Impaired immunosuppressive effect of bronchoalveolar mesenchymal stem cells in hypersensitivity pneumonitis: preliminary findings

Enikő Balogh[1,#], Béla Nagy Jr[2,#,*], Ágnes Gyetvai[1], Zsolt Bene[3], Zoltán Hendrik[4], Viktória Jeney[1,6], Péter Nagy[5], Ágnes Papp[3], József Balla[1,6], György Balla[3,6], János Kappelmayer[2], Béla Nagy[3]

[1]Department of Internal Medicine, Division of Nephrology, [2]Department of Laboratory Medicine, [3]Department of Pediatrics, Division of Pulmonology, [4]Department of Pathology, [5]Department of Biophysics and Cell Biology, Faculty of Medicine, University of Debrecen, [6]MTA-DE Vascular Biology, Thrombosis and Hemostasis Research Group, Hungarian Academy of Sciences, Debrecen, Hungary

[#]These authors equally contributed to this manuscript.

Running title: Abnormal immunosuppressive properties of BALF MSCs in HP

Key terms: mesenchymal stem cells, hypersensitivity pneumonitis, bronchoalveolar lavage, T-lymphocyte proliferation and activation

Conflict of interest: The authors have declared that no conflict of interest exists.

***Correspondence to:**

Béla Nagy Jr, MD, PhD, Department of Laboratory Medicine, Faculty of Medicine, University of Debrecen, Nagyerdei körút 98. H-4032 Debrecen, Hungary

E-mail: nagyb80@gmail.com

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process which may lead to differences between this version and the Version of Record. Please cite this article as an 'Accepted Article', doi: 10.1002/cyto.b.21490

ABSTRACT

Background: Bronchoalveolar mesenchymal stem cells (MSCs) play an important role in the maintenance of lung integrity. Therapeutic application of bone marrow-derived MSCs reduced chronic bronchial inflammation in idiopathic pulmonary fibrosis, and improved the ratio of survivors in sepsis with pneumonia. This study investigated the effect of MSCs from bronchoalveolar lavage fluid (BALF) of hypersensitivity pneumonitis (HP) on T-cell function under *in vitro* conditions.

Methods: Bronchoalveolar MSCs were obtained via bronchoscopy with BAL from children with severe subacute HP. As control, BALF MSCs were assessed from children without any inflammatory lung disease. Isolated MSCs were characterized via immunophenotyping by flow cytometry and confocal laser scanning microscopy. HP-derived and healthy separated peripheral blood mononuclear cells (PBMCs) were stimulated by 5 μg/mL phytohemagglutinin in the presence of HP-derived or control MSCs in 5-day cultures. Proliferation and activation of T-cells were characterized by the mean fluorescence intensity (MFI) of 5,6-carboxyfluorescein-diacetat succinimidyl ester (CFSE) and CD25, CD69 as well as HLA-DR surface positivities, respectively.

Results: HP-derived MSCs showed significantly lower level of CD73, CD90 and CD105 expression compared to control MSCs in both flow cytometric and confocal microscopic experiments. MSCs from HP did not reduce T-cell proliferation based on CFSE MFI values, while the level of CD25 expression on both control and HP-derived CD4+ and CD8+ T-cells was significantly reduced by normal MSCs, while HP-derived MSCs did not have any significant effect. The level of other activation markers was not markedly modulated by MSCs.

Conclusions: BALF MSCs from HP are unable to downregulate the proliferation and activation of T-cells that may support the development of recurrent intrapulmonary inflammation in HP.

Introduction

Mesenchymal stem cells (MSCs) are multipotent progenitor cells that were originally identified in the bone marrow (BM) (1), and since then these cells have been detected and isolated from many tissues including the lungs (2). Lung residual MSCs are present at the bronchoalveolar duct junctions and in the alveolar walls (3), and can be cultured from bronchoalveolar lavage fluid (BALF) samples (4). Isolated MSCs can be easily identified by flow cytometry via their positivity for CD105, CD73, and CD90 as well as the absence of CD45 and CD34 expression (5). These cells inhibit inflammation, contribute to epithelial tissue repair, and maintain tissue integrity in severe conditions such as sepsis (6). Normal MSCs possess immunomodulatory properties including the suppression of T- and B-cell proliferation, alteration of dendritic cell function, and modulation of natural killer cells and macrophages (7). For instance, in a murine asthma model, BM-derived MSCs markedly suppressed chronic bronchial inflammatory processes (8). Hence, according to the current understanding MSCs hold great promise for the treatment of severe inflammatory disorders (9). On the contrary, abnormal MSCs from BM of hematological disorders showed decreased CD90 positivity with a loss of immunosuppressive activity versus healthy counterparts (10).

Hypersensitivity pneumonitis (HP) is a spectrum of relatively rare diffuse, inflammatory, immune-mediated interstitial lung disease (ILD). In children, it is usually caused by the repetitive inhalation of two major environmental agents: bird (feather) allergens and antigens consisting of fungal spores (11). Analysis of cellular content in BALF improves the diagnostic accuracy in these diseases by revealing the predominately high percentage of CD8+ T-lymphocytes, the alteration in CD4/CD8 ratio with increased levels of immunoglobulins and albumin in BALF (11). The exacerbated immune response to HP-causing antigens is a pivotal event in the pathomechanism of HP modulated by CD4+/CD25+/FoxP3+ regulatory T cells (Treg) without a substantial activity to suppress lymphocyte proliferation. Consequently, the fraction of CD4+/CD25+ T-cells in the whole CD4+ population in BALF was highly elevated in active HP subjects, and the maturation of

dendritic cells caused pro-inflammatory mediator production (e.g. IL-17 and tumor necrosis factor-α) that also inhibited Treg cell function (12). Beside these facts, further investigations are required to elucidate the role of other impaired immunological events (e.g. defective bronchoalveolar MSCs) in HP.

The objective of the present study was to reveal whether BALF MSCs obtained from children with severe subacute HP had any immunomodulatory effect in comparison to normal MSCs from control patients.

Methods

Patients and BALF samples

The National Ethics Committee, Medical Research Council, Budapest, Hungary approved the trial (number: 7270-1-2012/EKU) in accordance with the Declaration of Helsinki. Written informed consent was obtained from the parent or legal guardian of each child before any investigation. Three children (2 females, 1 male, aged 6-10 years) with subacute clinical forms of HP were recruited into this study, who had specific serum antibodies to feather or moulds, and ground glass and linear opacities were observed on HRCT. Bronchoscopy and BAL were performed as described previously (13). The cellular analysis was performed immediately after the BAL procedure. As control, three children (2 females, 1 male, aged 3-16 years) were involved with suspicious foreign body aspiration or psychogenic cough. Bronchoscopy and BAL excluded any chronic immune-mediated disorders or diffuse parenchymal lung diseases without signs of any type of inflammation.

Isolation and culturing of lung-derived MSCs

Recovered BALF samples were first filtered through a sterile strainer to remove non-cellular particulate materials, and the cell pellet was recovered by centrifugation at 800 g for 10 minutes. 6 x 10^5 cells in BALF samples were seeded in a T-25 tissue culture flask (Biotech-

Hungary, Budapest, Hungary), and incubated in RPMI-1640 medium (Sigma-Aldrich Co. Ltd., Dorset, England) supplemented with 1.0% fetal bovine serum (Sigma-Aldrich), sodium-pyruvate (Sigma-Aldrich), and penicillin-streptomycin-amphotericin (Sigma-Aldrich) at 37°C in 5% CO_2 / 95% air atmosphere. Medium was changed every 3 days, and after 2-3 weeks of incubation, BALF cells gave rise to a confluent cell layer. By consecutive passages most non-adherent contaminating cells, i.e. macrophages were effectively removed and the adherent fibroblastoid colonies were expanded by weeks 3-4.

Identification of bronchoalveolar MSCs by flow cytometry and CLSM

MSC colonies were harvested by treatment with trypsin (Gibco, Life Technologies, Vienna, Austria) at day 6 to 10 from the initiation of culturing (passage 2-3) and were aliquotted at the concentration of 0.5 x 10^5/mL. Cells were labeled with fluorochrome conjugated anti-human CD105-APC, CD73-PE, CD90-PerCP-Cy5.5 and CD34-FITC monoclonal antibodies or isotype-matched control mouse IgG (BD, San Jose, CA) at the recommended concentration (5), then washed in PBS and fixed in 1% PFA, and surface positivities were evaluated using a flow cytometer (FACSCalibur; BD) (Figure 1A). Before flow cytometric analysis, Calibrite beads (BD) (a three-color kit supplemented with APC- and PerCP-Cy5.5-labeled beads) were used monthly to adjust instrument settings, to set fluorescence compensation and to check instrument sensitivity. Cells were considered to be positive for a given antigen if their fluorescence intensity was higher than the 95th percentile of cells stained with an isotype control antibody. The mean fluorescence intensity (MFI) of positive cells was determined by subtracting the MFI of the control sample from the MFI of cells stained with the appropriate antibody. MSCs were also investigated by confocal laser scanning microscopy (CLSM; FluoView FV1000, Olympus, Tokyo, Japan) to study the level of coexpression of their receptor profile using the same set of antibodies. CD105-APC was excited at 633 nm and its fluorescence was detected in the spectral range between 650-750 nm. CD73-PE and CD90-

PerCP-Cy5.5 were excited at 488 nm and their emission was measured between 500-600 nm and 650-750 nm, respectively. Due to the absorption of Cy5.5 in the tandem dye PerCP-Cy5.5 at 633 nm, the overspill of PerCP-Cy5.5 intensity to the APC channel was strong. Therefore, the overspill compensation factor was determined using cells labeled with CD90-PerCP-Cy5.5 only and pixel-by-pixel correction was performed for samples labeled with both CD90-PerCP-Cy5.5 and CD105-APC using DipImage (University of Technology, Delft, The Netherlands) in Matlab (Mathworks, Inc., Natick, MA). Ten visual fields were observed at random in each cell culture (Figures 1B and C).

Coculture of PHA-stimulated PBMCs and MSCs

PBMCs were separated from the buffy-coat of healthy volunteers using Ficoll-Histopaque-1077 density gradient (Sigma-Aldrich). HP-derived or control MSCs (2 x 10^4 cells/well) were adhered on a 96-well plate (Sigma-Aldrich) for 4 hours, and treated with mitomycin C (10 μg/mL) for 2 hours. These cells were washed three times in PBS. Normal and HP-derived isolated PBMCs (2 x 10^5 cells/well) were resuspended in 400 μL DMEM (Gibco, Life Technologies) with 10% autologous serum and then stimulated by phytohemagglutinin (PHA, Sigma-Aldrich) at the final concentration of 5 μg/mL. HP and control MSCs were separately cultured with either normal or HP-derived PBMCs in RPMI-1640 medium at 37°C, 5% CO_2 for 5 days. As control samples, untreated and PHA-stimulated PBMCs were plated into the wells in the absence of MSCs.

Analysis of T-cell activation

After culturing for 5 days both types of PBMCs were simultaneously harvested, washed twice in PBS, and stained by anti-CD4-FITC and anti-CD8-PE antibodies along with anti-CD25-APC, anti-CD69-PerCP-Cy5.5, and anti-HLA-DR-PerCP antibodies (BD) followed by flow cytometry to analyze T-cell activation in both subgroups.

T-cell proliferation assay

To monitor lymphocyte division, both HP and control PBMCs were stimulated with 5 µg/mL PHA in the presence or absence of either abnormal or healthy MSCs for 5 days and were stained with 1.7 µM 5,6-carboxyfluorescein N-succinimidyl ester (CFSE) (Sigma-Aldrich) according to the manufacturer's instructions. PBMCs were then harvested, washed in fresh culture media, and after using anti-CD4-APC and anti-CD8-PE antibodies (BD) for 15 minutes, the alteration of mean fluorescence intensity (MFI) of CFSE in CD4+ and CD8+ T-cells was measured in the FL1 channel with a 530/30 filter.

Statistical analysis

Results were expressed as mean±standard deviation (SD). Normally distributed variables were analyzed by using two-tailed Student's independent samples *t* test. Differences in various activation markers among samples at different time points were tested by analysis of variance (ANOVA) followed by Tukey's HSD test. P values <0.05 were considered statistically significant. All analyses were performed using the SPSS Statistics software, version 19.0 (IBM Corp., Armonk, NY, USA).

Results

Characteristics of BALF samples

Total cell count in HP BALF samples was 9.5-13.0 x 10^5/mL (normal value: 1.0-1.5 x 10^5/mL) with elevated number of lymphocytes (3.6-8.3 x 10^5/mL) and macrophages (2.7-7.5 x 10^5/mL) and moderately altered neutrophil count (0.1-1.3 x 10^5/mL). According to flow cytometry, 61±11%, 38±24% and 23±12% of the lymphocytes were positive for CD3, CD4 and CD8, respectively. Control patients had normal BALF cytology (1.0 x 10^5/mL

macrophages, 0.1 x 10^5/mL lymphocytes, 0.01 x 10^5/mL neutrophils) at mean total cell count of 1.15 x 10^5/mL.

Identification and characterization of MSCs from HP and normal BALF

Both in control subjects and HP patients, MSCs showed positivity for CD73 (93.2±2.1% vs. 97.3±3.7%), CD90 (90.6±1.3% vs. 99.2±0.4%) and CD105 (89.5±5.7% vs. 99.4±0.2%), and were negative for CD34 (data not shown). However, the MFI values in the positive cells were significantly ($P<0.05$) lower in the HP samples, compared to the controls, for CD73 (132.7±11.3 vs. 290.7±15.6), CD90 (72.4±8.9 vs. 217.1±16.3) and CD105 (36.5±5.2 vs. 60.7±3.8) (n=4-6) (Figure 1A). Furthermore, the expression pattern of the three MSC-specific antigens appeared to show substantially less overlap in HP (Figure 1C) vs. control MSCs (Figure 1B) when visualized by confocal microscopy (14) after staining any pairwise combination of CD73, CD90 and CD105. Assuming that the expression of these antigens is required for the development of the MSC phenotype, the decreased coexpression of the three antigens may lead to impaired MSC functions, which may even be associated with the impaired immunosuppressive effect of HP MSCs as suggested by Campioni *et al.* (10).

HP-derived MSCs failed to inhibit T-lymphocyte proliferation

The ratio of proliferating PBMCs in HP and control samples was determined based on the CFSE MFI values with the positive control sample (PBMCs with PHA) considered as 100%. Compared to the unstimulated PBMC-sample, 5 μg/mL PHA stimulation induced maximal proliferation of PBMCs in the absence of MSCs. The proliferation of normal and HP-derived T-cells was downregulated in the entire (CD3+) population in the presence of healthy MSCs to 33% and 45%, respectively, of the positive control sample (Figure 2A). Although this effect was statistically significant ($p<0.01$), this type of MSCs did not completely prevent proliferation since the CFSE MFI values of untreated negative control samples of normal and

HP-derived T-cells were 7-8% of the positive control. In contrast, MSCs from HP could not suppress T-lymphocyte proliferation induced by this mitogenic agonist since the CFSE MFI values were 89% and 86% of the positive control sample for normal and HP-derived T-cells, respectively. Statistical analysis revealed a significantly higher fraction of proliferating lymphocytes in the presence of HP-derived MSCs compared to control MSCs ($P<0.05$) (Figure 2A). Selective evaluation of the proliferation of CD4+ and CD8+ T-cells revealed that control MSCs could also inhibit the proliferation of T-lymphocytes, while HP-derived MSCs were without effect (data not shown).

T-cell activation was not suppressed by MSCs from HP in contrast to normal MSCs

We intended to analyze whether MSCs in HP were unable to modulate the activation of either normal or HP-derived CD4+ and CD8+ T-cells. First, PHA stimulation caused a significant elevation in the fraction of CD25+ cells among CD4+ T-cells from healthy donors (17.2±2.5% after PHA stimulation vs. 1.9±0.9% in unstimulated cells) and children suffering from HP (19.8±3.4% after PHA stimulation vs. 2.5±0.8% in unstimulated cells; Figures 2B and C). While normal MSCs significantly lowered the CD25 positivity on CD4+ T-lymphocytes from both healthy donors (5.7±1.2%) and HP patients (9.5±0.8%), the impact of MSCs from HP was not statistically significant (Figure 2B). Simultaneously, CD8+ T-cells were also analyzed, and in the same fashion, healthy MSCs depressed CD8+ T-cell activation shown by the reduced fraction of CD25+ cells in samples from healthy donors (8.2±1.8% and 4.1±0.6% in the absence and presence of normal MSCs, respectively; $P<0.05$) and in samples from HP patients (7.9±1.9% and 4.6±1.2% in the absence and presence of normal MSCs, respectively; $P<0.05$). At the same time, abnormal HP-derived MSCs were unable to modify the activation of T-cells demonstrated by the fact that the CD25+ positivities in the absence of MSCs were similar to those measured in the presence of HP-derived MSCs cocultured with CD8+ T cells from healthy donors (7.8±1.6%) and HP patients (9.1±1.7%). Hence, there was

a significant difference between the effect of control and HP MSCs in this regard as well ($P<0.05$). In parallel, there was a similar but only minor change in CD69 and HLA-DR expression on both types of T-lymphocytes from normal and HP samples (data not shown).

Discussion

MSCs are in the focus of interest due to their regulatory role in immune responses (6-9). Most data are available from animal models using human BM-derived MSCs in asthma (8), in acute lung injury (15), in pulmonary fibrosis (16) and in acute respiratory distress syndrome with sepsis (6). However, there is no data about the immunoregulatory activity of BALF MSCs in HP.

Due to the impaired lymphosuppressive function of Treg cells showing decreased FoxP3 expression lymphocytes are free to proliferate and accumulate in the lung (12). We believed that nonfunctional lung resident MSCs might be also involved in the impaired immune tolerance. First, we characterized BALF MSCs via immunophenotyping and decreased level of CD105, CD73, and CD90 was observed in HP MSCs by flow cytometry and CLSM similarly to MSCs from BM of hematological diseases showing a loss of immunosuppressive activity (10). Then we investigated the immunoregulatory effect of MSCs obtained from BALF samples from patients with subacute HP in comparison to that of normal cells. T-cells upregulate surface activation markers CD25, CD69 and HLA-DR induced by PHA (17), thus we studied these markers of T-lymphocytes cocultured with MSCs. Isolated MSCs from HP had a significantly lower effect on T-cell function than normal MSCs, which substantially reduced CD4+ and CD8+ T-cell proliferation and activation with decreased CD25 positivity. However, the level of other activation markers was not markedly affected. Furthermore, healthy BM-derived MSCs (18) and normal BALF MSCs (19) induced a similar degree of division arrest anergy in both subtypes of T-cells. In contrast to our data, Glennie *et al.* did not observe an alteration in CD25 positivity by healthy BM-derived MSCs (18). In BALF

samples from active HP patients, the level of CD4+/CD25+ T-cells was much higher supporting the significance of these lymphocytes in this ILD (12). Since we found a significant difference in the ratio of reactive CD25+ T-cells in the presence and absence of functional BALF MSCs in cell cultures, we presume that nonfunctional MSCs in HP may highly participate in processes leading to the raised level of disease-associated reactive T-cells in the lung.

The rarity of HP in childhood prevented us from recruiting more patients into this study, which limits statistical robustness of the conclusions. However, we had striking differences between the control and HP-derived MSCs on T-cell function *in vitro*. These preliminary data also demonstrate that healthy MSCs can even influence HP-derived activated T-lymphocytes suggesting a potential therapeutic approach for HP by administrating isolated normal MSCs to these patients (20). However, further studies are required to confirm these alterations and to analyze the precise mechanism of abrogated lymphomodulatory effect of BALF MSCs in HP, e.g. in connection with their altered level of surface antigens (10).

In conclusion, to the best of our knowledge, this is the first report to demonstrate the abnormal inhibitory capacity of bronchoalveolar MSCs on T-cell responses in this type of ILDs. These results suggest an additional cellular mechanism that allows a pro-inflammatory environment in HP.

Acknowledgements

The study was supported by the TÁMOP-4.2.2.A-11/1/KONV-2012-0045 project. The project was implemented through the New Hungary Development Plan, co-financed by the European Social Fund and the European Regional Development Fund. Béla Nagy Jr was supported by the Lajos Szodoray Grant of the University of Debrecen. The laboratory assistance of Dr. Dávid Pethő is gratefully acknowledged.

References

1. Caplan AI. Molecular and cellular differentiation of muscle, cartilage, and bone in the developing limb. Prog Clin Biol Res 1986;217B:307-318.
2. Sabatini F, Petecchia L, Tavian M, Jodon de Villeroché V, Rossi GA, Brouty-Boyé D. Human bronchial fibroblasts exhibit a mesenchymal stem cell phenotype and multilineage differentiating potentialities. Lab Invest 2005;85:962–971.
3. Kim CF, Jackson EL, Woolfenden AE, Lawrence S, Babar I, Vogel S, Crowley D, Bronson RT, Jacks T. Identification of bronchioalveolar stem cells in normal lung and lung cancer. Cell 2005;121:823-835.
4. Lama VN, Smith L, Badri L, Flint A, Andrei AC, Murray S, Wang Z, Liao H, Toews GB, Krebsbach PH, Peters-Golden M, Pinsky DJ, Martinez FJ, Thannickal VJ. Evidence for tissue-resident mesenchymal stem cells in human adult lung from studies of transplanted allografts. J Clin Invest 2007;117:989-996.
5. Dominici M, Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini F, Krause D, Deans R, Keating A, Prockop Dj, Horwitz E. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy 2006;8:315-317.
6. Mei SH, Haitsma JJ, Dos Santos CC, Deng Y, Lai PF, Slutsky AS, Liles WC, Stewart DJ. Mesenchymal stem cells reduce inflammation while enhancing bacterial clearance and improving survival in sepsis. Am J Respir Crit Care Med 2010;182:1047-1057.
7. De Miguel MP, Fuentes-Julián S, Blázquez-Martínez A, Pascual CY, Aller MA, Arias J, Arnalich-Montiel F. Immunosuppressive properties of mesenchymal stem cells: advances and applications. Curr Mol Med 2012;12:574-591.
8. Bonfield TL, Koloze M, Lennon DP, Zuchowski B, Yang SE, Caplan AI. Human mesenchymal stem cells suppress chronic airway inflammation in the murine ovalbumin asthma model. Am J Physiol Lung Cell Mol Physiol 2010;299:L760-770.

9. Ménard C, Tarte K. Immunoregulatory properties of clinical grade mesenchymal stromal cells: evidence, uncertainties, and clinical application. Stem Cell Res Ther 2013;4:64-71.
10. Campioni D, Rizzo R, Stignani M, Melchiorri L, Ferrari L, Moretti S, Russo A, Bagnara GP, Bonsi L, Alviano F, Lanzoni G, Cuneo A, Baricordi OR, Lanza F. A decreased positivity for CD90 on human mesenchymal stromal cells (MSCs) is associated with a loss of immunosuppressive activity by MSCs. Cytometry B Clin Cytom 2009;76:225-230.
11. Schuyler M, Cormier Y. The diagnosis of hypersensitivity pneumonitis. Chest 1997;111:534-536.
12. Girard M, Israël-Assayag E, Cormier Y. Impaired function of regulatory T-cells in hypersensitivity pneumonitis. Eur Respir J 2011;37:632-639.
13. Papp Á, Bene Z, Gáspár I, Nagy B Jr, Kádár L, Márialigeti T, Bánfi A, Baktai G, Balla G, Nagy B. Decreased VEGF level is associated with elevated ferritin concentration in bronchoalveolar lavage fluid of children with interstitial lung diseases. Respiration 2015;90:443-450.
14. Bolte S, Cordelieres FP. A guided tour into subcellular colocalization analysis in light microscopy. J Microsc 2006;224:213-232.
15. Fang X, Abbott J, Cheng L, Colby JK, Lee JW, Levy BD, Matthay MA. Human Mesenchymal Stem (Stromal) Cells Promote the Resolution of Acute Lung Injury in Part through Lipoxin A4. J Immunol 2015;195:875-881.
16. Jun D, Garat C, West J, Thorn N, Chow K, Cleaver T, Sullivan T, Torchia EC, Childs C, Shade T, Tadjali M, Lara A, Nozik-Grayck E, Malkoski S, Sorrentino B, Meyrick B, Klemm D, Rojas M, Wagner DH Jr, Majka SM. The pathology of bleomycin-induced fibrosis is associated with loss of resident lung mesenchymal stem cells that regulate effector T-cell proliferation. Stem Cells 2011;29:725-735.
17. Caruso A, Licenziati S, Corulli M, Canaris AD, De Francesco MA, Fiorentini S, Peroni L, Fallacara F, Dima F, Balsari A, Turano A. Flow cytometric analysis of activation markers

on stimulated T cells and their correlation with cell proliferation. Cytometry 1997;27:71-76.

18. Glennie S, Soeiro I, Dyson PJ, Lam EW, Dazzi F. Bone marrow mesenchymal stem cells induce division arrest anergy of activated T cells. Blood 2005;105:2821-2827.
19. Jarvinen L, Badri L, Wettlaufer S, Ohtsuka T, Standiford TJ, Toews GB, Pinsky DJ, Peters-Golden M, Lama VN. Lung resident mesenchymal stem cells isolated from human lung allografts inhibit T cell proliferation via a soluble mediator. J Immunol 2008;181: 4389-4396.
20. Lee SH, Jang AS, Kim YE, Cha JY, Kim TH, Jung S, Park SK, Lee YK, Won JH, Kim YH, Park CS. Modulation of cytokine and nitric oxide by mesenchymal stem cell transfer in lung injury/fibrosis. Respir Res 2010;11:16-30.

Legend for Figures

Figure 1. Flow cytometry (A) and CLSM (B, C) analysis of a population of BALF MSCs obtained from control (B) and HP (C) patients. Typical surface expression of CD73, CD90 and CD105 were detectable by both techniques. Histograms of fluorescence intensity values of each parameter depicted that HP-derived MSCs showed a significantly lower level of these markers compared to control MSCs (A). Double staining of HP MSCs (C) and control cells (B) against the aforementioned antigens confirmed the flow cytometric results regarding lower expression in HP-derived MSCs.

Figure 2. Analysis of the proliferation (A) and activation of PHA-stimulated CD4+ (B) and CD8+ (C) lymphocytes in the presence of normal and HP-derived MSCs. Via measuring CFSE positivity by flow cytometry, the proliferation of PBMCs stimulated by PHA was analyzed with HP-derived or normal bronchoalveolar MSCs (A). While normal MSCs were able to reduce proliferation of T-cells, HP-derived MSCs did not show a marked immunosuppressive effect on either normal or HP T-lymphocytes. Results were expressed as the ratio of proliferating PBMCs based on CFSE MFI values calculated from the positive control with 100% (mean±SD, n=3/experiment). T-cell activation was studied by flow cytometric detection of CD25 expression on CD4+ and CD8+ cells (B, C). When T-cells were cocultured with normal or HP MSCs, we found that normal MSCs significantly reduced the fraction of CD25+, activated T-cells, while HP-derived MSCs did not have such an effect under the same experimental conditions. Results are expressed in percent positivity (mean±SD, n=3/experiment).

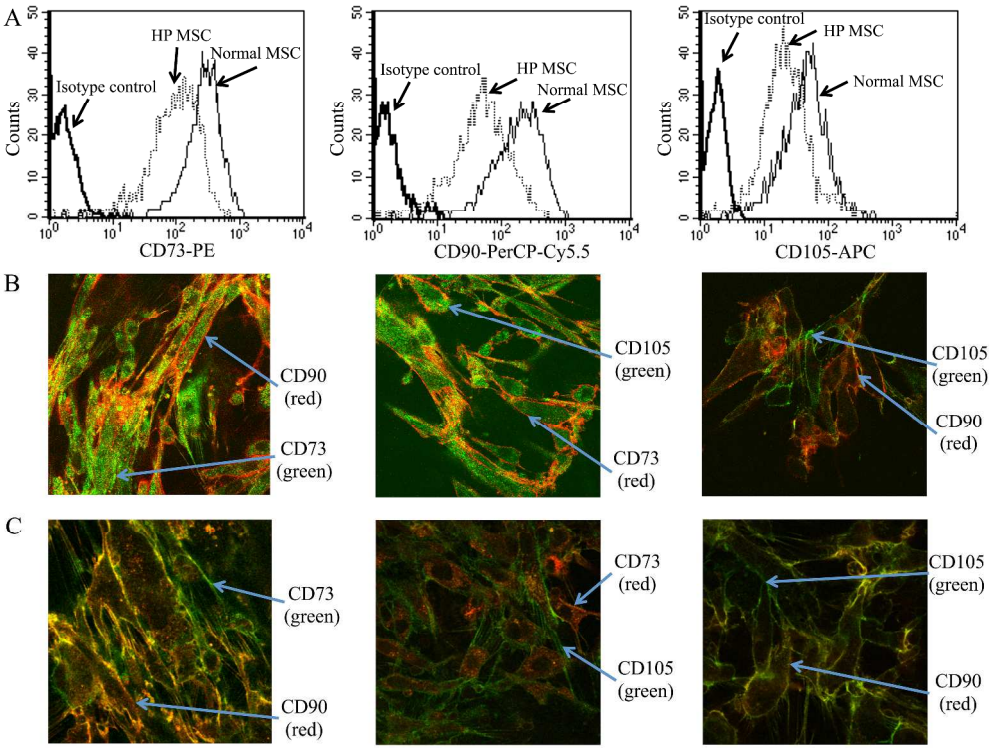

Figure 1.

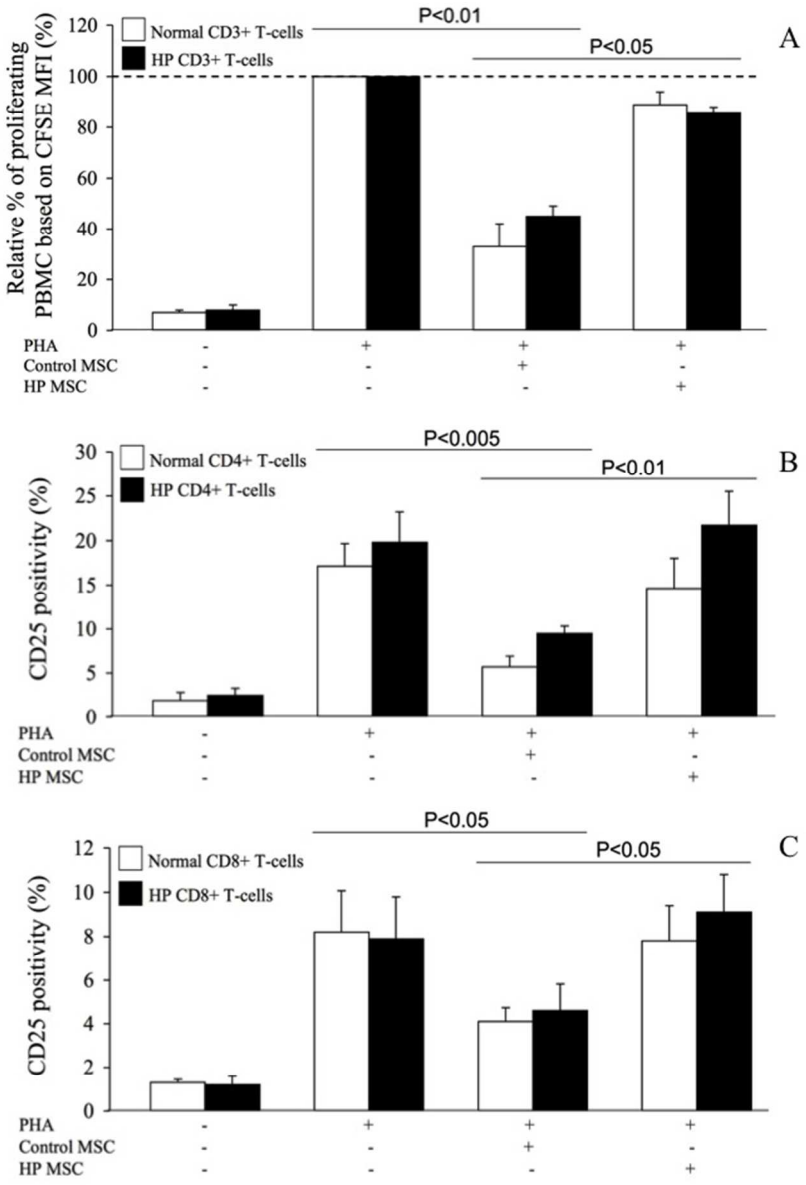

Figure 2.

233x337mm (72 x 72 DPI)