



# TNFR2 Is a Crucial Hub Controlling Mesenchymal Stem Cell Biological and Functional Properties

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Mesenchymal stem cells (MSCs) have drawn lots of attention as gold standard stem cells in fundamental and clinical researches during the last 20 years. Due to their tissue and vascular repair capacities, MSCs have been used to treat a variety of degenerative disorders. Moreover, MSCs are able to modulate immune cells' functions, particularly T cells while inducing regulatory T cells (iTregs). MSCs are very sensitive to inflammatory signals. Their biological functions could remarkably vary after exposure to different pro-inflammatory cytokines, notably TNF $\alpha$ . In this article, we have explored the importance of TNFR2 expression in a series of MSCs' biological and functional properties. Thus, MSCs from wild-type (WT) and TNFR2 knockout (TNFR2 KO) mice were isolated and underwent several *ex vivo* experiments to investigate the biological significance of TNFR2 molecule in MSC main functions. Hampering in TNFR2 signaling resulted in reduced MSC colony-forming units and proliferation rate and diminished the expression of all MSC characteristic markers such as stem cell antigen-1 (Sca1), CD90, CD105, CD44, and CD73. TNFR2 KO-MSCs produced more pro-inflammatory cytokines like TNF $\alpha$ , IFN $\gamma$ , and IL-6 and less anti-inflammatory mediators such as IL-10, TGF $\beta$ , and NO and induced Tregs with less suppressive effect. Furthermore, the TNFR2 blockade remarkably decreased MSC regenerative functions such as wound healing, complex tube formation, and endothelial pro-angiogenic support. Therefore, our results reveal the TNF $\alpha$ -TNFR2 axis as a crucial regulator of MSC immunological and regenerative functions.

**Keywords:** mesenchymal stem cells, immunoregulation, immune-checkpoint, tissue regeneration, angiogenesis, TNF $\alpha$ -TNFR2 signaling pathway

**Abbreviations:** BM-MSCs, bone marrow-derived mesenchymal stem cells; CD, cluster of differentiation; CPDT, cell population doubling time; CFSE, carboxyfluorescein succinimidyl ester; CFUs, colony-forming units; CM, conditioning media; EPCs, endothelial progenitor cells; HGF: hepatocyte growth factor; IFN $\gamma$ , interferon gamma; IGF-1, insulin-like growth factor 1; IL, interleukin; iPSCs, induced pluripotent stem cells; iTreg, induced regulatory T cell; MACS, magnetic-activated cell sorting; MDCs, myeloid-derived suppressive cells; MFI, mean fluorescence intensity; MSCs, mesenchymal stem cells; P, passage; Sca1, stem cells antigen-1; Tconv, conventional T cell; TNFR1, tumor necrosis factor receptor 1; TNFR2 KO, tumor necrosis factor receptor 2 knockout; TNFR2, tumor necrosis factor receptor 2; TNF $\alpha$ , tumor necrosis factor alpha; Treg, Regulatory T cell; VEGF, vascular endothelial growth factor.

## INTRODUCTION

In 1968, for the first time, a heterogeneous population of bone marrow (BM)-derived stem cells were identified and introduced to the scientific world, referred to as mesenchymal stem/stromal cells (MSCs) (Friedenstein et al., 1968). These cells bear different important potentials like self-renewal and proliferation and can differentiate into multilineage cell types of different origins such as endodermal, ectodermal, and mesodermal including (but not limited to) myocytes, cardiomyocytes, neural cells, hepatocytes and endothelial cells (ECs), adipocytes, osteocytes, and chondrocytes (Han et al., 2017; Khosravi et al., 2018a; Afshari et al., 2020; Zhang et al., 2020).

MSCs demonstrated three principal biological characteristics that make them specifically interesting for cell therapy applications: (1) differentiation potential, (2) secretion of different mediators, and (3) immunoregulatory properties (Khosravi et al., 2017, 2018b,c; Beldi et al., 2020). Based on these features, in addition to their ability to be simply expanded and manipulated *ex vivo*, MSC fundamental and clinical research has evolved rapidly during the last decade.

Despite our advanced knowledge, there currently exist no distinctive markers to phenotypically identify and characterize MSCs or their subpopulations. However, depending on their origin species, they are expected to express stem cell antigen-1 (Sca1), CD44, CD105, CD73, and CD90 and must not express CD14 monocyte, CD34 hematopoietic, and CD45 common leukocyte markers (Dominici et al., 2006; Maleki et al., 2014).

MSCs are sensitive and responsive to microenvironment changes. Their biological properties are strongly influenced by the interaction with other cells, extracellular matrix, soluble bioactive factors like cytokines, and most importantly the presence and intensity of the surrounding inflammation (Chen et al., 2015; Broekman et al., 2016; Daneshmandi et al., 2017; Zhou et al., 2017; Bai et al., 2018; Noronha et al., 2019). The modulation of several biological and/or biochemical elements has been proven to augment MSC therapeutic capacity through impacting MSC's fate, differentiation, and function (Nava et al., 2012; Huang et al., 2015). Phenotypical profile and immunomodulatory functions of MSCs could also be altered by exposure to an inflamed environment (Pourgholaminejad et al., 2016). Many studies previously evidenced that treating MSCs with pro-inflammatory cytokines like IL-1 $\beta$ , IL-17, IFN $\gamma$ , and TNF $\alpha$  could modify their differentiation, proliferation, migration, immunomodulation, and cytokine production capacities via a range of signaling pathways particularly NF- $\kappa$ B and STAT3 activation (Noronha et al., 2019). Priming enhances MSC anti-inflammatory and immunosuppressive functions via upregulating important immunoregulatory elements, such as IDO, PGE2, hepatocyte growth factor (HGF), CCL2, TGF $\beta$ , and IL-10 (Prasanna et al., 2010; de Witte et al., 2015; Baldari et al., 2017; Vigo et al., 2017; Bai et al., 2018; Hu and Li, 2018; Noronha et al., 2019). Moreover, TNF $\alpha$  pre-treatment increases MSC ability to induce monocyte differentiation into IL-10 secreting immunosuppressive M2 macrophages (François et al., 2012). Equally, primed MSCs have enhanced T cell immunosuppressive effect that is associated with increased nitric oxide (NO)

production, a major murine MSC immunosuppressive molecule (Szabó et al., 2015; Pourgholaminejad et al., 2016; Noronha et al., 2019).

By binding to its receptors (TNFR1 and TNFR2), TNF $\alpha$  exerts miscellaneous and complex biological activities. Per contra to TNFR1 ubiquitous expression on every cell, TNFR2 has rather restricted expression notably by ECs, neural cells, immune cells, and MSCs (Kelly et al., 2010; Chen and Palmer, 2013; Salomon et al., 2018; Yang S. et al., 2018). Even though TNF $\alpha$  recognizes both receptors, its interaction with either of them could end in an entirely different biological outcome.

Previous researches on MSCs established that the TNF $\alpha$ -TNFR2 signaling pathway leads to pro-angiogenic, protective, and survival mechanisms, while TNF $\alpha$ -TNFR1 signaling generally terminates in harmful and pro-apoptotic mechanisms (Faustman and Davis, 2013). For instance, administration of BM MSCs (BM-MSCs) harvested from TNFR2 knockout (KO) mice in acute ischemia model displayed impaired or no myocardial recovery function, which was followed by elevated pro-inflammatory factors and reduced vascular endothelial growth factor (VEGF) production (Kelly et al., 2010; Tan et al., 2010). Accordingly, other studies showed that TNF $\alpha$  primed human BM-MSCs have enhanced capacity to produce important growth factors such as HGF, insulin-like growth factor 1 (IGF-1), and VEGF via a TNFR2-dependent mechanism (Crisostomo et al., 2008; Zhang et al., 2010). Besides, TNFR2 upregulation in rat and human BM-MSCs effectively increased their therapeutic potential in cardiac ischemia (Bao et al., 2008, 2010) and inflammatory disorders like rheumatoid arthritis (RA), which was accompanied by decreased IL-6, IL-1 $\beta$ , and TNF $\alpha$  secretion (Liu et al., 2013; Park et al., 2017).

Our cumulative research works from the last decade demonstrate a straight association between the TNFR2 expression and the immunomodulatory properties of different cells including regulatory T cells (Tregs), myeloid-derived suppressor cells (MDSCs), Bregs, MSCs, and endothelial progenitor cells (EPCs) (Polz et al., 2014; Leclerc et al., 2016; Ticha et al., 2017; Beldi et al., 2020; Naserian et al., 2020). Accordingly, the same phenomenon was speculated for neural stem/progenitor cells, which are the other subcategory of rare TNFR2-expressing cells (Shamdani et al., 2020).

Recently, in an attempt to investigate the immunological importance of TNFR2 expression on MSCs and to evaluate its role in MSC/T cell cross talk, we revealed TNF $\alpha$ -TNFR2 as an indispensable immune checkpoint signaling pathway involved in MSC immunomodulatory effect against T cells. We have reported that unlike TNFR2 KO BM-MSCs, their TNFR2-expressing wild-type (WT) counterparts were remarkably more efficient in T cell suppression, down-modulating T cell activation markers, reducing T cell pro-inflammatory factors, and increasing their anti-inflammatory cytokine production. Moreover, blocking TNFR2 signaling caused a significant decrease in MSC ability to convert conventional T cells (Tconv) to induced Tregs (iTregs) (Beldi et al., 2020).

Here, in this complementary study, we emphasize more specifically on the impact of TNFR2 expression on a series of MSC biological and functional properties that have not been

revealed yet. Therefore, we aimed to investigate (1) if MSCs from WT or TNFR2 KO mice have different morphological, colony forming, proliferation, and differentiation properties; (2) if MSCs from WT or TNFR2 KO mice express different levels of characteristic markers; (3) if the absence of TNFR2 could alter MSC cytokine secretion and NO production pattern and eventually; and (4) if Tregs induced from WT and TNFR2 KO mice have different immunosuppressive efficiency. Concerning the indispensable MSC regenerative properties, we have evaluated the impact of TNFR2 blockade on (1) MSC wound healing capability of a scratched monolayer, (2) MSC ability to form complex 3D tubular structures on extracellular matrix component, and finally (3) MSC ability to support the pro-angiogenic function of human umbilical vein ECs (HUVECs).

## MATERIALS AND METHODS

### Mesenchymal Stem Cell Isolation

MSCs were isolated from total BM harvested from femurs and tibias of 6–8 weeks old C57BL/6 WT (Charles River and Envigo) and C57BL/6 TNFR2 KO mice (B6.129S2-Tnfrsf1b<sup>tm1Mwm/J</sup>, The Jackson Laboratory) housed under pathogen-free conditions as already described (Beldi et al., 2020). The TNFR2 KO mice did not demonstrate any different phenotypes as compared with WT mice. They had normal lymphoid tissues and did not develop any spontaneous pathology. Harvested cells were cultured in 25 cm<sup>2</sup> flasks in Dulbecco's modified Eagle's medium (DMEM) medium (Gibco) supplemented with 1% GlutaMAX, 10% fetal bovine serum (FBS) (Eurobio, ref CVFSVF00-01), and 1% penicillin–streptomycin–neomycin (P/S/N) (Gibco), hereinafter referred to as complete DMEM and incubated at 37°C in 5% CO<sub>2</sub>. Every 8 h, non-adherent cells were eliminated, and the remaining cells were washed carefully with phosphate-buffered saline (PBS) 1×, and then the fresh complete medium was added. The initial colonies of MSCs (passage 0) appeared after 4–5 weeks of culture. The best and purest colonies according to their morphological appearance, homogeneity, and the speed of colony growth were selected and detached using cell dissociation reagent TrypLE (Gibco). From passage 1, cells were seeded at 5,000 cells/cm<sup>2</sup>. After the expansion phase, all cells were evaluated for the expression of MSC phenotypical characterization markers as explained in the next section. Furthermore, MSCs underwent a series of quality controls for evaluation of their functional features, such as colony-forming unit (CFU), immunosuppression, and differentiation capacities. Merely, the qualified cells were passed and/or stored at –150°C for further experimental procedures. Passages 1–3 of WT and TNFR2 KO-MSCs were used throughout the experiments depending on the experimental requirements.

### Mesenchymal Stem Cell Phenotypical Characterization

A total of 10<sup>5</sup> WT and TNFR2 KO-MSCs were seeded in 96-well round-bottom plates (Falcon). Thereafter, they were immunostained using the following Abs: CD44-PE-Vio770, CD105-FITC, Sca1-APC, CD73-PE, CD90-Biotin or PE, anti-biotin-PE or VIOBLUE, CD45-VIOBLUE, CD34-Biotin or FITC

(Miltenyi), and streptavidin-PeCY5/CY7 (eBioscience). We used isotypes Abs (Miltenyi) and unstained MSCs as controls for our experiments.

For F-actin staining, we have used F-actin Staining Kit, Green Fluorescence, Cytopainter (Abcam) according to supplier's protocol. Briefly, we have seeded 10<sup>5</sup> WT and TNFR2 KO MSCs in 96-well plates, treated them with 100 μl/well of Green Fluorescent Phalloidin Conjugate solution, and stained them in room temperature for 30 min. We then washed the cells with PBS 1× and analyzed in FITC channel using LSRFORTESSA flow cytometer (BD Biosciences). Data were then analyzed using FlowJo software v10 (FlowJo, LLC).

### Mesenchymal Stem Cell Colony Forming Unit Assays

A total of 200 WT or TNFR2 KO-MSCs (P1) were seeded in 25 cm<sup>2</sup> flasks in 5 ml of complete DMEM. After 7 days, culture media were changed, and flasks were re-incubated for another 3 days. The staining solution was prepared with a crystal violet solution (Sigma Aldrich) diluted 1/20 with osmosis water. Culture media were removed, and flasks were rinsed twice with PBS 1× without calcium and magnesium. Staining solution was added (3 ml/flask) and left for 20 min at room temperature. Then the flasks were rinsed twice with osmosis water and left to dry. To count the number of CFUs, high-resolution pictures were taken and analyzed in a double-blinded manner using Image J software (National Institutes of Health, Bethesda, MD, United States).

### Mesenchymal Stem Cell Proliferation Measurement

WT and TNFR2 KO-MSCs from different passages (i.e., P1, P2, and P3) were seeded in 6-well plates (Falcon) with an initial density of 3 × 10<sup>4</sup> cells/well. Cells were cultured in complete DMEM at 37°C and 5% CO<sub>2</sub>. The cell culture medium was changed once every other day for a duration of 5 days. Every 24 h cells were detached using TrypLE (Gibco). We determined the cell numbers with an automated cell counter apparatus (Countess II). Cell population doubling time (CPDT) was calculated using the following formula: CPDT = (t – t<sub>0</sub>) × log<sub>2</sub> × (log[N/N<sub>0</sub>]) – 1, with t as time and N as cell number, according to already published articles (Zhou et al., 2008). Data were analyzed using FlowJo software v10 (FlowJo, LLC).

### Mesenchymal Stem Cell Viability Measurement

A total of 2 × 10<sup>5</sup> WT or TNFR2 KO-MSCs (P1) were seeded in 25-cm<sup>2</sup> flasks. Cells were cultured untreated or after a pre-treatment with 0.1, 1, 5, 10, and 100 ng/ml of premium-grade mouse TNFα (Miltenyi) for a duration of 24 h. Then, they were detached using cell dissociation reagent TrypLE (Gibco). The percentage of viable cells was determined by the absence of the expression of annexin V and propidium iodide (PI) markers using an annexin V-FITC kit (Miltenyi) with respect to the supplier's protocol. Events were acquired on a LSRFORTESSA flow cytometer (BD Biosciences) and analyzed using FlowJo software v10 (FlowJo, LLC).

## Mesenchymal Stem Cell Differentiation Assay

The induction of adipogenic differentiation was performed by culturing the cells in a homemade adipogenic differentiation medium for 21 days as already described (Doucet et al., 2005). Thereafter, we stained the cells using Oil Red for a duration of 5 min. For osteocyte differentiation, we purchased a specific medium containing P/S at 1:100 dilution + StemXVIVO Mouse/Rat Osteogenic supplement 20 × (R&D Systems). WT and TNFR2 KO-MSCs were cultured for 17 days. Then, they were stained for 3 min using 2% Alizarin red. Samples were visualized by the objectives of 4 × and 10 × of the microscope (EVOS XL Core, Invitrogen by Thermo Fisher Scientific).

## Mesenchymal Stem Cell Cytokine Quantification

A total of  $5 \times 10^5$  WT or TNFR2 KO-MSCs were seeded in 75 cm<sup>2</sup> flasks and were activated by the addition of 10 ng/ml of premium-grade mouse TNF $\alpha$  (Miltenyi) in a total volume of 10 ml. After 36 h, MSCs were treated with 1  $\mu$ l/ml of Golgi-Plug (protein transport inhibitor) (BD Biosciences) and incubated for another 12 h. Cells were then stained with following Abs: CD44-PE-Vio770, CD73-PE, IFN $\gamma$ -APC, IL-6-PE or FITC, TNF $\alpha$ -FITC, IL-10-APC (Miltenyi), and anti-TGF $\beta$ -PE (BioLegend). Events were acquired on a LSRFORTESSA flow cytometer (BD Biosciences) and analyzed using FlowJo software v10 (FlowJo, LLC).

## T Cell Isolation

T cell isolation was performed as previously reported (Beldi et al., 2020). Briefly, we utilized mouse Pan T Cell Isolation kit (Miltenyi) in order to separate CD3<sup>+</sup> T cells from pooled lymph nodes and spleens of 6–12 weeks old WT C57BL/6 female mice (Charles River and Envigo). CD25<sup>+</sup> subpopulation was depleted from the total CD3<sup>+</sup> T cells by means of the anti-CD25 biotin-conjugated antibody (BD Biosciences), tailed by anti-biotin microbeads immunostaining (Miltenyi). Afterward, magnetic-activated cell sorting (MACS) was used for the cell isolation process. The sorted CD3<sup>+</sup>CD25<sup>-</sup> T cells with higher than 94% purity were co-cultured with WT or TNFR2 KO-MSCs.

## Co-culture of Mesenchymal Stem Cells and T Cells

The co-culture of MSCs with T cells was performed as previously reported (Beldi et al., 2020). Briefly, WT or TNFR2 KO-MSCs were seeded either into 75 cm<sup>2</sup> flasks or into 6 or 12-well plates and incubated for 6–24 h, in complete DMEM. Then, depending on the experimental conditions, different ratios of CD3<sup>+</sup>CD25<sup>-</sup> T cells were added in Roswell Park Memorial Institute (RPMI) medium supplemented with 10% FBS (Eurobio, ref CVFSVF00-01), 1% P/S/N, 1% HEPES, and  $5 \times 10^{-5}$  M of  $\beta$ -mercaptoethanol, hereinafter referred to as complete RPMI. All co-culture experiments were performed in a mixture of 50% DMEM and 50% RPMI media. Suspending T cells were collected by gentle aspiration based on MSC's ability to adhere to plastic.

## Regulatory T Cell Induction and Mixed Lymphocyte Reaction Assay

Freshly isolated CD3<sup>+</sup>CD25<sup>-</sup> mouse T cells were activated by mouse anti-CD3/CD28 beads (Dynabeads, Gibco) with respect to the supplier's protocol. A total of  $5 \times 10^5$  WT or TNFR2 KO-MSCs were seeded in 75 cm<sup>2</sup> flasks for a duration of 6 h. Afterward, they were co-cultured with  $5 \times 10^6$  of bead-activated mouse CD3<sup>+</sup>CD25<sup>-</sup> T cells (1/10 MSC/T cell ratio) in a final volume of 10 ml using a mixture of 50% RPMI and 50% DMEM media. We have used  $5 \times 10^5$  non-activated and activated CD3<sup>+</sup>CD25<sup>-</sup> mouse T cells cultured alone as negative and positive controls, respectively. After 72 h, the CD4<sup>+</sup>CD25<sup>+</sup> mouse regulatory T cell isolation kit was utilized to isolate iTregs according to the manufacturer's instructions (Miltenyi). The purity of CD4<sup>+</sup>CD25<sup>+</sup> iTregs (more than 90%) was determined via flow cytometric analysis using the following Abs: CD4-VIOBLUE, CD25-PE-Vio770, CTLA4-PE, TNFR2-APC (Miltenyi), and Foxp3-PE-Cy5 (eBioscience). A total of  $2 \times 10^4$  iTregs were then set in a mixed lymphocyte reaction (MLR) assay in a 96-well plate with  $10^5$  freshly isolated activated CFSE<sup>+</sup>CD3<sup>+</sup>CD25<sup>-</sup> responder T cells (Tconvs) in a fixed 1/5 iTreg/Tconv ratio. Cells were kept in a final volume of 200  $\mu$ l of complete RPMI medium at 37°C in 5% CO<sub>2</sub>. We used  $10^5$  non-activated and activated mouse CFSE<sup>+</sup>CD3<sup>+</sup>CD25<sup>-</sup> T cells alone as negative and positive controls, respectively. After 72 h, we collected the cells and immunostained them using CD4-VIOBLUE and CD8 $\alpha$ -PE-Vio770 antibodies. The percentage of proliferating CD4<sup>+</sup> and CD8<sup>+</sup> T cells was analyzed by measuring the carboxyfluorescein succinimidyl ester (CFSE) dilution using LSRFORTESSA flow cytometer (BD Biosciences). Data were then analyzed using FlowJo software v10 (FlowJo, LLC).

## Nitric Oxide Measurement

Nitric oxide was quantified using DAF-2/DA probe according to supplier's instructions (Eugene, Oregon, United States). A total of  $10^5$  MSCs were seeded into 12-well plates and incubated with 5  $\mu$ M of DAF-2/DA in complete DMEM medium containing 10% FBS or in DMEM medium containing 0.5% FBS for a duration of 1 h at 37°C. Plates were kept away from light exposure during the experiment. Cells were washed to remove excess probe and detached using cell dissociation reagent TrypLE (Gibco). Thereafter, cells were re-suspended in PBS 1× supplemented with 0.5% bovine serum albumin (BSA) and immediately detected in FITC channel using LSRFORTESSA flow cytometer (BD Biosciences) and analyzed by FlowJo software v10 (FlowJo, LLC).

## Wound Healing Assay

Wound healing assays were conducted by means of the specific culture insert with respect to the supplier's instructions (IBIDI, Germany). WT and TNFR2 KO-MSCs were seeded in the two separate wells of the insert at  $15 \times 10^3$  cells/ml in a final volume of 70  $\mu$ l/well, to acquire a confluent layer within 24 h. To create a 500  $\mu$ m homogeneous cell-free gap, culture inserts were carefully removed. The wounded area appearance

was instantaneously captured by taking pictures (Nikon D3100, Japan) using objectives 4× and 10× of the microscope (Nikon ECLIPSE T5100) and continued every 2 h, until complete closure in WT-MSC group. Cells were incubated at 37°C in 5% CO<sub>2</sub>. The decrease in wounded area was analyzed by measuring the scratch area size using the plugin MRI Wound Healing Tool for Image J (NIH, United States).

## Network Formation Assay

MSC network formation capacity was assessed by seeding WT or TNFR2 KO-MSCs (10<sup>5</sup> cells/well) on phenol red-free Matrigel™ (BD Biosciences) in 24-well plates for a duration of 24 h, at 37°C in 5% CO<sub>2</sub>. Cells were cultured in complete DMEM or EGM-2MV medium, Endothelial SingleQuots™ Kit (Lonza).

Pictures were taken every 2 h, till 24 h, using a camera (Nikon D3100, Japan) installed on an inverted microscope (Nikon ECLIPSE T5100) with 4× and 10× objectives in phase-contrast mode.

For evaluating the impact of MSCs on HUVEC angiogenesis, 5 × 10<sup>5</sup> WT or TNFR2 KO-MSCs were seeded in 75 cm<sup>2</sup> flasks and cultured for 48 h, in EBM2 medium containing 5% FBS (Lonza) and 1% P/S/N (Gibco). After 2 days, conditioning media (CM) from both WT and TNFR2 KO-MSC cultures were taken and filtered through a 0.45 μm filter to eliminate the remaining MSCs and were added to HUVECs (P5). HUVECs cultured in EBM2 basal endothelial medium (Lonza) or EGM-2MV complete endothelial medium (Lonza) were used as negative and positive controls, respectively. Then, we analyzed the images for evaluating the tube length and the network structural complexity (number of branches, number of sides, ability to construct closed networks, and tube length) by means of the angiogenesis analyzer that is developed for the Image J software as previously described (Chevalier et al., 2014).

## Statistical Analysis

Prism (GraphPad) software was utilized for statistical analysis. To assess the normal distribution of data, the Shapiro–Wilk normality test was performed. Thereafter, depending on the number of comparatives, Student's *t*-test or one-way ANOVA with *post hoc* analysis was used. Regarding cytometry analysis, we have normalized the mean fluorescence intensity (MFI) values with WT-MSC or T cell alone groups depending on the experimental conditions. This was followed by unpaired, two-tailed Student's *t*-test, or one-way ANOVA for *p*-value generation. Data are presented as mean value ± SEM. \**P* < 0.05, \*\**P* < 0.01, \*\*\**P* < 0.001, \*\*\*\**P* < 0.0001.

## RESULTS

### Mesenchymal Stem Cell Characterization and Differentiation Capacity

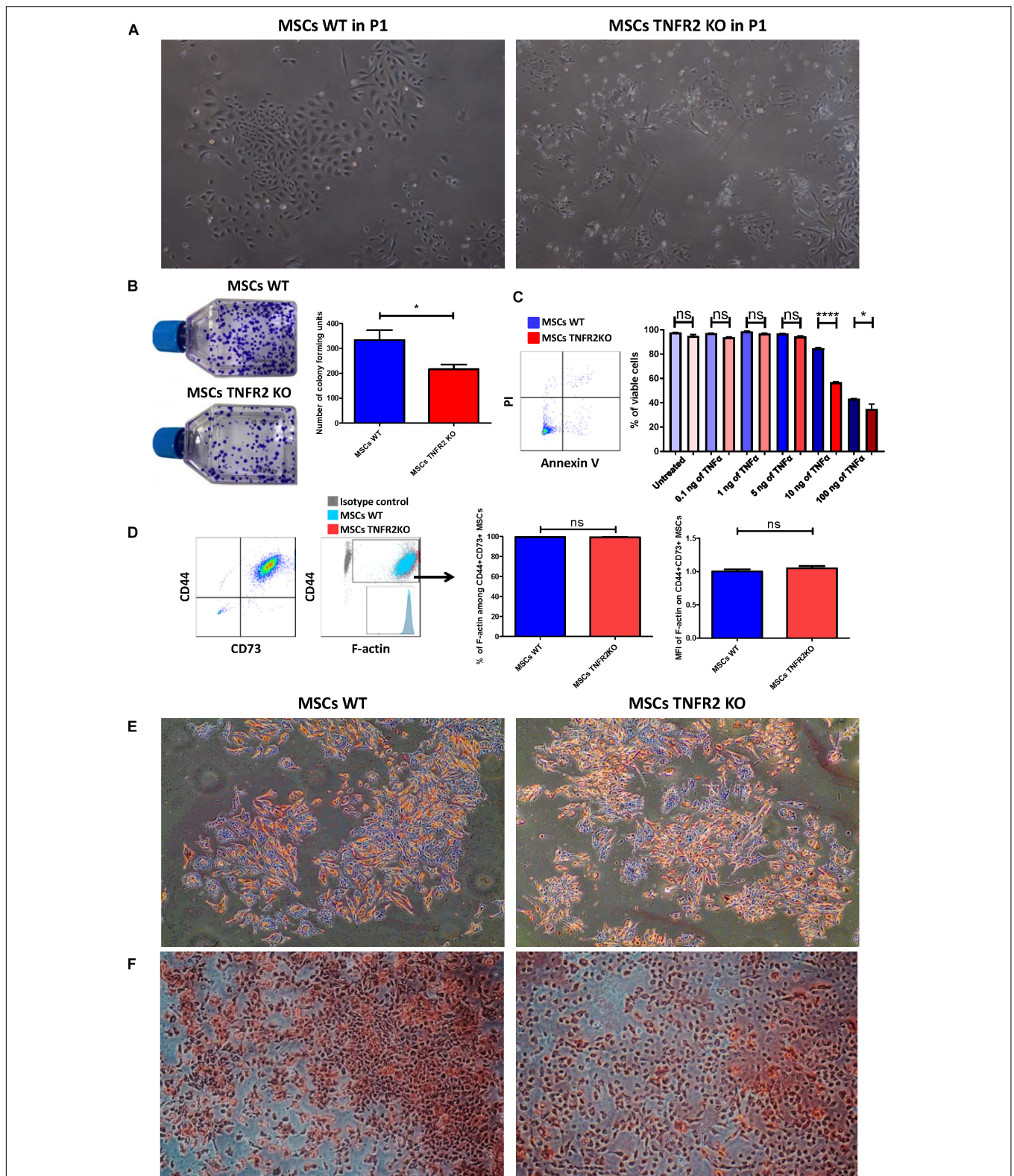
We have recently reported that BM-MSCs harvested from WT and TNFR2 KO mice have both normal physiological capabilities such as adherence to plastic plates and differentiation toward

adipocytes and osteocytes (Beldi et al., 2020). Nevertheless, clear morphological differences were noticed notably in their early passages. TNFR2 KO-MSCs were more heterogeneous with considerably smaller cytoplasm. Moreover, they had less number of cells in each initial colony compared with WT-MSCs (Figure 1A). Assessing the ability of MSCs to form CFUs in passage 1 revealed that WT-MSCs formed significantly more colonies than TNFR2 KO-MSCs (Figure 1B). This was in accordance with their lower proliferation rate at P1, P2, and P3 (Supplementary Figure 1A). In this case, CPDT was longer for TNFR2 KO-MSCs compared with WT-MSCs in P1 (27.04 ± 0.27 h, versus 23.35 ± 0.04 h, respectively) and in P2 (27.17 ± 0.59 h versus 22.39 ± 0.08 h, respectively) (Supplementary Figure 1B). The TNFR2 KO-MSCs' CPDT reached that of WT-MSCs in passage 3 (13.37 ± 0.01 h vs. 12.46 ± 0.01 h, respectively) (Supplementary Figure 1B). In order to evaluate the cell viability in the early passage (P1) after isolation, we have measured the expression of annexin V and PI among both MSC types. Our results demonstrated a high percentage of viable cells in untreated basal conditions among both WT and TNFR2 KO-MSCs with 97.76 and 95.06% of viability, respectively (Figure 1C). In addition, to explore the impact of the TNFα–TNFR2 axis on MSC viability we have treated them with five increasing doses of TNFα (i.e., 0.1, 1, 5, 10, and 100 ng/ml) for a duration of 24 h. We did not notice any difference in their viability until 5 ng/ml of TNFα treatment; however, in 10 and 100 ng/ml conditions, WT-MSCs were significantly more viable than their TNFR2 KO counterparts (Figure 1C).

Observing the morphological differences, we investigated whether there is a difference in their cytoplasmic microfilament constitution. Therefore, we measured filamentous-actin expression (F-actin) and surprisingly observed no difference in neither the percentage of expression nor the MFI of this protein (Figure 1D). We then explored if the absence of TNFR2 could change MSC differentiation capacity. While we did not notice a significant dissimilarity between their adipogenic differentiation potential (Figure 1E), we found a remarkable reduction in TNFR2 KO-MSC osteogenic capacity (Figure 1F).

### The Expression of Mesenchymal Stem Cell Characteristic Markers Is Diminished in the Absence of TNFR2

Our previous studies revealed that both WT and TNFR2 KO-MSCs express mouse MSC characteristic markers like CD44, Sca1, CD73, CD105, and CD90 and do not express CD45 common leukocyte and CD34 hematopoietic markers (Beldi et al., 2020). Accordingly, here we demonstrate that both BM-MSCs were able to express those markers (Figure 2). However, our further experiments to compare the expression level of each MSC characteristic marker showed that except for the percentage of CD44, the percentage and MFI of all other cytoplasmic markers were significantly diminished in TNFR2 KO-MSCs in comparison with WT-MSCs (Figure 2). Moreover, we did not see any CD45 or CD34 expression in neither of the MSCs (Supplementary Figure 2).



**FIGURE 1 |** Mesenchymal stem cell (MSC) characterization and differentiation capacity. **(A)** Wild-type (WT)-MSCs in P1 demonstrate regular fibroblast-like form with spindle-shaped morphology (4 $\times$ ), while a more heterogeneous appearance with smaller cytoplasm is observed by their TNFR2 knockout (KO) counterparts in P1 (4 $\times$ ). **(B)** Colony-forming unit (CFU) assay reveals higher efficiency of WT-MSCs to form colony units in comparison with TNFR2 KO-MSCs. Results are collected from two independent experiments ( $n = 14$ ). **(C)** Flow cytometric analysis of MSC viability assessment reveal the equivalent percentage of viable cells among (Continued)

**FIGURE 1 | Continued**

untreated MSCs. Upon TNF $\alpha$  treatment for a duration of 24 h, WT-MSCs show more viability (annexin V<sup>-</sup> PI<sup>-</sup> population) in a dose-dependent manner. Cells were gated on total MSCs' population ( $n = 6$ ). **(D)** Flow cytometric analysis of the F-actin expression in WT and TNFR2 KO-MSCs (P3). Cells were gated on CD44<sup>+</sup>CD73<sup>+</sup> MSCs. Mean fluorescence intensity (MFI) values have been normalized with WT-MSC group. Results are collected from two independent experiments ( $n = 6$ ). **(E)** To evaluate adipogenic differentiation capacity, both MSC types (P3) were incubated in proper adipogenic differentiation medium for a duration of 3 weeks and then stained with Alizarin red S (4 $\times$ ). **(F)** To evaluate osteogenic differentiation, both MSC types (P3) were incubated in proper osteogenic differentiation medium for a duration of 17 days and then stained with Oil-Red-O (4 $\times$ ).

## TNFR2 Expression Modulates Mesenchymal Stem Cell Capacity to Produce Anti- and Pro-inflammatory Cytokines

Afterward, we tested whether blocking the TNF $\alpha$ -TNFR2 signaling pathway impacts MSC cytokine production pattern. WT and TNFR2 KO-MSCs were first activated by the addition of 10 ng/ml of TNF $\alpha$ . After 48 h, their capacity to produce different anti-inflammatory and pro-inflammatory cytokines was analyzed. Cells were initially gated on CD44, since this marker was merely equally expressed on both MSC types (**Figure 3A**). We first investigated the capacity of MSCs to produce IL-10 and TGF $\beta$  anti-inflammatory cytokines. Interestingly, we observed a dramatic decrease in the capacity of TNFR2 KO-MSCs to produce both cytokines (**Figure 3B**). Contrariwise, hampering the TNF $\alpha$ -TNFR2 axis led to a significant increase in IFN $\gamma$ , TNF $\alpha$ , and IL-6 production by TNFR2 KO-MSCs compared with their WT counterparts (**Figure 3C**).

## Expression of TNFR2 by Mesenchymal Stem Cells Is Associated With Their Higher Nitric Oxide Production

One of the main mediators of murine MSC immunosuppressive and immunomodulatory effect is the NO production (Ren et al., 2008). Therefore, we investigated if there is a liaison between the TNFR2 expression and NO production by MSCs. It was already evidenced that MSCs do not secrete elevated amounts of NO in non-stressed conditions (Rahmat et al., 2013). Accordingly, we identified limited percentages of NO producing MSCs at basal level; however, even in this condition, WT-MSCs were more capable of NO production (**Figure 4A**). In order to create cellular stress, we have starved MSCs by growing them in a medium containing 0.5% FBS. This condition effectively enhanced NO production by both WT-MSCs (from 12.7% before starvation to 45.1% after starvation) and in TNFR2 KO-MSCs (from 1.8% before starvation to 18.5% after starvation). Due to the absence of TNFR2 pro-survival factor, in addition to the absence of the indispensable mediators present in FBS, starvation might have caused more cellular stress leading to a more effective increase in NO production by TNFR2 KO-MSCs; nevertheless, a more statistically significant NO production was observed by WT-MSCs (**Figure 4A**). We have recently demonstrated that TNFR2 KO-MSCs had a hampered ability to suppress T cells. In order to evaluate how MSCs react in the presence of T cells, we have created an inflammatory condition by co-culturing both MSCs with six increasing ratios of activated T cells (1/1 up to 1/10 MSC/T cell ratio). Our data showed that although both MSCs

were able to produce more NO with regards to increasing T cell numbers, this effect was substantially more enhanced for WT-MSCs than TNFR2 KO-MSCs (**Figure 4B**). Comparing MSCs in each T cell dose revealed that the blockade of TNFR2 resulted in less NO production in every single ratio (**Figure 4C**).

## TNFR2 Expression by Mesenchymal Stem Cells Results in the Induction of More Immunosuppressive Tregs

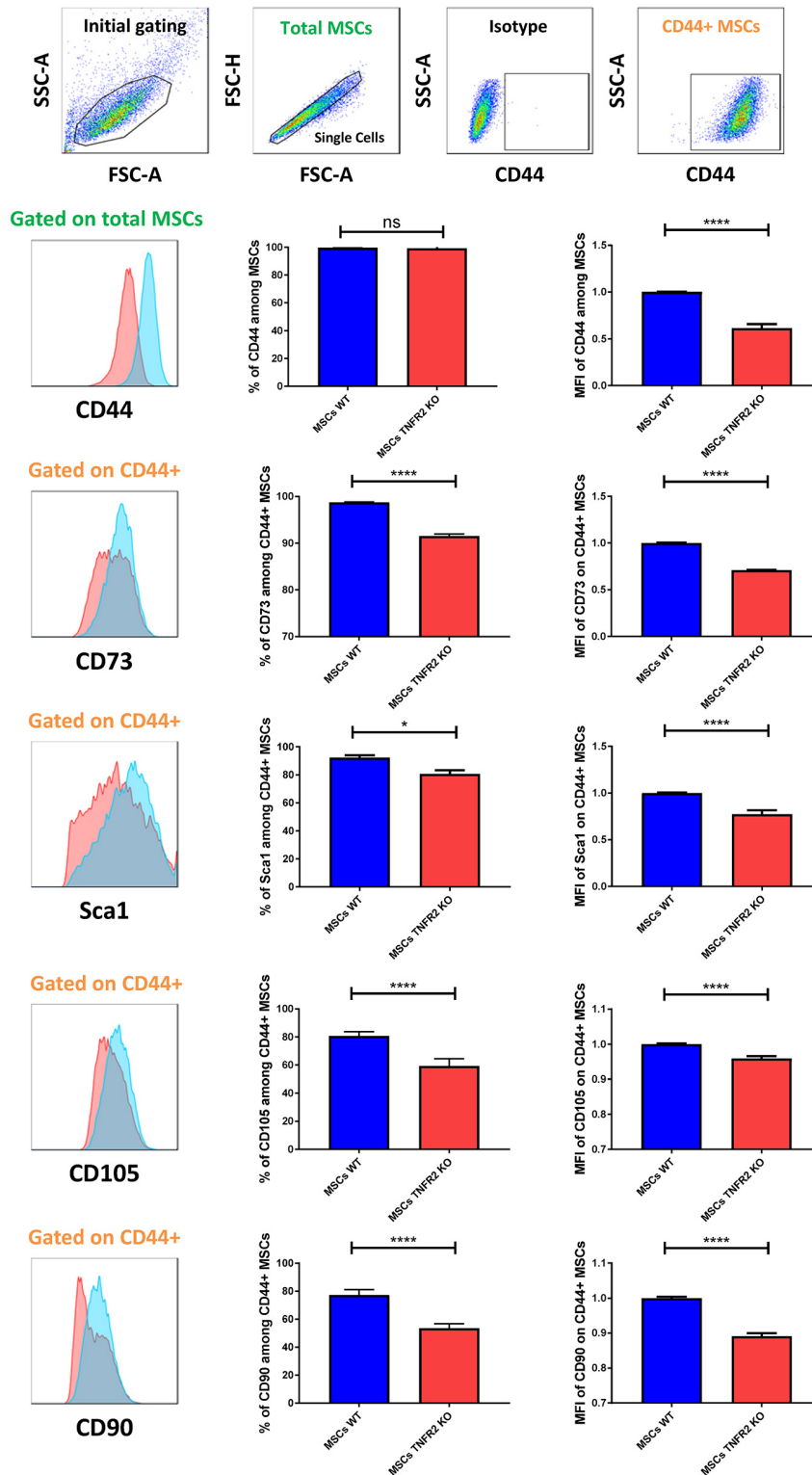
We have already depicted that the expression of TNFR2 is directly related to MSC ability to induce CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> and CD8<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Tregs (Beldi et al., 2020). Since TNFR2 KO-MSCs had less IL-10, TGF $\beta$ , and NO production rate, we have assessed if iTregs derived from these cells display less immunosuppressive effect. Therefore, T cells were freshly isolated and depleted from the CD25 subpopulation. This step was performed to eliminate natural Tregs and highly activated T cell populations. CD3<sup>+</sup>CD25<sup>-</sup> Tconvs were then co-cultured with WT and TNFR2 KO-MSCs in 1/10 MSC/T cell ratio. After 72 h, CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> iTregs generated in those co-cultures were evaluated with an MLR test with freshly isolated, CFSE-labeled activated mouse CD3<sup>+</sup>CD25<sup>-</sup> Tconvs in a fixed 1/5 iTreg/Tconv ratio (**Figure 5A**). In this setting, iTregs derived from WT-MSCs were significantly more immunosuppressive against both CD4<sup>+</sup> and CD8<sup>+</sup> Tconvs than iTregs derived from TNFR2 KO-MSCs (**Figure 5B**). iTregs derived from WT-MSCs suppressed 58.91% of CD4 and 38.16% of CD8 T cell proliferation. These values were 39.19 and 19.19% of CD4 and CD8 T cell suppression, respectively, after co-culturing with TNFR2 KO-MSCs (**Figure 5B**).

## Expression of TNFR2 by Mesenchymal Stem Cells Is Related to Their Increased Wound Healing Property

We then assessed the ability of MSCs to close a scratch wound in the monolayer cell surface. Our data revealed a much stronger wound healing capacity for WT-MSCs compared with TNFR2 KO-MSCs (**Figure 6A**). WT-MSCs achieved close to more than 99% of the scratched area within 30 h, while their TNFR2 KO counterparts reached only 69% within the same period of time (**Figure 6B**).

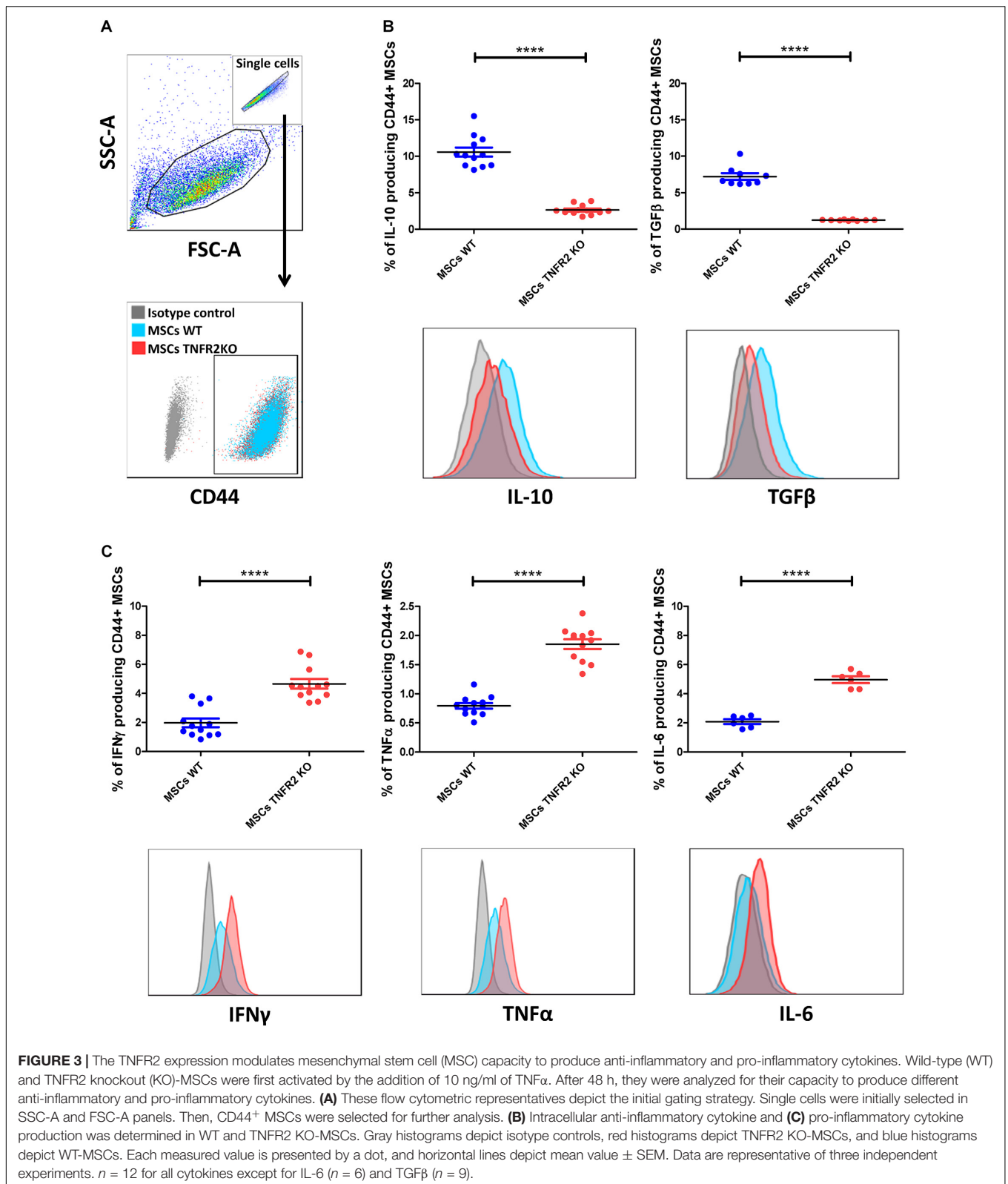
## Expression of TNFR2 by Mesenchymal Stem Cells Influences Their Tube Formation Property

One of the main characteristics of MSCs is their capability to exert pro-angiogenic functions through the construction of



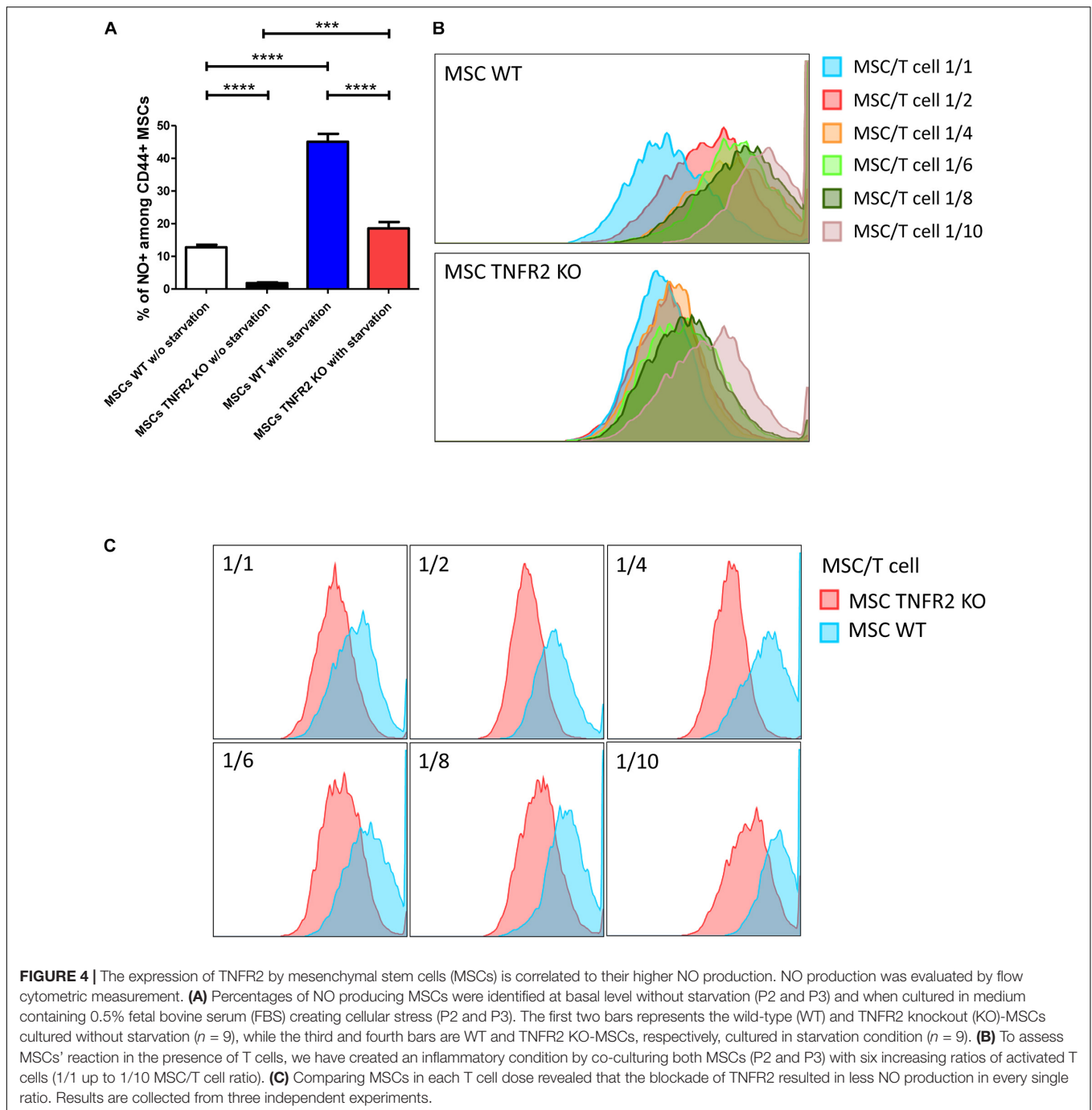
**FIGURE 2 |** Mesenchymal stem cell (MSC) characteristic markers' expression is diminished in the absence of TNFR2. This figure illustrates the surface expression of mouse MSC markers such as CD44, CD73, stem cell antigen-1 (Sca1), CD105, and CD90 in both MSC types (P3). To investigate the expression level of CD44 marker, cells were pre-gated on total MSCs (represented in green color). For the rest of the markers, cells were pre-gated on CD44+ MSCs (represented in orange color). Mean fluorescence intensity (MFI) values have been normalized with wild-type (WT)-MSC group. Red histograms depict TNFR2 knockout (KO)-MSCs, and blue histograms depict WT-MSCs. The results of CD44 ( $n = 25$ ) and Sca1 ( $n = 19$ ) expression are collected from four different experiments. The results of CD73, CD105, and CD90 markers ( $n = 13$ ) are collected from three independent experiments.





tubular complex structures in the presence of proper angiogenic and extracellular matrix mediators (Shen et al., 2015; Yu et al., 2018). To evaluate the involvement of TNFR2 in this

regenerative feature, WT and TNFR2 KO-MSCs were cultured on Matrigel using either DMEM standard medium or EC growth medium (EGM2) containing a variety of pro-angiogenic

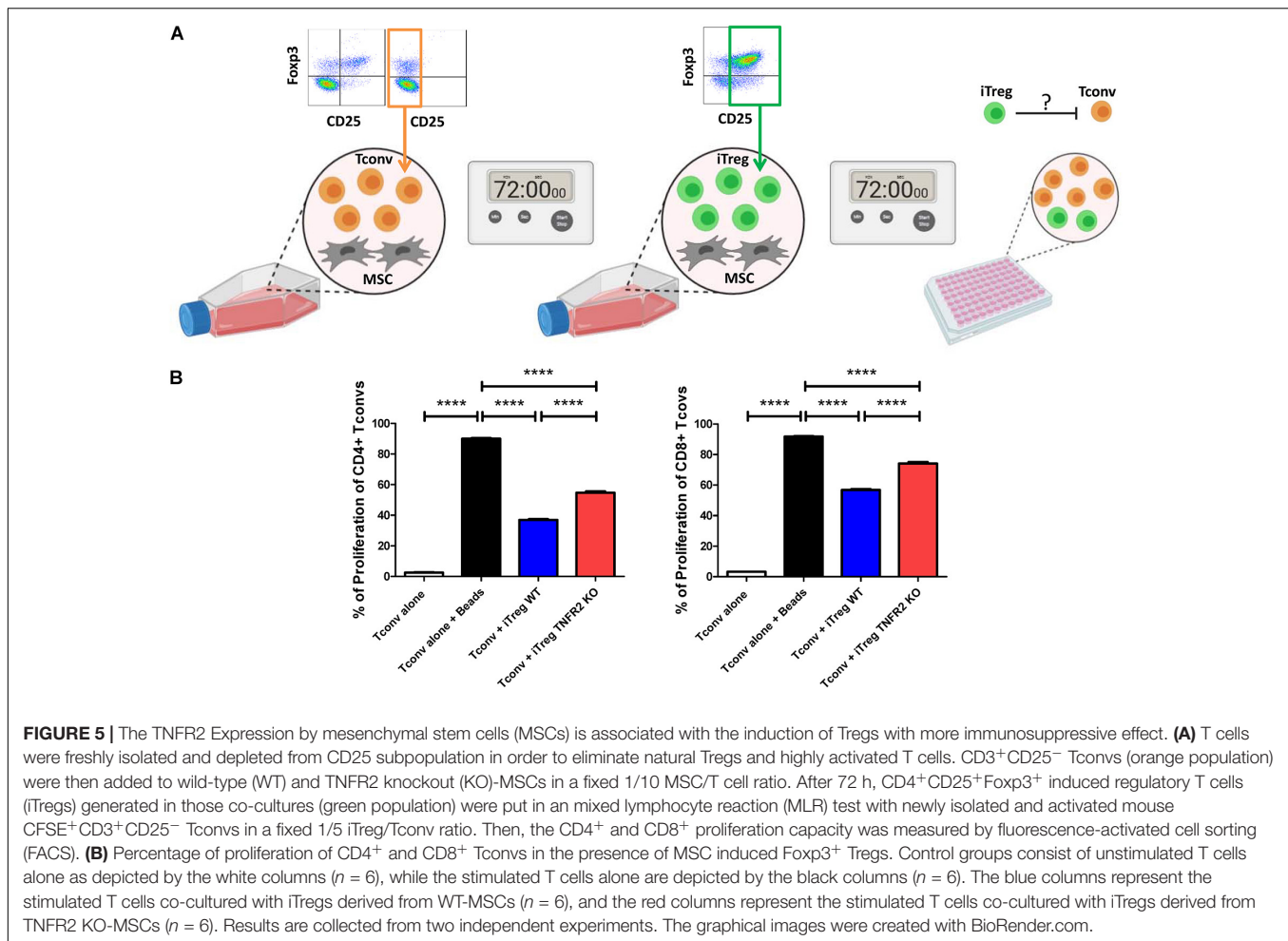


factors such as VEGF, FGF, EGF, and IGF. As expected, we noticed that neither of the MSCs could form tubular complex structures in the absence of angiogenic factors (Figure 7A). On the contrary, while culturing WT-MSCs in EGM2 led to complex tubular and network formation, TNFR2 KO-MSCs were significantly less performant (Figure 7A). According to our acquired results, both MSCs constructed tubes with similar lengths (Figure 7B). However, comparing their network complexity revealed a much more developed, multibranchial, and dense 3D structures for WT-MSCs, which were some of

the important features that were plainly deficient in TNFR2 KO-MSCs (Figure 7C).

### Expression of TNFR2 Is Crucial for Mesenchymal Stem Cells to Support Endothelial Cell Angiogenic Function

MSCs have been shown to have supportive and protective effects toward EC angiogenic function mostly through secretion of pro-angiogenic factors like VEGF, TGF $\beta$ , and HGF (Watt et al., 2013;



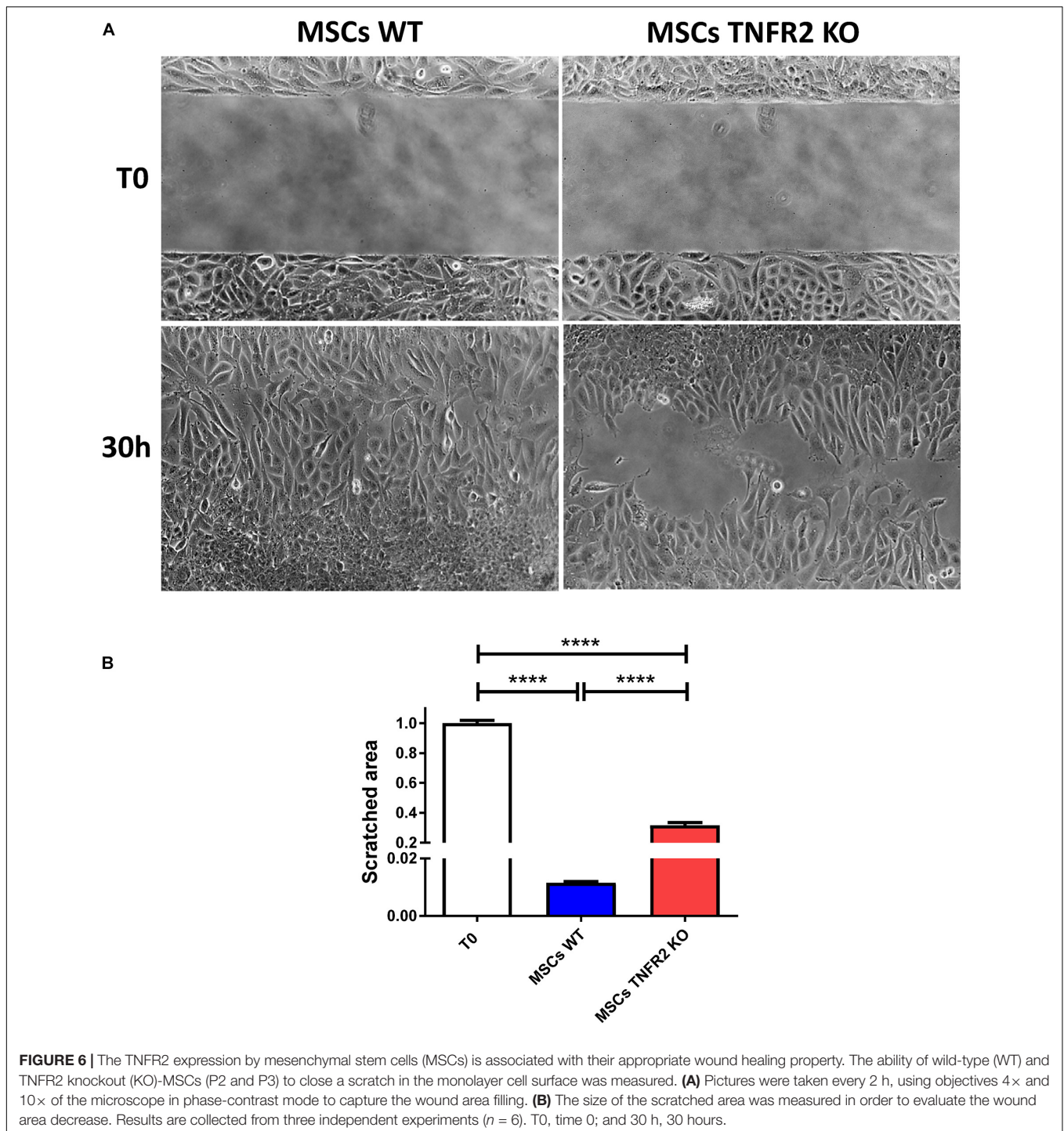
Beckermann et al., 2008; Batlle et al., 2019; Maacha et al., 2020). Here, we aimed to investigate whether there exists a relation between the TNFR2 expression and the MSC pro-angiogenic function. HUVECs were selected as the responsive cells to assess the tube formation function on Matrigel. Although HUVECs were unable to form tubular networks in endothelial basal medium (EBM2), the addition of pro-angiogenic factors (EGM2 medium) led to the formation of complex tubular structures (**Figure 8A**). We then evaluated the impact of CM of MSCs on HUVEC tube formation. WT and TNFR2 KO-MSCs were cultured in EBM2 medium; and after 48 h, their CM were collected and added to HUVECs. Surprisingly, while we witnessed a complete pro-angiogenic effect of CM from WT-MSCs, this effect was considerably impaired with CM of TNFR2 KO-MSCs (**Figure 8A**). HUVECs in CM of WT-MSCs formed significantly longer tubes (**Figure 8B**) and much more complex and closed network structures (**Figure 8C**).

## DISCUSSION

The combination of immunomodulatory and regenerative properties of MSCs made these cells the most frequently

investigated stem cells for clinical applications during the last couple of decades. This led to a large number of publications describing their therapeutic potential in a variety of disorders, often by suggesting new mechanisms of action from genomic to proteomic investigations. MSCs have been demonstrated to react unexpectedly in different inflammatory conditions. Endeavors to discover MSC behavior in presence of inflammatory cytokines such as TNF $\alpha$  surprisingly revealed enhanced immunosuppressive and regenerative effects in a compensatory feedback (English et al., 2007; Szabó et al., 2015; Broekman et al., 2016; Yang H.-M. et al., 2018; Noronha et al., 2019). As already mentioned in the *Introduction*, TNF $\alpha$  recognizes two transmembrane receptors (TNFR1 and TNFR2), which distinctly initiate downstream signaling that terminates in biological fates from one extreme to the other (Faustman and Davis, 2013; Wajant and Siegmund, 2019). TNFR1 is omnipresent on all cells, while TNFR2 is limitedly expressed on few cells including MSCs. Therefore, understanding the exact role of TNFR2 and the reason for its expression seems crucial.

Our previous attempts to unravel the role of TNF $\alpha$ -TNFR2 signaling pathway in other cell types led to the conclusion that this axis controls principal immunoregulatory and protective

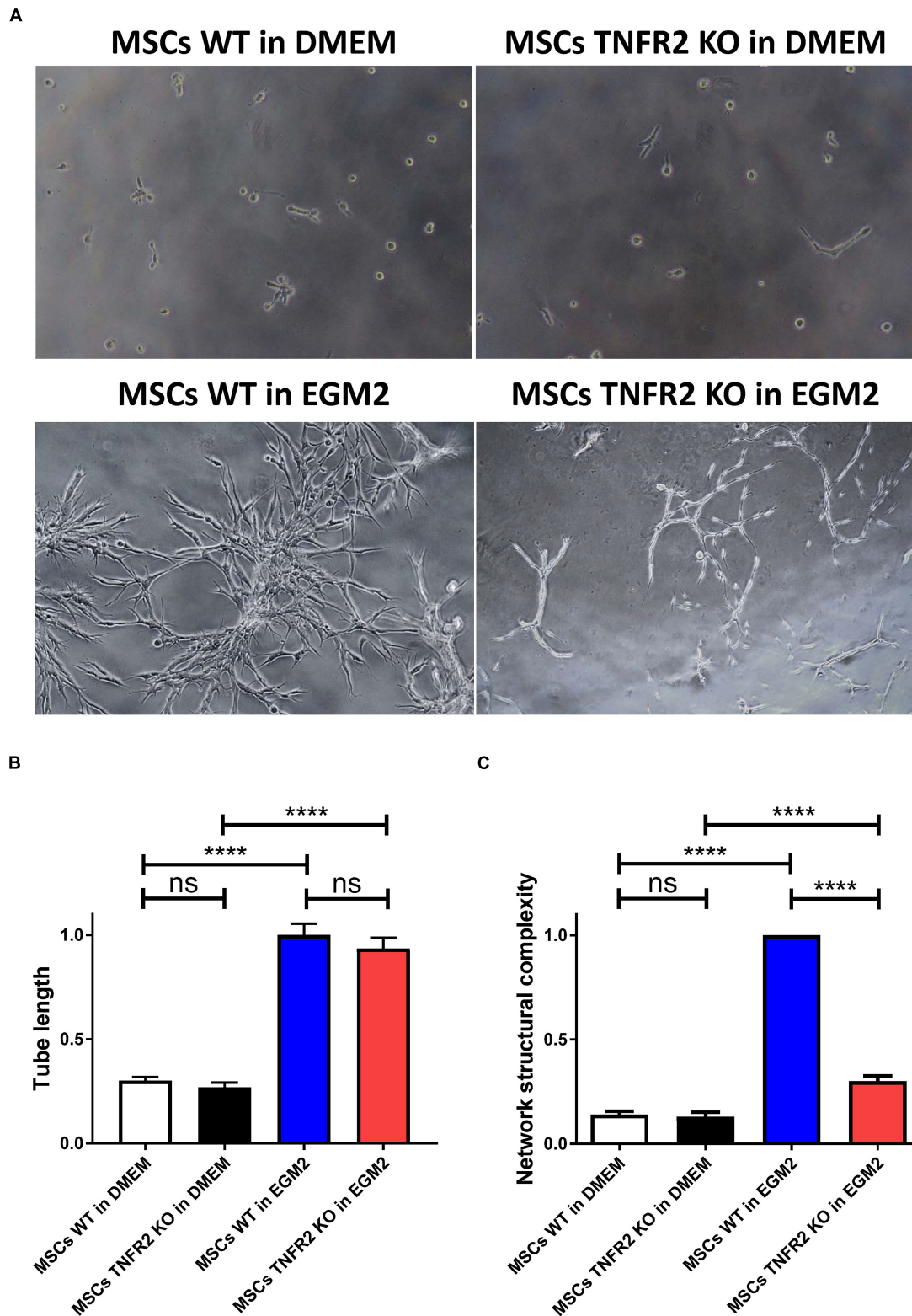


**FIGURE 6 |** The TNFR2 expression by mesenchymal stem cells (MSCs) is associated with their appropriate wound healing property. The ability of wild-type (WT) and TNFR2 knockout (KO)-MSCs (P2 and P3) to close a scratch in the monolayer cell surface was measured. **(A)** Pictures were taken every 2 h, using objectives 4× and 10× of the microscope in phase-contrast mode to capture the wound area filling. **(B)** The size of the scratched area was measured in order to evaluate the wound area decrease. Results are collected from three independent experiments ( $n = 6$ ). T0, time 0; and 30 h, 30 hours.

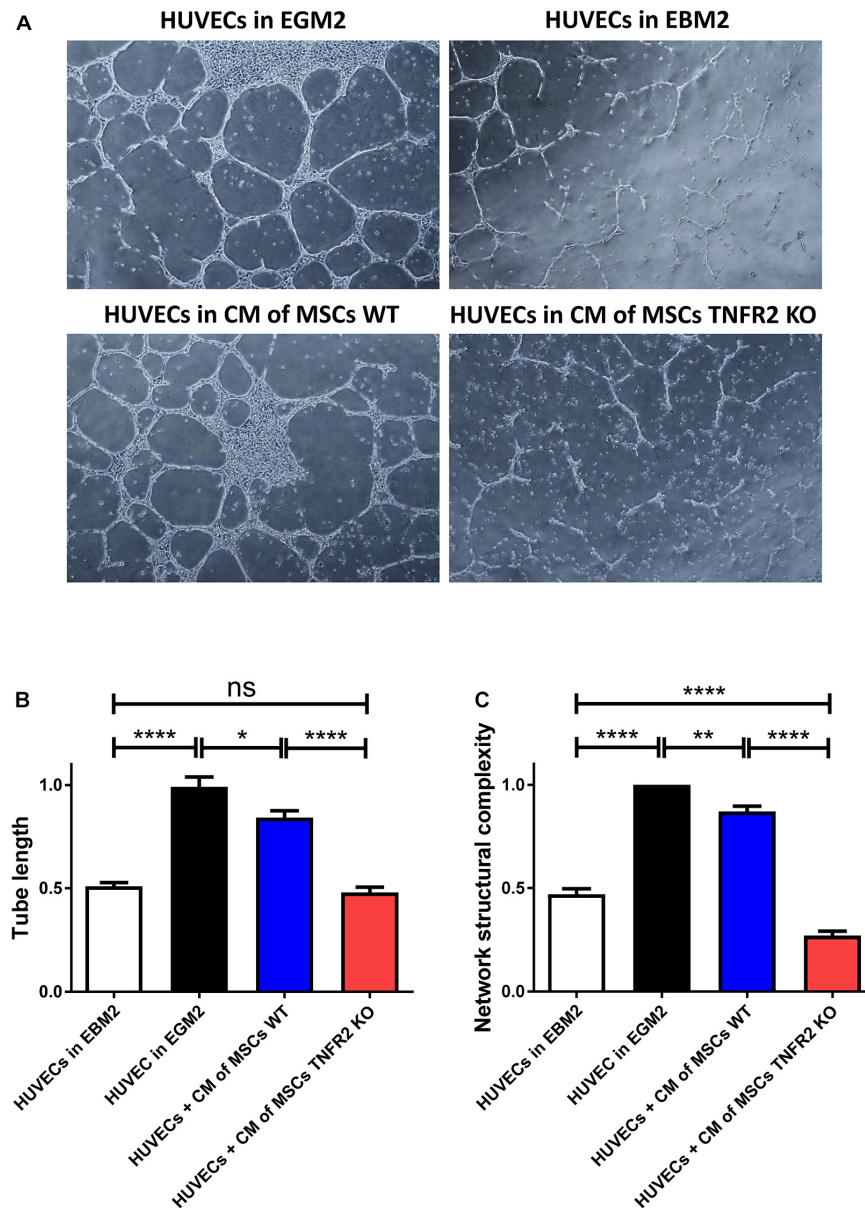
functions. For instance, it has been shown that TNFR2 controls Treg, Breg, MDSC, and EPC immunosuppressive effect (Polz et al., 2014; Leclerc et al., 2016; Ticha et al., 2017; Naserian et al., 2020). Interestingly, TNF $\alpha$  pre-treatment resulted in increased Treg and MDSC immunosuppressive property (Hu et al., 2014; Pierini et al., 2016). In case of MSCs, signaling through TNFR2 was shown to support regenerative functions and to be essential for their therapeutic effect in treating

inflammatory and autoimmune disorders (Kelly et al., 2010; Yan et al., 2018).

In this study, we wanted to understand more precisely the exact role of TNFR2 in MSCs. To this goal, we first compared the expression of MSC specific markers on BM-MSCs harvested from WT and TNFR2 KO mice. Our data showed that TNFR2 KO-MSCs had lower expression of CD44, Sca1, CD105, CD73, and CD90 principal markers. Despite these phenotypical



**FIGURE 7 |** The TNFR2 expression by mesenchymal stem cells (MSCs) is associated with their enhanced tube formation property. To evaluate the involvement of TNFR2 in MSC regenerative feature, wild-type (WT) and TNFR2 knockout (KO)-MSCs (P2 and P3) were cultured on Matrigel using either Dulbecco's modified Eagle's medium (DMEM) standard medium or EGM2 endothelial medium. **(A)** Pictures were taken every 2 h, using objectives 4 $\times$  and 10 $\times$  of the inverted microscope in phase-contrast mode. **(B)** The tube length and **(C)** network structural complexity of WT and TNFR2 KO-MSCs were further evaluated. Results are collected from three independent experiments ( $n = 10$ ).



**FIGURE 8** | Expression of TNFR2 is crucial for mesenchymal stem cells (MSCs) to support endothelial cell (EC) angiogenic function. To evaluate the impact of MSCs on human umbilical vein endothelial cell (HUVEC) angiogenic capacity, wild-type (WT) or TNFR2 knockout (KO)-MSCs (P2 and P3) were cultured in complete Dulbecco's modified Eagle's medium (DMEM) medium. After 2 days, CM were taken, filtered, and added to HUVECs on Matrigel. HUVECs cultured in EBM2 basal medium were used as negative control, and HUVECs cultured in EGM2 complete medium were used as positive control. **(A)** Pictures were taken every 2 h, using objectives 4× and 10× of the inverted microscope in phase-contrast mode. Images were further analyzed to evaluate **(B)** the tube length and **(C)** the network structural complexity. Results are collected from three independent experiments ( $n = 10$ ). CM, conditioned media.

differences, due to the absence of a specific marker, it is difficult to determine whether TNFR2 KO-MSCs have already committed toward another lineage. However, as supported by other research publications and the results of our study, we think that the reduction in the TNFR2 KO-MSC characterization markers is associated with less functionality linked to each or a combination of those markers rather than a conversion toward another lineage. For example, the expression of CD90 was mostly associated with MSC immunosuppressive effect

(Campioni et al., 2009). Accordingly, we reported that mouse TNFR2 KO-MSCs have significantly lower immunosuppressive and immunomodulatory effect against T cells (Beldi et al., 2020). CD73 marker was associated with MSC reparative and regenerative properties. The injection of CD73<sup>low</sup> MSCs into mouse heart was shown to be much less effective to repair myocardial infarction than CD73<sup>high</sup> MSCs (Tan et al., 2019). In agreement, TNFR2 expression was shown to be crucial in MSC cardiac protection following acute ischemia

(Kelly et al., 2010). CD105, CD73, and CD90 markers were also associated with MSC differentiation capacity (Arufe et al., 2010; Vaculik et al., 2012; Lv et al., 2014). Our results demonstrated that although we did not observe a clear difference in MSCs' adipogenic differentiation, the absence of TNFR2 caused a significant decrease in MSCs' osteogenic differentiation capacity. It would be interesting to further assess the role of TNFR2 in MSC differentiation capacity by studying the expression of different proteins and their genes such as PPAR $\gamma$ , BMPR1, and FABP4 that are shown to be upregulated in adipogenesis (Farmer, 2005; Moseti et al., 2016; Liu et al., 2019) and RUNX2 that is related to MSC osteogenesis (Thiagarajan et al., 2017). Our investigation on chondrogenic differentiation revealed a disrupted capacity for TNFR2 KO-MSCs (data not shown). Further studies are indeed necessary to confirm these preliminary results.

Our previous research works correlated the expression of TNFR2 to MSC immunosuppressive effect (Beldi et al., 2020). Besides, TNF $\alpha$  priming was shown to increase IL-10 and TGF $\beta$  secretion by MSCs (Putra et al., 2018). This encouraged us to evaluate the TNF $\alpha$ -TNFR2 involvement in the production of MSC immunomodulatory mediators like cytokines and NO. TNFR2 blockade led to increased levels of IFN $\gamma$ , TNF $\alpha$ , and IL-6 pro-inflammatory and decreased IL-10 and TGF $\beta$  anti-inflammatory cytokines and NO production. This is in accordance with other studies showing a direct correlation between the expression of TNFR2 and enhanced IL-10, TGF $\beta$ , and HLA-G secretion by EPCs (Naserian et al., 2020) and Bregs (Ticha et al., 2017).

The secretion of anti-inflammatory mediators by MSCs is one of the main mechanisms mediating their Treg induction capacity. We have recently reported that in comparison with WT-MSCs, TNFR2 KO-MSCs were considerably less able to induce CD4 $^{+}$  and CD8 $^{+}$  Tregs (Beldi et al., 2020). Here, we complete our preceding work by demonstrating that TNFR2 deficiency leads to the induction of Tregs with remarkably less immunosuppressive effect. This piece of information is specifically interesting since it highlights the importance of TNFR2 not only in MSC immunosuppressive effect but also in other cells that are in contact with them such as T cells. It seems essential to evaluate the role of TNFR2 on other cells such as tolerogenic dendritic cells (tDCs) and M2 macrophages that could be also induced from MSCs.

In the second part of this work, we aimed to investigate the involvement of TNFR2 in some principal MSC regenerative properties including wound healing, tube formation, and pro-angiogenic effects. We demonstrated that hampering TNFR2 molecule will cause diminished MSC wound healing and tube formation capacities.

MSC involvement in angiogenesis has been already proven. By a direct cellular contribution to vascular system, they can induce a protective vascular function. Rapidly after their injection, MSCs induce primal immature vascular tubes linked to the host circulation. Surprisingly, this phenomenon was shown to be even faster than host EC and smooth muscle cell (SMC) recruitment to neovascularization site (Dufourcq et al., 2008). The angiogenic efficiency of MSCs was reported

to be remarkably variant depending on their originated source, resulting in heterogeneous regenerative outcomes (Xu et al., 2017; Lu et al., 2018). Our data reveal TNFR2 as key regulator of MSC angiogenic properties. This point is interesting since enriching TNFR2 $^{+}$  MSCs could potentially provide MSCs with the most homogeneous and highest pro-angiogenic functions. Blocking TNFR2 by an appropriate antagonist, on the other hand, could efficiently hamper the MSC angiogenic and immunoregulatory functions. This is specifically crucial in cancer treatment where MSCs have been shown to be strongly involved in favoring tumor growth by supporting angiogenic and immunosuppressive microenvironment mostly via TGF $\beta$ -dependent mechanism (Zhang T. et al., 2013; Li et al., 2016; Trivanović et al., 2016; Yang et al., 2017; Battle et al., 2019; Lee et al., 2019), a cytokine that we showed to be significantly less produced by TNFR2 KO-MSCs.

MSCs can also favor angiogenesis through supporting EC function via promoting the secretion of pro-angiogenic factors (Fransson et al., 2015; Premer et al., 2015). Here, by evaluating the impact of CM of MSCs on HUVEC angiogenic capacity, we demonstrated that in complete opposite to WT-MSCs, CM from TNFR2 KO-MSCs that cannot support HUVEC tube formation. This is in accordance with other studies showing that the therapeutic effect of MSCs is through paracrine mechanism, which is strictly regulated by Rap1/NF- $\kappa$ B signaling pathway (Zhang Y. et al., 2013; Zhang et al., 2015). Interestingly, the TNF $\alpha$ -TNFR2 interaction can activate NF- $\kappa$ B signaling pathway through TNF receptor-associated factor (TRAF) involvement (Faustman and Davis, 2013). Indeed, previous studies have reported that the VEGFR2 signaling pathway is defective in TNFR2 KO mice (Luo et al., 2006). Additionally, interfering in the TNFR2 signaling pathway impacts the secretion of pro-angiogenic factors such as HGF, IGF-1, and VEGF by MSC (Wang et al., 2006; Crisostomo et al., 2008; Zhang et al., 2010; Yan et al., 2018). The same phenomenon was observed in other cells such as EPCs linking the TNFR2 expression to VEGF, IGF, HGF, and IL-8 production and consequently to their angiogenic function (Yoshida et al., 1997; Hoefer et al., 2002; Goukassian et al., 2007). The principal novelty of our work is that we have measured some other mediators that are as crucial as classical growth factors in angiogenesis such as NO and TGF $\beta$ . NO has a central role in starting and stimulating angiogenesis through a direct effect and via induction of VEGF and FGF (Kang et al., 2020; Yamamoto et al., 2020). To our knowledge, this is the first report that correlates TNFR2 expression by MSCs to their NO production capacity.

In this study, we have used MSCs harvested from the BM, which is considered as an adult source for these cells. Several disadvantages have been attributed to adult tissue sources of MSCs including the heterogeneity, batch to batch variations, cellular senescence, and limited proliferative potency (Lukomska et al., 2019; Musiał-Wysocka et al., 2019). MSCs can be also derived from induced pluripotent stem cells (iPSCs). It has been demonstrated that human iPSC-derived MSCs (iPSC-MSCs) possess higher proliferative and immunomodulatory potentials (Zhang et al., 2012; Bloor et al., 2020). Besides, due to the higher

expression of TNF $\alpha$ IP2, iPSC-MSCs are more responsive to TNF $\alpha$ -induced tunneling nanotube formation for mitochondrial transfer and have been shown to be more protective against cardiomyocyte damage (Zhang et al., 2016). Thus, it is worthy to assess the variation of expression rate and the exact implication of TNFR2 molecule in these MSC sources as well. Furthermore, it would be interesting to demonstrate if restoration of the TNFR2 expression in TNFR2 deficient MSCs can rescue their impaired regenerative and immunological functions. In the end, blocking the TNFR1 signaling pathway, sorting the TNFR2<sup>+</sup> MSCs, and upregulating the TNFR2 molecule via its specific agonist to assess the potentially increased MSC biological function are among the missing information in this current work. Altogether, this work supports the strong implication of the TNFR2 expression in MSC biological and functional properties, especially in their immunological and regenerative potentials. We strongly believe that this study paves the way for further *in vitro* and *in vivo* explorations in this field.

## DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

## ETHICS STATEMENT

All experimental procedures were performed in accordance with the European Community Council Directive (2010/63/UE) for the care and use of laboratory animals. Experimental protocols were approved by the local ethics committee “Comité d'éthique en expérimentation animal Charles Darwin No 5” under the number 02811.03 in compliance with European Union guidelines.

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## AUTHOR CONTRIBUTIONS

SN and GU conceived the study. GB, SB, CL, MNB, and SN performed the experiments. GB, SB, and SN analyzed the data. GB and SN wrote the manuscript. MEA, GU, and SN reviewed the manuscript. SN and GU revised the manuscript. All authors contributed to the article and approved the submitted version.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fcell.2020.596831/full#supplementary-material>

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**Conflict of Interest:** SN is the CEO of CellMedEx Company.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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