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Research paper

Transcriptomic analysis of BM-MSCs identified EGR1 as a transcription factor to fully exploit their therapeutic potential

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ABSTRACT

Bone marrow-mesenchymal stromal cells (BM-MSCs) are key components of the BM niche, where they regulate hematopoietic stem progenitor cell (HSPC) homeostasis by direct contact and secreting soluble factors. BM-MSCs also protect the BM niche from excessive inflammation by releasing anti-inflammatory factors and modulating immune cell activity. Thanks to these properties, BM-MSCs were successfully employed in pre-clinical HSPC transplantation models, increasing the rate of HSPC engraftment, accelerating the hematological reconstitution, and reducing the risk of graft failure. However, their clinical use requires extensive in vitro expansion, potentially altering their biological and functional properties. In this work, we analyzed the transcriptomic profile of human BM-MSCs sorted as $CD45^-$, $CD105^+$, $CD73^+$, and $CD90^+$ cells from the BM aspirates of heathy-donors and corresponding ex-vivo expanded BM-MSCs. We found the expression of immune and inflammatory genes downregulated upon cell culture and selected the transcription factor EGR1 to restore the MSC properties. We overexpressed EGR1 in BM-MSCs and performed in vitro tests to study the functional properties of EGR1-overexpressing BM-MSCs. We concluded that EGR1 increased the MSC response to inflammatory stimuli and immune cell control and potentiated the MSC hematopoietic supportive activity in co-culture assay, suggesting that the EGR1-based reprogramming may improve the BM-MSC clinical use.

1. Introduction

Bone marrow mesenchymal stromal cells (BM-MSCs) are a rare population of non-hematopoietic cells localized around the sinusoidal vessels of the BM niche supporting the homeostasis of hematopoietic stem and progenitor cells (HSPCs) together with other niche components [1–3]. BM-MSCs physically sustain HSPCs and control their maintenance and differentiation in response to different stimuli by direct contact and mainly through the secretion of paracrine factors [4–6], including hematopoietic supportive factors such as SDF-1α, SCF, and ANGPT1, which directly sustain the HSPC retention and maintenance within the BM niche. Moreover, BM-MSCs precisely control the BM microenvironment to indirectly protect HSPCs from aberrant differentiation [7]. Indeed, they display potent inflammatory sensing capacities through specific receptors [8] and activate an anti-inflammatory program by releasing indoleamine 2,3-dioxygenase (IDO), prostaglandin E2 (PGE2) and tumor necrosis factor-stimulated gene 6 protein (TSG6) preventing the inflammation-driven HSPC exhaustion and malignant transformation and protecting the BM niche cells from detrimental inflammation [9,10]. BM-MSCs also show immune regulatory function, regulating the activity of innate and adaptive immune cells [11,12]. In particular, BM-MSCs inhibit B-, T-, and natural killer (NK)-cell proliferation through the release of soluble molecules, such as Transforming Growth Factor-β (TGF-β), Hepatocyte Growth Factor (HGF), PGE2, IDO, nitric oxide (NO), and hemoxygenase (HO) [12–14]. Finally, BM-MSCs control the metabolic state of HSPCs, serving

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as ROS scavengers and favoring a glycolytic quiescent metabolism to prevent HSPCs differentiation [15,16]. Thanks to their biological and functional properties, BM-MSCs have been exploited in pre-clinical models of HSPC transplantation (HSCT), showing that BM-MSCs improve the long-term engraftment of human hematopoietic cells when co-infused in humanized models and in patients [17], mitigating the conditioning-induced inflammatory stress in the BM niche, and supporting the survival of transplanted cells [18–20]. In patients, the co-infusion of MSCs with HSPCs prevents acute graft-versus-host-disease (aGVHD) and chronic GVHD (cGVHD) [21–25], and the main complications occurring after allogeneic HSCT thanks to their immunomodulatory effects primed by the inflamed microenvironment [11]. In addition, MSCs ameliorate tissue toxicity following HSCT upon their recruitment to the site of inflammation mediated by inflammatory cytokines [26,27]. In vitro, BM-MSCs were employed to expand umbilical cord blood (UCB) HSCPs while preserving their stemness before transplantation, overcoming the limit of low number of HSPCs from UCB source [28]. Moreover, in our laboratory, we have recently demonstrated the supporting activity of BM-MSCs in the context of gene therapy, demonstrating the capacity of BM-MSCs to mitigate the DNA-damage response in UCB and mobilized peripheral blood (mPB) HSPCs in vitro upon gene editing, optimizing the transplantation outcome [29]. Finally, BM-MSCs have also been exploited clinically to sustain tissue regeneration since they favor the survival of tissue-resident stem cells upon injury and promote the inflammation process resolution [30–32]. However, the available data on BM-MSC functionality and clinical use have been obtained using ex-vivo expanded MSCs due to their low frequency in the human BM, representing 0.001–0.01 % of mononuclear cells (MNCs) [33]. Indeed, MSCs can be easily isolated from BM-MNCs by plastic adherence and expanded ex-vivo for several passages as fibroblast-like cells [34]. The clinical use of BM-MSCs requires extensive ex-vivo manipulation, which alters their biological and functional properties. Several works indicate that MSCs may become heterogeneous and acquire different properties upon plastic adherence and culture media exposure, undergoing clonal selection with altered capabilities [35–39]. Moreover, the MSC function derives from the coordinated interactions with the other BM niche components and may operate differently in vitro [39]. Recent transcriptome analysis has revealed a distinct transcriptional profile of human primary and in vitro cultured BM-MSCs, highlighting a mitigated expression of genes associated with functional properties of MSCs upon ex-vivo expansion [35]. To overcome these culture-associated issues, the manipulation of culture conditions, including cytokines, glucose concentration, oxygen tension, and culture as mesospheres, has been proposed as a strategy to maintain MSC native properties [40], including their capacity to support HSPCs. More recently, a transcription factor cocktail was employed to reprogram murine ex-vivo expanded BM-MSCs to a more primitive state [41]. In this work, we propose a transcription factor-mediated reprogramming of human BM-MSCs based on a global transcriptomic analysis of primary and corresponding ex-vivo cultured counterparts that highlighted a reduced expression of anti-inflammatory and supportive genes upon ex-vivo expansion. We identified EGR1 as a transcription factor to exploit BM-MSC functional properties and performed multiple experiments to prove the superior anti-inflammatory and immunoregulatory capacity of reprogrammed BM-MSCs compared to untransduced MSCs. We also tested the hematopoietic supportive capacity of reprogrammed BM-MSCs to expand and preserve untreated or gene-edited human HSPCs. Our results showed that novel strategies preserving MSC primary properties are desirable to optimize the clinical use of BM-MSCs.

2. Materials and methods

2.1. Isolation and culture of human BM-MSCs

Human MSCs were isolated from BM aspirate collection from healthy donors (median age: 12 years; range: 4–18 years), after obtaining informed consent according to the research protocol TIGET09 approved by the Ethics Committee of San Raffaele Hospital in 2017. Briefly, $CD34^-$ fraction was magnetically separated from the BM mononuclear cells and plated at a set concentration to isolate MSCs after 14 days of culture in proper MSC medium [34]. Retrived cells were plated at a density of 2000 cells/cm^2, expanded in culture, and used for experimental purposes between passage 2 and 5. The clonogenic capacity of MSCs was evaluated after 7 and 14 days of culture as CFU-Fs; $CD34^-$ cells were plated at the concentration of 70,000 cells/cm^2 in 35 mm petri dishes and CFU-F colonies were manually counted using 0.5 % Crystal Violet Solution. Cells were stained with the following antibody mix for 30 min at room temperature: anti human CD45 Pacific Blue (3ul/10^6) (Biolegend, 304,021), anti-human CD105 FITC (5ul/10^6 cells) (BD Biosciences, 561,443), anti-human CD90 APC (3ul/10^6) (Biolegend, 328,113), and ant-human CD73 PE (5ul/10^6) (BD Biosciences, 550,257). Cells were washed twice with PBS + 2 % FBS and resuspended into PBS for cell sorting using the BD FACSAria™ III Cell Sorter (BD Biosciences). Primary MSCs were selected as live cells, lacking the expression of CD45 and triple positive for CD105, CD90 and CD73, and collected into the RNeasy lysis buffer for total RNA extraction according to the manufactures' instructions (Qiagen, RNeasy Micro Kit, 74,004). RNA was processed for library preparation and sequencing.

2.2. Total RNA-Seq library preparation and bioinformatic analysis

Total RNA was purified 24 h after editing using RNeasy Micro Kit (QIAGEN). DNase treatment was performed using RNase-free DNAse Set (QIAGEN), according to the manufacturer's instructions. RNA was quantified with Qubit 2.0 Fluorometer (Thermo Fisher) and its quality assessed by a 2100 Agilent Bioanalyser (Agilent Technologies). Minimum quality was defined as RNA integrity number (RIN) > 8. 300 ng of total RNA were used for library preparation with TruSeq Stranded mRNA (Illumina) and sequenced on a NextSeq 500 High 75 (Illumina) by the San Raffaele Center for Omic Sciences (COSR). The quality of the input reads was assessed with FastQC (v0.11.6) and sequences were pre-processed with Cutadapt (v3.1) to get rid of low-quality base positions. Then, the resulting trimmed reads were aligned to the human genome assembly (GRCh38) using the STAR software (v2.7.6a) with standard parameters, and gene abundancies were computed exploiting the Subread featureCounts function (v2.0.1). Differential Gene Expression (DGE) analysis between primary and ex-vivo samples was performed using the R/Bioconductor package DESeq2 (v1.30.0), normalizing for library size using DESeq2's median of ratios. *p*-values were corrected using false discovery rate (FDR), and genes having FDR < 0.01 were considered to be differentially expressed. Post-analyses on the DGE results were performed with the R/Bioconductor package ClusterProfiler (v4.7.1), using the Hallmark collection from MSigDB and the Biological Processes (BP) and Cellular Component (CC) collections from Gene Ontology as reference databases.

2.3. Transduction of ex-vivo expanded BM-MSCs

BM-MSCs, reaching 70 % confluence, were transduced by overnight addition of LV-EGR1 (vector titer mean: $2{,}01 \times 10^9$ TU/ml) at different multiplicity of infection (MOIs, 10 and 25). After 12 h, medium was re-

plenished with fresh BM-MSC medium. LV-EGR1 transduced cells were functionally tested within 2 passages after transduction.

2.4. In vitro inflammatory stimulation

BM-MSCs reaction to inflammation stimuli was evaluated upon addition of specific inflammatory cytokines or upon irradiation at a dose of 8Gy. In detail, untransduced and LV-EGR1 transduced BM-MSCs (MOI 25) were exposed to LPS (1μg/ml) for 3, 6 and 24 h. Changes in the expression of anti-inflammatory genes (IL1A, IDO, PGE2, and IL10 genes) were determined by RT-qPCR upon cDNA pre-amplification.

2.5. Gene expression analyses

Total RNA was purified following the manufacturer's instructions of the Maxwell® RSC simplyRNA Cells and Tissue Kits (Promega, Cat. AS1340). After extraction, RNA samples were quantified using the NanoDrop 2000 (Thermo Scientific). 30 ng of RNA were reverse-transcribed with the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Cat. 4,368,814) and pre-amplified using the TaqMan™ Pre-Amp mix (2×) (Applied Biosystems, Cat. 4,488,593). In detail, 12.5 μl of the produced cDNA were mixed with 12,5 μl of 1 μM Primers Mix and 25 μl of TaqMan™ Pre-Amp mix (2×). cDNA was pre-amplified according to the following PCR reaction: 10 min at 95 °C, 14 cycles of 15 s at 95 °C, 4 min at 60C, and a final step at 95 °C for 10 min. The pre-amplified samples were diluted 1:20 into 950 μl H2O and used for Syber green based qPCR using QuantiFast SYBR Green PCR Kit (Qiagen, 1,039,712). 2,5 μl of pre-amplified cDNA was used for each reaction. The following primers were used:

IL6	FW 5' GATGGATGCTTCCAATCTGG 3' RW 5' TGTTCTGGAGGTACTCTAGG 3'
IL10	FW 5' TCTCCGAGATGCCTTCAGCAGA 3' RW 5' TCAGACAAGGCTTGGCAACCCA 3'
IDO	FW 5' TGGTGACTGCTTTGGTAGAG 3' RW 5' AATAGACAGTCTCAGTCGCTGC 3'
PGE2	FW 5' CAAATTGCTGGCAGGGTTGC 3' RW 5' AGGGCTTCAGCATAAAGCGT 3'
IL1A	FW 5' GACGCACTTGTAGCCACGTA 3' RW 5' GGCCATCTTGACTTCTTTGCT 3'
HEYL	FW 5' AGACCGCATCAACAGTAGCC 3' RW 5' GTGATCCACCGTCATCTGC 3'
EGR1	FW 5' CCCCGACTACCTGTT 3' RW 5' TGGGTTTGATGAGCT 3'
VCAM	FW 5' GACCTTCATCCCTACCATTG 3' RW 5' TTCACAGAACTGCCTTCCTCCA 3'
GAPDH	FW 5' CAAGAGCACAAGAGGAAGAGAG 3' RW 5' CTACATGGCAACTGTGAGGAG 3'
ACTB	FW 5' ACAGAGCCTCGCCTTTGCC 3' RW 5' GATATCATCATCCATGGTGAGCTGG 3'

2.6. HSPCs co-culture with BM-MSCs

BM-MSCs were plated at a density of 10,000 cells/cm^2 and expanded for 3 days in proper MSC medium. Twenty-four hours before starting the co-culture experiment, MSC medium was replenished with HSPC medium with or without proper pre-stimulating factors for conditioning. Mobilized peripheral blood (mPB) CD34$^+$ HSPCs (Stem Cell 70,060) were thawed at a density of 5 × 10 [5]/ml on an MSC feeder and expanded for 3 days on an MSC feeder in HSPC medium conditioned by MSCs. After editing, GE HSPCs were maintained in culture for 24 h or expanded for 96 h on an MSC feeder at a density of 5 × 10 [5]/ml in HSPC medium conditioned by MSCs. Human HSPCs edited according to standard protocol without the support of an MSC feeder were used as controls. Commercial mPB CD34$^+$ cells were thawed and expanded in StemSpan medium (StemCell Technologies 09600) supplemented with 100 IU/ml penicillin, 100 μg/ml streptomycin, 2 % glutamine, 300 ng/ml hSCF (Peprotech 300–07), 300 ng/ml hFlt3-L (Peprotech 300–19), and 100 ng/ml hTPO (Peprotech 300–18), 60 ng/ml hIL-3 (Peprotech 200–03) in the presence or absence of MSCs. Upon GE, GE-HSPCs were recovered in culture on an MSC feeder using HSPC medium conditioned from MSCs or on plastic using HSPC medium.

2.7. PHA-induced proliferation assay

Peripheral blood mononuclear cells (PBMCs) were purified by Ficoll density gradient centrifugation (20 min at 600 xg) from heparinized blood of healthy donors, after obtaining written informed consent. The proliferation of PBMCs, cultivated in RPMI 1640 medium (ThermoFisher, 11,875–093), supplemented with 10 % FBS, in response to PHA-P (Sigma-Aldrich, L8754), in presence or absence of MSCs, was evaluated. MSCs were seeded overnight at MSCs:PBMCs ratio of 1:5, 1:50, and 1:500. 1 × 10^5 PBMCs per well were added with or without PHA-P (4 μg/ml) and pulsed with 3H-thymidine (1 μCi/well, specific activity 6.7 μCi/mmole, Perkin Elmer, Waltham, MA) after 48 h of culture. PBMC proliferation was measured by 3H-thymidine incorporation assay 18 h later using a Microbeta Trilux 1450 detector. Results were expressed as mean percentage of stimulation index (SI = cpm stimulated/cpm unstimulated).

2.8. In vitro single-cell differentiation assay

Single-cell differentiation assay was performed according to a previously published protocol [42]. In detail, 2 h before CD34$^+$ cell seeding, non-tissue culture-treated 96-well plates were coated with StemSpan Differentiation Coating Material (Stem Cell Technologies) according to manufacturer specifications. Cells were seeded in SFEM II medium (Stem Cell Technologies) supplemented with hSCF (100 ng/ml), hFLT3 (10 ng/ml), hIL-7 (100 ng/ml), hIL-2 (10 ng/ml) (Novartis), hTPO (75 ng/ml), hIL-6 (40 ng/ml), hIL-3 (10 ng/ml), hIL-11 (50 ng/ml), hEPO (0.1 U/ml) (Peprotech), hIL-4 (10 ng/ml) (Miltenyi Biotec), hLDL (4 μg/ml) (Stem Cell technologies). Medium change was performed every 3–4 days. After 3 weeks of culture, cells were harvested and labeled with the multilineage antibody mix as previously described. The stained samples were acquired through BD FACS Symphony A5 (BD Biosciences) flow-cytometry after Rainbow bead (Spherotech) calibration.

2.9. Luminex assay for cytokine quantification

Cell supernatants from untransduced and EGR1 overexpressing BM-MSCs were collected 48 h after culture and irradiation (8Gy), stored at —20C and centrifuged for 5 min at 1500 rpm prior to use. Cytokines were quantified in undiluted samples by magnetic beads assay according to the manufacturer's instructions (Bio-Rad M5000031YV) using MAGPIX System (Luminex assay).

2.10. Statistical analyses

All data are represented as mean ± SEM. Statistical analyses and graphs were performed using the Prism software (Graphpad). One-tailed Mann-Whitney test was used for statistical evaluation (* $p \leq 0.05$, ** $p \leq 0.01$).

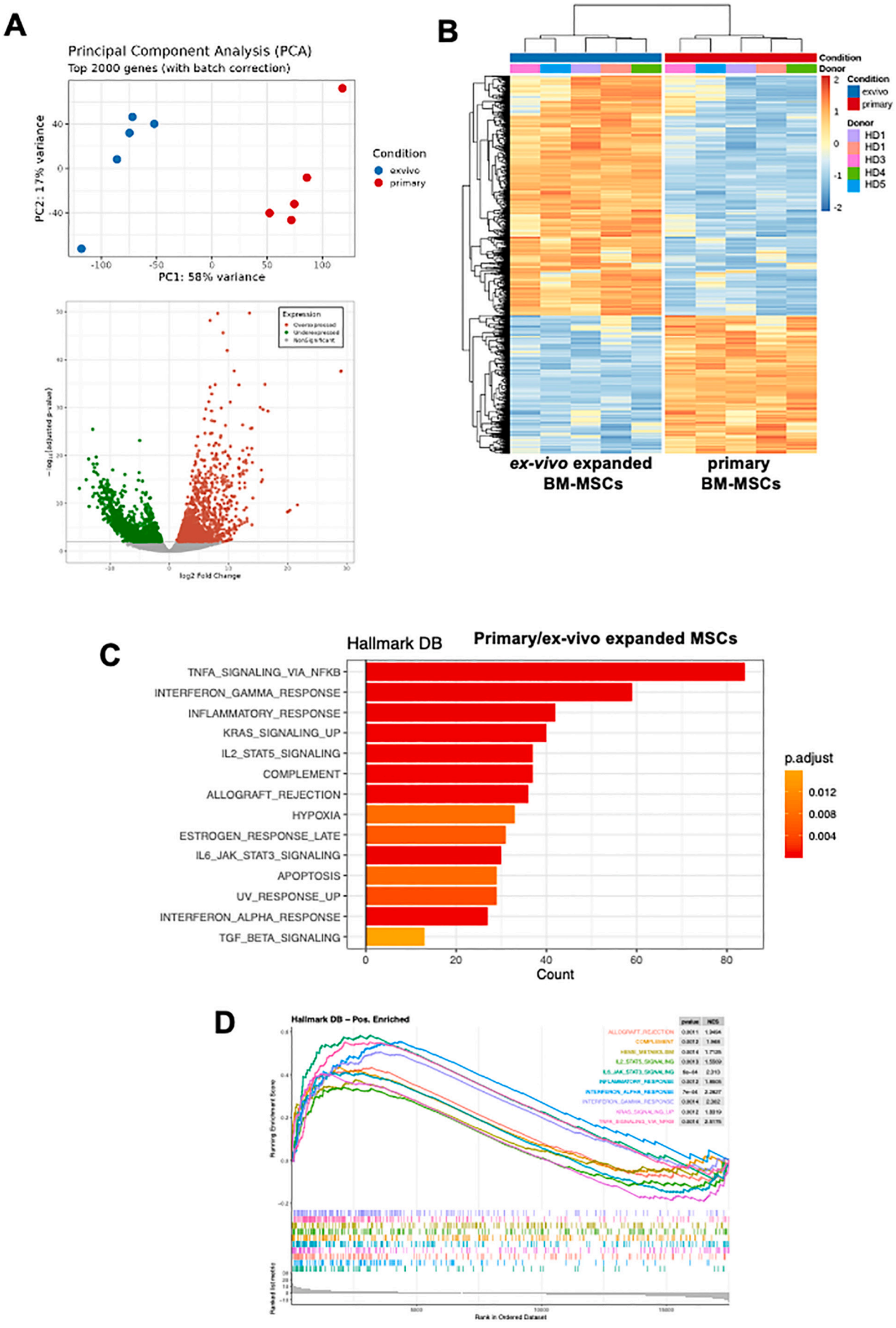

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Fig. 1. Exploratory analysis of transcriptomic changes in BM-MSCs upon ex-vivo expansion. A) Principal Component Analysis (PCA) of RNA-seq sample, colored by condition: ex-vivo (blue) and primary (red) MSCs (upper panel). Volcano plot showing the modulated gene expression of primary and ex-vivo expanded BM-MSCs (lower panel). B) Heatmap showing the expression of the differentially expressed genes (FDR < 0.01) in the comparison between primary and ex-vivo samples. Genes (rows) and samples (columns) are clustered in an unsupervised way. C) Barplot showing the significant enriched categories (adj. *p*-value <0.05) from the Hallmark MSigDB on upregulated genes. Terms on the y-axis are sorted according to the gene count (x-axis), while bars are colored by adj. p-value. D) Random walk plots of the significant categories from the Hallmark MSigDB, resulting from the GSEA.

3. Results

3.1. Human primary MSCs show an active anti-inflammatory, immunomodulatory and hematopoietic supportive gene profile

Despite the high number of clinical trials using ex-vivo expanded BM-MSCs, the impact of ex-vivo expansion on the biological and functional properties of MSCs has not been fully elucidated. We decided to exploit RNA sequencing for high throughput characterization of primary, freshly isolated BM-MSCs, which were sorted from the BM aspirates of pediatric healthy donors based on the expression of canonical MSC markers, allowing a direct comparison with ex-vivo cultured MSCs. In detail, after parental informed consent (Tiget09), we purified MNCs by density gradient centrifugation from residual BM samples of pediatric healthy donors (median age: 12 years, range 4–18 years). BM-MNCs were in part plated at a density of 2×10^5 cells/cm^2 for in vitro isolation and expansion according to standard protocols [34] and in part sorted as CD45neg cells co-expressing CD105, CD73, and CD90 cells (Supplementary Fig. 1A). We referred to freshly isolated MSCs as primary MSCs. As expected, the mean percentage of primary MSCs on the total BM-MNCs was 0,003 ± s.e.m. (Supplementary Fig. 1B). RNA was directly purified and sequenced from sorted MSCs, while MSC clones were expanded for 2 passages and characterized according to the criteria proposed by the International Society for Cellular Therapy before RNA isolation [43]. All the MSCs acquired a spindle-like morphology and expressed the canonical MSC surface markers (CD105, CD90, CD73) (Supplementary Fig. 1A, C). As expected, they differentiated in vitro into osteoblasts, adipocytes, and chondrocytes (Supplementary Fig. 1D). 100 pg of RNA from freshly isolated MSCs and corresponding ex-vivo expanded cells were used for RNA sequencing library preparation and sequenced using the Illumina platform. Principal component analysis (PCA) of the sequencing data showed a clear separation of the two MSC populations on PC1 (primary and ex-vivo expanded MSCs), which was also reflected by the high number of differentially expressed genes (DEGs) (Fig. 1A, B). We found ~3500 DEGs upon MSC ex-vivo isolation (FDR < 0.01) (GSE253276). In particular we found 1299 upregulated genes and 2247 downregulated genes in primary BM-MSCs as compared to ex-vivo expanded counterparts. Over Representation Analysis (ORA) demonstrated that the majority of genes upregulated in primary MSCs belonged to pathways involved in inflammatory responses, key immune regulatory axis, such as IL2, IL6, TNF, and INFs driven pathways, and in the control of allograft rejection (Fig. 1C, D). These categories of genes, which were significantly enriched in primary MSCs, suggest that the immunoregulatory and anti-inflammatory potential of MSCs may be mitigated by the ex-vivo culture (Fig. 1C, D). We selected specific genes (IL-7, TLR2 and ICAM1) described to mediate the MSC protective function on graft rejection and confirmed their expression by RT-qPCR (Fig. 2A) [44–46]. Several genes associated with the anti-inflammatory response were enriched in the primary MSCs (Fig. 2B). Among these, we checked the expression levels of IL1R1, encoding for IL1-β antagonist, a potent anti-inflammation mediator [47], and found a significant reduction in ex-vivo expanded MSCs compared to primary cells (Fig. 2B).

The enrichment of IFNα and γ-associated genes in primary BM-MSCs indicated a pre-activation state of MSCs possibly stimulated by the BM niche extracellular environment, which facilitated the production of anti-inflammatory molecules. Indeed, we observed an increased trend of production of IL10 by expanded MSCs after the in vitro treatment with IFNγ (Fig. 2C). We also found TNFα and TGFβ signaling genes were more expressed in primary MSCs (Fig. 1C). TNFα induces MSCs to adopt an immune-suppressive phenotype [48] similar to IFNs, suggesting that BM-MSCs may lose their pre-activation state in culture, preventing them from driving a prompt anti-inflammatory response. Moreover, TGFβ-primed MSCs showed superior efficacy in modulating graft rejection [49]. Of note, TGFβ activation also sustained the epithelial-mesenchymal transition (EMT) in primary cells [50] [51]. Primary cells expressed an EMT gene signature (SNAI1, TWIST2, and ZEB1), which was downregulated in ex-vivo expanded cells (Fig. 2D). On the contrary, Gene Set Enrichment Analysis (GSEA) on GO Biological Processes showed the activation of genes encoding for adherent junction and focal adhesion proteins in ex-vivo expanded MSCs, highlighting that adherence may convert MSCs toward an epithelial-like phenotype (Supplementary Fig. 2 A). Since the EMT transcription factors downregulated in culture are known to control cell migration, we performed a scratch assay on ex-vivo expanded MSCs stimulated with TGFβ and untreated MSCs as controls. The scratch percentage was reduced upon TGFβ treatment, indicating a superior migration capacity in treated cells, possibly due to the TGFβ-mediated reactivation of EMT genes (Supplementary Fig. 2B).

Finally, we interrogated the transcriptome profiling to evaluate the expression of hematopoietic supportive genes and found a significant Reads Per Kilobase Milion (RPKM) reduction of key bone marrow niche supporting genes, including CXCL12, ANGPT1, SCF, and VEGFA in ex-vivo expanded MSCs (Fig. 3A). We also observed a significant reduction of Angiogenin 1 (ANG1) expression in ex-vivo expanded MSCs, a niche-secreted ribonuclease promoting HSPC quiescence [16] (Fig. 3A). We concluded that the anti-inflammatory and HSPC-supportive properties of MSCs are globally impacted by the ex-vivo culture, and novel strategies to preserve MSC primary properties are warranted to fully exploit the clinical use of MSCs. A distinct gene expression pattern was shown in a recent work comparing the transcriptome of primary and adherent MSCs. In this study, they identified a cluster of genes involved in the immune response, mRNA processing, and antigen presentation enriched in primary cells, while a cluster of genes mainly involved in cell adhesion and cytoskeleton organization was overexpressed in adherent cells [35]. Using the gene signatures from these clusters, we were able to separate our samples, showing a similar expression profile for primary and ex-vivo expanded cells showed by Ghazanfari et al., despite the use of a different sorting strategy and method used to isolate MSCs (Fig. 3B, C). We also found the expression of Early Growth Response 1 (EGR1), a C2H2-type zinc-finger transcription factor described to control the paracrine activity of MSCs, reduced in MSCs upon in vitro culture [52] (Fig. 3D). EGR1 was also described to promote the hematopoietic supportive activity of MSCs [53]. We reasoned that EGR1 may exert control over several gene regulatory networks in MSCs and that the restoration of its expression in ex-vivo expanded MSCs might contribute to better exploit their biological properties. Using a global approach of TF-target gene prediction (JASPAR Predicted Transcription Factor Targets), we found that EGR1 controls the expression of 685 target genes, including mitogenic genes, PGE2 synthase, TNFalpha-associated factors, and mesenchymal cadherin. Enrichment analysis of EGR1 target genes highlighted the role of EGR1 in controlling signal transduction, cell-cell communication, and signaling by interleukins, all biological functions associated with MSCs (Supplementary Fig. 3A).

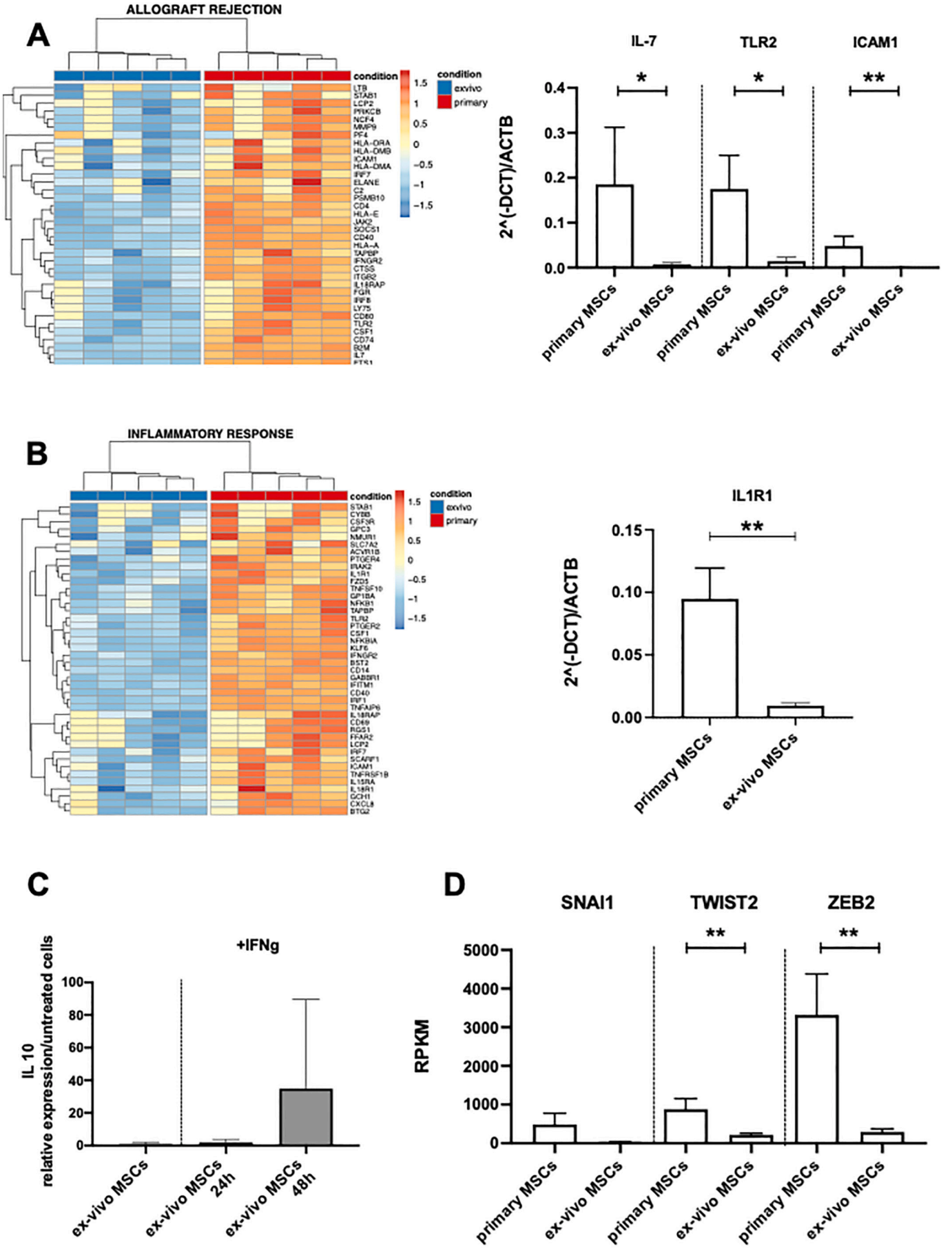

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Fig. 2. Specific analysis of biological pathways differentially utilized by BM-MSCs upon in vitro expansion. A) Heatmap showing the expression of the genes belonging the core-enrichment of the allograft rejection category from the enrichment analysis on the Hallmark MSigDB, in which genes (rows) and samples (columns) are clustered in an unsupervised way (left). On the right panel, qPCR expression analysis of IL7, TLR2, and ICAM1 in primary and ex-vivo BM-MSCs. Gene expression was calculated as 2^-DCT over the ACTB gene. Each error bar shows means ± SEM ($n \geq 4$). (*$p \leq 0.05$; ** $p \leq 0.01$). B) Heatmap showing the expression of the genes belonging the core-enrichment of the inflammatory response category from the enrichment analysis on the Hallmark MSigDB, in which genes (rows) and samples (columns) are clustered in an unsupervised way (left). On the right panel qPCR expression analysis of IL1R1 in primary and ex-vivo BM-MSCs. Gene expression was calculated as 2^-DCT over the ACTB gene. Each error bar shows means ± SEM (n ≥ 4). (** p ≤ 0.01). C) qPCR expression analysis of IL10 in ex-vivo BM-MSCs at basal level and upon treatment with IFNgamma for 24 and 48 h. Data are expressed as fold change over untreated BM-MSCs. DCT was calculated as Ct gene-Ct ACTB gene. Each error bar shows means ± SEM (n ≥ 4). D) Reads Per Kilobase Million (RPKM) of SNAI1, TWIST2, and ZEB2 in primary and ex-vivo BM-MSCs. Each error bar shows means ± SEM (n ≥ 4) (** p ≤ 0.01).

3.2. MSC reprogramming by EGR1 overexpression

To potentiate the MSC paracrine activity in vitro, we generated a lentiviral vector bearing the human EGR1 cDNA sequence under the control of the phosphoglycerate kinase (PGK) promoter to overexpress EGR1 in ex-vivo expanded human BM-MSCs (Fig. 4A). First, we set the conditions for BM-MSCs transduction, showing a significant induction of EGR1 expression in MSCs transduced with LV-EGR1 at a Multiplicity of Infection (MOI) of 25 (mean: 6 ± s.e.m.) (Fig. 4B). EGR1 was also detected by Western Blot in transduced cells (Fig. 4C), which overexpressed EGR-1 target genes (HEYL and VCAM) (Fig. 4D). We found that EGR1 overexpressing MSCs expressed significantly higher levels of the anti-inflammatory factor IL-10 and IL1R1 at steady-state compared to untransduced (UT) MSCs (Fig. 4E, F), similar to freshly isolated MSCs (Fig. 3B). In LV-EGR1 transduced MSCs, we also observed a statistically significant higher expression of IL6, known to maintain MSC stemness [54], as compared to UT MSCs (Fig. 4G).

3.3. Analysis of the anti-inflammatory and immunomodulatory properties of EGR1 overexpressing MSCs

Based on these results, we functionally tested the anti-inflammatory properties of EGR1-reprogrammed MSCs by in vitro stimulation with different factors and upon irradiation. We also performed phytohemagglutinin (PHA)-mediated stimulation of peripheral blood mononuclear cells (PBMNCs) to analyze the immunoregulatory effect of reprogrammed MSCs. Finally, we studied the hematopoietic supportive capacity of EGR1 overexpressing MSCs in vitro through a co-culture assay of human mPB HSPCs expanded on an MSC feeder, in normal conditions or upon gene editing.

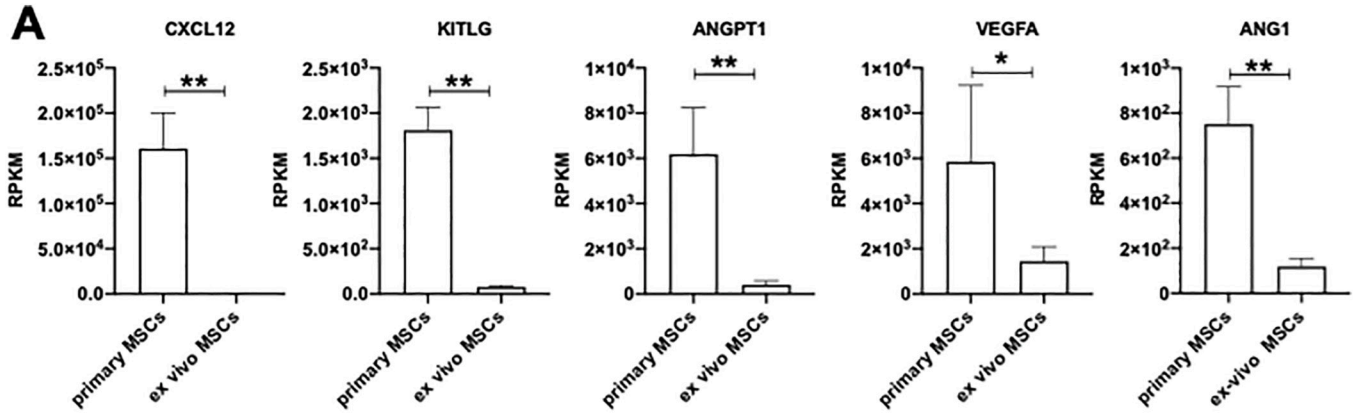
We stimulated MSCs with LPS (1 μg/ml) and analyzed the expression of inflammatory genes at different points post stimulation since LPS is known to induce pro-inflammatory or anti-inflammatory MSC phenotype following a short- or long-term exposure [55]. MSCs transduced with LV-EGR1 induced IL1A expression at significantly higher levels compared to control cells (UT) after short-term stimulation (3 and 6 h), while the level of IL1A returned to basal level after 24-h stimulation (Fig. 5A). Moreover, EGR1 overexpressing MSCs activated the expression of IDO at significantly higher levels compared to treated UT MSCs at 3 and 6 h upon stimulation, while we observed a trend of IDO upregulation at 24 h in EGR1 overexpressing MSCs (Fig. 5B). We observed a time-course increase of PGE2 expression in EGR1 overexpressing MSCs, with the pick of expression occurring at 24 h. On the contrary, PGE2 expression reached a plateau at 6 h in LPS-treated UT MSCs (Fig. 5C). The expression of IL10 was higher at 6 h post-treatment in EGR1 overexpressing MSCs compared to control UT cells (Fig. 5D). These data confirmed a superior paracrine activity of LV-EGR1 transduced MSCs in response to external stimuli, sustaining the wide range of genes controlled by the transcription factor activity of EGR1.

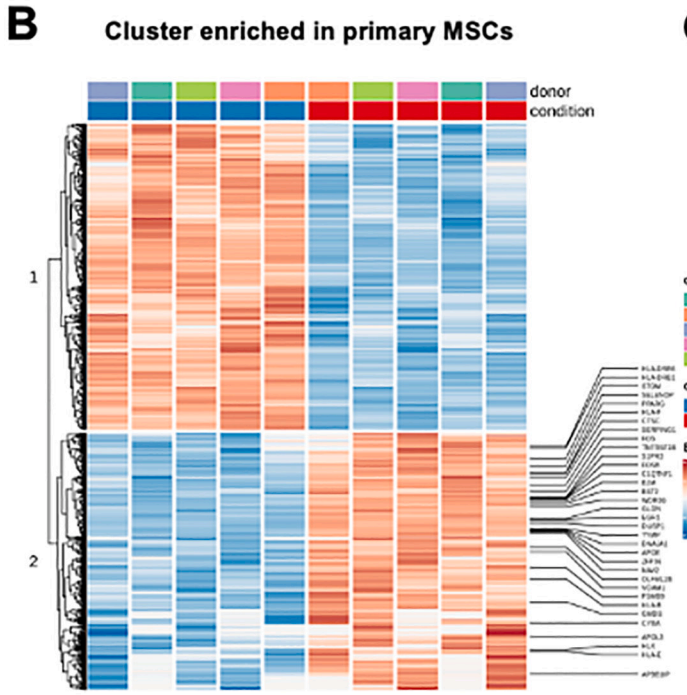
To test the anti-inflammatory capacity of MSCs in response to external inflammatory signals, mimicking the typical conditioning regimen-induced BM niche damage, we in vitro irradiated EGR1 overexpressing MSCs and control cells at a dose of 8Gy. Only IDO was robustly activated by EGR1 overexpressing MSCs (Fig. 5E), while a trend of PGE2 overexpression was observed both at steady state and upon irradiation (Fig. 5F). We could not detect IDO and PGE2 using ELISA in the cell supernatant of both untransduced and EGR1 overexpressing BM-MSCs (data not shown), either at baseline and after exposure to 8Gy irradiation. However, we found increased levels of IL-7 in the cell supernatant from MSCs + EGR1 using Luminex technology. This difference was consistent after irradiation (Fig. 6A). On the contrary, we observed reduced levels of IL-8 in the cell supernatant from EGR1 overexpressing MSCs both before and after irradiation (Fig. 6B). Additionally, the presence of EGR1 stimulated the release of G-CSF, which increased in the cell supernatant of BM-MSCs overexpressing EGR1 after irradiation (Fig. 6C). We further tested the immunoregulatory capacity of MSCs upon EGR1 overexpression in a T-cell proliferation assay. In this assay, the MSC immunoregulatory capacity is evaluated in terms of T-cell proliferation inhibition upon PHA stimulation. The level of T-cell proliferation in the absence of MSCs was evaluated as a positive control (-MSCs). Our results indicated that T-cell proliferation was robustly inhibited by EGR1 overexpressing MSCs plated at a ratio MSC:PBMC of 1:5 and this was statistically different as compared with UT MSCs. This suggests that EGR1 overexpressing MSCs display superior immunoregulatory capacity as compared with UT counterparts at a ratio of 1:5. As also reported in the literature [56], the MSC-mediated inhibitory effect was dependent on the ratio with lower MSC:PBMC ratios being associated with reduced inhibitory effects on PBMCs by both LV-EGR1 transduced and UT MSCs (Fig. 6D). Altogether these results demonstrated that the restoration of EGR1 expression improved the anti-inflammatory and immunomodulatory properties of ex-vivo cultured MSCs, possibly potentiating specific gene activation in response to external stimuli.

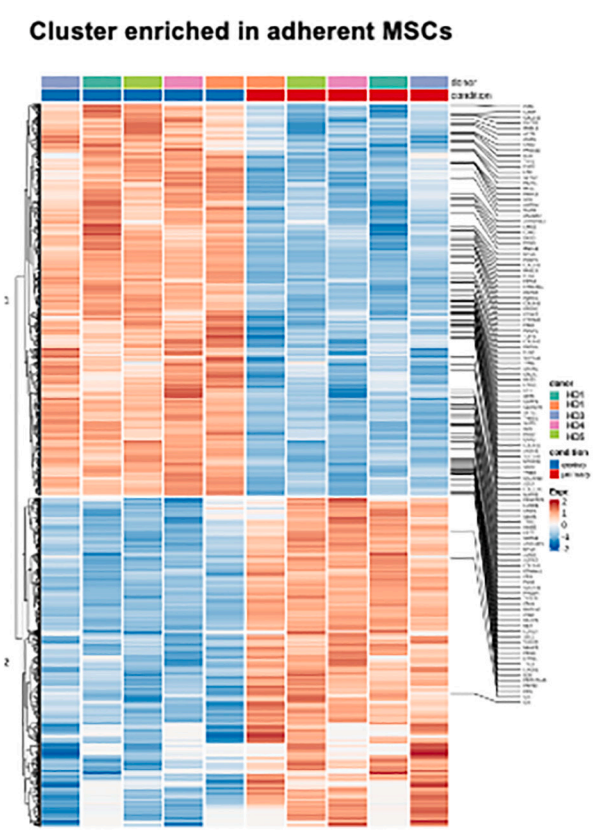
3.4. Analysis of the hematopoietic supportive capacity of EGR1 overexpressing MSCs

Considering that our transcriptomic data highlighted a reduced expression of hematopoietic supportive genes in ex-vivo expanded MSCs, we evaluated the capacity of EGR1 overexpressing MSCs to favor HSPCs expansion and maintenance in a 2D co-culture system previously set in our laboratory. Human mPB HSPCs were cultured for 72-96 h on UT MSCs and MSCs overexpressing EGR1. mPB HSPCs expanded on plastic were used as controls. We determined the total counts of HSPCs after co-culture and found similar effects of UT MSCs and EGR1 overexpressing MSCs on total $CD34^+$ cell proliferation (Fig. 7A). However, we found an increased clonogenic capacity of HSPCs co-cultured with LV-EGR1 transduced cells as compared with UT MSCs, despite the detrimental culture-induced effects on the HSPC primitive compartment (Fig. 7B). Co-cultured HSPCs were also analyzed for their differentiation capacity through the single-cell multi-lineage differentiation assay previously described [42]. After 3 weeks of culture, the HSPCs expanded in the presence of MSCs + LV-EGR1 differentiated into myeloid, lymphoid, erythroid, and megakaryocyte lineages with a similar efficiency compared to HSPCs maintained in culture on plastic or co-culture with UT MSCs. Despite the EGR1-mediated maintenance of $CD34^+$ cells, the presence of EGR1 overexpressing cells did not impact the proper differentiation of expanded HSPCs (Fig. 7C).

Finally, to study the hematopoietic supportive capacity of EGR1 overexpressing MSCs in the presence of an external stress signal, we







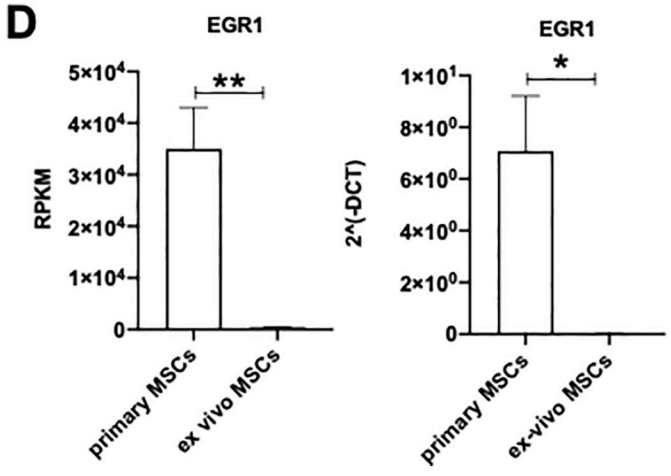


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Fig. 3. Expression analysis of the hematopoietic supportive genes in ex-vivo expanded BM-MSCs compared to primary BM-MSCs. A) Values of Reads Per Kilobase Million (RPKM) of the hematopoietic supportive genes CXCL12, KITL, ANGPT1, VEGFA, and ANG1 in primary and ex-vivo BM-MSCs. Each error bar shows means ± SEM ($n \geq 4$) (*$p \leq 0.05$; ** $p \leq 0.01$). B) Heatmaps showing the expression of the differentially expressed genes (FDR < 0.01) in the comparison between primary and ex-vivo samples, highlighting genes from the cluster enriched in primary (left) and adherent (right) MSCs from (36). Genes (rows) and samples (columns) are clustered in an unsupervised way. C) Values of Reads Per Kilobase Million (RPKM) and D) qPCR expression analysis of EGR1 gene calculated as 2^-DCT over ACTB in primary and ex-vivo BM-MSCs (right panel). Each error bar shows means ± SEM ($n \geq 4$) (*$p \leq 0.05$; ** $p \leq 0.01$).

employed MSCs as feeders in support of HSPC during gene-editing (GE), as we previously demonstrated that MSCs preserved the fitness of HSPCs undergoing gene-editing [29]. We used the same protocol of GE previously reported [29] including both untransduced and EGR1 overexpressing MSCs as feeders. We found that both feeders favored the recovery of GE cells maintained in culture for 72 h upon editing (Fig. 7D). A similar number of $CD45^+$ cells was detected upon co-culture of GE mPB HSPCs with UT MSCs and EGR1 overexpressing MSC that was significantly higher compared to the number of HSPCs retrieved upon recovery of GE cells on plastic (Fig. 7D). All together these data show a trend of increase of absolute number of phenotypically primitive HSPCs in the presence of EGR1 overexpressing cells. We confirmed the capacity of MSCs to preserve the most primitive compartment, despite no significant differences observed compared to GE HSPCs recovered on untransduced MSCs (Fig. 7E). We further analyzed the expression of inflammatory cytokines and found IL1A significantly reduced in GE HSPCs cultured in the presence of EGR1 overexpressing MSCs (Fig. 7F), highlighting the robust anti-inflammatory potential driven by EGR1 which mitigated the inflammation driven by the GE procedure. We also found a trend of reduced p21 activation in GE HSPCs recovered on LV-EGR1 transduced cells compared to cells maintained on the UT MSC feeder (Fig. 7G). Overall, our data functionally confirmed the reduced hematopoietic supportive capacity of ex-vivo expanded MSCs. The restoration of EGR1-driven paracrine activity possibly potentiates the hematopoietic support in terms of clonogenic capacity and differentiation of HSPCs. Importantly, the presence of EGR1 overexpressing MSCs strongly mitigated the IL1A expression in GE-HSPCs.

4. Discussion

In this work, we analyzed the transcriptomic profile of human primary BM-MSCs and their corresponding ex-vivo expanded counterpart. The clinical use of BM-MSCs requires extensive ex-vivo manipulation to reach the numbers which are required for therapeutic applications; this is known to alter the MSC intrinsic biological and functional properties and possibly induce the loss of their primitive in vivo functions, thus limiting their therapeutic potential [23,57,58]. In order to investigate the effect of culture conditions on the original properties of BM-MSCs, we performed a high throughput characterization of primary, freshly isolated BM-MSCs, sorted from the BM aspirates of pediatric healthy donors, versus their ex-vivo cultured counterpart. We confirmed a global gene profile significantly altered upon in vitro culture, as previously described by other groups [35]. The majority of genes upregulated in primary MSCs belonged to pathways involved in inflammatory responses, immune regulatory control, and hematopoietic supportive capacity, highlighting that the original properties of BM-MSCs are impacted by the ex-vivo culture. In particular, genes associated with inflammatory responses and immune regulation, such as IL2, IL6, TNF, and INFs, were found to be upregulated in primary BM-MSCs, together with genes involved in the MSC protective effect on graft rejection, i.e. IL-7, ICAM1, and TLR2. Moreover, the expression of hematopoietic supportive genes, including SCF, ANGPT1, CXCL12, VEGFA and ANG1, was significantly higher in primary BM-MSCs, while it was reduced in ex-vivo expanded cells. Our results parallel well the recently published data from Ghazanfari et al., who reported a distinct transcriptional profile of human primary and ex-vivo cultured MSCs, highlighting a reduced expression of genes associated with the hematopoietic supportive functional properties of BM-MSCs upon ex-vivo expansion [35]. In our approach, we aimed to enhance the sensing, signaling, and paracrine activity of cultured MSCs by modulation of gene expression. To achieve this goal, we focused on overexpressing transcription factors (TF) in cultured MSCs, as these factors regulate multiple target genes, amplifying the effects of gene modulation, similar to in vitro cell reprogramming. Furthermore, previous data from mouse studies demonstrated that a cocktail of TFs effectively enhances the MSC hematopoietic supportive function [41]. In line with this, we also found that the expression of EGR1, a transcription factor able to control the paracrine activity of MSCs [52,59], was reduced in BM-MSCs upon in vitro culture. Since paracrine factors have been reported to be critical in the ability of MSCs to control inflammatory and immune responses both in vitro and in vivo in the clinical scenario [8,9], we aimed at modifying ex-vivo cultured human BM-MSCs in order to restore their biological features, similar to primary MSCs. Therefore, we overexpressed EGR1 in ex-vivo expanded BM-MSCs by a lentiviral vector bearing the human EGR1 cDNA with the aim of restoring the original MSC properties and maximizing their paracrine activity. In line with this, Nakahara et al. identified a transcription factor cocktail successfully employed to reprogram murine ex-vivo expanded MSCs to a more primitive state [41]. We tested in vitro the functionality of EGR1 overexpressing MSCs through stimulation with different signals intended to mimic in vitro typical clinical situations, such as responses to inflammatory signals, i.e. infections or Graft-versus-Host-Disease, and conditioning regimen-induced niche damage. We demonstrated an anti-inflammatory primed state similar to primary MSCs, with IL10 and IL1R1 genes overexpressed in reprogrammed MSCs compared to control cells. We also showed, for the first time, that, upon stimulation with LPS, used to reproduce in vitro the inflammatory effects of cytokines, the EGR1 overexpressing MSCs robustly activated the expression of IDO, PGE2, and IL10. Moreover, in response to irradiation-associated stress, we also found a superior expression of IDO compared to LPS-treated untransduced BM-MSCs. Even though we could not detect higher levels of IDO in the cell supernatant from MSCs+EGR1 compared to UT cells possibly due to the low correlation of certain gene transcripts with protein levels [60], we observed a higher amount of IL-7 and G-CSF in MSCs overexpressing EGR1, which may contribute to immunomodulate the inflammatory milieu [61–63] and protect from conditioning induced tissue damage [64,65]. EGR1 may also contribute to reduce the levels of inflammatory cytokines. Indeed, we found a lower level of IL-8, a potent inflammatory cytokine, in the cell supernatant from EGR1 overexpressing MSCs both before and after irradiation, suggesting a potential mitigation effect on radiation-induced injury [66]. Finally, we also tested the effect of EGR1 overexpression in the control of immune cells by performing a PHA-induced T cell proliferation assay, showing an improved immunoregulatory potential of EGR1 overexpressing BM-MSCs at a ratio of 1:5.

We concluded that the restoration of EGR1 expression improved the anti-inflammatory and immunomodulatory properties of ex-vivo cultured MSCs, suggesting that EGR1 overexpression in MSCs might be employed to optimize clinical outcomes in the setting of HSPC transplantation and regenerative medicine where anti-inflammatory and immunoregulatory signals are required to control excessive inflammatory responses. Furthermore, to avoid any risks associated with the use of gene-modified cells, the characterization of the EGR1-associated MSC secretome would be fundamental for future clinical

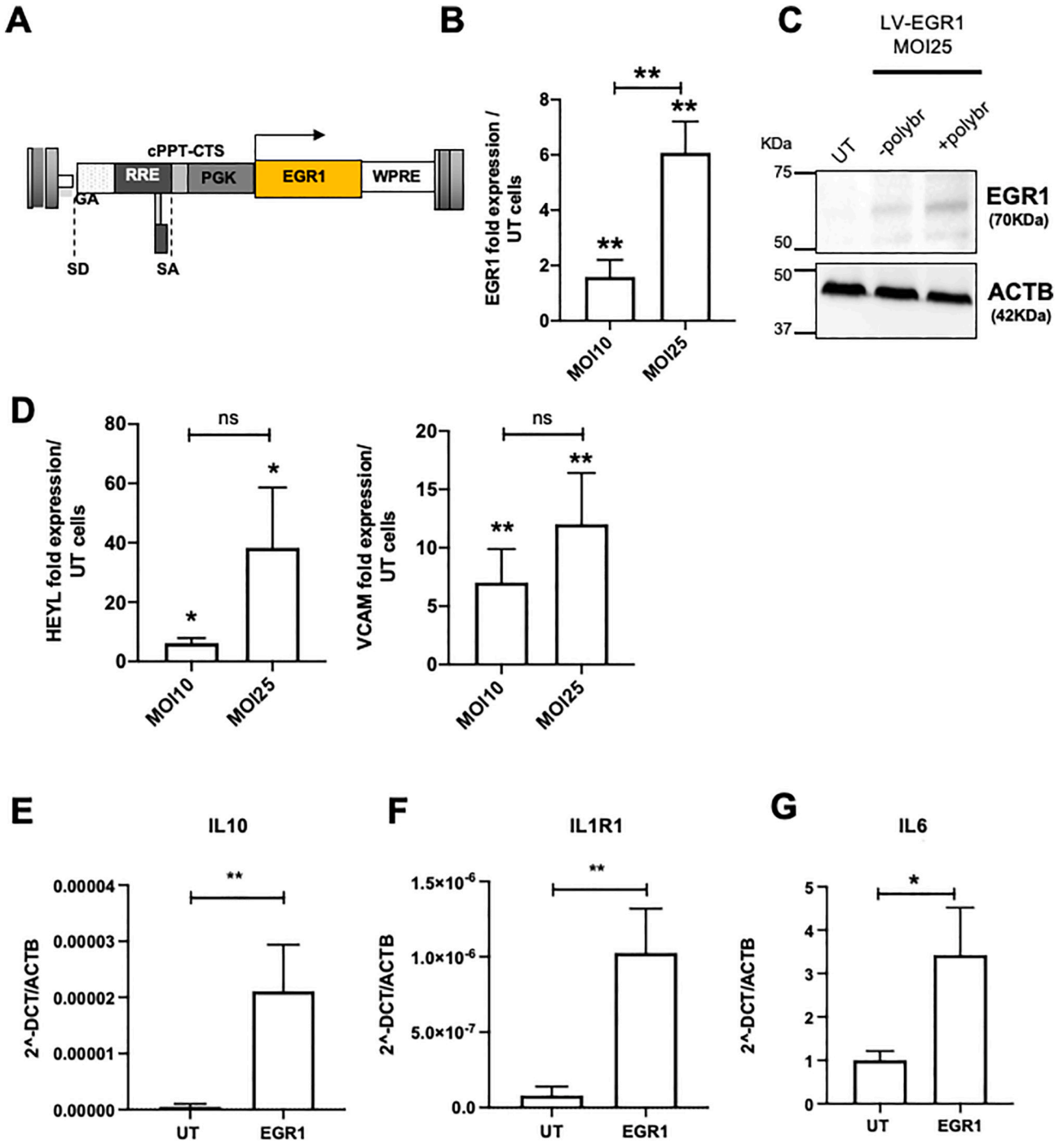

Fig. 4. In vitro analysis of EGR1 overexpressing BM-MSCs A) Schematic representation of the third-generation lentiviral vector bearing the EGR1 human transgene (LV-EGR1) under the control of the PGK promoter used to transduce ex-vivo expanded BM-MSCs at different MOIs (10, 25). B) qPCR analysis of EGR1 expression in untransduced and LV-EGR1 transduced BM-MSCs. Each error bar shows means ± SEM ($n \geq 4$) (** $p \leq 0.01$). Data are represented as fold change over untransduced cells. DCT was calculated as Ct gene - Ct ACTB gene. C) Western blot analysis of EGR1 expression in the protein extract from BM-MSCs transduced with the LV-EGR1 at an MOI of 25 in the presence/absence of polybrene. The protein extract from untransduced cells was used as a control. ACTB protein expression was evaluated as a reference. D) Expression analysis of the EGR1 target genes, HEYL and VCAM, in BM-MSCs transduced with the LV-EGR1 at an MOI of 10 and 25. Each error bar shows means ± SEM ($n \geq 4$). (*$p \leq 0.05$; ** $p \leq 0.01$). *E*-G) Expression analysis of the IL10 (E), IL1R1 (F), and IL6 (G) genes in BM-MSCs transduced with the LV-EGR1 at an MOI of 25. Untransduced cells were used as controls. Each error bar shows means ± SEM ($n \geq 4$). Data are represented as 2^-DCT, with DCT calculated as Ct gene - Ct GAPDH (*$p \leq 0.05$; ** $p \leq 0.01$).

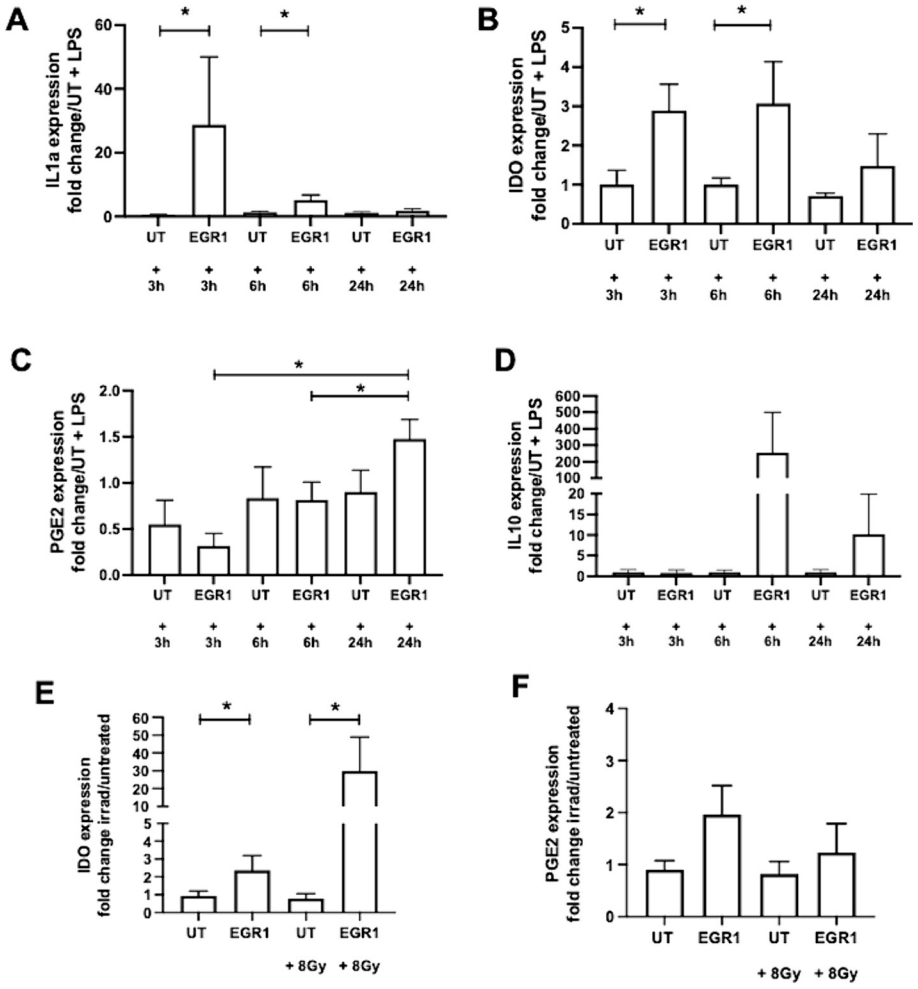

Fig. 5. EGR1 overexpressing BM-MSCs showed superior anti-inflammatory and immunomodulatory properties. (A-D) qPCR expression analysis of IL1 A (A), IDO (B), PGE2 (C), and IL10 (D) in untransduced (UT) and LV-EGR1 transduced BM-MSCs upon treatment with LPS (1μg/ml) for 3–6-24 h. E, F) qPCR expression analysis of IDO (E) and PGE2 (F) in untransduced (UT) and EGR1 overexpressing BM-MSCs irradiated in vitro with a dose of 8Gy. In all panels, results are shown as fold change over untransduced cells. DCT was calculated as Ct gene – Ct GAPDH. Each error bar shows mean ± SEM ($n \geq 4$). (*$p \leq 0.05$; ** $p \leq 0.01$).

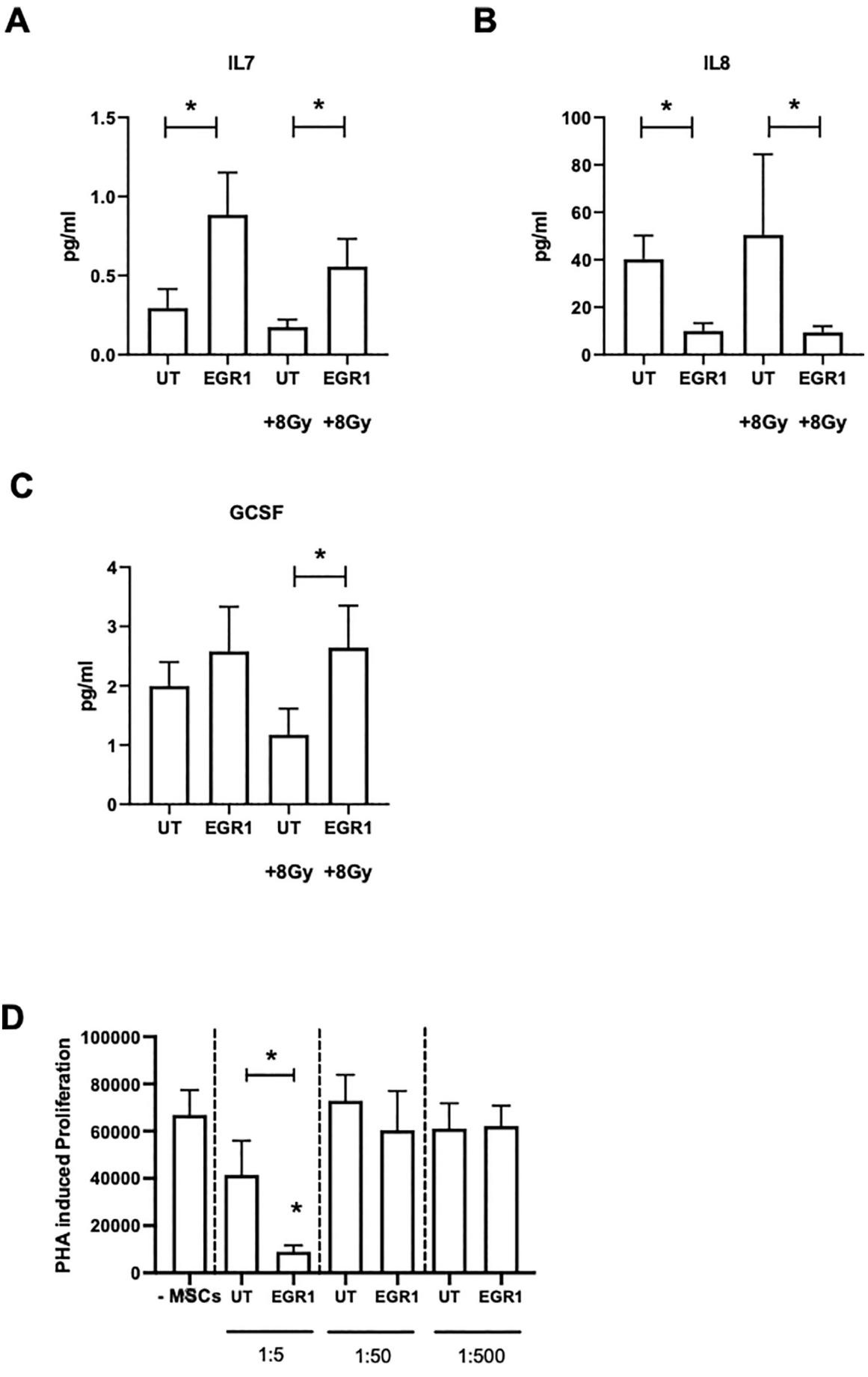

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Fig. 6. Immunomodulatory function of EGR1 overexpressing BM-MSCs. The levels IL-7 (A), IL-8 (B) and G-CSF (C) were determined in the cell supernatant from untransduced and EGR1 overexpressing BM-MSCs cultured for 48 h and after in vitro irradiation with a dose of 8Gy (48 h after treatment) using Luminex technology. Each error bar shows means ± SEM ($n \geq 3$). (*$p \leq 0.05$). (D) PHA-induced lymphocyte proliferation assay on lymphocyte from healthy donors in the presence of different doses (1:5, 1:50, and 1:500) of untransduced and LV-EGR1 transduced BM-MSCs. Lymphocytes stimulated in the absence of BM-MSCs were used as controls. Each error bar shows means ± SEM ($n \geq 4$).

applications. The use of MSC-derived secretome as a lyophilized medical product could overcome any concerns regarding the infusion of ex-vivo expanded cells, thawed and genetically manipulated MSCs, offering several biological and technological advantages, including the availability of an off-the-shelf product and repeatable dosing [67–69].

Another relevant innate property of MSCs is their hematopoietic supportive ability [58] for which MSCs have been successfully employed in the setting of HSPC transplantation to facilitate engraftment of HSPCs and prevent graft rejection [25,70]. Since the expression of some of the key BM niche-supporting genes was found to be significantly reduced in ex-vivo expanded MSCs, we tested whether the overexpression of EGR1 could directly or indirectly improve the hematopoietic supportive capacity of BM-MSCs in vitro co-culture systems with unmanipulated and GE-HSPCs. When EGR1-MSCs were co-cultured with unmanipulated HSPCs we observed an increased clonogenic capacity of $CD34^+$ cells as compared with HSPCs cultured on untransduced MSC feeder, in the presence of an adequate differentiation of expanded HSPCs. When EGR1-MSCs were co-cultured with GE-HSPCs we confirmed the capacity of transduced MSCs to preserve the most primitive compartment to a similar magnitude of untransduced ones, however MSC-mediated EGR1 overexpression was associated with a significantly higher reduction of IL1A expression and a reduced p21 activation in GE-HSPCs compared to cells maintained on the untransduced MSC feeder. Importantly, IL1A was found to cause aberrant myeloid differentiation, altering the hematopoietic reconstitution capacity of human HSPCs [71]. The presence of EGR1-MSCs prevented IL1A expression in GE-HSPCs preserving their fitness. This result confirms the superior anti-inflammatory capacity of EGR1-overexpressing cells and indicates that their use in a GE in vitro co-culture system may protect GE-HSPC from stressful genetic modifications and ex-vivo culture and help to maintain a better fitness of HSPCs [29], potentially translating into improved clinical outcomes in terms of probability of engraftment and kinetics of hematopoietic recovery in transplanted patients. Our data corroborate what already observed by Li et al. who reported that EGR1 overexpression in MSCs was able to induce in vitro a potent hematopoietic supportive effect and was accompanied by increased expression of some hematopoietic supporting genes [59].

In conclusion, our results confirm the deep, culture-induced gene expression changes occurring in primary BM-MSCs after ex-vivo expansion, possibly limiting their therapeutic potential, and points to further investigate the mechanisms regulating these changes in view of optimizing methods to maintain primary MSCs in culture. Moreover, altogether our data indicate that EGR1 is a key stromal transcription factor with a dual role in regulating the hematopoietic support and the anti-inflammatory/immune-regulatory properties of MSCs. Based on this, we speculate that the restoration of EGR1 expression in ex-vivo expanded MSCs might contribute to better exploit their biological properties. If translated to the clinical setting, the use of EGR1-MSCs may result favorable not only in the context of HSPC transplantation to better control GvHD and prevent graft rejection, but also in the setting of regenerative medicine where several chronic inflammatory conditions may benefit from the increased ability of EGR1-MSCs to mediate an anti-inflammatory response. Finally, a future deep characterization of the EGR1 overexpressing MSC secretome will highlight alternative cell-free therapeutic strategies that may offer biological and practical advantages, including the administration of a ready to use off-the-shelf product.

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Informed consent statement

Informed consent was obtained for all human samples involved in the study. Protocol Tiget09 approved by the Ospedale San Raffaele Ethical Committee on 11-05-2017.

CRediT authorship contribution statement

Ludovica Santi: Writing – review & editing, Writing – original draft, Methodology, Formal analysis, Data curation, Conceptualization. **Stefano Beretta:** Writing – review & editing, Writing – original draft, Methodology, Formal analysis, Data curation. **Margherita Berti:** Methodology, Data curation. **Evelyn Oliva Savoia:** Data curation. **Laura Passerini:** Methodology, Investigation. **Marilena Mancino:** Data curation, Conceptualization. **Giada De Ponti:** Writing – original draft, Data curation, Conceptualization. **Gaia Alberti:** Writing – original draft, Data curation. **Pamela Quaranta:** Data curation. **Luca Basso-Ricci:** Data curation. **Maria Antonietta Avanzini:** Writing – original draft, Data curation. **Ivan Merelli:** Supervision. **Serena Scala:** Data curation. **Samuele Ferrari:** Writing – review & editing, Writing – original draft, Data curation, Conceptualization. **Alessandro Aiuti:** Writing – review & editing, Supervision. **Maria Ester Bernardo:** Writing – review & editing, Writing – original draft, Supervision, Funding acquisition, Conceptualization. **Stefania Crippa:** Writing – review & editing, Writing – original draft, Validation, Supervision, Conceptualization.

Declaration of competing interest

The authors declare no conflicts of interest.

Data availability

Data available on request.

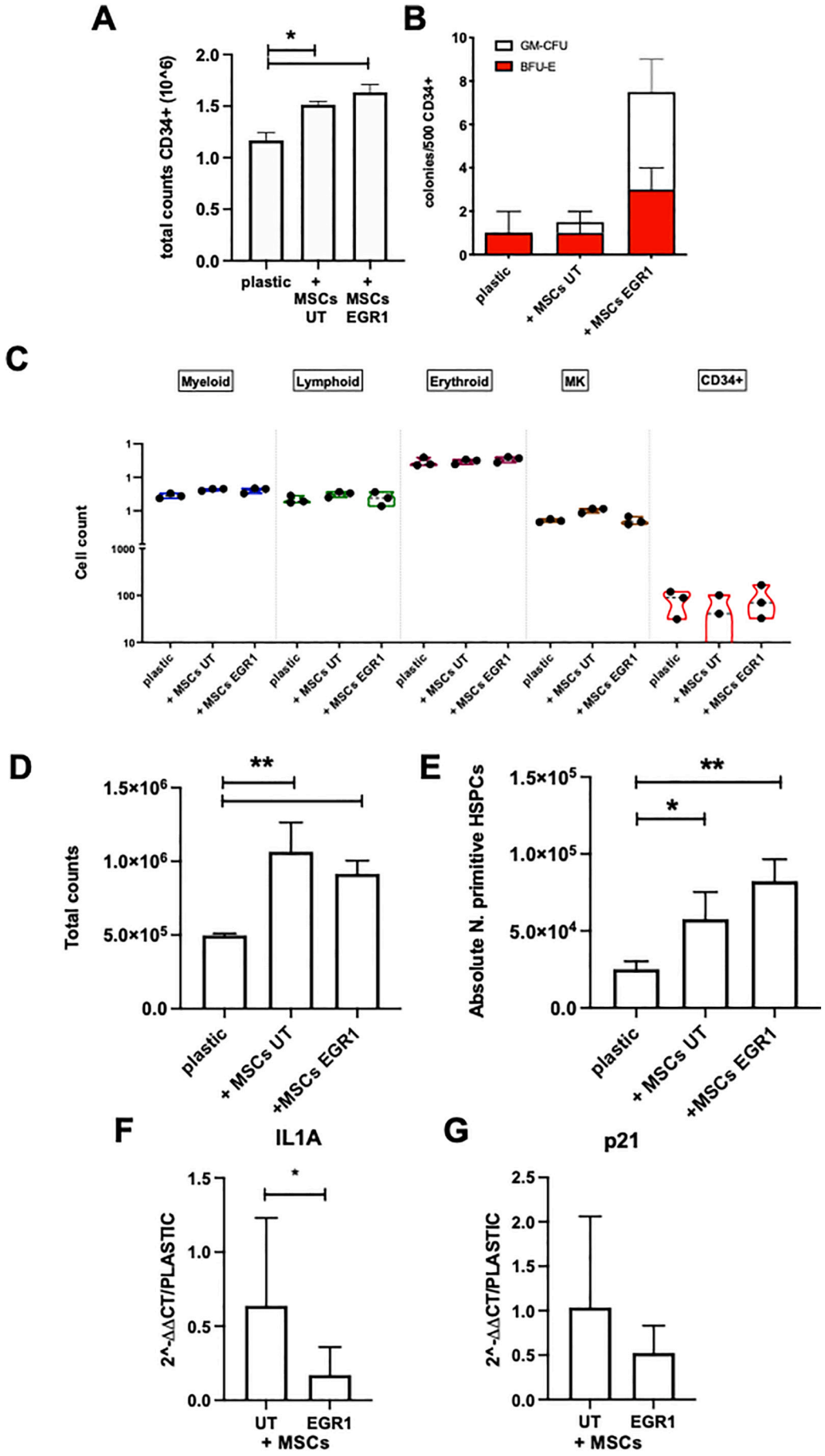

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Fig. 7. Evaluation of the hematopoietic supportive properties in EGR1 overexpressing BM-MSCs. A, B) Total counts of CD34+ cells (A) and clonogenic assay (B) after 72-h culture on BM-MSC feeders (untransduced or LV-EGR1 transduced BM-MSCs). CD34+ cells maintained in culture on plastic dishes were evaluated as controls. Each error bar shows means ± SEM ($n \geq 4$).(*$p \leq 0.05$). C) Single-cell differentiation assay performed on CD34+ cells maintained in culture on plastic dishes or on BM-MSC feeders (untransduced or LV-EGR1 transduced BM-MSCs). D, E) Gene-editing CD34+ cells from mobilized peripheral blood were recovered in culture for 72 h on plastic dishes or on BM-MSC feeders. Total CD34+ cell counts (D) and absolute number of CD34+ with a phenotypically primitive phenotype (E) were determined. F, G) qPCR expression analysis of IL-1A (F) and p21 (G) in gene-edited mPB human HSPCs recovered in culture on untransduced or EGR1 overexpressing BM-MSCs. Data show the expression fold change over gene-edited cells maintained in culture on plastic dishes. In all panels, each error bar shows means ± SEM ($n \geq 4$). (*$p \leq 0.05$; ** $p \leq 0.01$).

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbamcr.2024.119818.

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