Title: The impact of IFN-γ licensing on mesenchymal stem cells molecular signatures.

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# Abstract

Over the past few years, the immunomodulatory properties of mesenchymal stem cells (MSCs) have been target of extensive investigation. Therapeutically this potential is not widely explored principally due to some fundamental concerns regarding to MSC activation which remain to be investigated. IFN-γ is considered a major inflammatory mediator and plays a pivotal role in the MSC functionality. However the molecular mechanism involved in the MSC activation needs to be elucidated. Hence the aim of this present study was investigate the global molecular signature of MSCs under IFN-γ stimulus. The IFN-γ-licensed MSCs showed similar morphologic, phenotypic, proliferative and viability aspects to normal control MSCs. Moreover, when we compared the multipotent differentiation capabilities, the adipogenic potential decreased in IFN-γ-activated MSCs than control MSCs. In addition, the gene expression profile demonstrated that IFN-γ stimulation promoted a strong up-regulation of genes related to immunomodulation signaling pathways (e.g. IDO, MHC II, IL-1R, IRF-1, STAT-1 and HLA-G) and down-regulation of genes linked to extracellular matrix organization and cell adhesion molecules (e.g. SFRP4, SCG2, COL3A1 and MGP). Interestingly, the global DNA methylation was little affected by IFN-γ licensing, suggesting that a transcriptional regulator signal is mandatory for MSC activation. Thus, the precise understanding of MSC molecular signature during activation can elucidate some important aspects associated to its immunomodulatory potential, which is essential for its future establishment in the clinical practice.

# Introduction:

# Results:

# Discussion:

# Methods:

Mesenchymal stem cell isolation, characterization and culture.MSCs samples were isolated from femoral head of 12 individuals (aging 33-63) undergoing orthopaedic surgery after written consent using guidelines approved by the Ethic Committee of the University of Aachen (permit number: EK128/09). MSCs were cultured in Dulbecco’s Modified Eagle Medium (DMEM; 1 g/l glucose; PAA, Pasching, Austria), supplemented with 1% L-glutamine (PAA), 1% penicillin/streptomycin (PAA), 0.1% heparin (Ratiopharm, Ulm, Germany) and 10% of pooled human platelet lysate (hPL). The cells were expanded at a density of 5,000 cells/cm2 until 80% of confluence and only MSCs in passages P1-P2 were considered in further experiments. The differentiation assays into mesodermal lineages *in vitro* (i.e., adipocytes and osteoblasts) was performed conforming described in Frobel, 20141. The immunophenotyping profile was performed using specific sets of antibodies (i.e., CD90, CD105, CD73, CD45, CD34, CD14, CD31, CD29, HLA-DR, BD Bioscience, USA), according to the manufacturer's recommendations. A FACSCanto II flow cytometer (BD, Beckton Dickson) was used for cell acquisition and the FlowJo software was used for data analysis.

Interferon gamma (IFN-γ) treatment. MSCs were incubated at different time points (3, 6, 9 and 15 days) with human recombinant IFN-γ (100 ng/mL; R&D Systems, USA) and after the cells from both groups (treated or not with IFN-γ, n=6 by group) had its RNA and DNA collected for further analysis.

Morphology, proliferation and cell death assays.MSCs were seeded at a density of 3,000 cells/cm2 in 6-well plates (Corning, USA) and after 24 h they were stimulated or not with IFN-γ as previously mentioned. After 3, 6, 9 and 15 days, the proliferation rates were measured using Alamar Blue cell viability kit (1:10 dilution) (Invitrogen, USA) and FDA (5 mg/mL) + PI (2 mg/mL) reagents (Invitrogen, USA) were used for apoptosis analysis, according to the manufacturer's specifications. Fluorescence and phase contrast images of the cells were obtained using EVOS Cell Imaging equipment and EVOS FL Auto software (Life Technologies, USA).

Gene expression analysis. The total RNA was extracted from MSCs after 15 days in culture with or not IFN-γ stimulus, , using nucleoSpin RNA isolation kit (Macherey-Nagel, Germany), following the manufacturer's recommendations. The concentration of RNA was checked by Nanodrop spectrophotometer (Thermo Scientific, USA). Further, the reverse transcription was performed using the High Capacity kit (Life Technologies, USA), according to the manufacturer's specifications. Gene expression levels were measured by real-time PCR (RT-qPCR) using the TaqMan system for IDO (Hs00984148\_m1), IRF-1 (Hs00971960\_m1) and PDL-1 (CD274, Hs01125301\_m1) (Life Technologies, USA). The endogenous actin-β gene (Hs99999903\_m1) was used as an internal housekeeping control. Finally, the PCR reaction was carried out using the Applied Biosystems StepOne Plus device (Life Technologies, USA). The results were analysed with the method of relative quantification (10,000/2^ΔCt) with SDS software (Life Technologies, USA). The microarray analysis was run with HumanHT-12 v4 expression BeadChip platform (Illumina, San Diego, USA). All experimental array procedures were stablished and performed by Life & Brain GmbH Genomics (Bonn, Germany).

DNA methylation profile. The total DNA was extracted from MSCs after 15 days in culture with or not IFN-γ stimulus, using nucleoSpin DNA isolation kit (Macherey-Nagel, Germany), following the manufacturer's recommendations. The concentration of DNA was checked by Nanodrop spectrophotometer (Thermo Scientific, USA). The global methylation profile was run with HumanMethylation450 DNA Analysis BeadChip platform (Illumina, San Diego, USA). All DNA methylation procedures were stablished and performed by Life & Brain GmbH Genomics (Bonn, Germany).

RNA and DNA data analysis. First, for both array analyses the row data were normalized by quantile-normalization method. For gene expression, the data were expressed such as fold change (mean o relative expression of IFN-γ-treated group / mean of relative expression of untreated group). Furthermore, the Limma paired adjusted t-test was carried out using R software to select more significant genes differentially expressed. For DNAm profile, the data was provided as β values ranging from 0 (non-methylated) to 1 (100% methylated). The differences in DNAm levels were expressed considering the mean of β values for IFN-γ-treated group - mean of β values for untreated group). Then, differentially methylated CpGs-sites were selected using a cut-off of 5% of methylation and normal paired t-test. Gene Ontology enrichment classification of genes differentially expressed in the array as well as of genes associated to hypermethylated or hypomethylated CpGs were performed with GoMiner web software ([*http://discover.nci.nih*](http://discover.nci.nih)*. gov/gominer/*). For Heatmap analyses were used Mev Multiple Array Viewer software. All graphics were performed using Graphpad Prism software and for.

Statistical Analysis. The data sets were tested for a Gaussian distribution by a Kolmogorov-Smirnov test and further statistical inference with parametric methods was performed for all analyses. Student’s t and Two-way ANOVA (followed by Bonferromi post-test) tests were used to determine significant differences between two or more independent sample groups or variables, respectively. For specific association between two different variables a person correlation was executed. In this study all experimental data were represented by the mean and standard deviation (± SD), and statistical analyses were conducted considering a minimal statistical significance (α) at 5% (p<0.05).

Accession Numbers.Gene expression and DNAm data have been deposited at NCBI’s Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/) with accession numbers GSEXXXX, (gene expression) and GSEXXX (DNAm).

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# Figure Legends:

# Figures:

# References:

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