ORIGINAL ARTICLE

Inflammation and Hypoxia Negatively Impact the Survival and Immunosuppressive Properties of Mesenchymal Stromal Cells *In Vitro*

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ABSTRACT

Mesenchymal stromal cells (MSC) are nonhematopoietic cells with fibroblast-like morphology and multipotent capacity that are widely used in pre-clinical and clinical investigations. Unfortunately, the efficiency of MSC treatment is hindered by the poor survival rate after transplantation at the damaged tissue.

The goal of this study was to investigate the fate of MSC exposed to various stimuli mimicking the *in vivo* microenvironment post transplantation. To this aim, murine bone marrow–derived MSC were stimulated with IFNγ and TNFα under low oxygen (hypoxia) or atmospheric (normoxia) conditions for 24 to 72 hours, in order to better mimic an ischemic injury.

The results showed that MSC pre-stimulation with TNFα and IFNγ enhanced immunosuppressive pathways by over-expression of NOS2, IDO, COX2 and production of NO. However, MSC viability was affected by these two cytokines in dose-dependent and time-dependent manners. Besides, priming with TNFα and/or IFNγ under low oxygen concentrations revealed that significantly increased cell mortality rate and decreased NO production.

Our data suggest that both hypoxia and inflammation could impact the cell survival after transplantation and reinforces the necessity of further investigations to better understand MSC behavior after transplantation in order to identify the MSC-based strategies with the highest therapeutic potential.

Keywords: Mesenchymal stromal cells, hypoxia, inflammation, ischemic injury.

REZUMAT

Celulele stromale mezenchimale (MSC) sunt celule multipotente, non-hematopoietice, cu aspect morfologic de fibroblaști, ce sunt utilizate pe scară largă în cercetările preclinice și clinice. Din păcate, însă, eficiența tratamentului cu MSC este diminuată de rata scăzută de supraviețuire a acestora atunci când sunt transplantate într-un țesut lezat.

Scopul acestui studiu a fost de a investiga soarta MSC expuse la diferiți stimuli ce imită micromediul *in vivo* de după transplantare. Pentru aceasta, MSC izolate din măduva osoasă de la șoareci au fost stimulate cu TNFα și IFNγ în condiții de oxigen scăzut (hipoxie) sau de oxigen atmosferic (normoxie), timp de 24 până la 72 de ore, pentru a imita mai bine o leziune ischemică.

Rezultatele au arătat ca stimularea in vitro a MSC cu TNFα și IFNγ a activat căile moleculare implicate în imunosupresie, prin supraexprimarea de NOS2, IDO, COX2 și producția de NO. Cu toate acestea, viabilitatea MSC a fost afectată de aceste două citokine în moduri dependente de doză și de timp. În plus, stimularea cu TNFα și/sau IFNγ în prezența hipoxiei a crescut semnificativ rata mortalității celulare și a scăzut producția de NO.

Datele noastre sugerează că atât hipoxia, cât și inflamația pot influența supraviețuirea celulelor după transplant și întăresc necesitatea unor investigații suplimentare pentru a întelege cât mai bine comportamentul MSC *in vivo,* cu scopul de a identifica strategiile bazate pe MSC ce au cel mai mare potențial terapeutic.

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INTRODUCTION

Mesenchymal stromal cells (MSC) are nonhematopoietic cells with fibroblast-like morphology and multipotent capacity, being able to differentiate in osteoblasts, adipocytes and chondrocytes, under specific culture conditions[1]. These cells are widely distributed throughout the body and can be isolated from various sources, such as bone marrow, adipose tissue or blood[2]. In the bone marrow, MSC are known to reside in a complex microenvironment and together with haematopoietic stem cells (HSC) form a unique bone marrow niche[3,4]. HSC produce all blood cell lineages during homeostasis and stress in a highly dynamic program being tightly regulated by an interdependent network with MSC[4].

Among various stem cells proposed for cell therapy[5], MSCs have several advantages, which include convenient isolation, reduced immunogenicity, lack of ethical controversy, trophic activity[6,7], as well as potent immunomodulatory properties[8,9], and the potential to differentiate into specific cell types[1,10,11]. Moreover, other studies sustain the existence of a remote blood-borne–mediated pathway activated by transplanted MSC with no need of homing to the site of injury[12-15].

MSC are widely used in pre-clinical and clinical investigations, and according to the National Institute of Health, the cardiovascular diseases sum up almost 15% from the total number of studies which used MSC for cellular therapy[16,17]. Recently, MSC emerged as potent modulators of the immune system[18]. However, the mechanisms of action are complex and not fully understood. When injected directly into the ischemic myocardium, MSC preferentially polarized the adjacent macrophages (Mac) towards an anti-inflammatory M2 phenotype and improved the cardiac repair process[19].

Unfortunately, the efficiency of MSC treatment is hindered by the poor survival rate after transplantation at the damaged tissue[20]. One possible explanation on transplanted cell death is that MSC are fragilized during the manipulations preceding their use in therapy, by exposure to fluctuating oxygen concentrations: extraction from the original site (e.g. bone marrow) where the oxygen pressure is low (1.5-4.2% O_2 within bone marrow niches), followed by *in vitro* culture (usually under 21% O_2) and, finally, transplantation into the injured tissue (often in a hypoxic environment)[21]. In addition to that, the damaged tissue is characterised by an inflammatory microenvironment, abundant in proinflammatory molecules, like TNFα and IFNγ[22].

In vitro stimulation or priming with proinflammatory cytokines prior to transplantation gives MSC a short-term memory by mimicking the pro-inflammatory microenvironmental stimuli[23]. MSC exposed to TNFα and IFNγ alone or in combination, stimulate the immunosuppressive function by inducing the production of various molecules, with indoleamine-pyrrole 2,3-dioxygenase (IDO) and nitric oxide (NO), among many others[24,25]. IDO is involved in the tryptophan catabolism with direct effects on natural killer (NK) cell activation, proliferation and functional activity[26]. NO is produced by the inducible form of nitric oxide synthase (NOS2) and serves to inhibit activation and proliferation of T cells and reduce cytokine production[27].

Here, we show that MSC pre-stimulation with TNFα and IFNγ enhances immunosuppressive pathways by over-expression of NOS2, IDO, cyclooxygenase-2 (COX2) and production of NO. However, MSC viability is affected by these two cytokines in dose-dependent and time-dependent manners, therefore caution is warranted in MSC priming approaches. Besides, priming with TNFα and/or IFNγ under low oxygen concentrations is thought to improve MSC effective properties[23], but our results reveal that it also increases cell mortality rate and decreases the production of NO.

MATERIALS AND METHODS

Cell culture. MSC were isolated from mouse bone marrow as previously described[14]. Cells were grown in low-glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% MSC-qualified fetal bovine serum (FBS; Gibco™, Thermo Fisher Scientific) and 1% antibiotic-antimycotic at 37°C in a cell incubator, at 95% humidity, in 5% CO_2 concentration. For experiments, cells were seeded 24 hours before at a density of 25.000 cells/cm^2 to reach 70-80% confluency. MSCs were cultivated at atmospheric oxygen concentration (21%). To expose the cells to hypoxia, MSC were grown in 2% O_2, 5% CO_2 and 78% humidity in Whitley H35 Hypoxystation (Don Whitley Scientific).

MSC stimulation with TNFα and IFNγ. In order to mimic the proinflammatory environment *in vitro,* MSC were stimulated with IFNγ (20 ng/ml) and TNFα (1, 10 or 20 ng/ml) for 24 to 72 hours, followed by testing the viability and the anti-inflammatory properties of MSC. Recombinant mouse IFNγ and TNFα were purchased from R&D Systems.

Gene expression analysis. Total RNA was extracted using Trizol reagent (ThermoFisher Scientific, 15596026) and the reverse transcription reaction was performed using the High-Capacity cDNA Reverse Transcription kit (ThermoFisher Scientific, 4374966). Real-time RT-PCR was performed using gene-specific primers and SYBR™ Select Master Mix (ThermoFisher Scientific, 4472919).

The nucleotide sequence of each primer can be provided on request. The comparative CT method was used to quantify the data, and 18S rRNA or ACTB mRNA level was used for normalization.

xCELLigence analysis. The effect of the proinflammatory cytokines on the MSC cellular index was monitored with the xCELLigence system (Roche Applied Science), as previously described[28]. Briefly, MSC were detached from cell-culture plates and were seeded at 5.000 cells/well on 16-well E-plates in complete growth medium and cell index was recorded for 24 hours. The next day, the medium was changed with growth medium containing the proinflammatory cytokines (TNFα, IFNγ) at the specific concentration. The cell index was measured for another 2 or 3 days. The instrument read the electric impedance every 10 minutes for the first 3 hours and every hour thereafter.

Griess assay. NO production was detected in the supernatant of MSC cultures by measuring its stable end product nitrite using a Griess reagent according to manufacturer's indications (ThermoFisher Scientific, G-7921). This kit includes two components, A and B. Component B and nitrite form diazonium salt which, in presence of component A, produce an azo dye that can be spectrophotometrically quantified based on its absorbance at 548 nm. Briefly, culture medium was collected at a specific time after cytokine stimulation and was centrifuged at 400 x g for 5 minutes. The culture supernatant was collected and centrifuged again at 2.000 x g for 30 minutes to remove all cellular debris which can interfere with the reaction. A final volume of 130 μl supernatant was transferred to a 96-well plate in triplicate and 20 μl of Griess reagent was added. After 10 minutes of incubation, the absorbance was read at 548 nm with an Infinite M200 microplate reader from Tecan. The nitric oxide synthase inhibitor L-NAME (N omega-nitro-L-arginine methyl ester) was used to downmodulate the NO production in MSC stimulated with proinflammatory cytokines. Nitrite concentration was calculated with reference to a

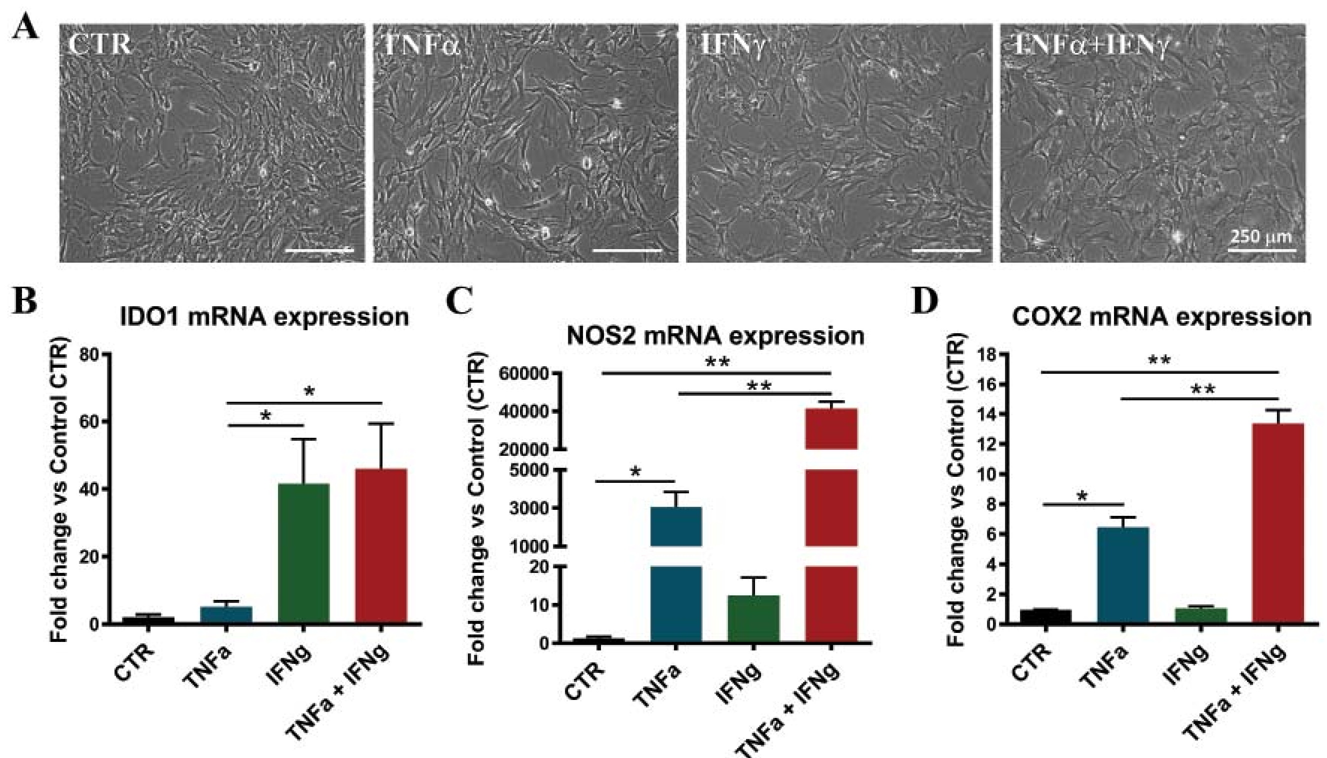

Figure 1. TNFα and IFNγ differentially regulate immunomodulatory factors in murine MSC. **A.** Phase contrast microscopy images illustrating the morphology of MSC after *in vitro* stimulation with TNFα and IFNγ for 24 hours. **B-D.** Gene expression levels of IDO1, NOS2 and COX2 in MSC stimulated with TNFα and IFNγ for 24 hours.

standard curve of six 2-fold serial dilutions of freshly prepared sodium nitrite (0 to 100 μM), and the culture medium alone was used as a blank control. The results are showed as concentration of NO_2 and are expressed in μM.

Statistical analysis. The results were expressed as the mean ± S.E.M. of at least three independent experiments. Statistical analysis was performed using GraphPad Prism 7 and one-way ANOVA followed by Tukey's post-test was used to assess statistical significance (*p < 0.05, **p < 0.01, ***p < 0.005).

RESULTS

The effect of *in vitro* MSC priming with TNFα and IFNγ

Activated MSC were reported to fulfil their immunosuppressive functions mainly through secretion of immunomodulatory factors[6]. Therefore, the benefits brought by the priming with proinflammatory cytokines over the naïve cells were assayed by determination of IDO1, NOS2, and COX2 expression levels, as major components of the immunomodulatory pathways activated in primed MSC.

First, MSC were seeded on tissue culture plates and 24 hours later, they were stimulated with TNFα and IFNγ alone or in combination (20 ng/ml each). After another 24 hours, the cells were imagined using phase contrast microscopy and no major morphological changes were observed under these experimental conditions (Figure 1A). Subsequently, cells were used for RNA extraction and qRT-PCR analysis. The results showed that TNFα and IFNγ had different effects on the expression levels of three important mediators of pathways associated to immunomodulation. Thus, a statistically significant overexpression of IDO1 was obtained in MSC after 24-hour stimulation with IFNγ, while TNFα did not produce any effect, either alone or in the presence of IFNγ (Figure 1B). However, stimulation with TNFα alone significantly increased the expression of NOS2 and COX2 and in the presence of IFNγ the effect was synergistic (Figure 1C-D).

The morphological changes were also followed with xCELLigence system. MSC stimulation with proinflammatory cytokines was performed under the same experimental conditions as described above, and the cell index was determined at 10-min interval for 48 hours. The xCELLigence analysis showed that IFNγ stimulation caused a slight decrease of the cellular index, most likely as a result of reduced cell proliferation. However, TNFα, either alone or in combination with IFNγ, caused a much more abrupt collapse of the cell index (Figure 2A). These results were confirmed by phase contrast microscopy images, which proved morphological alterations of TNFα-stimulated cells and increased mortality in the cytokine mix culture condition (Figure 2B).

As TNFα has important impact on cell index without affecting cell viability within the first 48 hours of the treatment, the next step was to evaluate the effect of an extensive treatment of MSC with different doses of TNFα. To this aim, MSC were stimulated with different concentrations of TNFα (1, 10 and 20 ng/ml) and cell index was continuously recorded for 72 hours, at 1-hour intervals. Of note, the xCELLigence recording showed a significant decrease in cell index even for the lowest dose of TNFα (Figure 2C), which apparently did not impact the cell viability, as concluded by phase contrast microscopy (Figure 2D). However, stimulation of MSC with higher TNFα concentration (10 and 20 ng/ml) for 3 days resulted in significant cell mortality (Figure 2D).

Nitric oxide production by MSC in the presence of proinflammatory cytokines and hypoxia

The above results showed that MSC stimulation with TNFα and IFNγ induced overexpression of NOS2, in parallel with an increase in cell mortality which suggested that the NO produced by NOS2 could induce cytotoxicity in cultured cells. In order to test this hypothesis, NO quantification was performed in control MSC cells stimulated for 48 hours with TNFα and IFNγ alone or in combination. The nitric oxide synthase inhibitor L-NAME was used to validate the specificity of the cellular response. NO is a relatively unstable molecule which suffers a spontaneous oxidation under physiological conditions, thus producing nitrite/nitrate, which can be determined in the culture medium by Griess reaction[29]. Phase contrast microscopy images illustrated that NOS2 inhibition partially reduced the mortality of cells stimulated with TNFα+IFNγ (Figure 3A). The quantification of nitrite in the culture medium was correlated with NOS2 gene expression in the cells and showed that TNFα stimulation was able to produce nitrite accumulation while IFNγ alone had no effect. Moreover, the combination of TNFα and IFNγ produced 4 times more nitrite than TNFα alone. Interestingly, NOS2 inhibition by L-NAME completely reduced nitrite accumulation in TNFα-stimulated cells, while only partially, yet significantly, reversed the increase in nitrite under TNFα+IFNγ stimulation (Figure 3B).

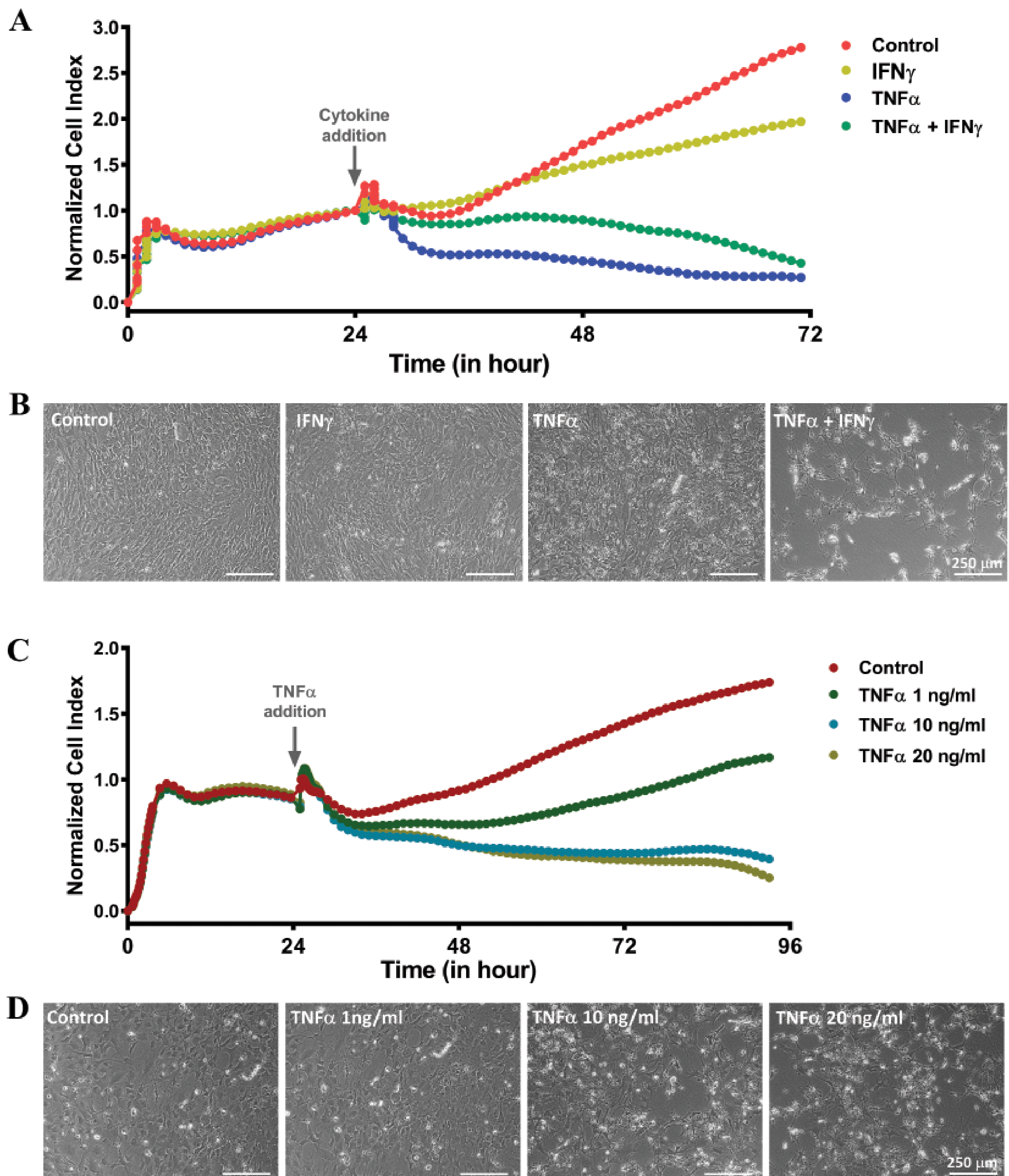

Figure 2. Prolonged inflammation affects the morphology and viability of MSC. **A)** xCELLigence analysis on MSC viability, correlated with the cellular index variation **B)** Phase contrast microscopy images showing the cellular morphology of MSC after stimulation with TNFα and IFNγ after 48 hours of cytokine treatment; **C)** xCELLigence analysis of MSC viability after stimulated with three different doses of TNFα; **D)** Phase contrast microscopy images showing the MSC viability after 72-hour stimulation with TNFα at different concentrations.

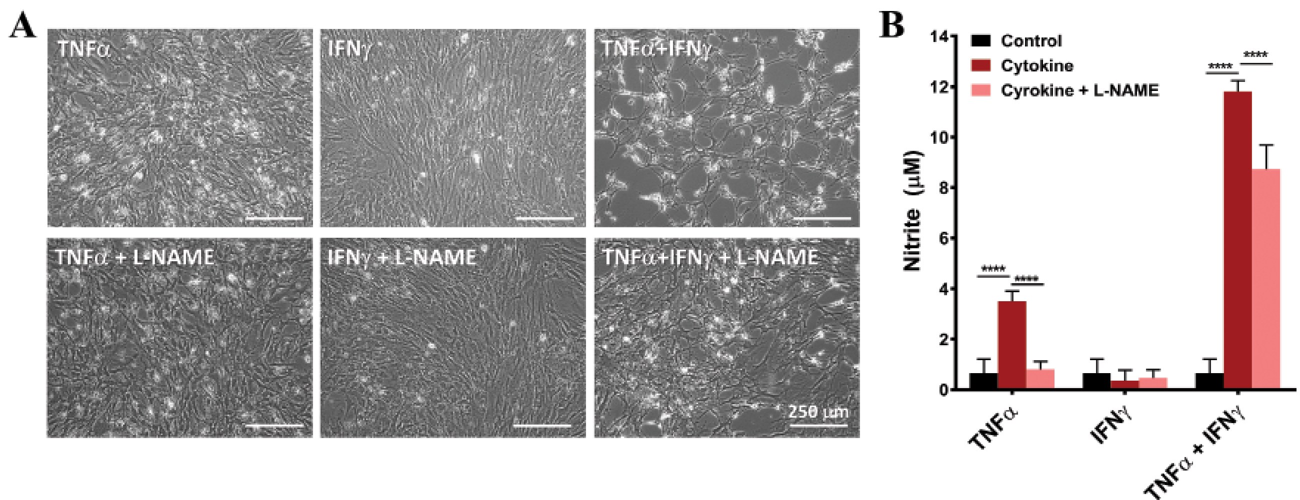

Figure 3. L-NAME effect on MSC mortality and NO production. **A)** Phase contrast microscopy images showing the cellular morphology of MSC after stimulation with TNFα and IFNγ, alone or in combination, in the presence or absence of NOS2 inhibitor L-NAME. **B)** Quantitative determination of nitrite produced by MSC after stimulation with TNFα and IFNγ.

Since hypoxia and inflammation were recently reported to play important roles in modulating the fate of the transplanted cells[30]. Cell survival and nitrite production were determined in MSC cultured for 24 hours under normoxic (21% O2) or hypoxic (2% O_2) conditions, in the presence or absence of TNFα+IFNγ stimulation (with or without L-NAME). As expected, phase-contrast microscopy images showed that hypoxia alone did not affect cell viability, however the combination of hypoxia with the proinflammatory cytokines resulted in the death of almost all cells (Figure 4A). Regarding NO production, hypoxia alone did not change nitrite level in the culture medium, but when combined with TNFα + IFNγ, it significantly reduced the level of NO production, probably due to increased cell mortality. NOS2 inhibition by L-NAME partially reversed cell survival in the presence of the both cytokines and hypoxic conditions, in addition to the partial reduction of nitrite level in the medium (Figure 4A-B). Taken together, our data suggest that both hypoxia and inflammation impact the cell survival after transplantation, in part by inducing NOS2 overexpression followed by NO-induced cytotoxicity.

DISCUSSION

The approach of MSC *in vitro* priming in order to obtain enhanced beneficial effects in various pathologies is currently being widely tested[31-34]. Therefore, a better understanding of how the in vivo microenvironment impacts MSC survival, as well as their therapeutic properties could be helpful for developing more effective in vitro priming strategies for MSC. The novel findings of our study are: (i) prolonged (more than 48 hours) stimulation of MSC with a combination of proinflammatory cytokines *in vitro* induces NOS2 overexpression followed by NO-mediated cytotoxicity, and (ii) hypoxia accelerates cell death in the presence of proinflammatory cytokines.

Several studies have shown that MSC-derived NO is a major contributor to the immunosuppression function of the cells[27,35,36]. However, the NO production in cytokine-activated MSC lead to cell apoptosis[24,37]. In line with these observations, we showed that major immunomodulatory genes were significantly induced in MSC by short priming (24 hours) of the cells with proinflammatory cytokines TNFα and IFNγ, and these cytokines produced independent, yet synergistic, effects on gene expression. However, prolonged stimulation of the cells, mimicking the continuous *in vivo* exposure to an inflammatory microenvironment, resulted in decreased cell viability. Importantly, the TNFα and IFNγ pretreatment may be responsible for effective inhibition of MSC repair ability and accelerating inflammation and the disease progression[38].

We have previously reported that MSC homing to the site of injury after transplantation is not required for their therapeutic efficacy in acute myocardial infarction[13], as they can mediate their actions systemically from remote sites[14]. Although there are still open questions regarding the mechanisms underlying cell migration and survival post-transplantation, we and other indicated that the short lifespan of transplan-

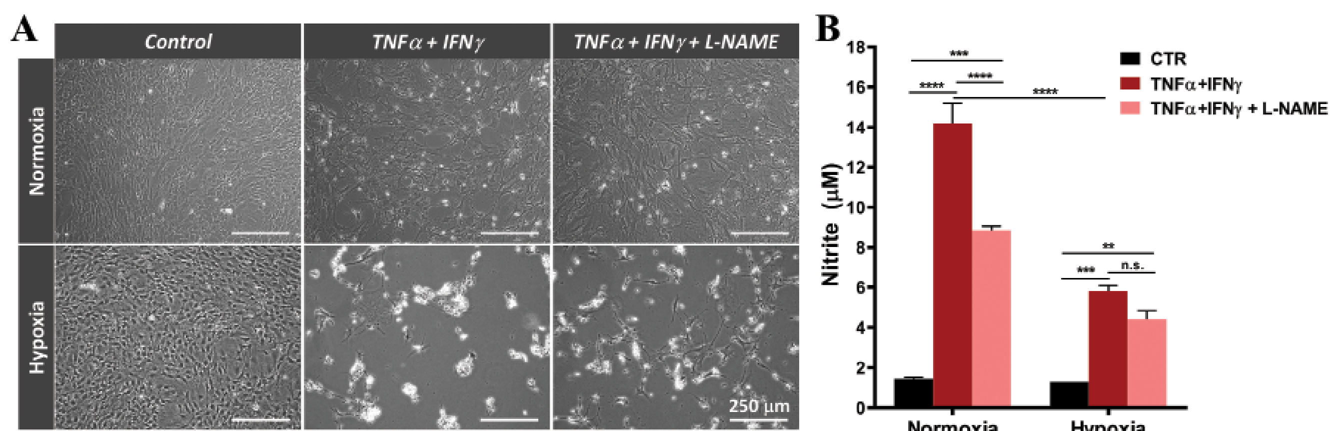

Figure 4. Low oxygen levels and cytokines affect MSC viability. **A)** Phase contrast microscopy images showing the cellular morphology of MSC after stimulation with TNFα and IFNγ in 21% (Normoxia) vs 2% (Hypoxia) O_2. **B)** Quantitative determination of the L-NAME MSC produced when stimulated with TNFα and IFNγ in 21% vs 2% O_2.

ted MSC does not impede their therapeutic effects, as these cells act through a "hit and die" mechanism, so that their viability was not entirely required for positive therapeutic outcomes[30,39-41].

Recently, numerous experimental data endorsed the idea of MSC cultured in low oxygen concentrations to simulate the in vivo microenvironment[42]. This preconditioning enhances the therapeutical properties of MSC, including those involved in immunomodulation[23,43]. In bone marrow, MSC are known to reside in a complex microenvironment and together with HSC form a unique bone marrow niche[3,4]. Naturally, they reside at 1-4% O_2, but when shifted *in vitro* they are exposed to 21% O_2. This transition may determine the alteration of many stem cell parameters which are not fully understood, therefore it is expected that a more physiologically relevant phenotype to be obtained by culture the cells under physiological O_2 *in vitro*[42,44], instead of atmospheric O_2. Here we showed that short-term exposure of MSC to mild hypoxia (2% O_2) did not altered cellular viability, however when hypoxia was combined with proinflammatory cytokines, which mimic a pathological ischemic microenvironment faced after transplantation *in vivo,* they underwent a significantly increased cell mortality indicating that their therapeutic effects cannot outlast.

It is recognized that poor survival of transplanted cells remains a limitation for clinical practice and priming of MSC via different signals (such as hypoxia and cytokines) could offer superior MSC-based therapies[23]. However, our data strengthen the idea of the requirement of further investigations to better understand MSC behavior after transplantation in order to identify the MSC-based strategies with the highest therapeutic potential.

CONCLUSIONS

Our study provides evidence that in vitro MSC priming with pro-inflammatory cytokines activates potent immunomodulatory pathways in the cells. However, by adding hypoxia and long-term cytokine exposure, there is an increase in the mortality rate, suggesting possible adverse consequences of locally transplanted MSC into ischemic tissues. Such insights may advance the understanding of MSC behavior and fate after transplantation and help to identify the optimal priming conditions for MSC in order to fully exploit their therapeutical capabilities.

Conflict of interest: none declared.

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