IMPACT OF DONOR AGE ON BIOLOGICAL AND IMMUNOGENIC PROPERTIES OF MESENCHYMAL STROMAL CELLS

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Running Head: Aging of MSCs

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Author contributions

M.S. performed cell culture, as well as the differentiation studies, flow cytometry assays, proliferation and migration assays, ELISPOT assays, and immunoblotting, analyzed the data, and edited the manuscript. E.N. designed the studies, analyzed the data, and wrote the manuscript. T.D. designed the studies, analyzed the data, and wrote the manuscript. D.M. performed cell culture as well as the adhesion assays and membrane arrays. C.L. performed differentiation assays and analyzed data. H.R. edited the manuscript. R.C.R. and R.E.B. contributed to the study design, edited the manuscript and secured the funding. H.D.V. designed the studies and edited the manuscript S.S. designed the studies, secured the funding, analyzed the data, and wrote the manuscript.

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Abstract—Over the last decades, mesenchymal stromal cells MSC have emerged as a promising therapeutic tool for cell-based therapies. However, their biological characteristics and beneficial potential may decline with patients' age. Because most patients amenable for regenerative medicine have increased age, this could dramatically limit the autologous use of MSCs. We therefore sought to characterize the biological, immunological, and angiogenetic capacities of infant (iMSCs) versus aged MSCs (aMSCs). Both iMSCs and aMSCs showed typical spindle-shaped morphology, plastic adherence, and the same potential to differentiate into osteogenic, chondrogenic and adipogenic lineages. iMSCs and aMSCs expressed MSC-typical surface markers such as CD90 and CD105 and were negative for CD45, CD34, CD31 and CD117. iMSCs showed faster *in vitro* proliferation ($p<0.001$), migration ($p<0.001$), and stronger endothelial adhesion ($p=0.018$) than aMSC. There were no differences in HLA class I, β2 microglobulin, HLA class II, and co-stimulatory molecule expression between iMSCs and aMSCs. Both immunogenic HLA class II and CD54 and tolerogenic HLA-E expression were upregulated by IFNγ. iMSCs showed significantly higher CD29 signals and stronger endothelial adhesion than aMSCs. Interestingly, iMSCs outperformed aMSCs in different angiogenesis parameters. If not used in an autologous setting, iMSCs and aMSCs similarly provoked T_H1 and T_H2 responses in unidirectional ELISPOT assays and donor-specific IgM antibody production. Our results thus suggest that donor age does not affect immunogenicity and immunomodulatory properties, but markedly reduces biological properties of proliferation, migration, adhesion, and limits their angiogenic effects.

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1. INTRODUCTION

Peripheral arterial disease (PAD) is a major health care problem, affecting mainly elderly patients older than 65 years (1). As the world population ages, it is estimated that the prevalence of the disease will rise to 15-20% in patients above the age of 70 (2). PAD is a highly debilitating condition, associated with significant morbidity and mortality (3). Patients suffering from occlusive PAD and critical intermittent claudication often fail to respond to conservative therapy. Although surgical or endovascular revascularization techniques can be used to alleviate symptoms, about 30% of patients suffering from critical limb ischemia are not candidates for such interventions, because of diffuse peripheral disease (4). These patients may require amputation with a particularly dismal prognosis. In 2005, 1.6 million people were estimated to be living with limb loss in the United States alone; by 2050, the rate is predicted to double to 3.6 million. While in patients with cancer and following trauma the rate of amputations have decreased, it has increased in patients with vascular diseases (5). Therefore, new strategies are urgently needed to offer these patients a viable therapeutic option.

In recent years, mesenchymal stromal cells (MSC) were suggested to possess therapeutic potential for cell-based therapies, mainly due to their paracrine action (6). Their remarkable ability to secrete various angiogenic cytokines under strenuous hypoxic conditions has raised expectation for their beneficial therapeutic value in ischemic diseases. Scientists have thus attempted to treat myocardial infarction (7, 8) and PAD (6) with promising early results. The immune-regulatory capacity of the MSCs (9) makes them further potential candidates for the treatment of oncogenic or immunologic disorders like lymphoma (10) and graft versus host disease (11). MSCs can be isolated from a variety of tissues, including different compartments of the umbilical cord (12), peripheral blood (13), and adipose tissue (14), but the main source utilized is the bone marrow (15). MSCs have the potential to differentiate in many different mesenchymal cell types. Recent studies have shown that as a person ages the immune system undergoes significant changes referred as immunosenescence. Bustos *et al*, using young vs old mice demonstrated decrease in cytokine and chemokine receptors in aged BM-MSCs due to the effects of the aged milieu on MSCs (16). Recently Li *et al* have shown an increased apoptosis of aged MSCs triggered by H_2O_2 affecting their viability and engraftment (17). In addition to this it has been reported that their therapeutic effectiveness decreases in relation to donor age increase, however the underlying mechanisms has not been thoroughly investigated. Since aging is a complex process affecting every cell type in the body, the angiogenic and immune-modulatory function as well as their proliferation potential of MSCs may be impaired.. Since most PAD patients have increased age (18), this could dramatically limit the autologous use of MSCs compromising their protective role. Therefore in this study we attempt to characterize the biological, immunological, and angiogenic capacities and delineate the differences between young versus old MSCs derived from the same tissue source, evaluating their regenerative potential to replace damaged tissue.

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2. MATERIALS AND METHODS Animals

Male Balb/C mice (6-8 weeks old) were purchased from Charles River Laboratories (Sulzfeld, Germany) and housed under specific pathogen-free conditions in the animal care facilities of the University Hospital Hamburg-Eppendorf. All animals received humane care in compliance with University guidelines. Animals were randomly assigned to either receive iMSC or aMSC transplants (n=6 and n=12, respectively) by intramuscular injection. All animal experiments were performed in compliance with the German Animal Welfare Act (TierSchG §43) and were approved by the State Office of Health and Social Affairs Hamburg (Permit number: G13/062).

Bone marrow MSC isolation and cell culture

aMSCs were isolated from sternal bone marrow aspirates of three multimorbid patients above the age of 70 years. These patients underwent sternotomy for coronary artery bypass surgery and all procedures were in compliance with the University Heart Center Hamburg hospital guidelines**.** Participants provided their written consent, which was approved by the Hamburg Ethics Committee. Red blood cells in the aspirates were lysed (ACK lysing buffer, Lonza, Walkersville, MD) and the remaining cells were plated onto plastic flasks and cultured for several passages. MSCs isolated from an infant (iMSCs) were isolated according to the previously described protocol (19). All MSCs used for experiments were between passage numbers 5 and 10 and cultured in DMEM with 1g/L D-glucose, L-glutamin and pyruvate, supplemented with 20% heat-inactivated fetal calf serum (FCS), 1% pencillin/streptomycin (P/S) and 1% glutamine (Gibco, Paisley, UK) at 37°C in a humidified atmosphere of 5% CO_2 until 80-90% confluency.

MSC lineage differentiation

In order to confirm the multipotent nature of our bone marrow-derived iMSCs and aMSCs, both cell lines were differentiated into osteogenic, adipogenic, and chondrogenic lineages. For osteogenic and adipogenic differentiation, a total number of $6x10^4$ cells/well were plated onto 24-well plates and cultured for 48h in normal MSC growth medium (low glucose, DMEM supplemented with 20% heat-inactivated FCS, 1% P/S and 1% glutamine). Differentiation to adipocytes and osteocytes was induced via medium change to appropriate differentiation medium (PromoCell, Heidelberg, Germany). Chondrogenic differentiation was achieved by plating the cells at $1x10^5$ cells/well in 200µl medium in 96-well round bottom suspension plates (Sarstedt). After 24h, the MSC culture medium was changed to differentiation medium according to the company's protocol (PromoCell). Differentiation medium was changed every other day for 21 days (osteocytes) or 14 days (adipocytes, chondrocytes), respectively. Adipogenic differentiation was confirmed by the presence of lipid droplets visualized using Oil Red O staining while osteogenic differentiation was confirmed by staining for calcification of the extracellular matrix using Alizarin red stain as previously described (20). Differentiated chondrocytes formed spheres that stained positive for Toluidine Blue, indicating peri-cellular proteoglycan deposition.

Flow cytometry

MSCs were incubated with antibodies against different surface markers and characterized using flow cytometry. Phycoerythrin (PE)-conjugated antibodies were used against human CD29 (clone MAR4), CD31 (clone WM59), CD34 (clone 563), CD40 (clone 5C3), CD45 (clone H130), CD54 (clone H130), CD80 (clone L307.4), CD86 (clone 2331(FUN-1)), CD90

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(clone 5E10), CD105 (clone 166707), CD116 (clone hGMCSFR-M1), CD117/c-Kit (clone 104D2), and CD166 (clone 3A6; all BD Pharmingen, Minneapolis, MN), β2-microglobulin (clone TU99), MICA/B (clone 6D4), and HLA I (clone DX17; all BD Pharmingen, Minneapolis, MN), HLA II (clone WR18; Abcam, Cambridge, UK), and Hsc70/Hsp70 (clone C92F3A-5, Enzo Life Science, Farmingdale, NY). Unconjugated antibodies: HLA-E (clone MEM-E/06, Santa Cruz Biotechnologies, Santa Cruz, CA) and HLA-G (clone MEM-G/9, Santa Cruz Biotechnologies) were visualised using a PE-conjugated anti-mouse IgG2a secondary antibody (Santa Cruz Biotechnologies). Mouse isotype-matched IgG1, IgG2a, or IgM antibodies (clones: MOPC-21, MOPC-173, and G155-228, respectively; all BD Biosciences) were used as controls.

Human endothelial cells (ECs) were stained with an anti-human PE-conjugated antibody against the surface marker VCAM1 (CD106; clone 51-10C9; BD Biosciences) and analyzed using flow cytometry. Mouse isotype IgG1 was used as control.

All measurements were performed on a FACSCalibur flow cytometer using CellQuestPro software (BD Biosciences) and analysed using FlowJo (Tree Star, San Carlos, CA). Results are presented as histograms. The fold change in mean fluorescence intensity (MFI) compared to the negative isotype control is indicated in the right upper corner (if applicable).

In some experiments, MSCs were stimulated with 25ng/ml of recombinant human interferon-γ (IFNγ; PeproTech, Rocky Hill, NJ) or ECs were stimulated using 100ng/ml recombinant human tumor necrosis factor-α (TNFα; PeproTech). Each reagent was added to the cell culture medium 48 hours before flow cytometry.

Cell proliferation assay

A total of 4×10^3 cells/ well in 100µl medium were seeded on a gelatin-coated 96-well flat-bottom plate (Nunc, Roskilde, Denmark) and incubated at 37°C in 5% humidified CO_2. Medium was changed every other day and cell counts were quantified daily for up to 5 days using the MTT assay (CellTiter 96 Aqueous One Solution Cell Proliferation Assay, Promega, Madison, WI). Absorbance was measured at 492nm wavelength with a Magellan ELISA Reader (Tecan, San Jose, CA).

To visualize differences in cell proliferation between iMSCs and aMSCs, live cell imaging microscopy was performed for 48h. A total of 1×10^5 cells/well were plated onto 6-well plates. Using Improvision Spinning Disc (Perkin Elmer, Waltham, MA) 100x magnification pictures were taken at 10 min intervals.

Cell migration assay

The migration of MSCs was assessed by the wound closure assay. 1×10^5 cells/well were plated onto 0.1% gelatin-coated 12-well plates. When cells reached approx. 100% confluency, a scratch was made into the cell monolayer using a pipette tip. After 14h, cells were fixed with 10% paraformaldehyde (PFA). Phase contrast images (magnification 100x) were acquired using a Leica Microscope with QWin acquisition software (Leica, Wetzlar, Germany). Cells migrated into 300µm x 500µm scratch areas were counted. Ten areas were counted per experiment.

MSC *in vitro* adhesion assay

Human endothelial cells (ECs) isolated from the umbilical vein (PromoCell) were cultured in their cell-specific media (PromoCell) and used for experiments between passage 3 and 8. 5×10^5 To study MSC adhesion to ECs, ECs were seeded in 24-well plates. After 48h, the EC-monolayer was stimulated with 20ng/ml TNFα for 6h. 2×10^5

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PKH26-labeled MSCs (PKH26 Red Fluorescent Cell Linker Kit, Sigma-Aldrich, St. Louis, MO) were added on top of the EC monolayer. The plate was placed on a shaker for 15 min. Non-adherent MSCs were removed and plates were examined by phase-contrast and fluorescence microscopy (21).

Unidirectional ELISPOT assays

For immunization, a total of $1x10^6$ MSCs were injected into the gastrocnemius muscle of BALB/c mice. Spleens were harvested after 5 days and $1x10^6$ splenocytes were used as responder cells with $1x10^5$ mitomycinC-inhibited MSCs as stimulators. Unidirectional ELISPOT assays were performed according to the manufacturer's protocol (BD Biosciences) using IFNγ and interleukin-4 (IL-4)-coated plates. Spots were automatically enumerated using an ELISPOT plate reader (CTL, Cleveland, OH) for scanning and analyzing.

Donor-specific IgM antibodies

Serum was recovered from immunized animals on day 5, decomplemented for 30 min at 56°C, and incubated with $1.5x10^6$ donor MSCs. Cells were labeled using FITC-conjugated anti-mouse IgM (Sigma) and measured using a FACSCalibur flow cytometer. The results are presented as MFI values from n=4 (iMSC) and n=8 (aMSC) independent measurements.

Indolamine 2,3-dioxygenase (IDO) detection

MSCs were left untreated or stimulated with 500ng/ml recombinant human IFNγ (PeproTech) for 48 hours to upregulate IDO. Cellular proteins were extracted using RIPA buffer supplemented with protease inhibitor and phosphatase inhibitor cocktail (Sigma-Aldrich). Protein concentrations were quantified with the Pierce BCA protein assay kit (Thermo Scientific, Rockford, IL). Protein samples (10µg) were separated on a NuPAGE Novex 4-12% Bis-Tris gel (Invitrogen, Carlsberg, CA) and transferred onto a 0.2µm PVDF membrane (Invitrogen). IDO (Atlas Antibodies AB, Stockholm, Sweden) and actin (Abcam) were detected using monoclonal antibodies and the ECL Plus Western Blotting Detection Kit (Amersham Biosciences) with the IVIS 200 system (Xenogen, Alameda, CA).

Tube formation assay

ECs were labeled in green using CellTrace CFSE Cell Proliferation Kit (Molecular Probes, Eugene, OR) and MSCs were stained red using PKH26 Red Fluorescent Cell linker Kit (Sigma), according to the manufacturers` guidelines. $1.5x10^4$ CSFE-labeled ECs and $1.5x10^4$ PKH26-labeled MSCs were added into a matrigel-coated 24-well flat-bottom plate (10mg/ml; BD Biosciences). Cells were incubated for 24h at 37°C, 5% CO_2 atmosphere. Nuclei were stained blue using Hoechst 33342 dye (Invitrogen, Eugene, OR) at a concentration of 10ng/ml for 10 minutes. In each well one photo per quadrant was taken using a Leica fluorescence microscope (Leica DMIRE2; Leica Microsystems) at 300x magnification. For analysis branch-points and number of tubes per high power field (HPF) were determined and tube length in µm was measured in 18 wells per group (4 HPFs/well) using Leica QWin software.

Chorio-allantoic membrane (CAM) assay

Fertilized chicken eggs were incubated at 37°C 60% humidity for four days. The eggshell was then carefully fenestrated, and a sponge was placed onto the inner membrane and loaded with 10µl of conditioned medium generated from MSCs. For the preparation of conditioned media, MSCs were cultured in a 96-well plate at a density of $2x10^4$ cells/well for 48h with 100µl DMEM (Gibco) supplemented with 20% FCS, 1% P/S and 1% Glutamine (all Gibco). On day six, the sponge was loaded with 10µl of conditioned medium for the second time and on day eight, the chorio-

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allantoic membrane was harvested for microscopy. For analysis, the number of branch-points per HPF in the area of the sponge was determined at a 16x magnification.

Human angiogenesis membrane array

An antibody array for human angiogenesis factors was performed (Human Angiogenesis Antibody Array C Series 1000; Ray Biotech, Norcross, GA) according to the manufacturer's protocol using 1000µl of undiluted conditioned MSC media. For generation of conditioned media, MSCs were placed in a 10cm cell culture dish at 80% confluency and incubated with 8ml media (as described above). After 48h, media were obtained, centrifuged at 2000rpm for 5min, and the supernatant was stored at -20°C. The array was analyzed using Bioluminescence Imaging (IVIS 2000, Xenogen, CA). All experiments were carried out in quadruplicates.

Statistical analysis

The SPSS statistical software package 15.0 for Windows (SPSS, Chicago, IL) was used. Results are presented as mean±standard deviation. Analysis for statistical significance was performed using the Student t-test or one-way analysis of variance (ANOVA) with LSD post-Hoc test, as appropriate. Probability values (p) of less than 0.05 were considered significant.

3. RESULTS

Phenotypic and functional characterization of MSCs

In a head-to-head comparison, iMSCs and aMSCs were characterized and compared. Both MSC types were plastic adherent and exhibited fibroblast-like spindle shape morphology (Figure 1A). To confirm the multi-lineage differentiation potential of both MSC types, cells were differentiated into osteogenic, adipogenic, and chondrogenic lineages (Figure 1B-D). Differentiation was reliably achieved in both MSC types. No differences were noted between both MSC types regarding the time required for cell differentiation.

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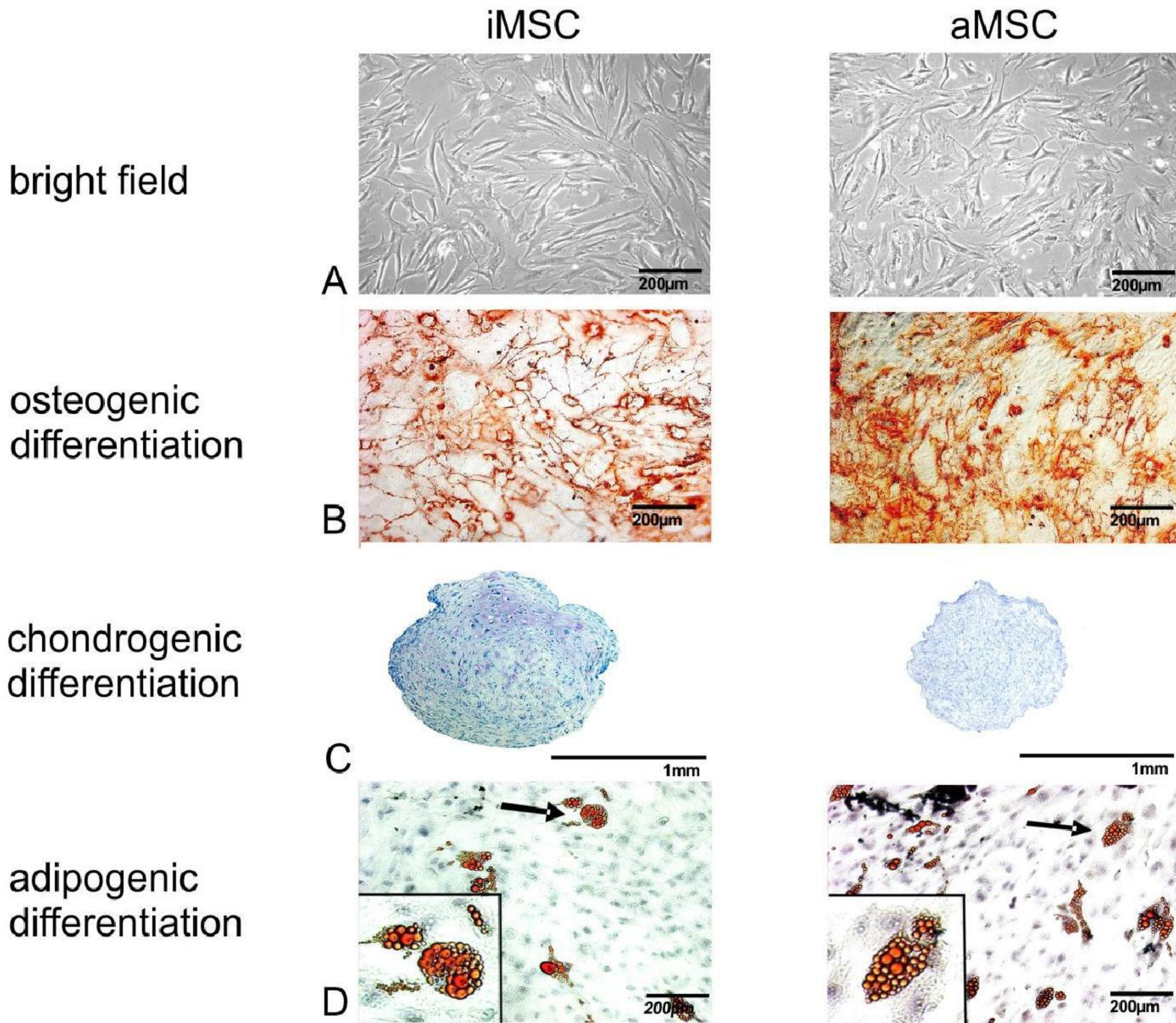

Figure 1. Bone Marrow derived MSC morphology and differentiation potential.
Phase- contrast images show both iMSCs and aMSCs are spindle shape and adhere to plastic. Scale bar 200 µm **(A)**. Both cell types were differentiated to into osteogenic **(B)**, chondrogenic **(C)**, and adipogenic **(D)** lineages.

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Surface expression of stem cell antigens and adhesion molecules was assessed by flow cytometry. Both iMSCs and aMSCs were positive for the mesenchymal stem cell markers CD90 and CD105 and negative for the hematopoietic stem cell markers CD34 and CD45. iMSCs and aMSCs were negative for the pluripotency marker CD117 and negative for endothelial CD31 (Figure 2).

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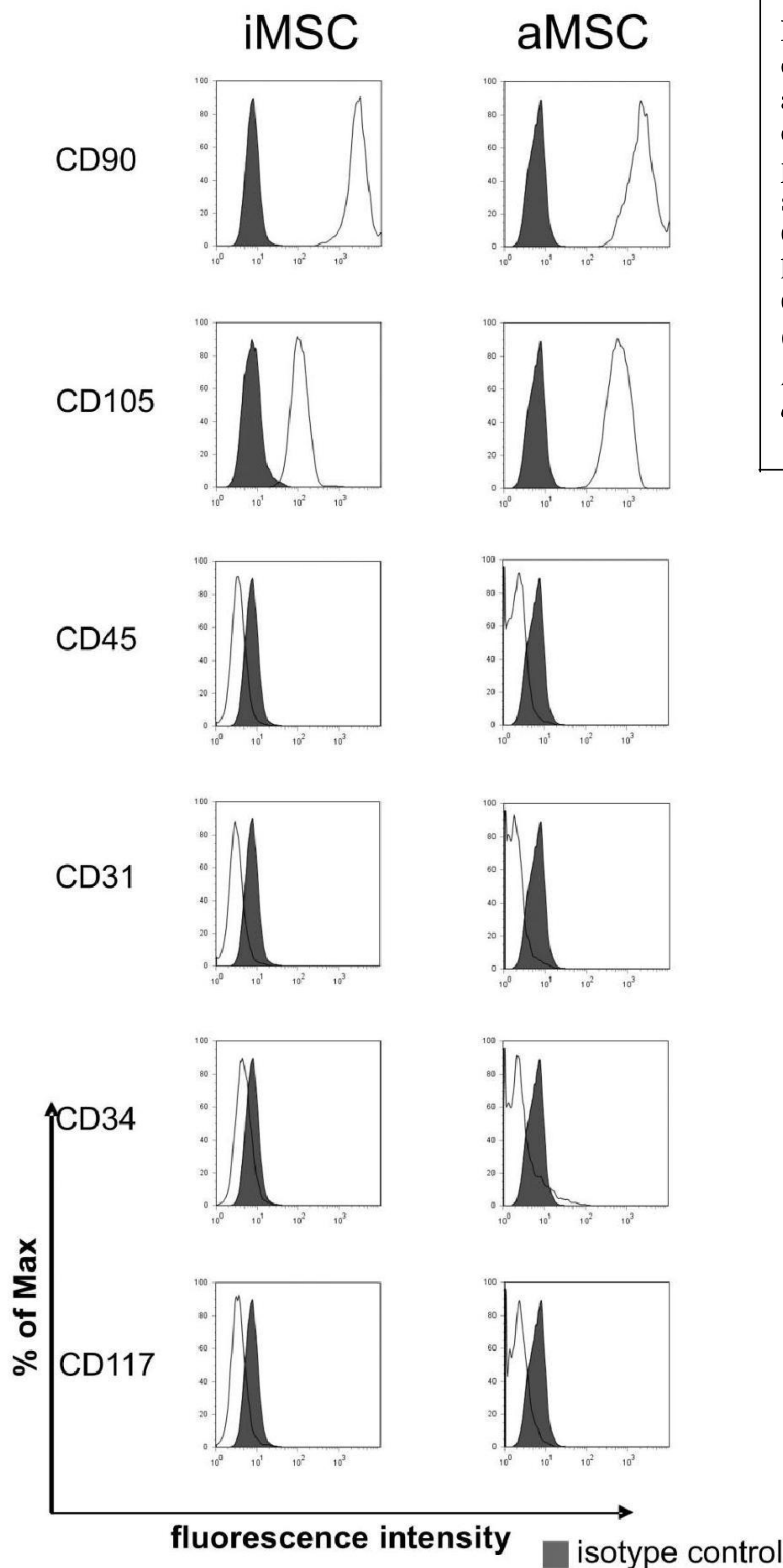

Figure 2. Functional characterization of both iMSC and aMSC cell types using flow cytometry. Both types were positive for mesenchymal cell surface markers CD90, CD105, and CD117, and negative for hematopoietic stem cell markers CD45, CD34, CD31, and CD117 (c-kit).

Isotype control: gray solid area, *antigens of interest:* solid line.

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Immunophenotype of iMSCs and aMSCs
The immunophenotype of iMSCs and aMSCs was analyzed by flow cytometry (Figure 3).

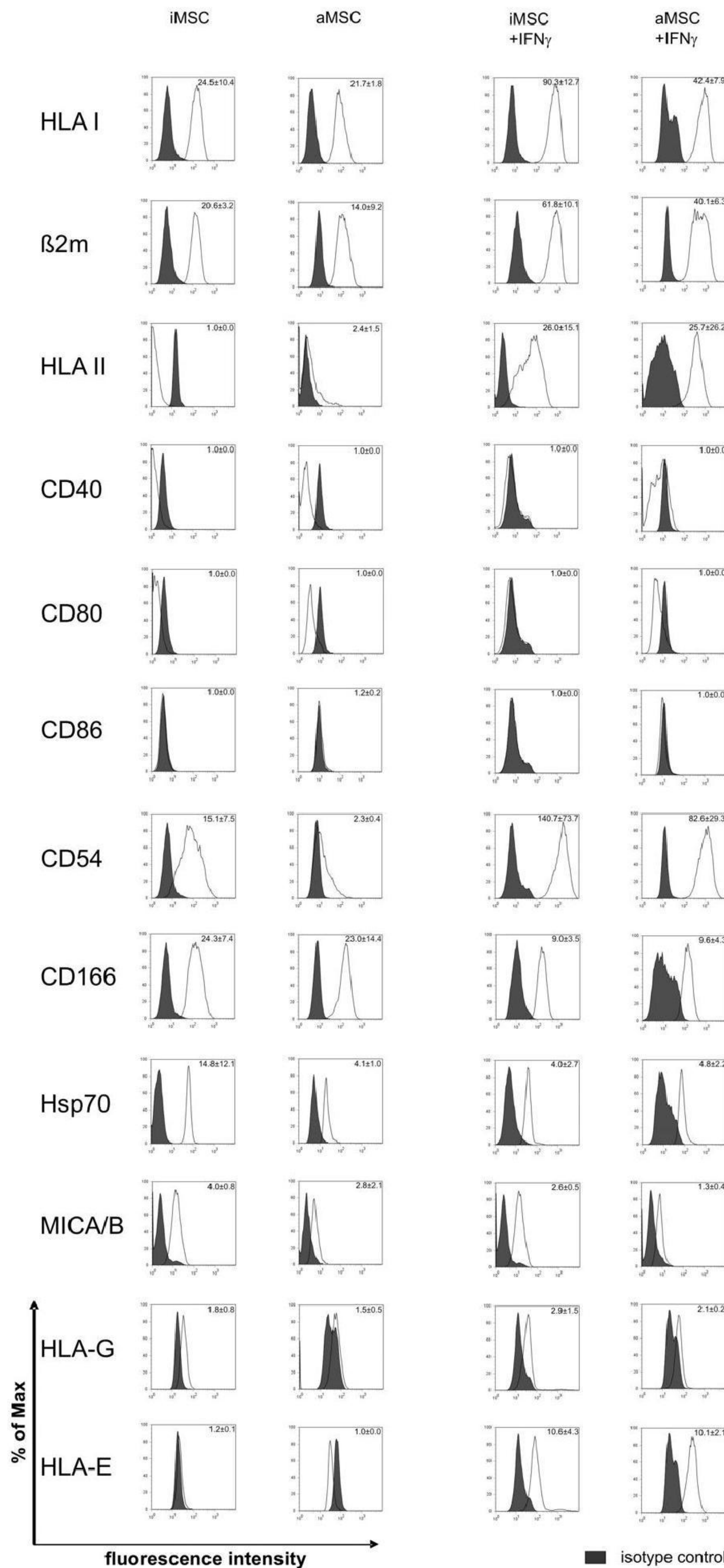

Figure 3. Antigenic, costimulatory, and tolerogenic immunophenotypes of iMSCs and aMSCs were assessed using flow cytometry pre and post stimulation with IFN-γ at 25 ng/ml. MSCs showed expression of β2-microglobulin and HLA class I. Both iMSCs and aMSCs were negative for HLA class II, and co-stimulatory molecules expression of CD40, CD80, or CD86. Both MSC types expressed the adhesion molecules CD54 and CD166. We detected only mild expression of the tolerogenic marker HLA-G on resting iMSCs or aMSCs. Post stimulation with IFN-γ for 48h both MSC types up-regulated their HLA class II and CD54 expression as well as the tolerogenic HLA-E was also markedly up-regulated. Isotype control: gray solid area, antigens of interest: solid line. The fold change in MFI compared to the negative isotype control is indicated in the right upper corner of each histogram (n=3 independent experiments).

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Both iMSCs and aMSCs were positive for HLA class I- and β2-microglobulin molecules and completely negative for HLA class II. The analysis for co-stimulatory molecules showed that they did not express CD40, CD80, or CD86. Both MSC types expressed the adhesion molecules CD54 and CD166, belonging to the integrin family and being implicated in cell transmigration. Ligands for NK cell activation (Hsp70, MICA/B) were detectable at moderate levels on iMSCs and aMSCs. We did detect only moderate expression of the tolerogenic markers HLA-G and HLA-E on resting iMSCs or aMSCs.

The immunophenotype was altered when MSCs were exposed to 25ng/ml of recombinant human IFNγ. Both MSC types largely up-regulated their HLA class II expression as well as CD54. On the other side, the tolerogenic HLA-E was also markedly up-regulated.

Thus, we show that iMSCs and aMSCs exhibited a similar immunophenotype that was affected by an inflammatory environment. Because immunogenic antigens and tolerogenic molecules were both up-regulated by IFNγ, their net effect required further immune activation assays.

Immunogenicity of MSCs

Five days after immunization, ELISPOT assays were carried out with Balb/C responder splenocyte and MSC stimulators. Both MSC types triggered a low-level cellular immune activation. Spot frequencies for IFNγ (Figure 4A) and IL-4 (Figure 4B) were similar for iMSCs and aMSCs. Accordingly, donor-specific IgM antibodies were detectable at similarly low levels (Figure 4C). Together, the data show that MSCs themselves are immunogenic and induce cellular and humoral immune responses.

On the other hand, it has previously been shown that MSCs have immunomodulatory effects and that IDO plays an important role in T cell inhibition by activated MSCs (22). MSCs were stimulated with 500ng/ml recombinant human IFNγ for 48 h to induce IDO upregulation. Both activated MSC types showed similar levels of IDO release (Figure 4D), suggesting similar immunomodulatory properties.

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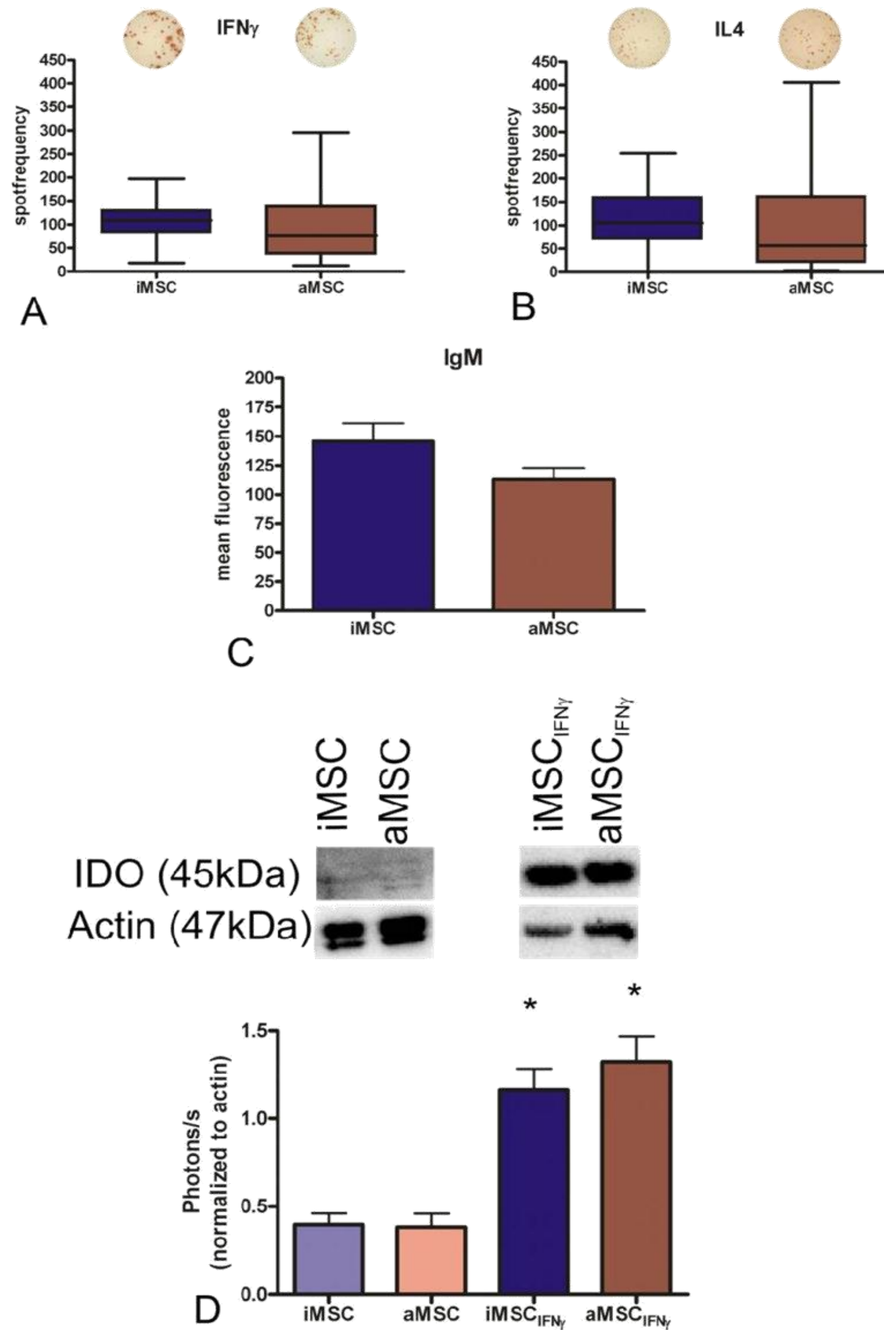

Figure 4. Immunogenicity of iMSCs and aMSCs. Five days after iMSC or aMSCs transplantation into Balb/ mice, Elispot assays for IFNγ (Th-1 response) **(A)** and IL-4 (Th-2 response) **(B)** demonstrated that both MSC types triggered a low level cellular immune activation (n=6 animals using iMSCs and 12 animals using aMSCs). Donor-specific IgM antibodies were detectable at similarly low levels **(C)** 5 days post cell transplantation (n=4 animals using iMSCs and 8 animals using aMSCs). Both MSCs types were stimulated with 500ng/ml recombinant human IFNγ for 48h to induce

Indolamine 2,3-dioxygenase (IDO) upregulation. Activated iMSC and aMSCs showed similar levels of significant increase of IDO compared to unstimulated cells ($p<0.001$ unstimulated versus stimulated iMSCs and aMSCs), suggesting similar immunomodulatory properties (n=4 independent experiments for iMSCs; n=3 independent experiments for aMSCs).

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Proliferation and migration of MSCs

iMSCs showed a significantly higher proliferation rate than aMSCs ($P<0.001$, Figure 5A). Similarly, iMSCs showed faster migration into a culture dish scratch area ($P<0.001$, Figure 5B) and achieved quicker wound closure

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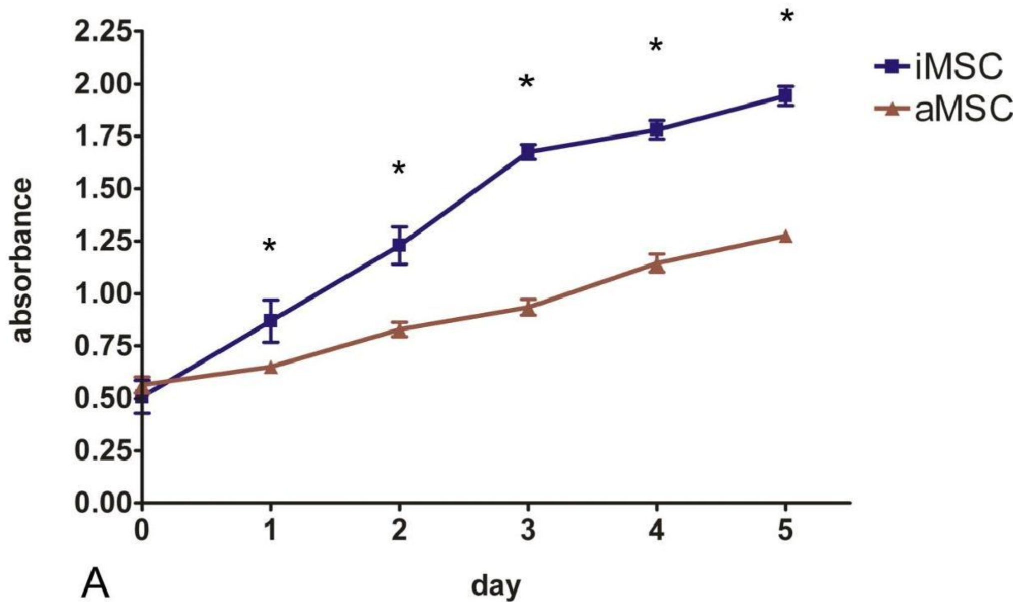

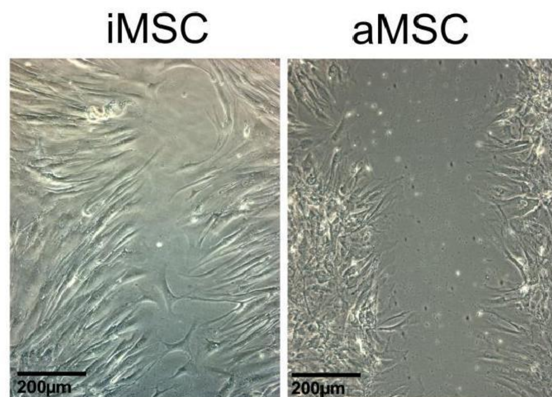

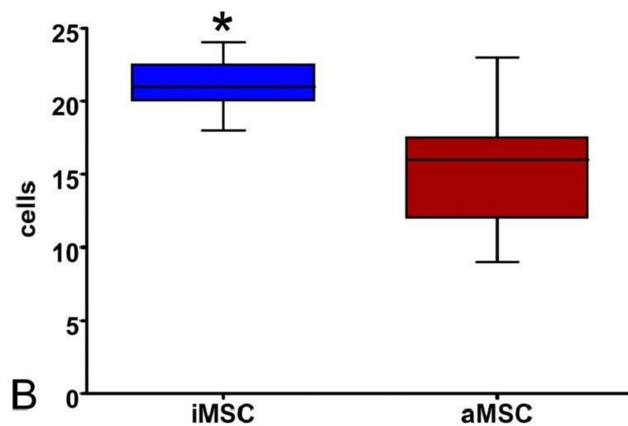

Figure 5. Differences in proliferation and migration potential of iMSCs compared to aMSCs. iMSCs showed a significantly higher proliferation rate than aMSCs ($P<0.001$) **(A)**. Graphs present the mean optical density (OD) at 492nm. Phase-contrast images of both types show the scratch area at 14h after initiation of the assay **(B)**. Each analysis was performed in quadruples using three independent biological samples per group (*$P<0.001$).

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Adhesion of MSCs to stimulated ECs

Cell-cell interaction via CD29 on MSCs and CD106 on ECs is important for MSC adhesion. When comparing MSC types, iMSCs showed significantly higher CD29 signals than aMSCs (Figure 6A). The counterpart of CD29, the integrin receptor CD106 could be upregulated on ECs by TNFα stimulation (Figure 6B). When MSCs were dropped onto EC monolayers placed on a shaker, the number of adhering iMSCs was significantly greater than for aMSCs ($p=0.018$, Figure 6C,D). Thus, increased adhesion of iMSCs to stimulated ECs might be caused by their increased expression of CD29.

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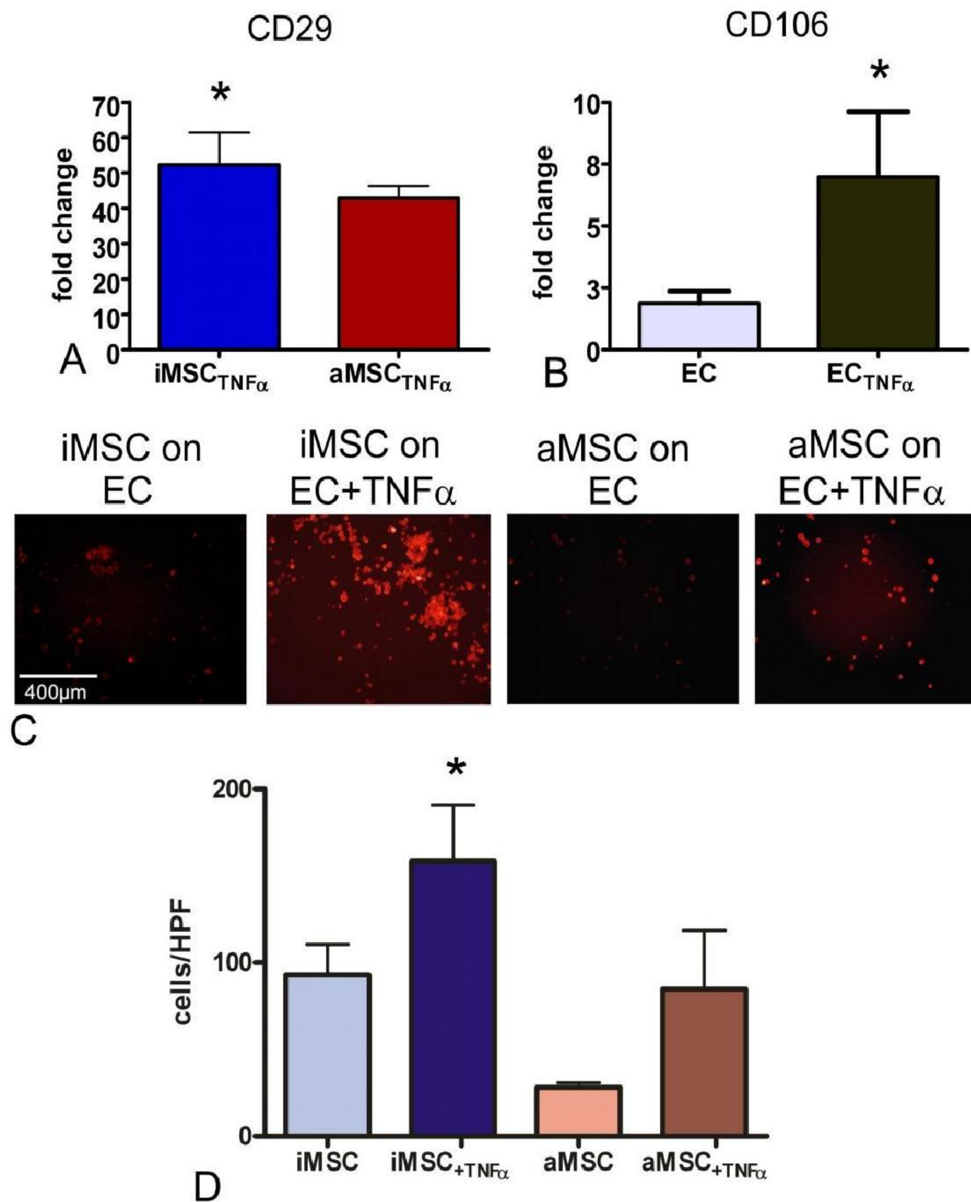

Figure 6. Adhesion of MSCs. CD29 expression on iMSCs was significantly higher than on aMSCs (P=0.02; n=4 independent experiments per group) **(A)**. The counterpart ligand of CD29, the integrin receptor CD106 is highly expressed on endothelial cells and was highly upregulated

6h post TNFα stimulation (P=0.01; n=4 independent experiments per group **(B)**. Fluorescence microscopic images of PKH labeled iMSCs and aMSCs adhered on unstimulated and TNFα-stimulated endothelial cells **(C)**. As expected from the flow cytometry data (A) and (B), the number of adhering iMSCs to stimulated ECs was significantly greater than for iMSCs to unstimulated ECs (P=0.017), as well as for aMSCs (P<0.001) **(D)**.

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Tube formation assay

To investigate whether there is any difference of the angiogenic potential in iMSCs and aMSCs *in vitro,* tube formation assays were performed (Figure 7). Figure 7A shows the capillary tube formation after 24h in culture. Tube formation by ECs+iMSCs was significantly higher compared to ECs+aMSCs (13±5 tubes versus 10±4, respectively (($P<0.001$), Figure 7B). Also, ECs+iMSCs showed increased branch point formation with 13±4 compared to ECs+aMSCs 10±4 ($P<0.001$).

CAM Assay

As shown in Figure 7C, the angiogenic potential of conditioned medium from iMSCs was significantly higher than that of aMSCs (62±7 branch points compared to 30±4, respectively; $P<0.001$).

Human angiogenesis membrane array

Human Membrane Arrays were used to identify relevant angiogenic factors released by both iMSCs and aMSCs. Out of 20 angiogenic factors contained in the assay kit, six angiogenic factors were significantly stronger secreted by iMSCs than aMSCs (Figure 7D).

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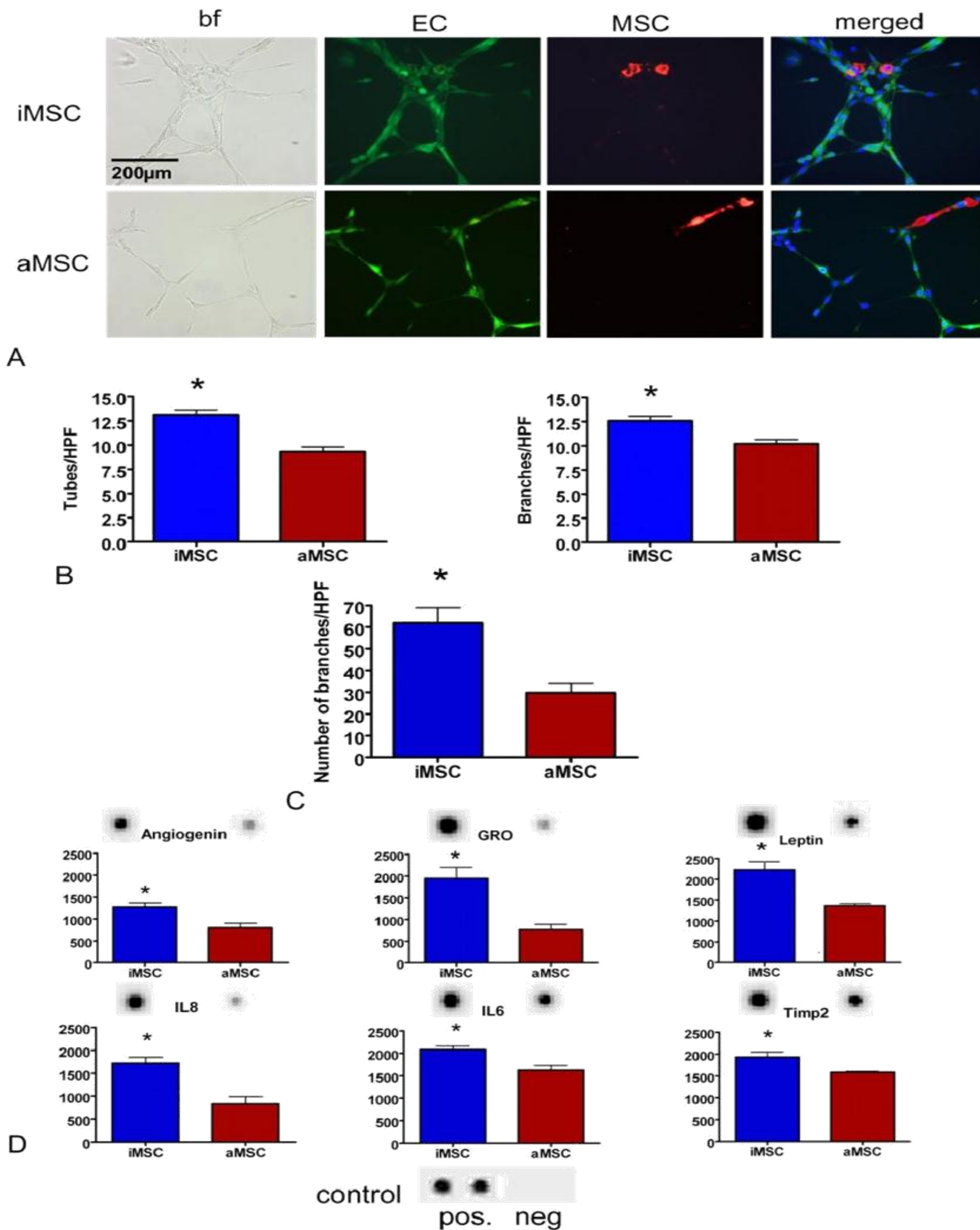

Figure 7. Angiogenic behavior of MSCs. ECs in co-culture with iMSCs showed significantly higher tube formatio capacity (P<0.001) as well as branching tendency (P<0.001) when compared to aMSCs **(A).** ECs were labeled green with CSFE, MSCs were labeled red with PKH26 and nuclei were stained blue using Hoechst for fluorescence microscopy (**B**). Representative pictures revealed that MSCs are not only stimulating EC-tube formation, but being also part of the tubes themselves. In the CAM assays, areas treated with iMSC-conditioned media showed significantly stronger branching than those treated with media obtained from aMSCs (P=0.0005) **(C).** Human angiogenesis antibody arrays revealed significantly higher secretion of angiogenin, GRO, leptin, IL8, IL6, and Timp2 by iMSC compared to aMSC (*P<0.05) **(D)**.

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4. DISCUSSION

The ability of human organs to regenerate fades over the years. There is a notion that this is because the functional regenerative properties of multipotent stem cells decrease with age. MSCs have been shown previously to participate in tissue repair and regeneration. Furthermore, MSCs have been shown to exert immunomodulatory function in immune conditions such as graft vs. host and autoimmune diseases (23). MSCs have therefore been attributed to counteract the progression of various diseases. Over the last five years, more than 200 clinical trials investigated the use of both autologous and allogeneic MSCs for a wide variety of diseases (24-26). Even though early results of preclinical studies and phase I & II clinical trials have been promising, most observed improvements have only been minor or transient. Some scientists believe that the beneficial paracrine or endocrine MSC effects (27) may be too limited to warrant their clinical use (23, 28), especially if used from elderly donors.

In our head-to-head comparison, both iMSCs and aMSCs showed the same phenotype with typical spindle-shape morphology, plastic adherence, and were similarly expressing MSC-characteristic surface markers that have been described in the literature (29). iMSCs and aMSCs showed an ability to differentiate into osteogenic, chondrogenic, and adipogenic lineages, thus meeting the MSC criteria previously defined by other researches in the field (30).

MSCs express adhesion molecules and our results show that iMSCs have a stronger expression of CD29 than aMSCs. CD29 is crucial for EC adhesion via cell adhesion molecule-1 CD106 (31). It was previously demonstrated that adhesion of circulating MSCs to injured areas is an endothelium-dependent process (32). Also, we show that the adherence of iMSCs to EC monolayers was superior to that of aMSCs, suggesting better cell-cell interaction and tissue invasion by iMSCs. *In vitro* assays demonstrated that iMSCs have a significantly higher proliferation rate as well as faster migration and invasion. In tube formation assays with co-cultured ECs, iMSCs showed a significant increase in several angiogenesis parameters. Other groups have also shown that young MSCs (33) outperformed old MSCs (34, 35) in various cell biology assays. In total, these data support the hypothesis that autologous aMSCs might have a limited therapeutic potential related to their impaired biological function (36).

Based on our flow cytometry results, both cell types showed expression of β2-microglobulin and HLA class I, while being completely negative for HLA class II. This low immunogenicity may explain their reduced immune recognition and delayed immune rejection previously reported (20). In addition, we found that both cell types did not express the co-stimulatory molecules CD40, CD80, and CD86. Exposure with the inflammatory cytokine IFNγ caused an up-regulation not only of HLA class II, but also of the tolerogenic HLA-E and IDO. This explains some of the MSC-typical immunomodulatory properties (20) since tryptophan which is an essential amino acid is crucial for T-cell proliferation and activation, and IDO catabolizes tryptophan (37). Therefore increased IDO expression thereby reduces T cell responsiveness. Furthermore, MSCs have been reported to inhibit dendritic cell maturation, B and T cell proliferation and differentiation, and to attenuate NK cell killing (38).

However, if not used autologously, our results show that both MSC types similarly trigger cellular immune activation, although at a relatively low level. The concomitant donor-specific IgM response was detectable

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at similar levels. Thus, despite their immunomodulatory function, we show that both MSCs types are immunogenic and induce cellular and humoral activation if used across HLA borders. Recently it has been shown that allogeneic MSCs response to local signals by releasing suppressive effects on innate and adaptive immunity. Although the use of allogeneic MSCs does provoke an immune response, MSC transplantation are slower rejected than other allogeneic cell types. To confirm the immunogenicity of allogeneic MSCs, Isakova *et al*, compared intracranial injections of allogeneic versus autologous MSCs in rhesus macaques. Animals receiving allogeneic MSCs rejected their cell transplants related to the degree of MHC class I and II mismatch between the donor and recipient (39). Furthermore, data from the POSEIDON clinical trial reported the generation of anti-donor antibodies in patients probably because MSCs retain some degree of immunogenicity that attenuate their beneficial effects and limits their survival (40-42). These data contradict previous assumptions that allogeneic MSC products could be used as an off the shelf treatment. It is therefore unlikely that allogeneic MSC use carries sustained potential for clinical tissue regeneration (43).

Overall, our results corroborate with those of other researchers studying age differences of MSCs which showed that adult MSCs showed a decrease ability to self-renew and properly differentiate (44). Conboy, et al had previously established a parabiotic pairing between young and old mice, that share circulatory system and came to the conclusion that the age related decline of progenitor cell activity is due to factors influence by age (45). Our experiments specified that iMSCs show better migration, proliferation and adhesion, although both cell types exhibit similar immunophenotypic and immunomodulatory characteristics. This might be why less than the expected functional outcomes were observed in most of the clinical trials using autologous MSCs from aged patients. Cell banking of umbilical cord blood iMSCs (46) might be an option to improve individual MSC-based treatment options. Another option may be the genetic modification and reprogramming of aMSCs to re-establish iMSC properties, as recently proposed (47).

5. CONCLUSION

MSCs are immunogenic and induce cellular and humoral activation if used across HLA borders, despite their immunomodulatory function. Although donor age does not affect immunogenicity and immunomodulatory properties of MSCs, aging reduces the biological properties of proliferation, migration, adhesion of MSCs and limits their angiogenic potential. Our results might explain why less than the expected functional outcomes were observed in most of the clinical trials using autologous MSCs from aged patients.

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DISCLOSURE
The authors have no conflict of interest.

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