gradient: 0–5 min at 10% A, 5–10 min at linear gradient from 10% to 20% A, 10–20 min at linear gradient from 20% to 100% A, 20–30 min at 100% A, 30–32 min at linear gradient from 100% to 10% A and 32–40 min at10% A.

2.9. Statistical analysis

Data are shown as mean ± SEM (standard error of mean) of $n = 3$ independent experiments from 3 distinct MSC-1 cell cultures. Differences between mean values were calculated by a 2-tailed Student *t*-test for independent samples; $p < 0.05$ was accepted as significant.

3. Results

3.1. Effects of L-Gln starvation on MSC-1 cell immunosuppressive activity

Culturing MSC-1 cells in the absence of L-Gln inhibited their growth; the specific growth rate decreased from 0.047 ± 0.001 h^{-1} in the control culture to 0.009 ± 0.002 h^{-1} (Table 1)without any significant effect on cell viability (data not shown). In fact, MSC-1 cells represent a heterogeneous population of immune cells, such as macrophages, granulocytes and dendritic cells [14], which are known to be proliferative and to depend on L-Gln to transit from G2 to M phase [6]. Therefore, the absence of L-Gln inhibited cell cycle progress and render MSC-1 cells quiescent. Contrarily to the control culture where nitrite and nitrate, both markers of iNOS activity, were continuously accumulated at a rate of 2.33 ± 0.09 μM h^{-1}, these remained quasi-stable at 81.78 ± 13.26 μM in the L-Gln-limited MSC-1 cell culture (Fig. 1A). However, ARG1 activity was not significantly affected in the absence of L-Gln (Fig. 1B). The cytotoxicity assay showed that MSC-1 cells cultured in the absence of L-Gln had a decreased ability

Table 1
Specific growth rate (μ_{max}, h^{-1}), specific oxygen consumption rate (q_{O2}, fmol $cell^{-1}$ h^{-1}), nutrients (glucose and glutamine) consumption and metabolites (lactate and ammonia) production rates (q_s, fmol $cell^{-1}$ h^{-1}) in: (i) MSC-1 cells grown in complemented culture medium (control culture), and (ii) MSC-1 cells grown in the absence of L-Gln.

	Control culture: MSC-1 cells	MSC-1 – L-Gln
μ	0.047 ± 0.001	0.009 ± 0.002*
q_{O2}	66.03 ± 4.52	82.86 ± 12.23
q_s, glucose	292.49 ± 18.22	119.78 ± 18.93*
q_s, glutamine	85.83 ± 4.36	4.00 ± 1.08*
q_s, lactate	510.75 ± 28.09	225.56 ± 33.97*
q_s, ammonia	144.73 ± 10.78	5.26 ± 2.80*

Experiments were performed in triplicate and errors are standard error of the mean, $^*p < 0.05$ when mean value is compared to that of the control culture.

to inhibit Jurkat cell growth. In fact, the ratio of Jurkat cell density was reduced by 26.17% ± 3.80% when co-incubated with MSC-1 cells that had been pre-cultured in the absence of L-Gln, whereas it was more reduced (37.08% ± 5.03%) in the presence of MSC-1 cells cultured in complemented culture medium (Fig. 1C). Moreover, L-Gln limitation seemed to have inhibited MSC-1 cells ability to induce Jurkat cell death, since the ratio of Jurkat cell viability remained higher than 98% after 32 h, compared to a value of 75% for the control culture in which cell viability rapidly decreased after 8 h (Fig. 1D). Therefore, the activity of ARG1, which is known to act on T-cell proliferation and immunomodulatory functions only [15], allowed MSC-1 cells to affect Jurkat cell growth, whereas the inhibition of iNOS activity, which is associated with T cell apoptosis when active [15], abolished MSC-1 ability to induce Jurkat cell death.

3.2. MSC-1 cell nutritional behavior

A limitation in L-Gln caused a decrease of glucose uptake from 292.49 ± 18.22 fmol $cell^{-1}$ h^{-1} in the control culture to 119.78 ± 18.93 fmol $cell^{-1}$ h^{-1} (Table 1). This was accompanied by a statistically not significant increase of the oxygen consumption rate, from 66.03 ± 4.52 fmol $cell^{-1}$ h^{-1} in the control culture to 82.86 ± 18.93 fmol $cell^{-1}$ h^{-1} (Table 1). Consistent with a lower glucose uptake rate in the L-Gln-limited MSC-1 cell culture, lactate accumulation rate decreased from 510.75 ± 28.09 fmol $cell^{-1}$ h^{-1} in the control culture to 225.56 ± 33.97 fmol $cell^{-1}$ h^{-1} (Table 1). The lactate production-to-glucose consumption ratio was high and similar in both culture conditions; i.e. 1.78 ± 0.18 in the control culture and 1.88 ± 0.11 in the L-Gln-limited culture. This suggests that L-Gln may play an important role in the production of lactate in the control culture and that most of the glucose-derived carbon is converted into lactate rather than entering the TCA cycle in the absence of L-Gln. This was confirmed by the concentrations of TCA cycle intermediates, α-ketoglutarate, fumarate and malate, which continuously increased in the control culture and remained at low levels in the absence of L-Gln (Fig. 2A–C, respectively). These low concentrations cannot be associated to a higher cell demand since cells cultured in the absence of L-Gln were quiescent and ana-

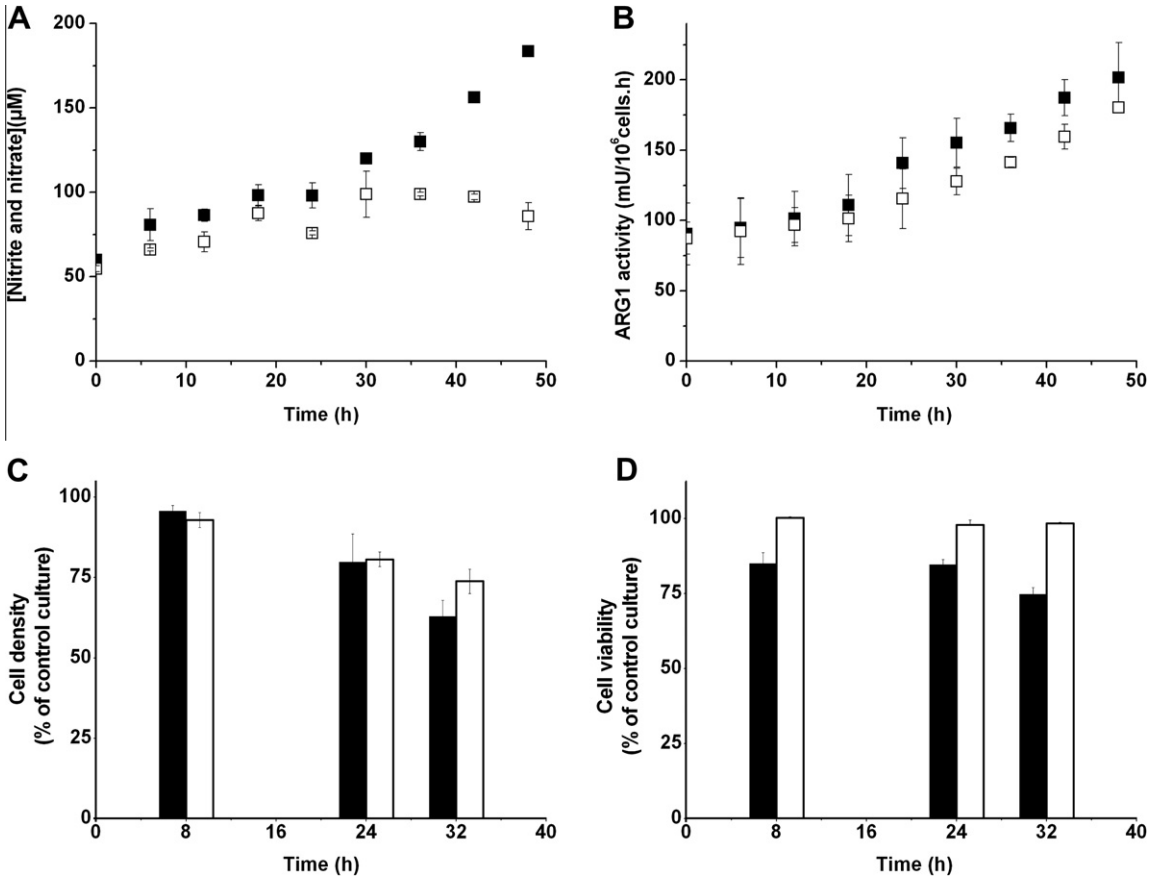

Fig. 1. Effect of L-Gln limitation on MSC-1 cell immunosuppressive activity. All data are presented as the mean of different experiments ($n = 3$) ± standard error of the mean. (A) Concentrations of nitrite and nitrate, marker of iNOS activity. (B) ARG1 activity. (■) Control culture: MSC-1 cells only, (□) MSC-1 cells cultured in the absence of L-Gln. Percentage of Jurkat cell density (C) and viability (D) referred to the control culture (Jurkat cells only). Jurkat cells were inoculated in the presence of: (i) MSC-1 cells in complemented culture medium (black bar) and (ii) conditioned medium recuperated after 12 h of MSC-1 cell culture performed in the absence L-Gln. Then, 2 mM of L-Gln were added to the culture to complement the medium (white bar).

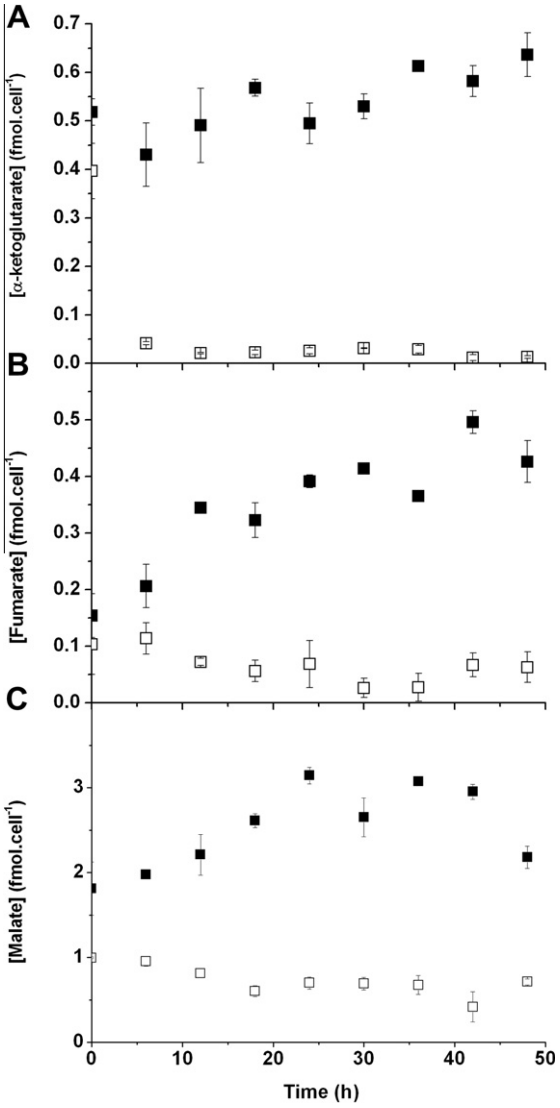

Fig. 2. Intracellular concentrations of α-ketoglutarate (A), fumarate (B), which is a by-product of the L-Arg production reaction, and malate (C), which is a precursor of pyruvate. Same symbols as in Fig. 1.

bolic-related biosynthesis reactions were probably at the maintenance level. The low TCA cycle activity has thus conducted to the down-regulation of L-Arg recycling pathway, as revealed by the decrease of fumarate concentration (Fig. 2B). Similarly, the low concentration of malate diminished lactate production through the glutaminolysis-TCA cycle pathway (Fig. 2C).

3.3. Effects of L-Gln deprivation on MSC-1 cell energetic profile

Although the oxygen consumption rate increased under L-Gln limitation, the cell specific ATP concentration progressively decreased at a rate of 0.022 ± 0.004 fmol cell^{-1} h^{-1}, whereas it was continuously accumulated at a rate of 0.033 ± 0.002 fmol cell^{-1} h^{-1} in the control culture (Fig. 3A). Similarly, the specific NADPH concentration decreased from 1.98 ± 0.13 fmol cell^{-1} (at inoculation) to a quasi-stable concentration of 0.82 ± 0.13 fmol cell^{-1} from 6 h, contrarily to the control culture where NADPH progressively accumulated at a rate of 0.034 ± 0.009 fmol cell^{-1} h^{-1} (Fig. 3B). However, the ATP-to-ADP ratio, a marker of respiration and energy consumption [16], was substantially similar in both cultures (Fig. 3C). This result was expected since a limitation in L-Gln and the resulting reduction of glucose uptake have down-regulated central carbon metabolism and so cell bioenergetic status. This was confirmed by the decrease (50% at the end of the culture compared to the control culture) of the AMP-to-ATP ratio (Fig. 3D), which is a marker of glycolysis and AMPK activity [17].

4. Discussion

The co-induction of ARG1 and iNOS by bacterial lipopolysaccharide (LPS) in macrophages has been shown to modulate central carbon metabolism and respiration [18,19], and therefore to affect cell bioenergetic state. Moreover, glutaminolysis has been shown to contribute to MDSC maturation process by providing energy and intermediates [5]. In fact, the L-Gln present in the tumour microenvironment has various roles. First, L-Gln is crucial for the proliferation of immune cells. Second, tumour cells metabolize L-Gln at high rate to support their fast growth. Third, L-Gln indirectly supports the tumour escape phenomenon by favoring MDSC maturation and ensuring the endogenous synthesis of its main substrate, L-Arg [18].

In this work, MSC-1 cell line, an *in vitro* model of MDSCs, were shown to present a reduced iNOS activity when cultured in the absence of L-Gln, as revealed by the decrease of nitrite and nitrate concentration from 18 h (Fig. 1A). Several studies have emphasized the role of L-Gln on iNOS mRNA expression level and the regulation of NO production. Indeed, L-Gln was shown to attenuate NO production and down-regulate iNOS mRNA level in IL-1β-activated hepatocytes [20]. In contrast, L-Gln did not trigger any significant effect on NO levels and iNOS gene expression in Caco-2 and HCT-8 cancer cells [21]. Our results are in agreement with those reported for LPS-treated macrophages, where NO production was reduced in the presence of an inhibitor of glutaminase [18]. Moreover, the conversion of glutamine to glucosamine in endothelial cells inhibits NO generation due to the reduction of NADPH availability [22]. Indeed, our results demonstrate that the absence of L-Gln does not affect ARG1 activity and MSC-1 cells are able to inhibit Jurkat cell growth, however to a lesser extent than for the control, without any noticeable effect on cell viability. In addition to the inhibition of the L-Gln-to-L-Arg pathway, due to reduced TCA cycle intermediates, the absence of L-Gln may have down-regulated the L-Arg recycling pathway since L-Gln was shown to regulate the gene expression of argininosuccinate synthetase, one of the enzymes implicated in endogenous production of L-Arg [23]. Thus, ARG1 activity may have been attenuated in a prolonged culture, due to the scarce availability of its substrate and considering the low affinity level of ARG1 for its substrate (~10 mM) [2].

We also showed that MSC-1 cells exhibit high glucose and L-Gln uptake rates, most likely to maintain high biosynthetic fluxes and energetic turnover rates that are required for their rapid growth. The high lactate-to-glucose ratio reported in the control culture (1.78 ± 0.18) suggested that MSC-1 cells re-direct most of the glucose-derived carbon as lactate, or that another source of carbon, such as L-Gln, contributes to the high lactate production rate. Culturing MSC-1 cells in a L-Gln-limited medium decreased lactate production rate by 50% due to the inhibition of the glutaminolysis-TCA cycle-$NADP^+$-dependent malic enzyme axis. Interestingly, MSC-1 cells cultured in the absence of L-Gln, which are characterized by an inactive iNOS enzyme, converted most of the glucose-derived carbon into lactate rather than entering the TCA cycle, as revealed by the high lactate production-to-glucose consumption rate (1.88 ± 0.01). Our results agree with study by Irace et al. who showed that NO regulates aconitase activity and α-ketoglutarate production and that the alteration of TCA cycle correlates with the inhibition of NO biosynthesis [24].

The global behavior observed in this work may be directly related to the cell immunosuppressive potential since MSC-1 cells

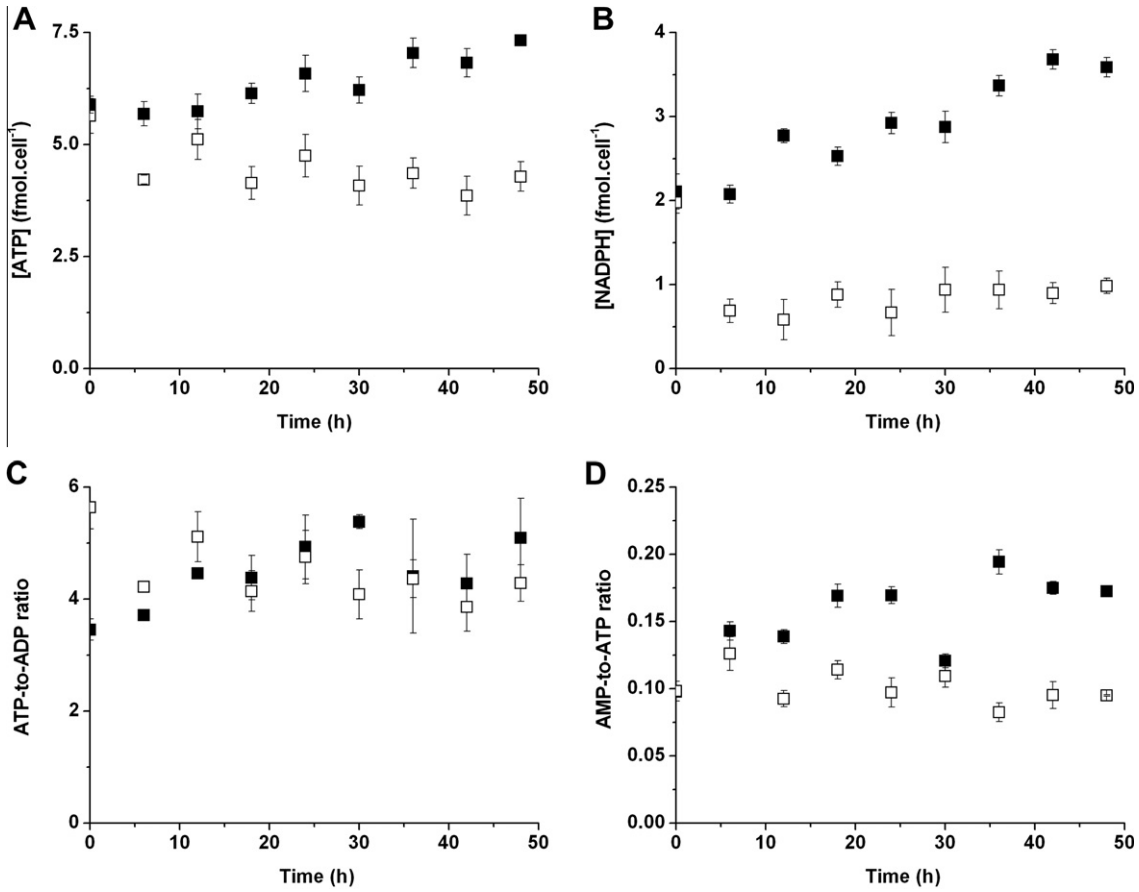

Fig. 3. MSC-1 cell energetic state. Specific concentrations of ATP (A) and NADPH (B) as well as behavioral markers: (C) the ATP-to-ADP ratio, a biomarker of respiration and energy consumption, (D) the AMP-to-ATP ratio, a biomarker of glycolysis and AMPK activity. Same symbols as in Fig. 1.

cultured in L-Gln-limited conditions are still immunosuppressive. Therefore, MDSCs contribute to the acidification of the tumoral microenvironment by supporting glycolytic metabolism and by converting part of the L-Gln-derived carbon into lactate.

Interestingly, the decrease of glucose uptake rate observed in the L-Gln-limited culture was accompanied by an increase of cell respiration. Although this increase was not statistically significant, these results may appear contradictory. However, recent studies showed that quiescent cells exhibit a high respiration rate, which was attributed to the energy and the metabolic activity required to maintain the cell redox potential status (pools of NAD^+ and $NADP^+$) as well as fatty acids synthesis [25,26]. Moreover, the inhibition of the production of NO derivatives abolish NO-associated down-regulatory effects on the respiratory chain [27]. MDSCs were also shown to express the hypoxia induction factor-1α (HIF-1α) to adapt to quasi-hypoxic conditions in the tumour [28,29]. However, fumarate, which is crucial for HIF-1α stability [30], was significantly decreased in the absence of L-Gln (Fig. 2B), enabling increased MSC-1 cell respiration. Although the respiratory capacity of the cells increased in the absence of L-Gln, the intracellular concentration of ATP decreased continuously (Fig. 3A); whereas the ATP-to-ADP ratio was substantially similar to that reported in the control culture (Fig. 3C). This may be due to the consumption of ATP by the maintenance processes. Furthermore, the decrease of the AMP-to-ATP ratio in L-Gln-limited culture showed that AMPK activity was reduced (Fig. 3D). In fact, AMPK is considered as an energy sensor in several metabolic disorders, such as cancer and diabetes, in which the enzyme switches cellular metabolism from anabolic to catabolic mode in reaction to deficit in cellular energy [31]. AMPK was shown to be implicated in the maturation process of MDSCs and the maintenance of their immunosuppressive activity [5]. In fact, the down-regulation of TCA cycle and $NADP^+$-dependent malic enzyme activity, in the absence of L-Gln, has not only decreased lactate production but also reduced NADPH and ATP concentrations. Although the absence of L-Gln deteriorates cell bioenergetics, AMPK activity was not enhanced to respond to energy deficit suggesting that iNOS, but not ARG1, and the related immune suppressive potential are probably associated to AMPK activity or that other substrates such as amino acids (unmeasured) may have been highly uptaken to compensate for the absence of L-Gln leading to the inactivation of AMPK. In addition to possible regulation of iNOS mRNA expression by L-Gln, iNOS activity may have been decreased due to the reduced availability of NADPH, which is a cofactor of iNOS.

In conclusion, controlling L-Gln availability or specifically inhibiting L-Gln transporters or glutaminolysis enzymes in MDSCs combined with immune cell stimulators, such as mTOR and interferon-γ, etc. [32], in tumour microenvironment, may thus offer a promising way to recover anti-tumour immune response.

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