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IFN β enhances mesenchymal stromal (Stem) cells immunomodulatory function through STAT1-3 activation and mTOR-associated promotion of glucose metabolism

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Abstract

Administration of mesenchymal stem cells (MSC) ameliorate experimental autoimmune encephalomyelitis (EAE), a mouse model of multiple sclerosis (MS), at both clinical and neuropathological levels. The therapeutic properties of MSC in EAE are mainly mediated by the modulation of pathogenic immune response, but other neurotropic effects, including decreased demyelination and axonal loss as well as promotion of tissue repair, play also a role. Properly controlled phase II clinical trials to explore the potential of MSC transplantation as a treatment for MS are underway. Interferon beta (IFN β) is an approved treatment for relapsing-remitting and secondary progressive MS. Here, we explored the possibility that IFN β might influence the therapeutic potential of MSC, in view of possible synergistic effects as add-on therapy. IFN β enhanced the immunomodulatory functions of MSC and induced the expression of secretory leukocyte protease inhibitor (*Sipi*) and hepatocyte growth factor (*Hgf*), two soluble mediators involved in immune and regenerative functions of MSC. At molecular level, IFN β induced a rapid and transient phosphorylation of STAT1 and STAT3, the transcription factors responsible for *Sipi* and *Hgf* induction. Concomitantly, IFN β dynamically affected the activity of mTOR, a key checkpoint in the control of metabolic pathways. Indeed, the impairment of mTOR activity observed early upon exposure to IFN β , was followed by a long-lasting induction of mTOR signaling, that was associated with an increased glycolytic capacity in MSC. When induced to switch their energetic metabolism towards glycolysis, MSC showed an improved ability to control T-cell proliferation. These results suggest that modifications of MSC energetic metabolism induced by IFN β may contribute to promote MSC immunomodulatory function and support a role for metabolic pathways in the therapeutic function of MSC. Altogether, these findings support the idea of a combined treatment for MS, in which the immunomodulatory and possibly regenerative activity of MSC could be enhanced by the administration of IFN β .

Introduction

Mesenchymal Stromal (Stem) Cells (MSC) are a heterogeneous subset of stromal progenitors of mesodermal lineage that have been isolated from almost every tissue, mainly the bone marrow and adipose tissue. MSC are defined on the basis of their capability to grow as adherent

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cells on plastic, to display a fibroblast-like morphology, to form colonies in vitro supporting hematopoiesis, to differentiate into cells of the mesodermal lineage and express stromal while lacking hematopoietic markers¹. Several studies showed that MSC possess immunomodulatory properties exerted on cells populations of both adaptive and innate immunity² and these features, together with their reported ability to protect neural cells from death and foster neural repair, account for their proposed therapeutic effect on multiple sclerosis (MS) and other neurological diseases³. Intravenous infusion of MSC improved the clinical course of EAE, inducing immune tolerance, reducing inflammation decreasing demyelination and promoting tissue repair^{4–9}. The mechanisms through which MSC exert their therapeutic function are heterogenous and probably pleiotropic. It is generally accepted that MSC immunomodulation is strongly influenced by cytokines in the inflammatory environment, particularly by IFN gamma (IFN γ)^{10,11} and that their therapeutic effect is mediated by paracrine mechanisms through the release of soluble factors². Particularly, intravenous injection of conditioned medium containing MSC-derived hepatocyte growth factor (HGF) promoted remyelination in vitro and tissue repair in vivo¹². Autologous MSC have been safely administered in a limited number of patients with MS¹³, and ongoing controlled clinical studies to explore the potential of MSC transplantation as a treatment for MS have been launched (Clinical Trial NCT01854957).

Interferon beta (IFN β) is an approved treatment for relapsing-remitting¹⁴ and secondary progressive multiple sclerosis (MS). Interferons are a family of cytokines secreted by various cell types of the innate and adaptive immune systems as well as by other tissues. One major pathway in IFN β signaling involves activation of Signal Transducer and Activator of Transcription (STAT) proteins and formation of complexes that translocate to the nucleus and bind to specific elements to regulate gene transcription¹⁵. Efficacy of IFN β for the treatment of MS is believed to be due to modulation of immune responses¹⁶. Among its functions, in MS subjects IFN β modulates dendritic cells¹⁷, T and B lymphocytes¹⁸, as well as regulatory NK cells and T regulatory cells¹⁹.

While the potent immunomodulatory effect of IFN β on cells of the immune system has been extensively studied, little is known on the interaction between IFN β and MSC. We recently demonstrated that the pro-immunomodulatory effect of IFN γ on MSC is mediated by the phosphorylation of STAT1 and STAT3 and by the inhibition of mammalian target of rapamycin (mTOR) activity¹¹. Thus, we sought to address the effect of IFN β on MSC immunomodulatory functions keeping in mind that, based on the possibility of a

synergic effect, these two treatments could be effectively associated to treat MS.

Our data showed that IFN β promoted the ability of MSC to control T-cell proliferation and enhanced the gene expression of *Hgf* and of secretory leukocyte protease inhibitor (*Slpi*), an important regulator of innate and adaptive immunity and a component of tissue regenerative programs²⁰. At molecular level IFN β induced a rapid activation of STAT1 and STAT3 in MSC, concomitant with an early inhibition followed, at 24 h, by a strong re-activation of mTOR pathway. The oscillatory switch of mTOR signaling induced by IFN β was associated with an increased glycolytic capacity of MSC, that improved their ability to control T-cell proliferation.

Materials and methods

MSC culture and treatments

MSC isolated from mouse bone marrow were cultured in Mesencult medium as described [8]. MSC were treated with 1500 UI/ml of IFN β (Rebif[®] 44, Merk Serono) for 1 h in serum-free RPMI (Gibco).

Gene silencing

siRNA transfection was carried out using Lipofectamine 2000 in serum free RPMI for 24 h in the presence of specific siRNA (20 pmol). STAT1:(gaaugaccuggagaccaccucuu/aagagagguggucuccaggucauc)

SLPI: (caagucgugaggguaua(dt)(dt)/uauaccucacagca-cuug(dt)(dt))

As negative control, MSC were transfected with AllStar siRNA (Qiagen) (CTRL-KD) according to manufacturer's instructions. The efficacy of siRNA knock-down was tested by real time PCR.

Collection of MSC-conditioned medium

Confluent MSC were primed with IFN β for 1 h in serum-free RPMI. MSC were washed and cultured for an additional 24 h in serum-free RPMI with glucose 2 or 4.5 g/L, and the medium was collected (conditioned medium, CM).

In vitro T-cell proliferation assay

MSC CM equivalent to that of 2×10^4 MSC was assessed for its effect on proliferation of 2×10^5 anti-CD3/CD28-activated spleen cells (1:10 ratio) labeled with CFSE (1 μ M; Molecular Probes). Equivalent amount of CM from 3T3 fibroblast culture was used as control. Where the CM of MSC was collected in RPMI with different concentrations of glucose (2 or 4.5 g/L), the final glucose concentration in the T-cell activation medium was 4.5 g/L. Alternatively, MSC CTRL-KD and SLPI-KD were irradiated (5000 rad) and added to 2×10^5 anti-CD3/CD28-activated spleen cells (1:10 ratio) labeled with CFSE (1 μ M) at the ratio of

1:40. After 72 h, splenocytes were collected and the mean fluorescence intensity of CD3-positive T cells was evaluated by flow cytometry. Statistical differences were evaluated using Student's *T*-test.

Real-time PCR

Total RNA was extracted with Trizol (Invitrogen) from MSC, MSC RAPA, MSC CUC, MSC CTRL-KD and STAT1-KD 24 h after treatment with IFN β . Real-time PCR experiments were conducted as per the manufacturer's instructions (SYBR Green I Master, Roche) using the following primers:

Cd274 (aaatcgtgggtccccaagc/tcctcatgttttgggaactatct)

Ccl2 (catccacgtgttgctca/gatcatcttgctggtgaatgagt)

Hgf (cacccttgggagtagtg/gggacatcagtcattcacag)

Il18bp (agctattcggggcttaggag/tgcaagcaagtctggtgtct)

Slpi (cttgctctggggatcctg/ggctccgatttggatgcat)

Gene expression values were calculated as $2^{(-\Delta CT)}$ with hypoxanthine guanine phosphoribosyl transferase (*Hprt*) (tctctcagaccgctttt/cttggttcatatcgtaatc) as reference gene. Statistical differences were evaluated using Student's *T*-test.

Western blot

Total cell lysates and western blot analysis were performed as previously described²¹. The blots were probed with antibodies specific for pSTAT1 (Tyr701), pSTAT3 (Tyr705), pS6 (all 1:1000 dilution and from Cell Signaling Technology, Beverly, MA) and with an actin antibody (1:1000 from Santa Cruz) to normalize for the amount of loaded protein.

Pathway inhibition by pharmacological agents

STAT3 inhibition was obtained by exposure of MSC to cucurbitacin I (CUC) (500 nM; Sigma Aldrich) for 3 h in serum-free RPMI; mTOR inhibition was obtained through exposure of MSC to rapamycin (100 nM; Sigma Aldrich) in serum-free RPMI for 1 h (RAPA). The inhibition of STAT3 and S6 phosphorylation following treatments was evaluated by Western blot analysis using phospho-specific antibodies (data not shown).

Immunofluorescence

MSC were fixed in 4% paraformaldehyde, permeabilized with 0.5% Triton and labeled with antibodies specific for mouse SLPI (1:100 dilution from Cell Signaling) followed by a secondary antibody conjugated with AlexaFluor-594. Nuclei were stained with DAPI. The slides were analyzed with a Olympus microscopy equipped with 405 and 561 nm filters.

Analysis of MSC metabolism

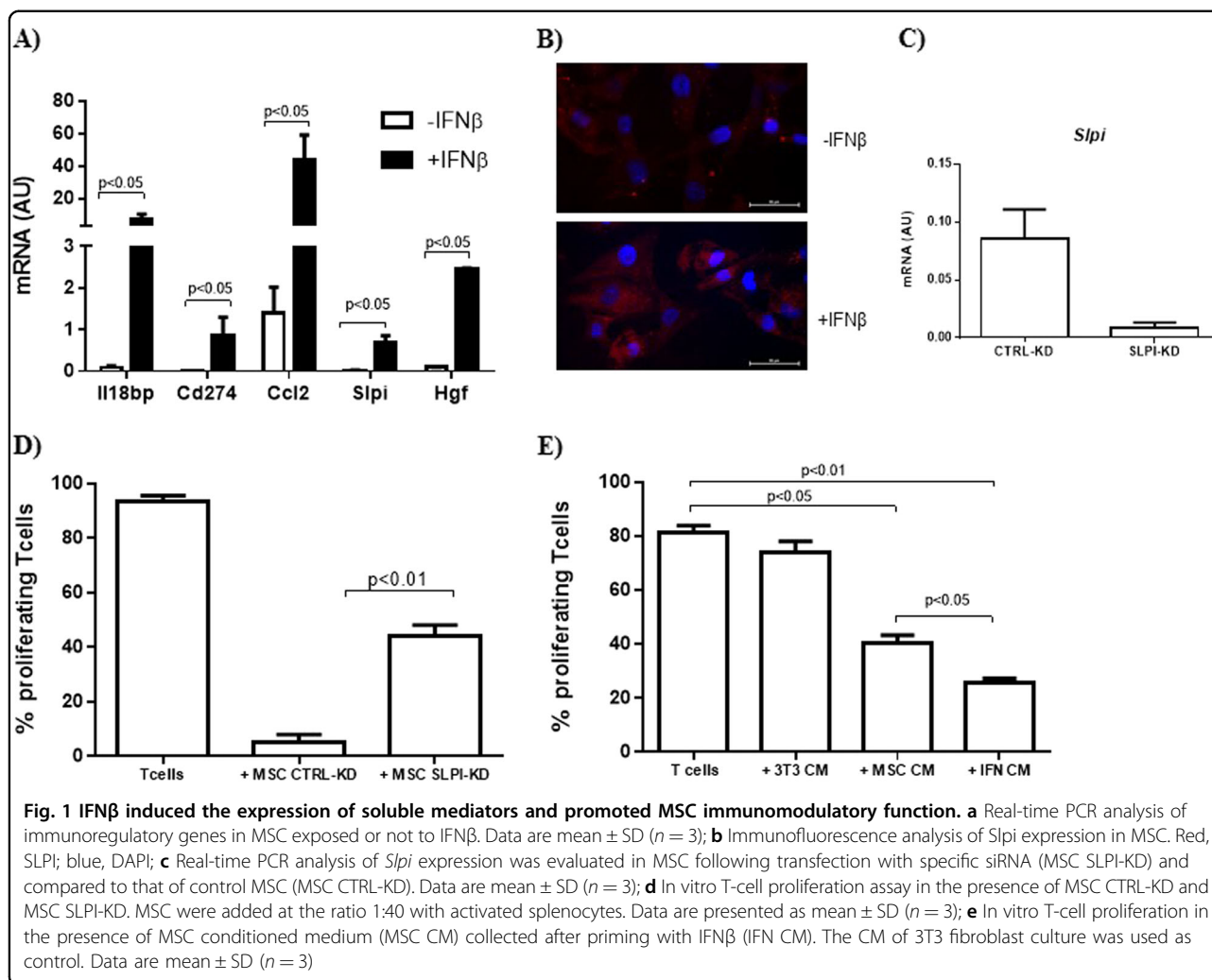
Real-time measurements of extracellular acidification rate (ECAR) and oxygen consumption rate (OCR) were

measured using an XFe-96 Extracellular Flux Analyzer (Seahorse Bioscience). Cells were counted using an automated Cell counter (Countess from Life Technologies), seeded in XFe-96 plates (Seahorse Bioscience) at the density of 2×10^4 cells/well and incubated overnight at 37 °C in 5% CO₂ atmosphere in the presence or absence of IFN β (1500UI/ml). ECAR was measured in XF media in basal condition and in response to 10 mM glucose, 4 μ M oligomycin and 100 mM of 2-Deoxy-D-glucose (2-DG). Basal glycolysis was calculated after glucose injection (subtracting the ECAR rate inhibited by 2DG). Maximal glycolysis was measured after oligomycin injection and glycolytic capacity as the difference of oligomycin-induced ECAR and 2-DG-induced ECAR. OCR was measured in XF media (non-buffered DMEM medium, containing 10 mM glucose, 2 mM L-glutamine, and 1 mM sodium pyruvate), under basal conditions and in response to 5 μ M oligomycin, 1.5 μ M of carbonyl cyanide-4-(trifluoromethoxy)-phenylhydrazine (FCCP) and 1 μ M of antimycin A and rotenone (all chemicals from Sigma Aldrich). Basal OCR was calculated as the difference between baseline measurements and antimycin A/rotenone-induced OCR; maximal OCR was measured as the difference between FCCP-induced OCR and antimycin A/rotenone-induced OCR. Finally, the ATP-linked parameter was calculated as the difference between baseline measurements, without injection, and oligomycin-induced OCR. Experiments with the Seahorse system were done with the following assay conditions: 3 min mixture; 3 min wait; and 3 min measurement. Then, metabolic parameters were calculated. Data are expressed as mean and S.E.M. from four independent experiments. Statistical differences were evaluated using the Wilcoxon matched-pairs test.

Results

IFN β promoted MSC immunomodulation inducing the expression of secreted mediators

To verify the possibility that IFN β may affect the expression of immunomodulatory factors secreted by MSC we analyzed, 24 h upon priming with IFN β , the mRNA levels of *Cd274*, *Ccl2*, and *Hgf*, which are known to act in a paracrine way on immune cells. We also assessed the gene expression of *Il18bp* and *Slpi*, that we recently identified as induced in MSC upon interaction with activated T cells¹¹. As shown Fig. 1a, IFN β induced a significant increase of mRNA level of all the genes analyzed. We then focused on *Slpi*, whose role in MSC immunomodulation has never been reported. We first confirmed that MSC exposed to IFN β increased *Slpi* expression at protein level (Fig. 1b). To get some insight into the role of *Slpi* on MSC immunoregulatory function, we reduced its expression in MSC through gene silencing (SLPI-KD) and used these *Slpi*-silenced cells to inhibit



T-cell proliferation (Fig. 1c). As shown in Fig. 1d, MSC SLPI-KD showed a reduced ability to inhibit T-cell proliferation in vitro as compared to MSC transfected with control siRNA (CTRL-KD).

Then we sought to verify if IFN β was able to affect the ability of MSC to control T-cell proliferation priming MSC with IFN β and testing the effect of the derived conditioned medium (CM) on activated T cells. As shown in Fig. 1e, IFN β significantly enhanced the constitutive immunomodulatory effect of MSC CM.

These results demonstrated that IFN β induced in MSC the expression of soluble immunomodulatory factors and concomitantly improved their ability to inhibit T-cell proliferation in vitro.

IFN β induced dynamic changes in STAT1, STAT3, and S6 phosphorylation

As activation of STAT1 and STAT3, and inhibition of mTOR pathway positively affects MSC immunomodulatory function upon administration of interferon gamma

(IFN γ)¹¹, we analyzed the activation state of these pathways following exposure to IFN β . Analysis of phosphorylated pathway components revealed that exposure to IFN β for 1 h dramatically increased the levels of phosphorylated STAT1 (pSTAT1) and STAT3 (pSTAT3) and concomitantly reduced the amount of phosphorylated S6 (pS6), used as read-out of mTOR activity (Fig. 2b). Upon a prolonged exposure (24 h), pSTAT1 and pSTAT3 decreased as compared to 1 h, indicating that IFN β induced a transient phosphorylation of STAT1 and STAT3. Concomitantly, the levels of pS6 strongly increased as compared to that observed upon exposure to IFN β for 1 h, revealing that IFN β induced an oscillatory modification of mTOR pathway activity (Fig. 2a, b).

IFN β -dependent expression of *Slpi* and *Hgf* was mediated by STAT1 and STAT3

Engagement of STAT1 and STAT3 induces the expression of immunomodulatory genes in MSC, that is further enhanced by inhibition of mTOR signaling¹¹. To assess

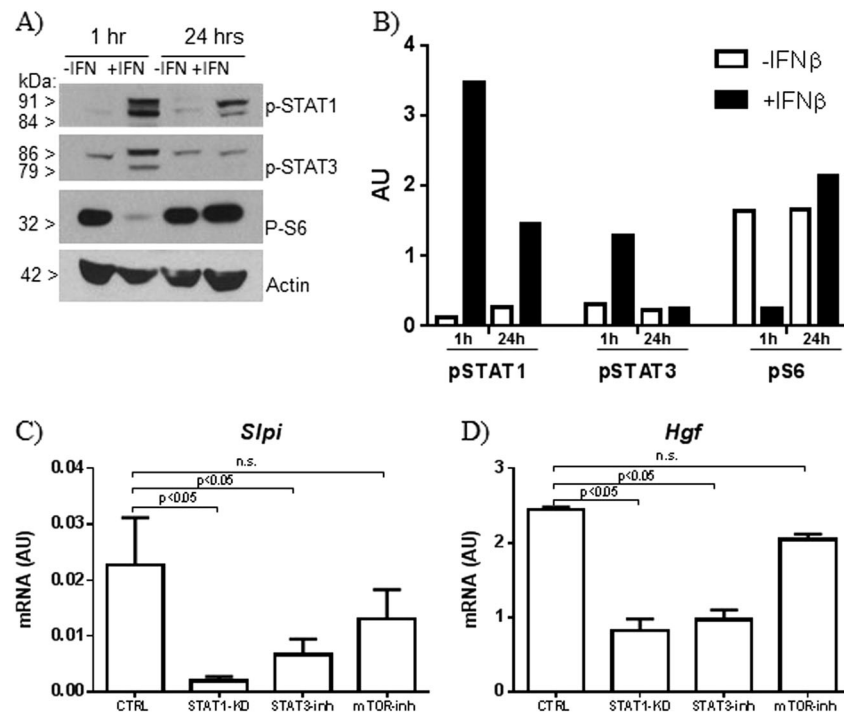


Fig. 2 IFN β induced dynamic changes in STAT1, STAT3, and S6 phosphorylation. **a** Western blot analysis conducted after 1 or 24 h of incubation with IFN β . Actin served as loading control; **b** relative densitometric quantitation of the shown gel, after normalization on actin; **c, d** Real-time PCR analysis of *Slpi* and *Hgf* expression in MSC exposed to IFN β upon pathway inhibition. Data are presented as mean \pm SD ($n = 3$)

the role of these pathways in the induction of *Slpi* and *Hgf* by IFN β , we inhibited STAT1 through gene silencing, STAT3 using the chemical inhibitor Cucurbitacin I (CUC) and mTOR with its inhibitor rapamycin (RAPA) before the exposure to IFN β and we analyzed *Slpi* and *Hgf* expression by real-time PCR. We observed that the induction of *Slpi* expression by IFN β was significantly impaired by STAT1 and STAT3 inhibition, while the blockade of mTOR pathway did not exert any effect on their expression (Fig. 2c). Similarly, we observed that impairment of STAT1 and STAT3 signaling, but not that of mTOR, reduced the induction of *Hgf* upon exposure to IFN β (Fig. 2d).

Exposure to IFN β modified MSC energetic metabolism

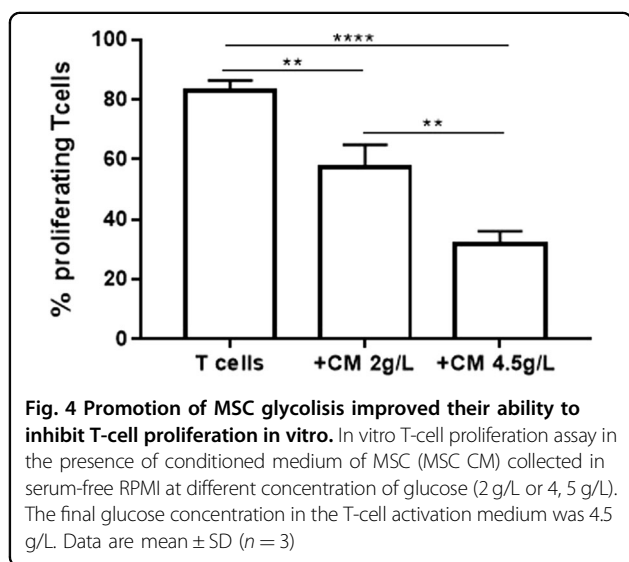
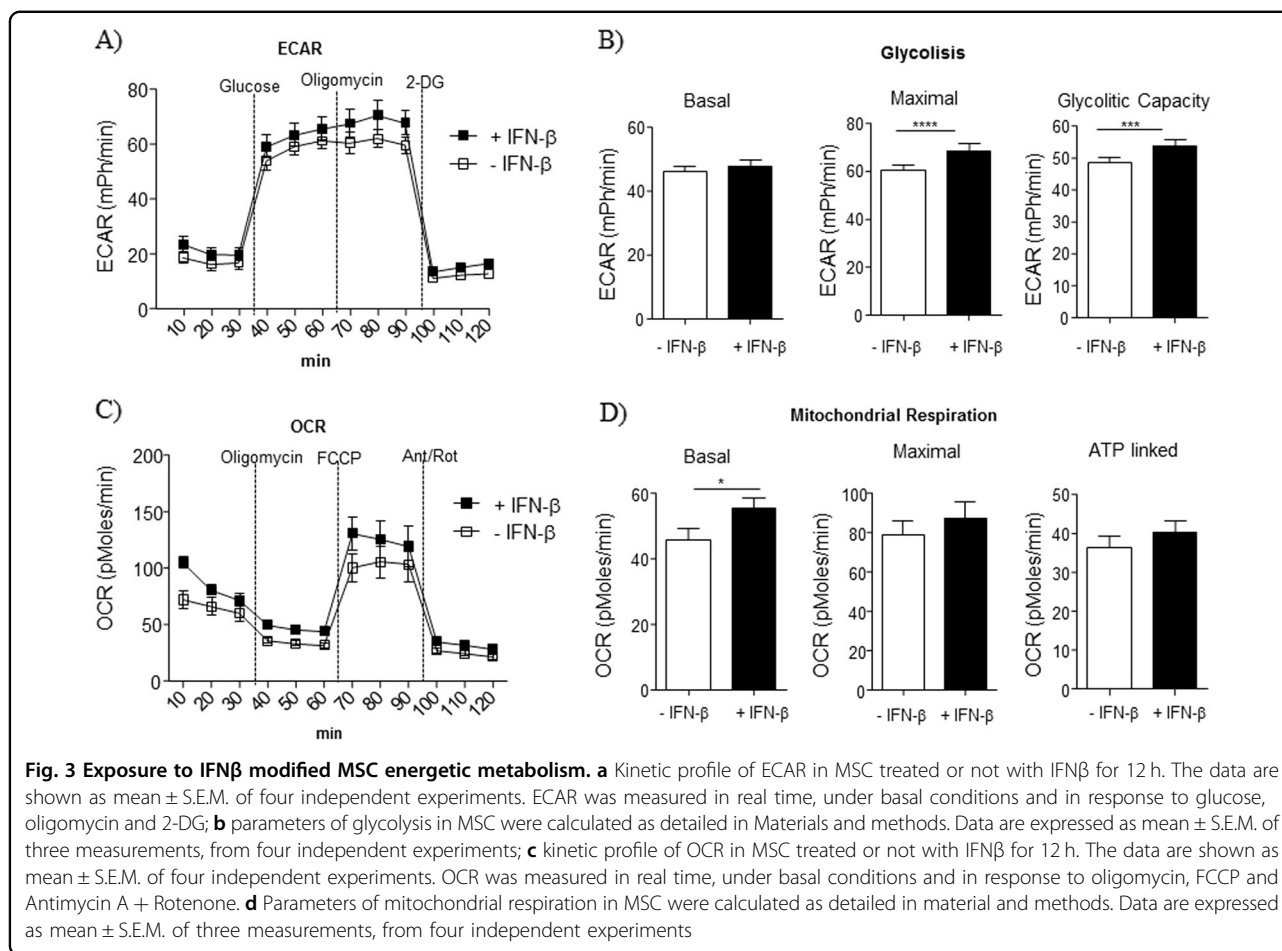
As IFN β affected the activity of mTOR pathway, a master regulator of intracellular metabolism, we assessed whether exposure to IFN β could impact on the bioenergetic profile of MSC, by measuring extracellular acidification rate (ECAR) as an indicator of glycolysis, and oxygen consumption rate (OCR) indicator of mitochondrial respiration. We observed that IFN β did not modify basal glycolysis in MSC (Fig. 3a, b). However, maximal glycolysis (induced by oligomycin injection) and glycolytic capacity were significantly increased by IFN β treatment, as compared to untreated MSC (Fig. 3a, b). Then we

investigated mitochondrial respiration in terms of OCR. IFN β did not affect maximal OCR and OCR coupled to ATP generation, but slightly enhanced the respiratory capacity of MSC in basal conditions (Fig. 3c, d), indicating that IFN β preferentially increased the glycolytic capacity of MSC.

Glycolysis engagement improved MSC immunomodulatory function

To link MSC glucose metabolism with their immunomodulatory function, we assessed whether or not improvement of MSC glycolytic rate may impact on their capacity to inhibit T-cell proliferation. We boosted glycolytic flux by growing MSC in high glucose containing medium (4.5 g/L), and we assessed the immunomodulatory properties of the resulting CM in T-cell proliferation assay. We obtained a significant improvement in the constitutive ability of MSC CM to inhibit T-cell proliferation by collecting MSC CM in high glucose-containing medium as compared to MSC CM prepared in normal medium (2 g/L) (Fig. 4).

These results clearly demonstrated that promoting glucose metabolism improved the immunomodulatory function of MSC, thus suggesting a new possible mechanism through which IFN β enhances MSC immunomodulation.



Discussion

While the potent immunomodulatory effect of IFN β on cell types of the immune system has been extensively studied²², little is known on the interaction between IFN β

and MSC. IFN β was shown to influence the expression of chemokines and their receptors in human MSC²³, but the effects of IFN β on MSC immunomodulatory function are largely unknown. Our data demonstrated that IFN β significantly increased the expression of molecules, secreted by MSC, with reported immunomodulatory activities. Among those, we observed that Slpi, a molecule never related to MSC biology, potentiated the ability of MSC to control T-cell proliferation in vitro.

Slpi is a polypeptide secreted by epithelial cells to protect tissue from damage by leucocyte proteolytic enzymes. Nevertheless, the regulatory function of Slpi on adaptive immunity is independent to its anti-protease activity. Slpi has been demonstrated to affect the monocyte pattern secretion by preventing the interaction of p65 with the NF- κ B binding sites²⁴, to inhibit effector T-cell proliferation²⁵ and to promote the generation of regulatory T cells²⁶. Slpi is upregulated in macrophages, activated microglia, neuronal cells and astrocytes during EAE attack²⁷. Interestingly, Slpi was shown to increase proliferation of adult neural stem cells promoting oligodendroglial differentiation, as incubation of adult neural stem cells with recombinant Slpi resulted in an increase of cell

proliferation and of differentiation towards oligodendrocytes²⁷. Whether *Slpi* secreted by MSC may play a role also in tissue repair promoted by MSC requires further investigation. Interestingly $\text{IFN}\beta$ significantly enhanced also the expression of *Hgf* a pleiotropic cytokine with neuroprotective which has been demonstrated to enhance axonal outgrowth and oligodendroglial maturation¹².

At molecular level, $\text{IFN}\beta$ induced a transient phosphorylation of STAT1 and STAT3, determining the induction of *Slpi* and *Hgf* expression, that was indeed impaired by both STAT1 and STAT3 inhibition. Concomitantly, $\text{IFN}\beta$ induced a dynamic modification of mTOR activity, that was early reduced and then increased. In line with the role of mTOR in the control of glucose metabolism, a prolonged exposure to $\text{IFN}\beta$ increased MSC glycolytic capacity, thus improving their ability to engage glycolysis to convert glucose to pyruvate or lactate. Energy metabolism and functional activation are fully integrated in immune cells to determine their ability to divide, differentiate, and carry out effector functions²⁸. Here we found that inhibition of T-cell proliferation by MSC was strongly enhanced by boosting MSC glycolysis. As ECAR, monitored as readout of MSC glycolytic activity, was mainly due to production of lactate, our data suggest the possible involvement of lactic acid in MSC immunomodulatory function. Lactic acid had been shown to suppress the proliferation and cytokine production of human cytotoxic T lymphocytes²⁹ and to inhibit TNF secretion and glycolysis of human monocytes³⁰.

Our data support that $\text{IFN}\beta$ can enhance MSC therapeutic function through two different molecular mechanisms: the early induction of STAT1-3-dependent immunomodulatory genes, and the long-lasting mTOR-associated promotion of glucose metabolism. Particularly, based on the finding that $\text{IFN}\beta$ induced the expression of cytokines with both immunomodulatory and neuroprotective features such as *Hgf* and *Slpi*, we speculate that MSC as add-on therapy to $\text{IFN}\beta$ treatment could promote tissue repair beyond modulation of inflammatory reactions. In line with these results, administration of $\text{IFN}\beta$ -secreting engineered MSC has been demonstrated to be more effective than wild-type MSC in the attenuation of EAE^{31,32}, further supporting the rationale for combined protocols to treat MS. Moreover, the finding that MSC immunomodulatory function is improved by glucose metabolism revealed a new possible mechanism underlying MSC therapeutic effect.

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Tiziana Vigo: conception and design, collection and/or assembly of data, data analysis and interpretation, manuscript writing, final approval of manuscript. Claudia La Rocca: collection and/or assembly of data, data analysis and interpretation, final approval of manuscript. Deriggio Faicchia: collection and/or assembly of data, data analysis and interpretation, final approval of manuscript. Claudio Procaccini: collection and/or assembly of data, data analysis and interpretation, final approval of manuscript. Maddalena Ruggieri: conception and design, final approval of manuscript. Marco Salvetti: conception and design, final approval of manuscript. Diego Centonze: conception and design, final approval of manuscript. Giuseppe Matarese: conception and design, data analysis and interpretation, manuscript writing, final approval of manuscript. Antonio Uccelli: conception and design, financial support, manuscript writing, final approval of manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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