Functional characterization of bone marrow-derived mesenchymal stromal cells from COPD patients

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Supplementary data

MATERIALS AND METHODS

MSC isolation and culture

Mesenchymal stromal cells were isolated and expanded from heparinized bone marrow aspirated from the iliac crest, under local (non-COPD) or general (COPD) anaesthesia. Mononuclear cells were isolated by Ficoll density gradient centrifugation at density 1.077 g/cm^3 and washed cells were plated at 0.16x10^6 cells/cm^2 in tissue culture flasks containing low-glucose Dulbecco's modified Eagle medium (LG-DMEM; Invitrogen, Breda, the Netherlands) supplemented with 100 U/ml penicillin and 100 µg/ml streptomycin (Lonza, Verviers, Belgium) and 10% [v/v] fetal calf serum (FCS; Hyclone, Logan, UT, USA). Cells were cultured at 37°C in a 5% CO_2 humidified incubator, and refreshed twice weekly. When cultures reached near-confluence (>80%), cells were transferred using Trypsin/EDTA (Lonza) and replated at 4000 cells/cm^2. At each passage, cell numbers harvested were counted to assess proliferation rates. For cryopreservation, a mixture of isotonic buffered saline with 10% dimethylsulfoxide was used.

The experiments were done with MSCs at passage three or four, and all cells were starved overnight in serum-free (SF) medium before experiments.

Characterization

Cell morphology was checked daily to assure that cells had a spindle-shaped form. Fluorescence-activated cell sorter analysis was done to assess expression of HLA class I (ABC) and II (DR), CD31, CD34, CD45, CD73, CD80, CD90 (all from Becton Dickinson Bioscience, Franklin Lakes, NJ, USA) and CD105 (Ancell, Bayport, MN, USA), using a FACS Calibur flow cytometer (BD Bioscience) and FlowJo Software (version 7.2.5; Treestar, San Carlos, CA, USA).

Differentiation assays and quantification

For differentiation assays, $0.02x10^6$ MSCs/cm^2 were plated in 24 wells plates in duplicates. After overnight adherence, medium was replaced by differentiation medium, i.e. LG-DMEM (with penicillin/streptomycin and 10% FCS) supplemented with 50 µM indomethacin, 0.25 µM dexamethasone, 0.5 mM IBMX and 1.6 µM insulin (all Sigma-Aldirch, St. Louis, MO, USA) for adipocyte differentiation, or α-MEM (Invitrogen) (with penicillin/streptomycin and 10% FCS) supplemented with dexamethaxone 10 nM and ascorbate 0.2 mM (both Sigma) for osteoblast differentiation, and for mineralization the osteoblast differentiation medium was used supplemented with 10 mM β-glycerophosphate (Sigma). Control cells were cultured in standard MSC medium on the same plate. All cultures were maintained during 21 days, refreshing medium twice weekly (for adipocyte differentiation 60% of medium was refreshed).

Oil-red O 0.3% [w/v] in 60% isopropyl alcohol was used to stain adipocytes, and alkaline phosphatase (AP substrate kit III, Vector Laboratories, Burlingame, CA, USA) to stain osteoblasts, following the manufacturer's protocol. To stain calcium in the mineralization assay, cells were fixed in 4% formaldehyde in PBS and incubated with 60 mM alizarin red (Sigma) in distilled water (pH 5.5 adjusted with 0.5% ammonia solution) at 37°C.

For quantification, cells were destained using 100% ethanol for oil-red O, 50 mM sodium hydroxide in 100% ethanol for alkaline phosphatase and 10% w/v cetylpiridinium (Sigma) in phosphate buffer for alizarin red. Sample duplicates were read at 550 nm on a microplate reader (Bio-Rad, Hercules, CA, USA).

Migration assays

MSCs were plated at $0.075x10^6$ cells/cm^2 in Electric Cell-substrate Impedance Sensing (ECIS) 8W1E arrays (Applied Biophysics, Troy, NY, USA). In assigned wells, an electric fence was activated upon inoculation to prevent cell adherence on the area of the electrode (0.049 mm^2) (1000 µA, 200 msec on/off, target capacitance 4.5 nF). When impedance in control wells was stabilized, cells were washed with PBS and medium was replaced by 250 µl of medium with 2% [v/v] FCS during at least 6

hours. Next, 250 µl serum free (SF) MSC medium was added resulting in a final volume of 500 µl medium per well, and the electrical fence was aborted to allow migration of cells over the surface area of the electrode. Electrical properties were measured using ECIS (Applied Biophysics) [1], measuring resistance at 500 Hz and capacitance at 40.000 Hz in duplicate wells during 15 hours. Normalized values were calculated by comparing the values obtained for resistance and/or capacitance in the well of interest to their respective negative control at time t=0.

Stimuli

Oxidative stress responses were investigated by incubating MSCs during 6 hours with 25 µM sulforaphane (Sigma) or different concentrations of cigarette smoke extract (CSE), which was freshly prepared shortly before experiments following a previously described protocol [2]. Briefly, cigarette smoke derived from two reference cigarettes (3R4F, filter removed) (University of Kentucky, Lexington, KY, USA) was drawn into 2 ml of PBS using a syringe. The solution was filtered through a 0.2 µm pore filter (Whatman GE Healthcare, Buckinghamshire, UK) and the maximal absorbance (ODmax) was measured in a 100-fold dilution to calculate the CSE concentration in arbitrary units (AU) per ml, on an Ultrospec 2100 pro UV/Visible Spectrophometer (GE Healthcare Life Sciences, Cleveland, OH, USA) at 200-350 nm. CSE was used in a concentration of 0.25, 0.5 and 1.0 AU/ml, based on prior dose response experiments.

MSC conditioned medium (MSC-CM) was collected from MSCs that were cultured during 24 hours in SF LG-DMEM (MSC-CM^{CTRL}) or in SF LG-DMEM supplemented with 20 ng/ml TNF-α/IL-1β (MSC-CM^{STIM}), after overnight starvation. MSC-CM was centrifuged to remove cell debris (230xG, 7 min) and aliquots of 2 ml were stored at -80°C until further use.

NCI-H292 airway epithelial cells

NCI-H292 airway epithelial cells were cultured in RPMI 1640 supplemented with penicillin/streptomycin, 2 mM glutamine (Bio Whittaker, Walkersville, MD, USA) and 10% [v/v] FCS

(Bodinco, Alkmaar, The Netherlands) until 80-90% confluent. Before experiments, cells were starved for growth factors by overnight incubation in SF- medium. NCI-H292 were incubated with MSC-CM diluted 1:1 in SF RPMI during 9 hours.

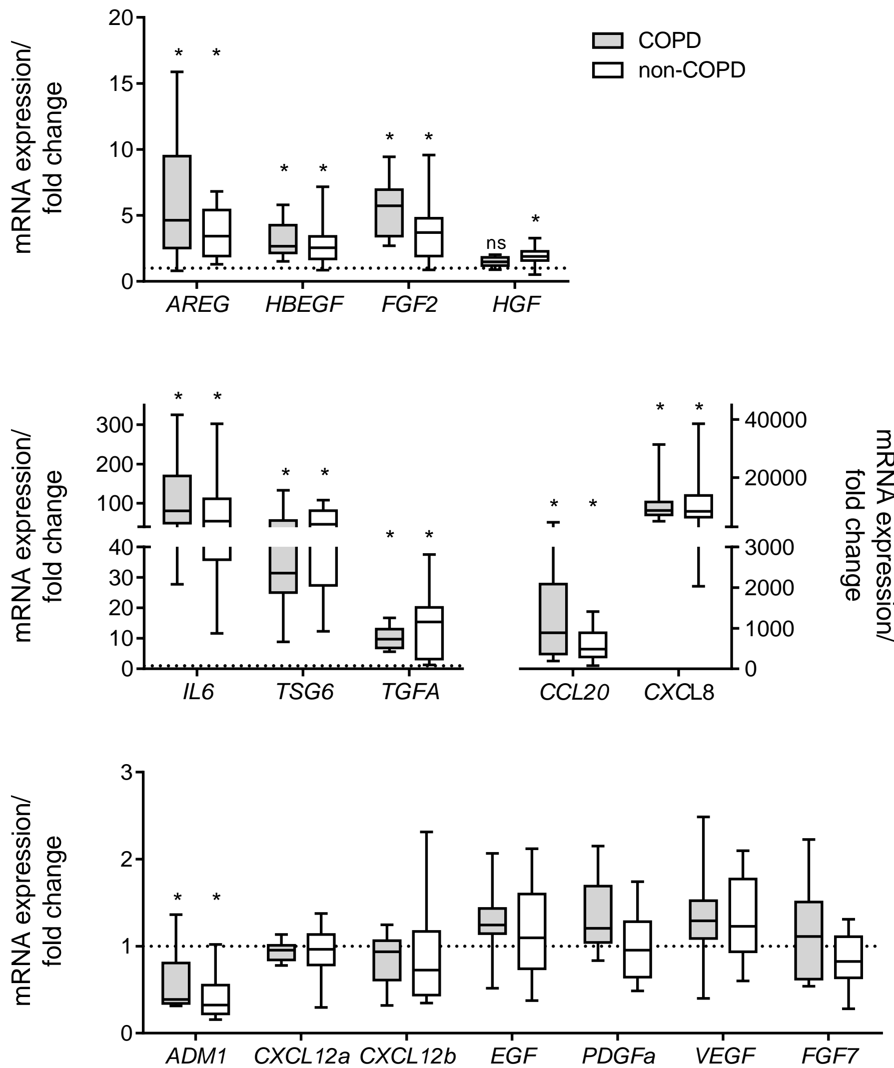
qPCR

Extraction of RNA was done using Maxwell® 16 RNA purification kit (Promega, Madison, WI, USA) and RNA concentration was assessed using Nanodrop ND-1000 UV Visible spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA). To generate cDNA, Oligo(dT) primers (Qiagen, Düsseldorf, Germany) and 10 nM dNTP mix (Promega) were added to the RNA sample and heated to 65°C for 5 minutes, followed by 5x 1st strand RNA buffer, RNasin and M-MLV (all Promega) at 37°C for 50 minutes. The last step consisted of heat inactivation of M-MLV at 70°C for 15 minutes.
Quantitative real-time PCR was performed using IQ SYBR Green supermix (Bio-Rad) and primers for target genes (see table 1) (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) in a final volume of 8µl/well and run in triplicate on a Bio-Rad CFX384™ real-time PCR system (Bio-Rad). Arbitrary mRNA concentrations were calculated by the Bio-Rad software. For relative gene expression, this concentration was normalized against two housekeeping genes that were selected based on GeNorm analysis (Genorm, South Hampton, UK), i.e. *B2M* and *RPS29* for MSCs and *ATP5B* and *RPL13A* for NCI-H292. Single data points were removed from the analysis if the variance within triplicates was above 10%.

Supplementary table E1 Primer sequence for target genes

GENE	Primer sequence FW	Primer sequence RV
ATP5B	5’-TCACCCAGGCTGGTTCAGA-3’	5’-AGTGGCCAGGGTAGGCTGAT-3’
B2M	5-‘GATCGAGACATGTAAGCAGC-3’	5’-TCAAACATGGAGACAGCAC-3’
RPL13A	5’-AAGGTGGTGGTCGTACGCTGTG-3’	5’-CGGGAAGGGTTGGTGTTCATCC-3’
RPS29	5’-GCACTGCTGAGAGCAAGATG-3’	5’-ATAGGCAGTGCCAAGGAAGA-3’
ADM1	5’-ATGAAGCTGGTTTCCGTCG-3’	5’-GACATCCGCAGTTCCCTCTT-3’
AREG	5’-GGTGGTGCTGTCGCTCTT G-3’	5’-AGGTGTCATTGAGGTCCAATCC-3’
CCDN1	5’-CAATGACCCCGCACGATTTC-3’	5’-CATGGAGGGCGGATTGGAA-3’
CCL20	5’-GCAAGCAACTTTGACTGCTG-3’	5’-TGGGCTATGTCCAATTCCAT-3’
CXCL12 -1	5’-CTACAGATGCCCATGCCGAT-3’	5’-GTGGGTCTAGCGGAAAGTCC-3’
CXCL12 -2	5’-GTAGCCCGGCTGAAGAACAA-3’	5’-GCGTCTGACCCTCTCACATC-3’
EGF	5’-TGCAGAGGGATACGCCCTAA-3’	5’-CAAGAGTACAGCCATGATTCCAAA-3’
FGF2	5’-TGGCTATGAAGGAAGATGGAAGA-3’	5’-TCCAATCGTTCAAAAAAGAAACAC-3’
FGF7	5’-TCCTGCCAACTTTGCTCTACA-3’	5’-CAGGGCTGGAACAGTTCACAT-3’
GPX2	5’-GAATGGGCAGAACGAGCATC-3’	5’-CCGGCCCTATGAGGAACTTC-3’
HBEGF	5’-TGGACCTTTTGAGAGTCACTTTATCC-3’	5’-CGTGCTCCTCCTTGTTTGGT-3’
HGF	5’-TCCAGAGGTACGCTACGAAGTCT-3’	5’-CCCATTGCAGGTCATGCAT-3’
HMOX1	5’-AACCCTGAACAACGTAGTCTGCGA-3’	5’-ATGGTCAACAGCGTGGACACAAA-3’
IL6	5’-CAGAGCTGTGCAGATGAGTACA-3’	5’-GATGAGTTGTCATGTCCTGCAG-3’
CXCL8	5’-CAGCCTTCCTGATTTCTGC-3’	5’-ACTTCTCCACAACCCTCTGC-3’
NQO1	5’-GAAGAGCACTGATCGTACTGGC-3’	5’-GGATACTGAAAGTTCGCAGGG-3’
PDGFA	5’-CACCACCGCAGCGTCAA-3’	5’-CCTCACCTGGACTTCTTTTAATTTTG-3’
SCAL1	5’-GGCATTTACCAGCTGAGGGA-3’	5’-TACCCCTACCTAGCACAGCA-3’
TGFA	5’-AGGTCCGAAAACACTGTGAGT-3’	5’-AGCAAGCGGTTCTTCCCTTC-3’
TSG6	5’-AGAATTTGTGAGCAGCCCCT-3’	5’-TGTATTTGCCAGACCGTGCT-3’
VEGF	5’-CGAGGGCCTGGAGTGTGT-3’	5’-TGGTGAGGTTTGATCCGCATA-3’

Supplementary figure



Supplementary figure E1. mRNA expression of immune mediators and growth factors in response to proinflammatory cytokines.

mRNA expression upon stimulation of MSCs with TNF-α and IL-1β, calculated as fold change compared to mRNA expression in unstimulated MSCs from the same donor (at t=6 hours). Box whiskers represent median, interquartile range and minimum and maximum values. n = 9 per group.

References supplement

1. Wegener J, Keese CR, Giaever I. Electric Cell–Substrate Impedance Sensing (ECIS) as a Noninvasive Means to Monitor the Kinetics of Cell Spreading to Artificial Surfaces. *Experimental Cell Research* 2000; 259: 158-166.

2. Luppi F, Aarbiou J, van WS, *et al.* Effects of cigarette smoke condensate on proliferation and wound closure of bronchial epithelial cells in vitro: role of glutathione. *Respir Res* 2005; 6: 140.