ONLINE DATA SUPPLEMENT

Bone Marrow Stromal Cells Attenuate Lung Injury in a Murine Model of Neonatal Chronic Lung Disease

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SUPPLEMENTARY MATERIALS AND METHODS

Cell Isolation, Culture and Differentiation

Bone marrow stromal cells (BMSCs) were isolated from the femurs and tibiae of 5-7 week old FVB mice as previously described (1-3). Briefly, the ends of each tibia and femur were clipped to expose the marrow and the bones inserted into adapted centrifuge tubes. The tubes were centrifuged for 1 minute at 400*g* to collect the marrow. The pellet was resuspended in 3 mL α-MEM medium through a 21-gauge needle followed by filtration through a 70-μm nylon mesh filter. The marrow cells were layered on a Ficoll-Paque (Amersham, Piscataway, NJ) density gradient, centrifuged and plated. Plastic adherent cells were maintained in culture with media changed every 2-3 days. Following 2-3 passages, immunodepletion was performed as per published protocols and the International Society for Cellular Therapy (ISCT) guidelines (4). The cells were negatively selected for CD11b, CD14, CD19, CD31, CD34, CD45, and CD79α antigens using the appropriate fluorescent-tagged antibodies (BD Biosciences, Pharmingen, San Diego, CA) in a fluorescence-activated cell sorter (MoFlo, Beckman-Coulter), further propagated, and then positively selected for CD73, CD90, CD105, c-kit and Sca-1 antigens, as above.

The differentiation potential of BMSC cultures was assessed following published protocols (2, 3): Adipogenesis was induced by culturing BMSCs in complete α-MEM medium, supplemented with 5 μg/ml Insulin, 50 μM Indomethacin, 1 μM Dexamethasone and 0.5 μM 3-isobutyl-1 methylxanthine (IBMX) with media changed three times per week. After three weeks, the cells were fixed with 10% Formalin and stained with 0.5% Oil Red O in Methanol. For osteogenesis, cells were supplemented with 20 mM β-glycerol phosphate, 50 ng/ml thyroxine, 1 nM

Dexamethasone and 0.5 μM ascorbate 2-phosphate with media change three times per week. At the end of three weeks, the cells were fixed with 10% Formalin and stained with Alizarin Red S. Primary mouse lung fibroblast (MLF) and pulmonary artery smooth muscle cell (PASMC) cultures were derived according to standard methods (5, 6). All reagents were obtained from Sigma.

BMSC Transplantation

A suspension of 5 x 10^4 BMSCs in 50 μl phosphate buffered saline (PBS) was injected via the superficial temporal vein on postnatal day 4. Higher volumes of cell suspension or higher cell numbers were technically challenging for intravenous administration given the small size and blood volume of the neonatal mouse pup. For control, mice were injected either with PBS alone, or a suspension of PASMCs in PBS or MLFs in PBS. Under these conditions, survival of injected pups was greater than 90%. After an additional 10 days in hyperoxia, animals were anesthetized with pentobarbital (60 mg/kg i.p.) and perfused with PBS through the right ventricle at a constant pressure of 15-20 cm H_2O. The tracheas were cannulated and bronchoalveolar lavage performed three times with PBS. Lungs were inflated to a fixed pressure of 15-20 cm H_2O with 4% paraformaldehyde, post-fixed overnight at 4^oC and paraffin embedded for sectioning. Hearts were excised and weighed for assessment of Fulton's Index (right ventricle/[left ventricle+septum]) as previously described (7). In separate experiments, animals were exposed to shorter periods of hyperoxia (one to five days) to assess kinetics of donor cell engraftment after transplantation. All animal experiments were approved by the Children's Hospital Boston Animal Care and Use Committee.

Assessment of BMSC Engraftment

The number of male donor BMSCs retained in female recipient lungs was determined through real-time polymerase chain reaction (qPCR) assays for Y–chromosome sequences, on total lung DNA from female recipients that received BMSCs from male donors. Lungs were harvested one or ten days post BMSC injection in both the normoxia and the hyperoxia groups. Total genomic DNA was isolated using the DNeasy Tissue Kit (Qiagen, Valencia, CA), as per manufacturer's instructions and multiplex real-time PCR was performed for Y-chromosome sequences using forward primers 5'- TTT TGC CTC CCA TAG TAG TAT TTC CT - 3'; reverse primer, 5' - TGT ACC GCT CTG CCA ACC A - 3'; TaqMan probe, 5' (FAM) - AGG GAT GCC CAC CTC GCC AGA - (TAMRA) - 3'. Glyceraldehyde phosphate dehydrogenase (GAPDH) served as the internal control, and the primers used for the reaction were: forward, 5' - GGC AAA TTC AAC GGC ACA GT - 3'; reverse, 5'- AAG ATG GTG ATG GGC TTC CC - 3'; TaqMan probe, 5' (HEX) – AGG CCG AGA ATG GGA AGC TTG TCA TC - (TAMRA) 3'. Standard curves were generated by serial dilutions of male mouse genomic DNA in H_2O. The experiments were carried out using Mx4000® Multiplex Quantitative PCR System (Stratagene, La Jolla, CA).
In parallel experiments, fluorescence *in-situ* hybridization (FISH) for the Y chromosome was performed in lung sections from female recipient mice to localize male donor-derived cells. Tissue sections were deparaffinized in xylene, rehydrated in decreasing ethanol series, and incubated at 80°C in 1M sodium thiocyanate (Sigma) for 10 minutes, washed in PBS, followed by digestion with 0.4% w/v Pepsin in 0.1 M HCl at 37°C for 10 minutes. The reaction was quenched with 0.2% glycine in PBS followed by PBS rinse and dehydration through graded alcohol washes. Fifteen µl of the FITC-labeled Y paint (Cambio, Cambridge, UK) probe mixture were added to the sections, sealed with glass cover slip using rubber cement, heated for 10

minutes at 60°C and incubated overnight at 37°C in a humidified chamber. Negative controls included sections covered with hybridization buffer alone without added probe. Slides were washed and air-dried, and Vectashield hard mounting medium with 4',6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Burlingame, CA) was applied. Slides were examined using a Nikon Eclipse 80*i* fluorescence microscope (Nikon Instruments, Melville, NY) with Nikon ACT-1 software version 2.63. Images were merged to colocalize Y signal with the DAPI nuclear signal.

Bronchoalveolar Lavage and cytokine profiling

Animals were anesthetized with sodium pentobarbital (60 mg/kg i.p.) and the tracheas were cannulated. Bronchoalveolar lavage fluid (BALF) was collected using PBS. The BALF was centrifuged at 400g for 5 minutes and the supernatant removed. The cells were resuspended in the staining solution and the total cell count was performed via Kimura stain using a hemocytometer (8). Differential cell count for neutrophils and macrophages within the BALF was performed via flow cytometry, using Ly-6G / Ly-6C (neutrophils) and CD11c (macrophages) fluorescent-labeled antibodies (BD Biosciences, Pharmingen, San Diego, CA). Cytokines, Chemokine and Growth factor levels in the BALF were measured using Luminex 200™ System (Luminex Corporation, Austin, TX). A total of 21 proteins were analyzed using a standard 20-plex mouse cytokine kit (FGFβ, INFγ, IL-1β, IL-1α, IL-2, IL-6, IL-4, IL-5, IL-13, IL-17, IP-10, KC, GM-CSF, MCP-1, MIG, MIP-1α, IL-10, TNFα , IL-12, VEGF) supplemented with reagents specific for interleukin 1 receptor antagonist (IL-1ra) (InVitrogen Corporation, Carlsbad, CA). BALF was concentrated 4-fold using Microcon Centrifugal Filter Devices (Millipore Corporation, Billerica, MA) with a molecular weight cut-off of 3kD (Ultracel YM-3)

and the absolute amount of cytokines in each sample was determined using the standard Luminex protocol and cytokine standards.

Tissue and Vessel Morphometry

For lung morphometry sections 5μm thick were stained with Hematoxylin and Eosin and viewed under bright light microscopy using Nikon Eclipse 80i microscope. At least four representative lobes were selected from each animal. Images intended for morphometric analysis were captured at 100x magnification and analyzed using the software package MetaMorph Imaging System version 62r.4 (Molecular Devices, Sunnyvale, CA). Underinflated areas and fields containing large airways and vessels were excluded from the analysis. The volume density of alveolar wall tissue (VD_{awt}) was determined by a point-counting method using a computer-generated 30 x 30 grid superimposed to each image. The volume density ($VD_{awt)}$ was estimated as the ratio of points that fell on the alveolar wall tissue compartment (P_{awt}) to total test points ($P_{t)}$ determined by the grid ($VD_{awt} = P_{awt}/P_t$) (9). The thickness of the alveolar septum was calculated by measuring the fiber breadth (area/length) using Metamorph. For vessel morphometry, α-smooth muscle actin (α-SMA)-stained wall thickness was measured in vessels less than 100μm diameter in sections captured under 400X magnification, using either the NIH Image J program or Metamorph and compared between groups using the following equation: Medial thickness index = $[(area_{ext} - area_{int})/area_{ext}] \times 100$, where $area_{ext}$ and $area_{int}$ are the areas within the external and internal boundaries of the α-SMA layer, respectively.

Immunohistochemical Analysis

Lung tissue sections were deparaffinized in xylene and rehydrated. Immunohistochemical analysis was performed by incubating with the indicated primary antibody at a dilution of 1:125 (α-SMA, Sigma), and 1:50 (Mac-3, BD Pharmingen, San Diego, CA) overnight at 4°C after 1 hour of blocking at room temperature to reduce nonspecific binding. Endogenous peroxidase activity was inhibited with 3% H_2O_2 in methanol (Sigma). Secondary antibodies and peroxidase staining was performed according to manufacturer's instructions (Vector laboratories, Burlingame, CA). Slides were counterstained with hematoxylin. The density of Mac-3 positive cells was determined by counting the number of cells per 200X magnification field using Metamorph.

Proteomic Analysis of Conditioned Media

Proteins in conditioned media from BMSCs or PASMCs were separated by SDS-PAGE and subsequently digested with 12.5 ng/μl sequencing grade trypsin (Promega, Madison, WI) in 100 mM NH_4HCO_3 at 37 °C overnight. Peptides were extracted with 100 mM NH_4HCO_3 and acetonitrile, lyophilized, resuspended in 5% acetonitrile, 5% formic acid and injected into an LC/MS system comprising a micro-autosampler, Surveyor HPLC pump and Proteome X (LTQ) mass spectrometer (Thermo Scientific, Waltham, MA). Peptides were eluted with a 30 minute linear gradient and the most abundant selected for automated fragmentation. Product ion spectra were matched to a species specific database using Mascot (Matrix Science, London, UK).

Western Blot Analysis

40 μg of conditioned media protein were electrophoresed on 12% denaturing polyacrylamide gels before transfer to 0.45 μm PVDF membranes (Millipore) and the membranes were probed

with polyclonal goat anti-mouse osteopontin (Spp1) or M-CSF (Csf1) antibodies (R&D Systems). Briefly, after blocking with 5% nonfat dry milk in phosphate buffered saline (pH 7.4) containing 0.1% Tween 20 (PBST) for an hour at room temperature, the membranes were incubated with primary antibodies (diluted 1: 1,000) at 4°C overnight with rocking. The membranes were then incubated with 40 ng /ml of peroxidase-conjugated anti-goat secondary antibody (Santa Cruz Biotechnology) for an hour at room temperature followed by reaction with ECL substrate (Thermo scientific) and the blots were developed according to the manufacturer's instructions.

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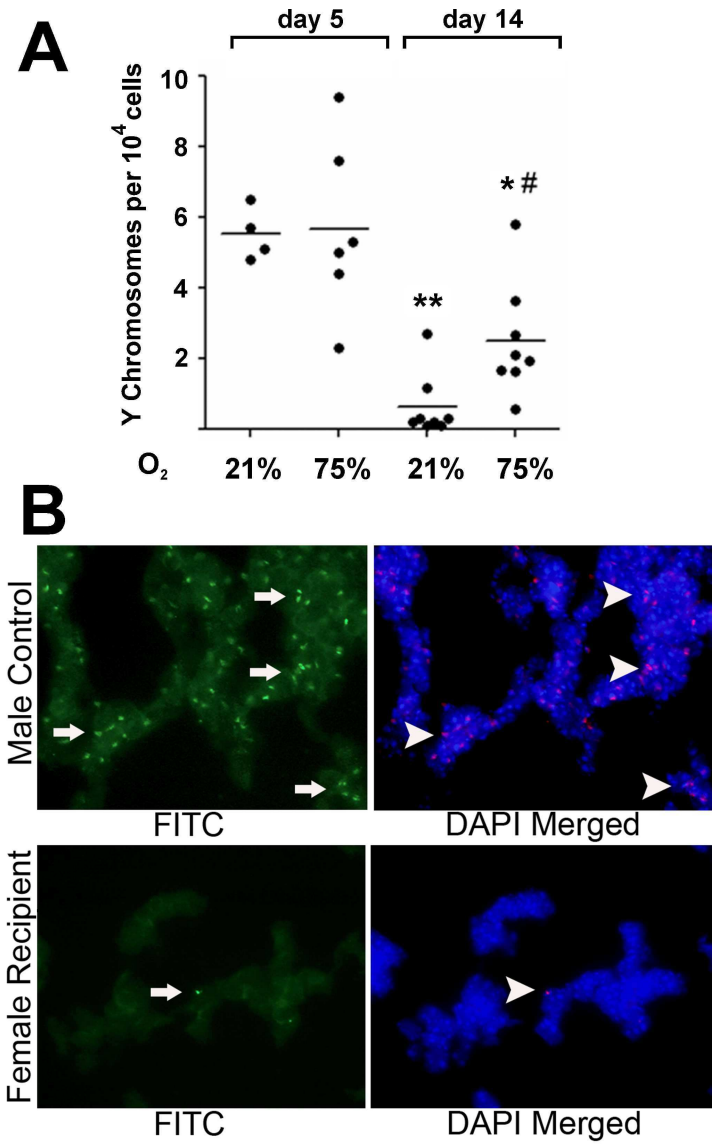
ON-LINE DATA SUPPLEMENT - Figure Legends

Figure E1. Male donor BMSCs retained in the lungs of recipient newborn female mice. (A) DNA isolated from lungs of recipient females that had been transplanted with male donor BMSCs on postnatal day 4 was analyzed using Multiplex Quantitative PCR System for Y chromosome and GAPDH-specific probes. Data are expressed as mean ± SEM. (*) $p < 0.05$, (**) $p < 0.01$, compared with day 5 samples; (#) $p < 0.01$, compared with the respective normoxia group. Each dot represents a single animal. (B) Deparaffinized lung sections were hybridized with a FITC-conjugated Y chromosome probe (arrows) and counterstained with DAPI. The arrowheads indicate Y chromosome within the DAPI-stained nuclei (DAPI merged). Upper panels: control male mouse lung sections. Lower panels: representative section of lung tissue from a hyperoxia-exposed female recipient at postnatal day 14.

Figure E2. Cytokine levels in BALF of BMSC-CM and PASMC-CM - treated animals and controls. Absolute levels of cytokines and related molecules in 4X concentrated BALF were quantified using a Luminex 20-plex cytokine kit supplemented with beads specific for IL1ra. IFNγ was below the level of detection in all samples. n = 4-6 animals per group. (*) $p < 0.05$, hyperoxia BMSC-CM treated group vs hyperoxia PASMC-CM treated group. Note that only the differences in IL-5, IL-17, TNFα and CXCL10 (IP-10) levels are statistically significant.

Figure E3. (A) Functional classes represented in the BMSC-CM proteome as defined by Ingenuity Pathways Analysis. (B) Western blot analysis for Csf1 and Spp1 on conditioned media produced by independently derived BMSC cultures. 1, 2 and 3 represent three independent BMSC cultures at passages 7, 9 and 8 respectively.



Aslam et al, Fig. E1

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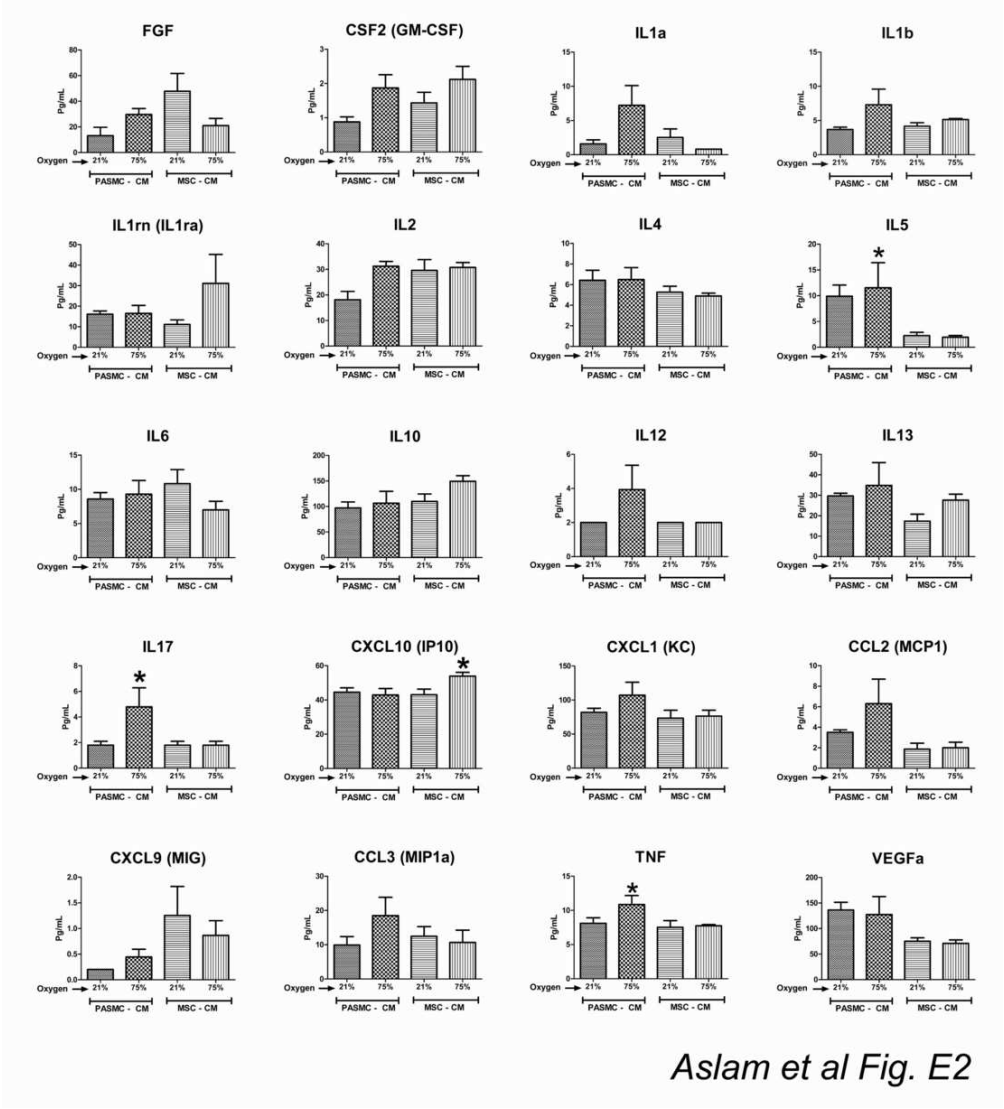

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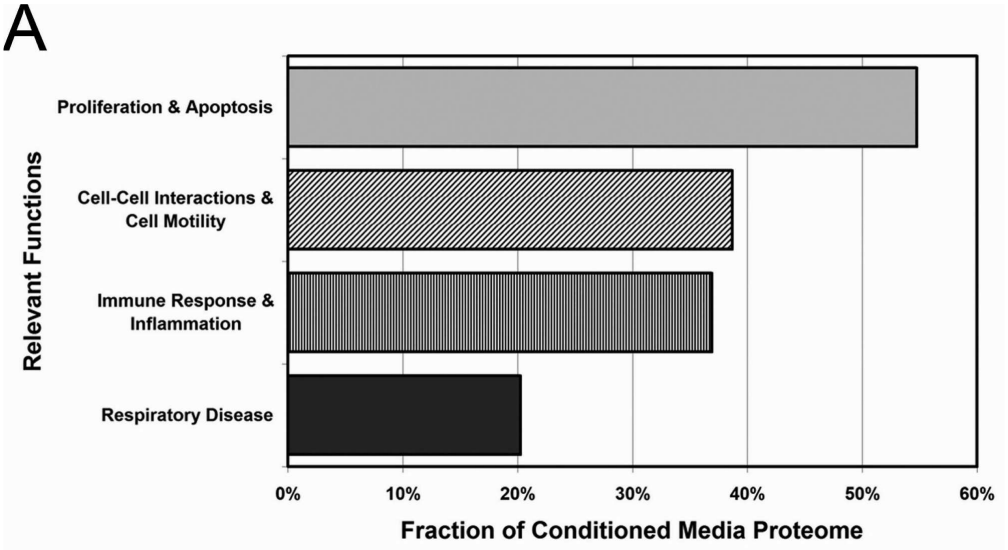

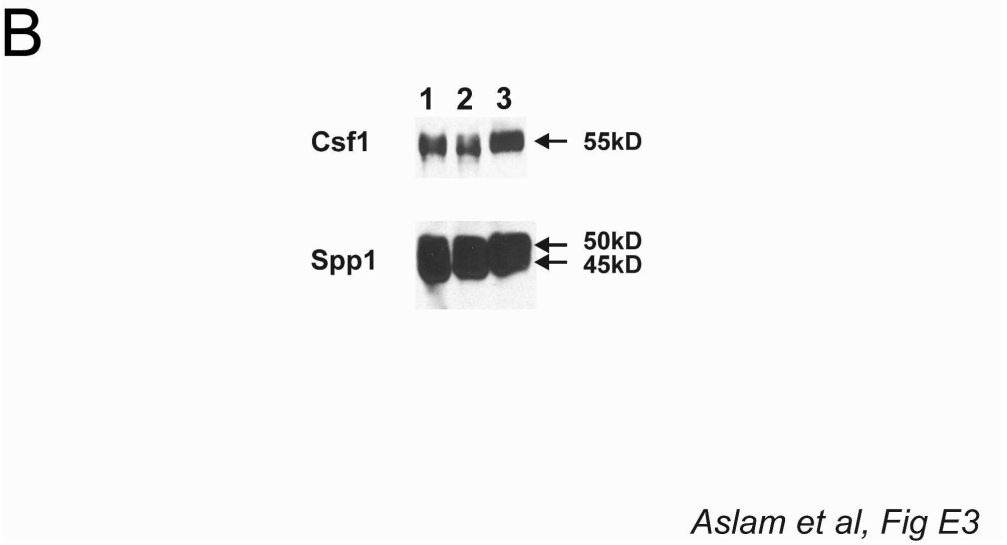

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