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## Article

# Mesenchymal Stem/Stromal Cells Induce Myeloid-Derived Suppressor Cells in the Bone Marrow via Activation of JNK Signaling Pathway

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**Abstract:** Our previous study demonstrated that mesenchymal stem/stromal cells (MSCs) induced the differentiation of myeloid-derived suppressor cells (MDSCs) in the bone marrow (BM) under inflammatory conditions. In this study, we aimed to investigate the signaling pathway involved. RNA-seq revealed that the mitogen-activated protein kinase (MAPK) pathways, among biochemical pathways, exhibited the highest number of differentially expressed genes in BM cells with MSC coculture relative to those without. Western blot confirmed strong phosphorylation of c-Jun N-terminal kinase (JNK) in BM cells cocultured with MSCs under granulocyte-macrophage colony-stimulating factor stimulation, whereas p38 kinase activation remained unchanged in MSC-cocultured BM cells. JNK inhibition by SP600125 abolished the expression of *Arg1* and *Nos2*, the hallmark genes of MDSCs, as well as *Hif1α*, a molecule mediating monocyte functional reprogramming into a suppressive phenotype, in MSC-cocultured BM cells. JNK inhibition also abrogated the effects of MSCs on the production of TGF-β1, TGF-β2 and IL-10 in BM cells. Furthermore, JNK inhibition increased TNF-α expression, while suppressing IL-10 production, in MSC-cocultured BM cells in response to lipopolysaccharides. Together, our results suggest that MSCs induce MDSC differentiation and promote immunoregulatory cytokine production in BM cells during inflammation, at least in part, through activation of the JNK MAPK signaling pathway.

**Keywords:** bone marrow; JNK; MAPK; mesenchymal stem/stromal cells; myeloid-derived suppressor cells

## 1. Introduction

The depletion of monocytes or macrophages abrogates the immunomodulatory and regenerative activities of mesenchymal stem/stromal cells (MSCs) in multiple disease models, pointing to the crucial role of innate immune cells as essential mediators of MSC action [1,2]. A number of studies have elucidated the actions and mechanisms of MSCs, revealing their capacity to suppress pro-inflammatory immune cells and induce immunosuppressive cells. Notably, among the suppressive immune cells induced by MSCs are myeloid-derived suppressor cells (MDSCs).

MDSCs constitute a heterogeneous group of myeloid cells with potent immunosuppressive activity. Recent data uncover that the development of MDSCs occurs in two partially overlapping phases [3,4]: One is the expansion and conditioning of myeloid cells in the bone marrow (BM) and spleen, and the other is the conversion of neutrophils and monocytes into pathologically activated MDSCs in peripheral tissues. MDSCs accumulate in tissues in various pathologic conditions, including cancer, infection, acute and chronic inflammation, and autoimmunity, and play an important role in regulating immune responses. The immunoregulatory activity of MDSCs can be detrimental or beneficial [3,4]. For instance, in cancer, MDSCs exacerbate the disease and are

generally associated with poor clinical outcome. In contrast, in autoimmune and inflammatory disorders, the immunosuppressive functions of MDSCs contribute to limiting disease severity.

Although emerging data have elucidated the genomic, proteomic and metabolic characteristics of MDSCs [3-5], the precise mechanisms that drive neutrophils and monocytes to differentiate into MDSCs remain unclear. Several molecular pathways that regulate the suppressive functions of MDSCs have been suggested, but they largely vary depending on the factors and tissue microenvironments inducing MDSC development [4-7]. Identifying the specific pathways leading to MDSC differentiation and activation would provide valuable information for potential clinical targeting of MDSCs for the treatment of various diseases.

In a previous study, our group demonstrated that MSCs directed the differentiation of BM cells from pro-inflammatory CD11b<sup>hi</sup>Ly6C<sup>hi</sup>Ly6G<sup>lo</sup> monocytes to CD11b<sup>mid</sup>Ly6C<sup>mid</sup>Ly6G<sup>lo</sup> MDSCs in granulocyte-macrophage colony-stimulating factor (GM-CSF) stimulation and in a mouse model of experimental autoimmune uveoretinitis [8]. In the present study, we explore the signaling pathway in BM cells through which MSCs induce MDSC differentiation and regulate their suppressive functions.

## 2. Results

### 2.1. MAPK-related genes are upregulated in MSC-induced MDSCs

In our prior study [8], MSCs were found to induce the expression of *Arg1* and *Nos2*, hallmark MDSC genes encoding the immunosuppressive enzymes arginase and inducible nitric oxide synthase (iNOS), respectively [3,4], in GM-CSF-stimulated BM cells. Also, MSCs elevated the production of immunosuppressive cytokines, such as IL-10, TGF- $\beta$ 1 and TGF- $\beta$ 2, in GM-CSF-stimulated BM cells, while suppressing TNF- $\alpha$  secretion [8]. Notably, the MSC-induced MDSCs exhibited a CD11b<sup>mid</sup>Ly6C<sup>mid</sup>Ly6G<sup>lo</sup> phenotype, distinct from the pro-inflammatory CD11b<sup>hi</sup>Ly6C<sup>hi</sup>Ly6G<sup>lo</sup> monocytes differentiated from GM-CSF-stimulated BM cells [8].

Building upon these findings, we conducted a screening for the signaling pathway involved in MSC-induced MDSC differentiation. We analyzed RNA-seq data from CD11b<sup>mid</sup>Ly6C<sup>mid</sup>Ly6G<sup>lo</sup> MDSCs derived from MSC-cocultured BM cells under GM-CSF stimulation and compared them with those of CD11b<sup>hi</sup>Ly6C<sup>hi</sup>Ly6G<sup>lo</sup> monocytes sorted from GM-CSF-stimulated BM cells without MSC coculture (ArrayExpress accession E-MTAB-8975) (**Figure 1A**).

A comparative Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis using the DAVID database revealed that the mitogen-activated protein kinase (MAPK) pathways exhibited the highest number of differentially expressed genes (DEGs) enriched in MSC-induced MDSCs compared to BM cells without MSC coculture. The identified target genes are listed in **Table 1**, and the pathways in which they are involved are illustrated in **Figure 1B**.

**Table 1.** Genes related to MAPK pathway that were upregulated in MDSCs differentiated from GM-CSF-stimulated BM cells by MSCs, relative to pro-inflammatory monocytes derived from GM-CSF-stimulated BM cells without MSC coculture.

Gene Description	Gene symbol	Fold Change
ELK1, member of ETS oncogene family	Elk1	13.873

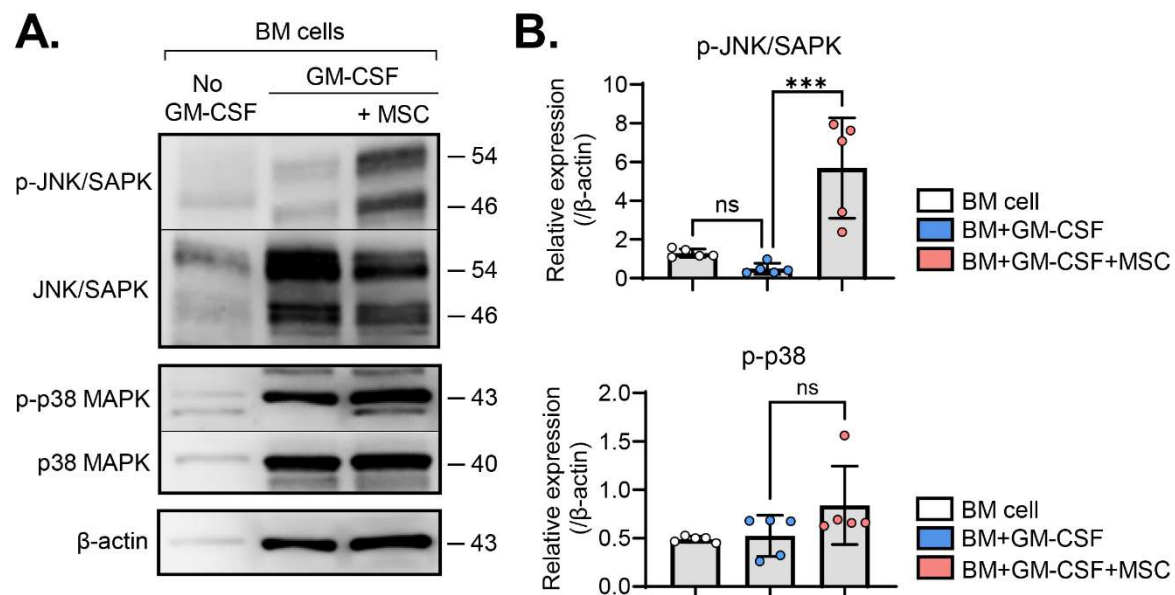
MAP kinase-activated protein kinase 5; similar to MK-5 type 2	Mapkapk5	12.104
RAS protein-specific guanine nucleotide-releasing factor 1	Rasgrf1	11.334
TNF receptor-associated factor 2	Traf2	10.991
activin A receptor, type IC	Acvr1c	239.476
brain derived neurotrophic factor	Bdnf	134.205
calcium channel, voltage-dependent, L type, alpha 1C subunit	Cacna1c	925.475
calcium channel, voltage-dependent, N type, alpha 1B subunit	Cacna1b	27.199
calcium channel, voltage-dependent, P/Q type, alpha 1A subunit	Cacna1a	123.802
calcium channel, voltage-dependent, T type, alpha 1G subunit	Cacna1g	20.065
calcium channel, voltage-dependent, alpha 2/delta subunit 2	Cacna2d2	293.280
calcium channel, voltage-dependent, alpha2/delta subunit 1	Cacna2d1	129.935
calcium channel, voltage-dependent, alpha2/delta subunit 3	Cacna2d3	111.485
calcium channel, voltage-dependent, beta 4 subunit	Cacnb4	20.455
calcium channel, voltage-dependent, gamma subunit 2	Cacng2	166.763
dual specificity phosphatase 10	Dusp10	119.544
ecotropic viral integration site 1	Mecom	221.142
fibroblast growth factor 12	Fgf12	1521.701
fibroblast growth factor 14	Fgf14	112.772
fibroblast growth factor 18	Fgf18	37.001
fibroblast growth factor 9	Fgf19	99.477
fibroblast growth factor receptor 1	Fgfr1	29.610
filamin, beta	Flnb	559.332
mitogen-activated protein kinase 13	Mapk13	54.817
mitogen-activated protein kinase 8 interacting protein 3	Mapk8ip3	31.535
mitogen-activated protein kinase kinase kinase 13	Map3k13	131.863
mitogen-activated protein kinase kinase kinase 4	Map3k4	21.317
mitogen-activated protein kinase kinase kinase kinase 3	Map4k3	12.556
neurofibromatosis 1	Nf1	179.681
nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 4	Nfatc4	20.130
phospholipase A2, group IID	Pla2g2d	131.904
phospholipase A2, group III	Pla2g3	33.243
protein phosphatase 3, catalytic subunit, gamma isoform	Ppp3cc	79.519
related RAS viral (r-ras) oncogene homolog 2	Rras2	21.369
serine/threonine kinase 3 (Ste20, yeast homolog)	Stk3	98.308
thymoma viral proto-oncogene 3	Akt3	112.279
transforming growth factor, beta 2	Tgfb2	51.476
transforming growth factor, beta 3	Tgfb3	38.421
v-crk sarcoma virus CT10 oncogene homolog (avian)	Crk	8.968



## 2.2. MSCs activate the JNK pathway in BM cells

Based on the observed upregulation of MAPK-related genes in MSC-induced MDSCs (**Figure 1**), we next investigated the activation of c-Jun N-terminal kinase (JNK) and p38 MAPK signaling pathways, the members of MAPK families [9], in BM cells with and without MSC coculture in the presence or absence of GM-CSF. Western blot analysis showed that GM-CSF did not significantly influence JNK or p38 phosphorylation in BM cells. Remarkably, MSC coculture induced robust phosphorylation of JNK in GM-CSF-stimulated BM cells (**Figure 2A, B**). In contrast, while JNK activation was prominent, p38 phosphorylation showed no significant increase in BM cells with MSC coculture (**Figure 2A, B**).

These findings indicate that, among biochemical pathways, MSCs specifically activate the JNK MAPK signaling pathway in GM-CSF-stimulated BM cells.



**Figure 2.** MSCs induce JNK activation in GM-CSF-stimulated BM cells. **(A) Representative western blot images.** BM cells were subjected to western blot analysis to assess total and phosphorylated forms of JNK/SAPK and p38 proteins under the indicated treatments. **(B)** Densitometric analysis of western blot images. The relative amounts of phosphorylated forms of specified proteins were quantitated as a ratio of each protein band relative to  $\beta$ -actin. Mean values  $\pm$  SD are shown. \*\*\* $p < 0.001$ , ns: not significant by one-way ANOVA with Tukey's multiple-comparison test (p-JNK/SAPK) or Kruskal-Wallis test followed by Dunn's multiple comparisons test (p-p38).

## 2.3. JNK inhibition abrogates MSC effects on MDSC induction

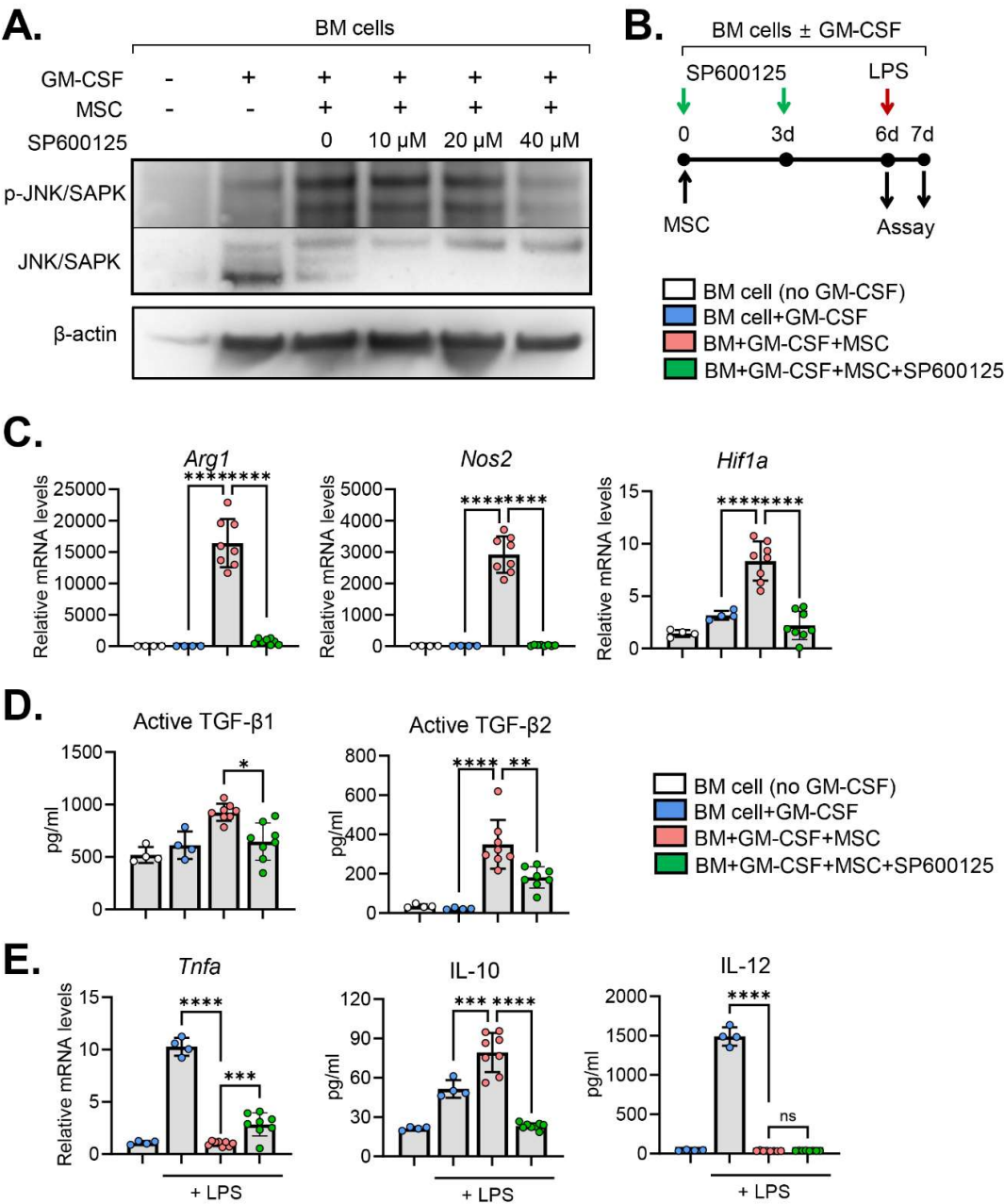
To assess the specific contribution of JNK in the MSC-induced differentiation of BM cells, we utilized the anthrapyrazolone inhibitor SP600125 as a JNK inhibitor [10]. Initially, we confirmed the efficacy of SP600125 in inhibiting JNK phosphorylation in a dose-dependent manner. Treatment of BM cells with 40  $\mu$ M SP600125 for 3 d negated the effect of MSCs on JNK activation in BM cells (**Figure 3A**).

Subsequently, we examined the impact of JNK inhibition on the expression of molecules mediating the immunosuppressive functions of MDSCs in BM cells cocultured with MSCs (**Figure 3B**). Results revealed that MSCs highly induced the expression of *Arg1*, *Nos2* and *Hif1a* in BM cells (**Figure 3C**). *Arg1* and *Nos2* are hallmark MDSC genes encoding the immunosuppressive enzymes, arginase and iNOS, respectively [3,4]. *Hif1a* encodes hypoxia-inducible factor (HIF)-1 $\alpha$  that has been shown to mediate the functional re-programming of monocytes into suppressive phenotype [11]. Interestingly, JNK inhibition by SP600125 significantly downregulated the expression of *Arg1*, *Nos2* and *Hif1a* (**Figure 3C**). Similar results were observed with the production of immunoregulatory cytokines. BM cells cocultured with MSCs exhibited elevated levels of immunosuppressive cytokines TGF- $\beta$ 1 and TGF- $\beta$ 2 compared to those without MSC coculture (**Figure 3D**). However, JNK inhibition

by SP600125 significantly reduced the secretion of TGF- $\beta$ 1 and TGF- $\beta$ 2 in BM cells cocultured with MSCs (**Figure 3D**).

We furthermore investigated the response of BM cells to lipopolysaccharides (LPS) stimulation (**Figure 3B**). As expected, LPS treatment markedly upregulated the expression of pro-inflammatory cytokines TNF- $\alpha$  and IL-12 in BM cells (**Figure 3E**). MSCs almost completely suppressed the upregulation of TNF- $\alpha$  and IL-12 in BM cells in response to LPS (**Figure 3E**). SP600125 treatment significantly reversed the effects of MSCs on TNF- $\alpha$  downregulation in BM cells, while the secretion of IL-12 remained unaffected by SP600125 (**Figure 3E**). In addition, MSCs induced IL-10 secretion in BM cells in response to LPS stimulation, which effect was significantly attenuated by SP600125 (**Figure 3E**).

Collectively, these results demonstrate that MSCs induce MDSC genes and immunosuppressive molecules in BM cells under GM-CSF stimulation and mitigate their pro-inflammatory activation in response to LPS. Inhibition of the JNK pathway in BM cells reverses these effects of MSCs on BM cells.



**Figure 3.** JNK inhibition reverses MSC effects on immunoregulatory molecules in BM cells. **(A) Representative western blot images.** BM cells were subjected to western blot analysis for total and phosphorylated forms of JNK/SAPK in BM cells under the indicated treatments. The cells were treated with incremental doses (10 ~ 40  $\mu$ M) of SP600125, a JNK inhibitor, for 3 d. **(B)** Experimental scheme. For JNK inhibition, GM-CSF-stimulated BM cells were treated with 40  $\mu$ M SP600125 every 3 d. For LPS stimulation assay, the cells were exposed to 100 ng/mL LPS for 18 h. **(C)** qRT-qPCR for MDSC hallmark and immunoregulatory genes *Arg1*, *Nos2* and *Hif1 $\alpha$*  in BM cells. The mRNA levels are presented as fold changes relative to the levels in GM-CSF-untreated and MSC-uncocultured cells. **(D)** ELISA for secreted levels of immunoregulatory cytokines TFG- $\beta$ 1 and TGF- $\beta$ 2 in the supernatant of BM cell culture. **(E)** qRT-qPCR for *Tnfa* and ELISA for IL-10 and IL-12 in BM cells in response to LPS stimulation. **Mean values  $\pm$  SD are shown.**  $p < 0.05$ ,  $**p < 0.01$ ,  $***p < 0.001$ ,  $****p < 0.0001$ , ns: not significant by one-way ANOVA with Tukey's multiple-comparison test or Kruskal-Wallis test followed by Dunn's multiple comparisons test (active TFG- $\beta$ 1).

### 3. Discussion

Our results demonstrated that a significant number of genes related to MAPK pathways were upregulated in MDSCs induced by MSCs in the BM. Specifically, the JNK pathway shows prominent activation in MSC-cocultured BM cells. Blockade of JNK activation in BM cells abolished the effects of MSCs on the induction of hallmark MDSC genes and immunosuppressive molecules mediating the MDSC function. Also, JNK inhibition abrogated the suppressive action of MSCs on the inflammatory activation of BM cells in response to LPS. Therefore, the results suggest that the JNK MAPK signaling pathway is crucial for MSCs' action to promote immunosuppressive molecules and induce MDSC differentiation in BM cells during inflammation.

The JNK signaling pathway is a member of the MAPK signal transduction pathways, which relay, amplify and integrate signals from a range of extracellular stimuli and elicit multiple physiological processes in mammalian cells, including cell proliferation, differentiation, apoptosis and inflammatory response [9]. The JNK pathway has been implicated in diverse innate immune responses. For example, JNK inhibition was reported to decrease IL-10 expression in stimulated human monocytes [12]. Also, JNK inhibition significantly reduced Pam3CSK4 (PAM3)-dependent generation of immunosuppressive macrophages in human monocytes [13]. Moreover, JNK inhibition was shown to increase the apoptosis of both granulocytic and monocytic MDSCs [14]. In our study, MSCs induced JNK activation in BM cells, thereby leading to the induction of immunoregulatory molecules (*Arg1*, *Nos2*, *Hif1 $\alpha$* , TGF- $\beta$ 1, TGF- $\beta$ 2 and IL-10) mediating the immunosuppressive function of MDSCs. These results indicate that JNK activation contributes to the action, survival and differentiation of MDSCs and immunosuppressive macrophages. Given that MDSCs and monocytes/macrophages are highly heterogeneous and context-dependent [7], it can be suggested that other signaling pathways, in addition to JNK pathway, are also involved in MDSC differentiation and functions. In our study, JNK inhibition did not influence the effect of MSCs on IL-12 expression in BM cells, while it significantly abrogated the effect on TNF- $\alpha$  and IL-10 levels, pointing to the involvement of other pathway in MSC-mediated IL-12 downregulation in BM cells. In a study by Bayik et al., JNK contributed to PAM3-driven generation of suppressive macrophages, but it was not involved in M-CSF-dependent suppressive macrophage polarization [13]. In fact, various pathways, including PI3K, Ras, Jak/Stat and TGF- $\beta$ , have been demonstrated to play a role in the generation of MDSCs from myeloid precursors in various disease conditions [5].

Conflicting results on the role of JNK in innate immune responses have also been reported. A study by Deng et al. demonstrated that JNK activation by synthetic bacterial lipoprotein, a TLR1/2 agonist, redirected differentiation of monocytic MDSC towards M1 pro-inflammatory macrophages [15]. Also, a study by Liu et al. reported that TNF- $\alpha$ -stimulated gene/protein 6 secreted by human umbilical cord-derived MSCs inhibited activation of both p38 and JNK MAPK pathways in peritoneal macrophages, and thus attenuated inflammation in the skin wound after burned injury [16]. These discrepancies suggest that the signaling pathways involved in the polarization of monocytes/macrophages and MDSC differentiation depend on the cell type and extracellular stimuli [13, 17], and that JNK activation can be pro-inflammatory or immunosuppressive according to cell



types and tissue microenvironments. As such, the identification of specific signals and pathways that direct MDSC differentiation and lead to suppressive modes in myeloid populations in different disease settings would be important for improving clinical outcomes of MDSC-based therapies.

The exogenous administration of MSCs has proven beneficial in ameliorating acute inflammatory pathology or autoimmune diseases, making MSC-based therapies the subject of extensive clinical trials [18]. One of key mechanisms underlying the therapeutic effects of MSCs is their ability to generate suppressive macrophages or MDSCs. However, the immunosuppressive environment created by MSCs raises concerns about potential tumor promotion in hosts receiving MSC therapy [19, 20]. Therefore, unraveling the molecular pathways involved in MSC-induced immunosuppressive actions, including generation of MDSCs and suppressive macrophages, would be essential for designing safe and effective regimens for the treatment of immune-related diseases and cancers. Our study reveals the JNK pathway as one such mechanism mediating MSC-induced MDSC differentiation in the BM. As mimetics and inhibitors targeting the JNK pathway become available, their combination with MSC therapy hold promise for enhancing therapeutic efficacy and mitigating side effects in patients.

In summary, our data demonstrate that MSCs activate the JNK MAPK pathway in BM cells under GM-CSF stimulation to induce the expression of immunosuppressive molecules, promote MDSC differentiation, and repress pro-inflammatory activation.

## 4. Materials and Methods

### 4.1. Cell culture

The experimental protocol was approved by the Institutional Animal Care and Use Committee of the Seoul National University Biomedical Research Institute (Seoul, Korea).

Single cell suspensions of BM cells were prepared by flushing the femur of 7-week C57BL/6 mice (Orient Bio, Seongnam, Korea). The cells were then filtered through a 70- $\mu$ m cell strainer (# 352350, Corning incorporation, Corning, NY) and collected through centrifugation. After red blood cell lysis, the resulting cells were cultured in RPMI1640 media (Welgene, Daegu, Korea) with 10% (vol/vol) heat-inactivated fetal bovine serum (FBS) (Gibco, Waltham, MA) and 1% penicillin-streptomycin (PS) (Lonza, Walkersville, MD) at 37°C in 5% CO<sub>2</sub>. For differentiation induction, the cells were treated with GM-CSF (40 ng/ml, GenScript, Piscataway, NJ) for 5 d.

In the LPS stimulation assay, GM-CSF-treated cells were exposed to 100 ng/mL LPS (InvivoGen, San Diego, CA) for 18 h.

For JNK inhibition, SP600125 (0, 10, 20 and 40  $\mu$ M) (#tlrl-sp60, InvivoGen) was added to BM cells for 3 d.

Human BM-derived MSCs were provided by the Center for the Preparation and Distribution of Adult Stem Cells (<http://medicine.tamhsc.edu/irm/msc-distribution.html>) and cultured according to the instructions. Briefly, passage 2 MSCs were cultured at 37°C in 5% CO<sub>2</sub> until reaching 70–80% confluency (approximately 7 d). The culture medium consisted of  $\alpha$ -minimal essential medium (Gibco), 17% FBS (Gibco), 1% PS (Lonza) and 2 mM L-glutamine (Lonza), and was changed every two days during culture. MSCs were harvested by incubating in 0.25% trypsin/1 mM ethylenediaminetetraacetic acid (EDTA) at 37°C for 2 min and cocultured with BM cells at a ratio of 1:5 (MSCs:BM cell) in the BM cell culture media.

### 4.2. RNA sequencing

CD11b<sup>hi</sup>Ly6C<sup>hi</sup> monocytes were isolated from GM-CSF-stimulated, MSC-uncocultured BM cells. CD11b<sup>mid</sup>Ly6C<sup>mid</sup> MDSCs were sorted from GM-CSF-stimulated, MSC-cocultured BM cells. For FACS-sorting, the BM cells were stained with anti-CD11b (clone M1/70, eBioscience, San Diego, CA) and anti-Ly6C (clone HK1.4, eBioscience) antibodies (Abs) and sorted using BD FACSARIA™ III cell sorter (BD Biosciences, San Jose, CA).

For RNA-seq with the sorted BM cells, 500 ng of total RNA was extracted, and library construction was performed with the QuantSeq 3' mRNA-Seq Library Prep Kit (Lexogen, Vienna,

Austria). High-throughput sequencing was conducted as single-end 75 sequencing using NextSeq 500 (Illumina, San Diego, CA). QuantSeq 3' mRNA-Seq reads were aligned using Bowtie2, and DEGs were determined based on counts from unique and multiple alignments using coverage in Bedtools. Gene classification was based on searches conducted by DAVID (<http://david.abcc.ncifcrf.gov/>) and Medline databases (<http://www.ncbi.nlm.nih.gov/>). The data were deposited at ArrayExpress accession E-MTAB-8975.

#### 4.3. Western blot assays

BM cells were lysed in RIPA Buffer (Biosesang, Seongnam, Korea) including protease inhibitor and phosphatase inhibitor (Thermo Fisher Scientific, Waltham, MA) and sonicated on ice. After centrifugation, clear cell lysates were acquired and assayed for protein concentration. Following denaturation, 40 µg protein was separated on a 4–12% SDS-PAGE gel (Invitrogen, Waltham, MA) at 150 V for 90 min and transferred to a methanol-soaked PVDF membrane (Invitrogen) at 40 V for 100 min. The membranes were blocked with 3% bovine serum albumin for 1 h and then incubated at 4°C overnight with anti-phospho-SAPK/JNK (Thr183/Tyr185) (#9255, Cell Signaling Technology, Danvers, MA), anti-phospho-p38 MAPK (Thr180/Tyr185) (#9216, Cell Signaling Technology), anti-SAPK/JNK (#9252, Cell Signaling Technology) and anti-p38 MAPK (#9212, Cell Signaling Technology) Abs. The membranes were then incubated with anti-mouse or anti-rabbit IgG Abs conjugated to horseradish peroxidase (HRP) at room temperature for 1 h. After stripping at 55°C for 30 min, they were incubated at room temperature for 1 h with anti-β-actin Ab (Santa cruz Biotechnology, Dallas, TX) and subsequently with anti-mouse HRP Ab (Cell Signaling Technology). The protein expression levels were normalized to the corresponding β-actin levels.

#### 4.4. Quantitative real-time reverse-transcription PCR (qRT-PCR)

BM cells were lysed in RNA isolation reagent (RNA Bee, Tel-Test, Friendswood, TX) and homogenized with an ultrasound sonicator (Ultrasonic Processor, Cole Parmer Instruments, Vernon Hills, IL). Total RNA was extracted using RNeasy Mini kit (Qiagen, Hilden, Germany), and double-stranded cDNA were synthesized by reverse transcription (High Capacity RNA-to-cDNA™ Kit, Applied Biosystems, Carlsbad, CA). Real-time PCR amplification was performed using TaqMan® Universal PCR Master Mix (Applied Biosystems) in an automated instrument (ABI 7500 Real Time PCR System, Applied Biosystems). Data were normalized to *Gapdh* and expressed as fold changes relative to controls. All PCR probe and primer sets were mouse-specific (TaqMan® Gene Expression Assay, Applied Biosystems).

#### 4.5. Enzyme-linked immunosorbent assay (ELISA)

The cell-free supernatants were collected by centrifugation and analyzed for concentrations of mouse-specific IL-10, active TGF-β1 and active TGF-β2 (DuoSet® ELISA, R&D Systems, Minneapolis, MN) according to the manufacturer's instructions.

#### 4.6. Statistical analysis

After a normality test, data were analyzed either by one-way ANOVA followed by Tuckey's Honestly Significant Difference test or Kruskal-Wallis test followed by Dunn's multiple comparisons test (GraphPad Prism Software, San Diego, CA). Data were presented as mean ± SD, and differences were considered significant at  $p < 0.05$ .

**Author Contributions:** Conceptualization, J.Y.O.; Methodology, H.J.L. and J.Y.O.; Formal Analysis, H.J.L. and J.Y.O.; Investigation, H.J.L. and J.Y.O.; Writing – Original Draft Preparation, H.J.L. and J.Y.O.; Writing – Review & Editing, H.J.L. and J.Y.O.; Visualization, J.Y.O.; Supervision, J.Y.O.; Project Administration, J.Y.O.; Funding Acquisition, J.Y.O.

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**Conflicts of Interest:** The authors declare no conflict of interest.

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