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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