ONLINE SUPPLEMENT

Mesenchymal stem cells improve murine acute Coxsackievirus B3-induced myocarditis

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Supplemental Materials and Methods

Mesenchymal stem cell isolation, characterization, and cell culture

Human adult MSCs were isolated from iliac crest bone marrow aspirates of normal male donors (n=6) after their written approval. The aspiration of iliac crest bone marrow was approved by the ethical committee of the Charité-Universitätsmedizin Berlin (EA1/131/07). Aspirates (3–5 ml) were washed twice with phosphate buffered saline (PBS) (Biochrom, Berlin, Germany), and resuspended in Dulbecco's Modified Eagle's Medium (DMEM; Biochrom) supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, 1% glutamine, 2% Hepes and 2 ng/ml of basic fibroblast growth factor (Tebu-bio, Offenbach, Germany). Cells were purified using a percoll gradient at a density of 1.073 g/ml (Biochrom). Next, cells were washed with PBS and then resuspended in complete DMEM. Cells were plated at a density of 3×10^5 cells/cm^2 and cultured under standard cell culture conditions. Medium was exchanged after 72 hours (h) and every 3 days thereafter. Reaching 90% confluence, cells were trypsinized and replated at a density of 5×10^3 cells/cm^2.

MSCs were characterized by flow cytometry analysis according to Binger *et al.*[1] with PE-labeled monoclonal mouse anti-human CD14, CD34, CD73, CD166 and FITC-labeled mouse anti-human CD44, CD45, CD90 and CD105 antibodies. Cells were washed with PBS-BSA 0.5%, resuspended in 100 µl of PBS-BSA 0.5% and incubated with titrated concentrations of antibodies at 4°C for 15 min. Prior to flow cytometry analysis, cells were washed with PBS-BSA 0.5%.

Coxsackievirus B3 timeframe experiment with mesenchymal stem cells

MSCs were plated into 6-well plates at a density of 200,000 cells/well. After 24 h of culture, MSCs were serum starved or infected with CVB3 under serum starvation conditions at a m.o.i. of 5 for 1 h. Then, cells were washed with PBS two times and complete DMEM medium was added. Phase contrast pictures at a magnification of 100x were taken 4 h, 12 h, 24 h, and 48 h after serum starvation or CVB3 infection with a Leica camera (Version Twain 7.0.0.0; Leica Microsystems, Wetzlar, Germany) connected with a Leica DMI 4000B

microscope (Leica Microsystems, Bensheim, Germany). At respective time-points, medium was collected for plaque assay and cells were collected in Trizol for RNA purposes.

MTS Viability Assay

10,000 MSCs were plated in each well of a 96-well plate. After 24 h of culture, cells were serum starved or incubated with CVB3 under serum starvation conditions at a m.o.i. of 5 for 1 h. Next, cells were 2 times washed in PBS (Biochrom) and 100 µl of medium was added. 4 h, 12 h, 24 h, and 48 h after serum starvation or CVB3 infection, 20 µl of the CellTiter 96® AQueous One Solution Reagent (Promega, Madison, USA) was directly added to the culture wells, and incubated for 2 h. The absorbance was recorded at 490 nm with a VersaMax microplate reader (Molecular Device GmbH, Munich, Germany).

Real-time PCR

To determine CVB3 copy number, quantitative real-time reverse transcriptase (RT)-PCR (ABI PRISM® 7900 HT Sequence Detection System software version 2.2.2., Perkin Elmer) was performed using absolute standard quantification. CVB3 was normalized towards human L32 in cDNA from MSCs. Following primer pairs were used: for CVB3: FOR 5´-CCCTGAATGCGGCTAATCC-3´ and REV 5´-ATTGTCACCATAAGC AGCCA-3´, for human L32: FOR 5´-AGGAGAGACACCGTCTGAACAAG-3´ and REV 5´-GAACCAGGATGGTCG CTTTC-3´. LV *tumor necrosis factor* (*TNF*)-*α* mRNA expression was normalized towards 18S. Murine TNF-α and 18S primers were bought from Applied Biosystems.

Co-culture of HL-1 with mesenchymal stem cells

Murine HL-1 cells were cultured in Claycomb medium (SAFC Biosciences, Kansas, USA) supplemented with 10% FBS, 1% penicillin/streptomycin, 100 µM norepinephrine (Sigma, Steinheim, Germany) and 2 mM glutamine. Unlabeled, DiO-labeled or DiI-labeled HL-1 were plated into 6-well plates at a density of 300,000 cells/well for RNA, Annexin V/7AAD or DCF flow cytometry, respectively. After 24 h of culture, HL-1 cells were serum starved or infected

with CVB3 under serum starvation conditions at a m.o.i. of 5 for 1 h. Then, cells were washed with PBS two times and complete Claycomb medium was added. Four hours after CVB3-infection or serum starvation, untreated MSCs or MSCs 24 h pre-treated with 10 mM of Nitro-L-Argininmethylesterhydrochloride (L-NAME; Sigma) were collected and added to the HL-1 cells for co-culture at a ratio of MSCs to HL-1 of 1 to 10, which is a commonly used ratio when MSCs are co-cultured in the presence of a target cell of interest[2,3]. To evaluate whether the MSCs require priming via IFN-γ, anti-mouse IFN-γ antibody (R&D systems, Minneapolis, USA) was added to the HL-1-MSC co-culture at a final concentration of 1 µg/ml at the moment of MSC supplemention. Supplementation of 1 µg/ml of anti-murine IFN-γ antibody to CVB3-infected HL-1 cells alone does not effect the condition of HL-1 cells (data not shown). Twenty-four hours after infection, phase contrast pictures were taken, supernatant was collected for plaque assay, NOx assay and IFN-γ ELISA, and cells were collected for RNA or flow cytometry purposes.

Annexin V/7AAD analysis

To be able to detect apoptosis specifically in HL-1 cells in the HL-1-MSC co-cultures, HL-1 cells were labeled with Vybrant® DiO Cell-labeling (Invitrogen, Heidelberg, Germany) before plating. Twenty-four h after CVB3 infection, cells were collected, fixated in paraformaldehyde and resuspended in PBS for flow cytometry analysis. Apoptosis was analyzed with the Annexin V-PE Apoptosis Detection Kit (BDSciences, Franklin Lakes, USA) by which Annexin V-positive and 7-Amino-Actinomycin (7-AAD) negative cells were considered as apoptotic cells. Annexin V/7AAD on DiO+ cells was analyzed by fluorescence-activated cell sorting (FACS) using a FACSScan flow cytometer and Cell Quest software (BD Biosciences, San Jose, CA, USA). Data are expressed as DiO+/Annexin V+/7AAD- cells (% gated).

Caspase 3/7 activity assay

10,000 HL-1 were plated in each well of a 96-well plate. After 24 h of culture, cells were serum starved or incubated with CVB3 at a m.o.i. of 5 for 1 h. Next, cells were 2 times

washed in PBS (Biochrom) and 100 µl of medium was added. Four hours after serum starvation or CVB3-infection under serum starvation conditions, untreated MSCs or MSCs 24 h pre-treated with 10 mM of L-NAME were collected and added to the HL-1 cells for co-culture at a ratio of MSCs to HL-1 of 1 to 10. To evaluate whether the MSCs require priming via IFN-γ, anti-mouse IFN-γ antibody was added to the HL-1-MSC co-culture at a final concentration of 1 µg/ml at the moment of MSC supplemention. Twenty-four hours after infection or serum starvation, caspase 3/7 activity was determined with a Caspase Glo 3/7 activity kit (Promega) according to the manufacturer´s protocol. Luminescence was measured with a luminometer (Berthold Technologies, LB 940 Multimode Reader Mithras, Bad Wildbad, Germany).

Reactive Oxygen Species analysis

Oxidative stress in HL-1 cells was determined via analysis of reactive oxygen species (ROS) by 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acety ester (CM-H_2DCFDA) (Invitrogen) flow cytometry. To be able to detect ROS specifically in HL-1 cells in the HL-1-MSC co-cultures, HL-1 cells were labeled with Vybrant® DiI Cell-labeling (Invitrogen) before plating. Twenty-four h after CVB3 infection, cells were collected, fixated in paraformaldehyde and resuspended in PBS for flow cytometry analysis. CM-H_2DCFDA on DiI+ cells was analyzed by FACS using a FACSScan flow cytometer and Cell Quest software (BD Biosciences). Data are expressed as DiI+/DCF+ cells (% gated).

Coxsackievirus B3 plaque assay

HeLa cells (DSMZ, Braunschweig, Germany) were plated in 6-well plates and cultured in RPMI 1640 (Invitrogen), supplemented with 10 % FBS and 1 % penicillin/streptomycin. To determine virion progeny release, titration dilutions of supernatant/medium from CVB3-infected MSCs and from co-cultures of CVB3-infected HL-1 cells with MSCs in the presence or absence of murine anti-IFN-γ, or with MSCs pre-treated with L-NAME, were prepared. To analyze intracellular viral particles[4], HL-1 cells, or HL-1 cells co-cultured with MSCs in the

presence or absence of murine anti-IFN-γ, or with MSCs pre-treated with L-NAME, were trypsinized 8 h after CVB3 infection. Next, cell pellets were resuspended in complete Claycomb medium. Then, cells were lysed by four freeze-thaw cycles by which each cycle consists of freezing the cells on dry ice and thawing them in a 37°C waterbath. Subsequently, cells were centrifuged for 5 min at 4,000 rpm and the supernatant were collected. For plaque assay, titration dilutions of the obtained supernatants were prepared.

After 24 h culture of HeLa cells, i.e. at 80% confluence, medium was removed and cells were washed with PBS. Next, 1 ml of the dilutions were added to each well and incubated for 30 min at 37°C. Then, 2 ml agar, consisting of an equal volume of MEM 2x, supplemented with 4% FBS, and of 1.3% noble agar, was added to each well. The plates were left under the hood for 15-20 min until the agar coagulated, and then kept in the incubator for 72 h. Finally, virus plaques were counted in n=6 wells/condition.

Evaluation of the difference between mesenchymal stem cells exposed and unexposed to Coxsackievirus B3

To evaluate whether MSCs exposed for 1 h with CVB3 change their functionality *in vitro* compared to MSCs unexposed to CVB3, 300,000 MSCs were plated for 24 h. Then, MSCs were serum starved for 1 h or infected with CVB3 at a m.o.i. of 5 for 1 h under serum starvation conditions. After washing with PBS, MSCs were further cultured for 24 h, and then supplemented to DiO- or DiI-labeled HL-1 cells, which were 4 h before infected with CVB3 at a m.o.i. of 5 for 1 h under serum starvation conditions, or serum starved for 1 h. Twenty-four h after CVB3-infection or serum starvation of DiO- or DiI-labeled HL-1 cells, medium was collected for plaque assay, and the effect of MSC supplementation on CVB3-induced apoptosis, oxidative stress and viral progeny release was determined, respectively.

Nitric oxide x analysis

NOx levels in medium were determined with a commercial NO kit according to the manufacturer´s protocol (Calbiochem, Darmstadt, Germany). Fluorescence was measured

with a fluorometer (Berthold Technologies, LB 940 Multimode Reader Mithras, Bad Wildbad, Germany) at excitation and emission wavelengths of 365 nm and 450 nm, respectively.

Quantification of murine interferon-γ levels

Murine IFN-γ levels present in medium of HL-1 cells, HL-1-MSC co-cultures, MNCs, and MNC-MSC co-cultures were determined with a commercial ELISA kit (R&D Systems, Minneapolis, USA), according to the manufacturer´s protocol.

Supplementation of murine interferon-γ on mesenchymal stem cells

10,000 MSCs were plated in each well of a 96-well plate. After 24 h of culture, cells were serum starved or incubated with CVB3 under serum starvation conditions at a m.o.i. of 5 for 1 h. Next, cells were 2 times washed in PBS (Biochrom) and 100 µl of medium was added. Four h, later, murine IFN-γ was added at a final concentration of 4 pg/ml, corresponding to the concentration of murine IFN-γ present in medium of HL-1 cells and HL-1-MSC co-cultures (see Supplemental Figure IIIB). Twenty-four h after CVB3 infection, medium was collected for NOx analysis.

Animals

To study the effect of MSC application on the progression of CVB3-induced myocarditis, 10^6 human MSCs or PBS was i.v. injected[5] in 6- to 8-week-old C57BL/6 mice, one day after i.p. infection with 5 x 10^5 plaque-forming units (p.f.u.) of CVB3 (Nancy strain) (CVB3-MSCs versus CVB3-PBS mice, respectively). Controls received PBS instead of CVB3. MSCs were administered one day post CVB3-infection since at this time-point CVB3 can already be retrieved in the spleen, pancreas, heart and blood[6], and virus-induced signalling which are important mediators of host susceptibility to CVB3, are already activated in the heart[7]. Furthermore, this time-point has been repeatedly used as starting point of pharmacologival treatment in murine acute CVB3-induced myocarditis[8,9]. Seven days after CVB3 infection, contractility parameters were analyzed as described previously[10], followed by harvesting of

the left ventricle, which was next snap-frozen for molecular biology purposes, and immunohistochemistry. The heart and spleen were isolated for mononuclear cell (MNC) isolation. The investigation was performed in accordance with the principles of laboratory animal care and the German law on animal protection.

Histology and TUNEL Staining

Hematoxylin and eosin staining was performed on 5 µm thick cryosections. Apoptotic cells were detected on 5 µm thick cryosections by end labeling the fragmented DNA using the DeadEnd Colorimetic TUNEL System (Promega) according to the manufacturer's instructions. Analysis of TUNEL positive cardiac cells (TUNEL/mm^2) was made in a blinded manner by digital image analysis on a Leica DMRB microscope (Leica Microsystems, Wetzlar, Germay) at 200x magnification.

(Cardiac) mononuclear cell, CD4-, and CD8-T cell proliferation

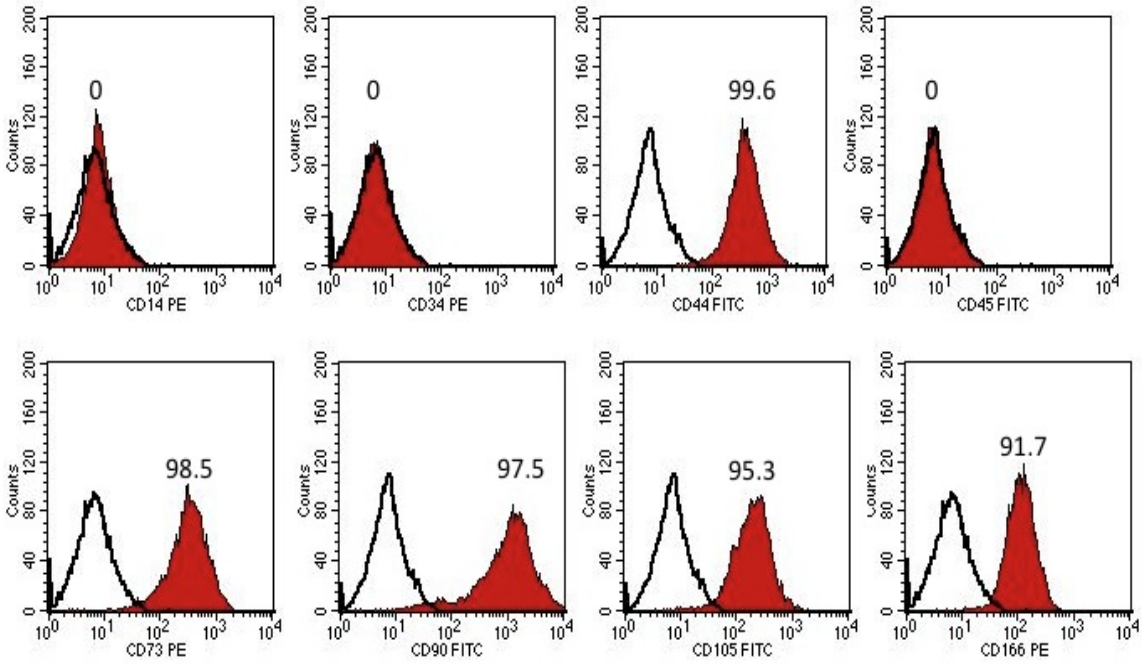
Cardiac MNCs were isolated from control-PBS, control-MSC, CVB3-PBS and CVB3-MSC mice, 7 days post-infection via trypsinization of heart pieces over night, followed by enzymatic digestion with collagenase type 2 (0.25 mg/ml; Sigma, Steinheim, Germany) and separation by density gradient sedimentation using Histopaque (Sigma). A minimum of n=8 hearts/group were pooled to perform the experiment. Cardiac MNCs were labeled with 10 µM of succinimidyl ester of carboxyfluorescein diacetate (CFSE Cell Tract^{TM}; Invitrogen, Carlbard, CA, USA) to be able to measure cell proliferation, indicative for MNC activation. Heat-inactivated CVB3[1] was used to stimulate cardiac MNCs *in vitro*. Cardiac MNCs were cultured in RPMI1640 medium (Invitrogen, Heidelberg, Germany), supplemented with 10% FBS and 1% penicillin/streptomycin for 72, followed by flow cytometry on a Becton Dickinson FACS Calibur and sample analysis with FlowJo 8.7. software (Tree Star, Ashland, OR, USA). To investigate how MSCs reduce MNC (CD4- and CD8-T cell) proliferation, splenocytes were isolated from control and CVB3-infected C57BL/6 mice according to De Geest *et al.*[11]. Next, MNCs were carboxyfluorescein succinimidyl ester-labeled and directly co-cultured, in

the presence or absence of inactivated CVB3, with or without MSCs (untreated or 24 h pre-treated with L-NAME) at a ratio of 10:1 in RPMI1640, 10% FBS, 1% penicillin/streptomycin, in the presence or absence of 1 μg/ml of anti-murine IFN-γ antibody for 72 h. Supplementation of 1 μg/ml of anti-murine IFN-γ antibody to stimulated splenocytes alone did not affect their proliferation (data not shown). Medium was collected for analysis of NOx and IFN-γ levels. Then, cells were stained with monoclonal anti-CD4 or anti-CD8 antibodies (BD Biosciences, Franklin Lakes, NJ, USA), followed by flow cytometry on a MACSQuant Analyzer (Miltenyi Biotec, Bergisch Gladbach, Germany) and analysis with FlowJo 8.7. software (Tree Star). The division index, the average number of cell divisions that the responding cells undergo. (i.e., ignores peak 0), was calculated.

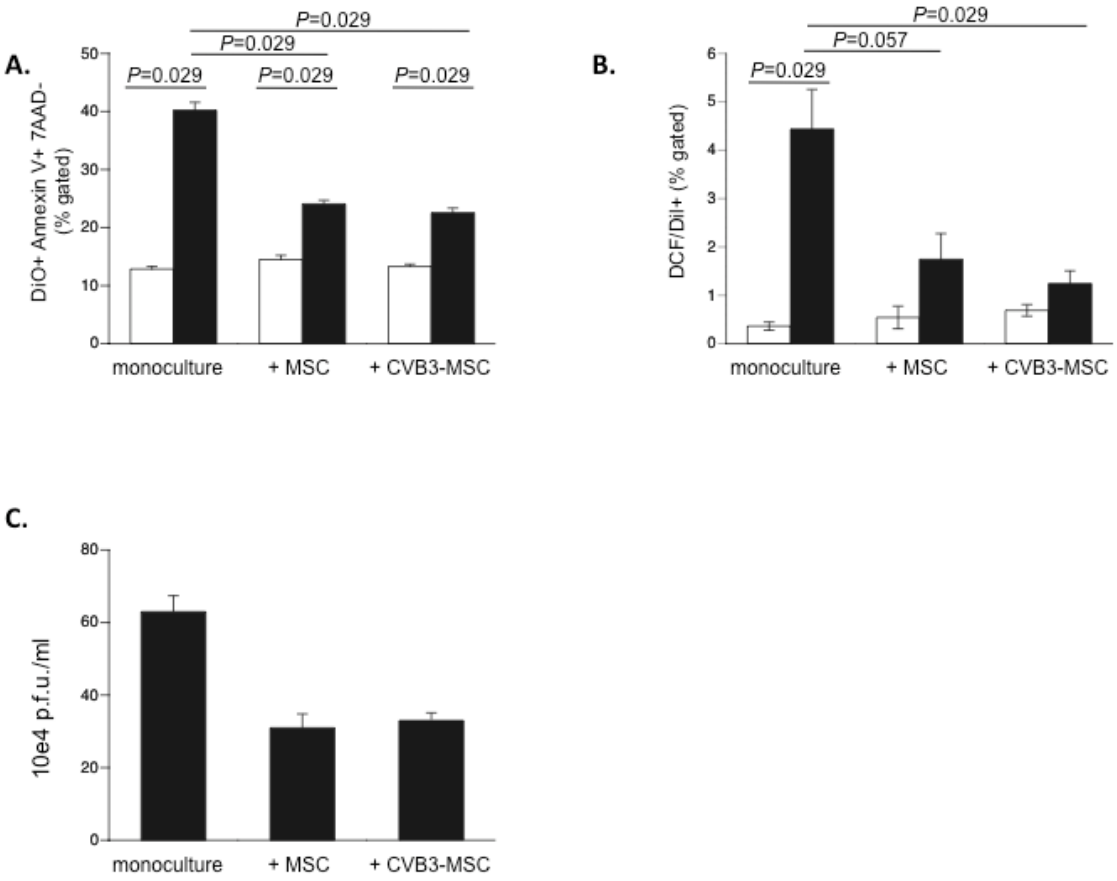
Statistical analysis

Statistical analysis was performed using GraphPad Instat 3.0a (GraphPad Software, Inc., La Jolla, USA). Assumption of Gaussian distribution was consistently tested by the method of Kolmogorov and Smirnov. Paired and unpaired Student's t tests were used for statistical analysis. When no Gaussian distribution was reached, a non-parametrical test was used. Data are presented as mean ± SEM. Differences were considered to be significant when the two-sided p-value was lower than 0.05.

Supplemental Figures

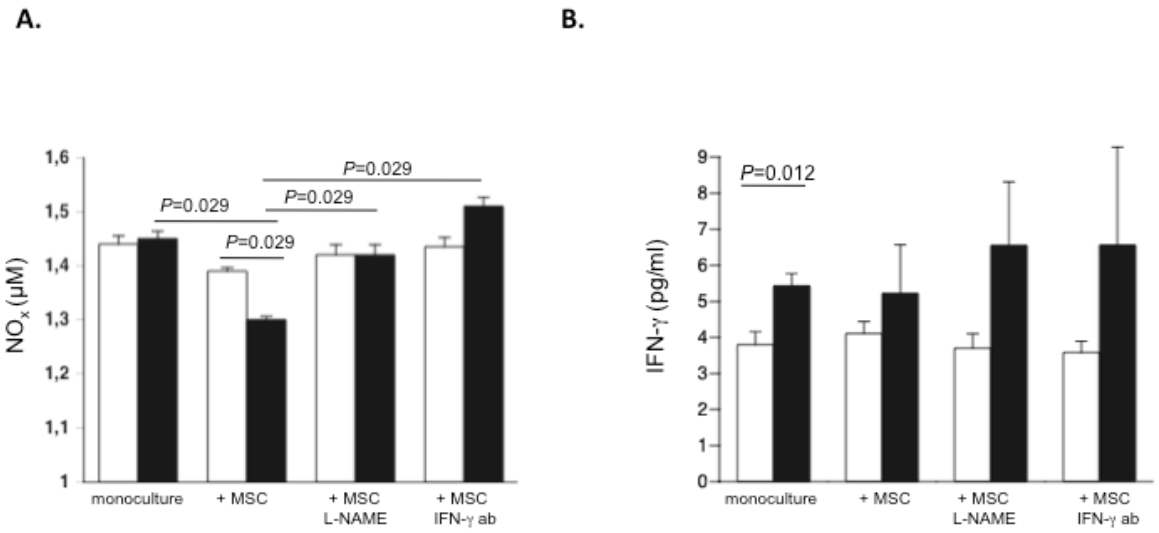


Supplemental Figure I. Representative flow cytometry analysis of mesenchymal stem cells. Flow cytometry histograms indicate that MSC are CD14-, CD34-, CD45- and CD73+, CD90+, CD105+ and CD166+. Numbers above the red histograms indicate the % of gated cells, which are positive.

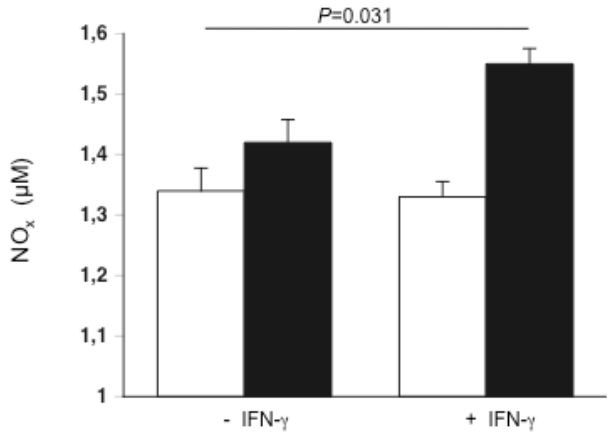


Supplemental Figure II. Mesenchymal stem cells exposed to Coxsackieviurs B3 do not loose their functionality compared to mesenchymal stem cells unexposed to Coxsackieviurs B3. MSCs exposed to CVB3 for 1 h under serum starvation conditions, or serum starved for 1 h, were 24 h later supplemented to DiO- or DiI-labeled HL-1 cells, which were 4 h before infected with CVB3 at a m.o.i. of 5 for 1 h under serum starvation conditions, or serum starved for 1 h. Twenty-four h after CVB3-infection or serum starvation of DiO- or DiI-labeled HL-1 cells, medium was collected for plaque assay, and the effect of MSC supplementation on CVB3-induced apoptosis, oxidative stress and viral progeny release was determined, respectively. Bar graphs represent the mean ± SEM of **A.** DiO+ Annexin V+/7AAD- or **B.** DCF+/DiI+ HL-1 cells in cultures of uninfected (open bar graph) or CVB3-infected (closed bar graph) HL-1 with or without MSCs exposed (CVB3-MSC) or not exposed to CVB3 (MSC); n=4/group. **C.** HeLa cells were incubated for 30 minutes with 1 ml of diluted medium. Then, cells were washed with PBS and covered with agar consisting of 50% 1.3%

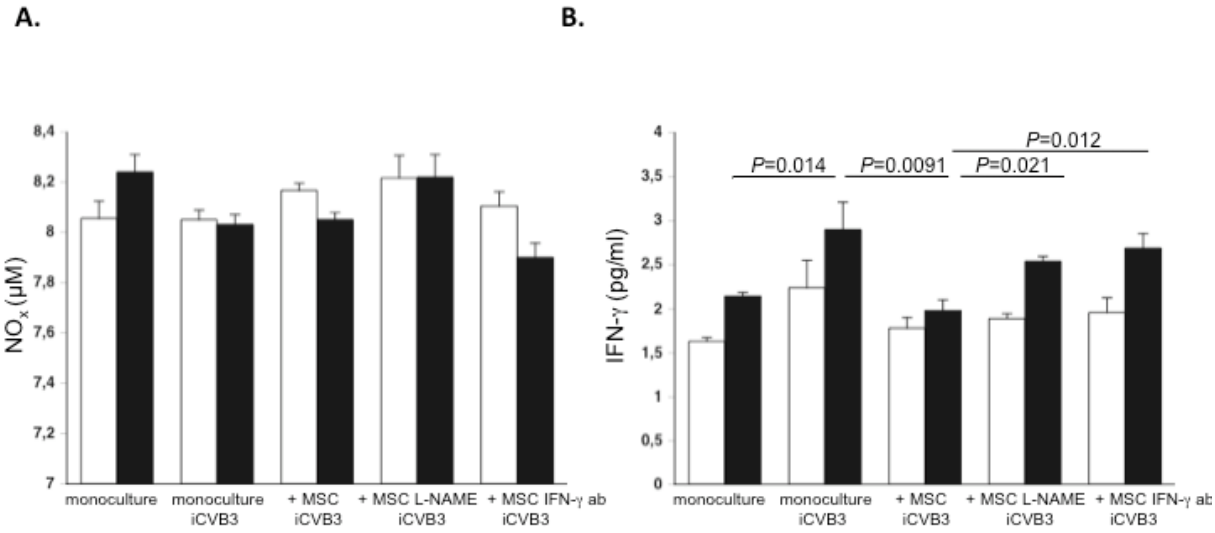
noble agar and 50% 2x MEM, supplemented with 4% FBS. Bar graphs represent the mean ± SEM of counted plaques in 3 wells/condition, 72 h after incubation with 10^{-4} diluted medium of cultures of CVB3-infected HL-1 with or without MSCs exposed (CVB3-MSC) or not exposed to CVB3 (MSC); n=3/group.



Supplemental Figure III. Nitric oxide x and murine IFN-γ **levels in HL-1 monoculture or co-cultures of HL-1 with mesenchymal stem cells.** Bar graphs represent the mean ± SEM of **A.** nitric oxide x (NOx) levels (µM) and **B.** murine IFN-γ levels (pg/ml) in the medium of uninfected (open bar graphs) or CVB3-infected (closed bar graphs) HL-1 with or without untreated or L-NAME treated MSCs or with MSCs in the presence or absence of 1 µg/ml of IFN-γ antibody (ab) with n=6/condition.



Supplemental Figure IV. Interferon-γ increases nitric oxide x levels in Coxsackievirus B3-infected mesenchymal stem cells. Bar graphs representing the mean ± SEM of nitric oxide x (NOx) levels (µM) in medium of non-infected (open bar graphs) and CVB3-infected (closed bar graphs) MSCs in the presence or absence of murine IFN-γ (4 pg/ml) with n=4-6/group.



Supplemental Figure V. Nitric oxide x and murine interferon-γ levels in mononuclear cell monoculture or co-cultures of mononuclear cells with mesenchymal stem cells. Bar graphs represent the mean ± SEM of **A.** nitric oxide x (NOx) levels (µM) and **B.** murine IFN-γ levels (pg/ml) in the medium of unstimulated or inactivated CVB3 (iCVB3)-stimulated splenocytes from control (open bar graphs) or CVB3-infected (closed bar graphs) mice with or without untreated or L-NAME treated MSCs or with MSCs in the presence or absence of 1 µg/ml of IFN-γ antibody (ab) with n=6/condition.

Supplemental References

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