Immunomodulatory Effects of Multipotent Mesenchymal Stromal Cells on T-Cell Populations at Tissue-Related Oxygen Level

A. N. Gornostaeva, P. I. Bobyleva, E. R. Andreeva, L. B. Buravkova

Abstract—Multipotent mesenchymal stromal cells (MSCs) possess immunomodulatory properties. The effect of MSCs on the crucial cellular immunity compartment – T-cells is of a special interest. It is known that MSC tissue niche and expected milieu of their interaction with T- cells are characterized by low oxygen concentration, whereas the *in vitro* experiments usually are carried out at a much higher ambient oxygen (20%). We firstly evaluated immunomodulatory effects of MSCs on T-cells at tissue-related oxygen (5%) after interaction implied cell-to-cell contacts and paracrine factors only. It turned out that MSCs under reduced oxygen can effectively suppress the activation and proliferation of PHA-stimulated T-cells and can provoke decrease in the production of proinflammatory and increase in anti-inflammatory cytokines. In hypoxia some effects were amplified (inhibition of proliferation, anti-inflammatory cytokine profile shift). This impact was more evident after direct cell-to-cell interaction; lack of intercellular contacts could revoke the potentiating effect of hypoxia.

Keywords—Cell-to-cell interaction, low oxygen, MSC immunosuppression, T-cells.

I. INTRODUCTION

MSCs possess immunomodulatory properties that are currently actively studied. It is shown that *in vitro*-derived stromal progenitors affect virtually all types of immune cells. MSC suppressive effect is manifested in inhibition of immune cells activation, proliferation, cytokine production, and others [1]-[3]. The effect of MSCs on T lymphocytes is of special interest because these cells play an important role in the adaptive immune response and are directly involved in the development of graft-versus-host disease. In most papers the suppression of T-cell immune response after interaction with MSCs had been described, but the effect appeared in different extent depending on the experimental conditions. MSC effect on T-cells is determined by many factors: the MSC/T-lymphocyte ratio, time of interaction, T-cell activation inducers, the presence/absence of

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direct cell-to-cell contact, microenvironment and other [1], [4], [5]. The oxygen concentration in local microenvironment, where the interaction occurs is an important parameter which remains virtually unexplored at the moment. In standard laboratory practice the experiments are carried out at the ambient O_2 in CO_2 incubators, although MSC tissue niche and implied foci of MSC-T-cell interaction in tissues are characterized by reduced oxygen concentration - 2-7% [6]. Besides it is shown that *in vitro* hypoxia significantly modifies the properties as MSCs [7]-[10], and lymphocytes [11], [12]. In particular, the significant changes in secretion of MSC paracrine factors have been described under hypoxic conditions [13], [14]. Many of which are actively involved in the implementation of immunosuppression. Therefore it is logic to suggest that immunomodulatory properties of MSCs under hypoxic conditions may change. We have performed a comparative evaluation of the immunomodulatory effects of MSCs on T-cells via direct interaction and paracrine mediators at the ambient O_2 and at the tissue-related "hypoxic" conditions (5% O_2).

II. MATERIALS AND METHODS

A. Isolation and Culture of Cells

MSCs were isolated from the stromal-vascular fraction of human adipose tissue by the method of Zuk et al. [15] in the modification of Buravkova et al. [7]. Immediately after isolation the one part of the cells were placed in a CO_2 incubator with atmospheric oxygen concentration (5% CO_2 + 95% air (20% O2), 37°C, 100% humidity), the other part – into Multigas CO_2-incubator Sanyo (Japan), where the concentration of 5% O_2 was maintained. MSCs were cultured in essential α-MEM with 10% fetal bovine serum (FBS, HyClone, USA), 2 mM L-glutamine (Gibco, USA), and 1% penicillin/streptomycin (Gibco, USA) and were passaged after reaching 70 - 80% of the confluence.

Mononuclear cells were isolated from peripheral blood of healthy volunteers by density gradient (ρ = 1,077, Histopaque-1077, Sigma-Aldrich, USA) according to manufacturer's instructions.

For each donor immune profile was determined by flow cytometry (Epics XL, Beckman Coulter, USA) using antibodies CD45-FITC/CD14-PE, CD3-FITC/CD19-PE, CD3-FITC/CD4-PE, CD3-FITC/CD8-PE, CD3-FITC/16+56-PE, (Immunotec, France) as a negative control IgG-PE/IgG-FITC (Immunotec, France). Isolation of CD3$^+$ cell population by

magnetic separation was performed on the magnetic column according to the manufacturer's procedure (Miltenyi Biotec, Germany). The isolated cells were cultured in RPMI 1640 medium (Gibco, USA) (2 mM L-, 1% penicillin/streptomycin), 5% heat-inactivated FBS. For T-cells activation phytohaemagglutinin (PHA, Sigma, USA) at a final concentration of 10 µg/ml was added into the culture medium.

B. MSC and T-Cell Coculture

T-lymphocytes were added to MSCs constantly cultured at 20 or 5% O_2, up to 70-80% of confluence. Then cells were cocultured for 72 hours in RPMI 1640 with PHA. Coculture of MSCs and T-cells was performed in two versions: the contact interaction ("monolayer") and noncontact ("transwell"). In the "monolayer" T-cells were added directly to the MSCs. To exclude cell contact, MSCs and T-cells were separated by a semipermeable membrane-insert "transwell" (0.4 micron pore diameter) (Costar, USA). Inserts with PHA-activated T-cells were placed over the monolayer MSCs in six-well plates.

The ratio of MSCs/T-lymphocytes in all experiments was about 1:10. Each experiment was reproduced 3-4 times.

C. T-Cell Assay

Using the flow cytometry (EPICS XL, Beckman Coulter; FACS Calibur, Becton Diskinson, USA) the following parameters were determined: viability of T-cells by using AnnexinV-FITC/PI kit (Immunotec (France); activation of T-cells by staining with antibodies against CD3, CD69, CD3/HLA-DR, CD3/CD25. FITC-labeled and PE, IgG (Immunotec (France) as isotype controls were used; the proliferative activity of T-cells using 5,6- carboxyfluorescein succinimidyl ester (CFSE, Invitrogen, USA) - intracellular covalently binding dye. T-lymphocytes were stained with CFSE (5 mM / mL) according standard technique (Suva D. et al., 2007), then the cells were grown in monoculture or with MSCs; the cytokines in the culture medium were evaluated with FlowCytomix human Th1/Th2 11 Plex (Bender MedSystems, Austria).

D. Statistical Methods

Statistical data processing was performed using the software package «Statistica 8.0». Significant differences were assessed using the Mann-Whitney test. Differences were considered significant at p <0.05 (*).The mean value and its standard deviation were calculated using Microsoft Excel.

III. RESULTS

A. Viability

After three days of culture, the share of alive T-lymphocytes in monoculture was averaged about 60% and did not depend on the oxygen concentration. In direct interaction under 5% O_2 MSCs maintained the viability of T-lymphocytes (it was 10% higher, than in T-cell monoculture, p<0,05), in the absence of cell contacts such effect was not found, the viability of T-cells remained at the control level (Table I).

TABLE I
VIABILITY OF T-CELLS AFTER INTERACTION WITH MSCS

Culture condition	Share of viability T-cells, %	
	20% O_2	5% O_2
T-cell monoculture	59,2±1,9	54,8±4,5
"Monolayer"	60,3±2,4	67,1±5,2 *
"Transwell"	64,0±1,9	62,9±0,7

* - significant difference (p<0,05) from T-cells in monoculture

B. Activation

The substantial alteration of T-cell activation was found out after interaction with the MSCs. Early T-lymphocyte activation was enhanced. The share of CD3$^+$/CD25$^+$ cells was increased in "monolayer" (p<0.05). The share of CD3$^+$/CD69$^+$ cells was higher twice on average (p<0.05). In "transwell" the effect was weaker (Fig. 1). Thus, the activation gain was not dependent on the O_2 concentration, but was more pronounced in the presence of cell-to-cell contacts.

On the contrary, the share of T-cells bearing HLA-DR marker of late activation was decreased. In "monolayer" the decline was 50% on average, and was the same at 20 and at 5% O_2. In the absence of direct cell-to-cell contact the effect was depend on the O_2 concentration, in "transwell" this effect in hypoxia was weakened (p<0.05), the difference was significant (Fig. 1).

C. Proliferation

MSCs possessed pronounced anti-proliferative effect. Under ambient O_2 the portion of dividing T-lymphocytes was decreased down to 30% on average. The effect did not depend on O_2 level. In "monolayer' reduced O_2 potentiated this effect - reduction reached 50% (Fig. 2).

D. The Cytokine Production

After 72 hours in T-cell monoculture, the concentration of IL-8 in conditioning medium (CM) was the same at 5 and at 20% O_2. IL-10 and IL-8 production was changed depending on the O_2 level and was significantly higher in hypoxia (Fig. 3).

After interaction with MSCs the secretion of pro-and anti-inflammatory cytokines changed. Given that hypoxia affected significantly the secretion of paracrine factors and their concentration in the CM varied depending on the donor, cytokine changes were assessed as change from of control - PHA T-lymphocytes monoculture. The concentration of cytokine in the T-lymphocyte CM was taken as 100% (Fig. 4).
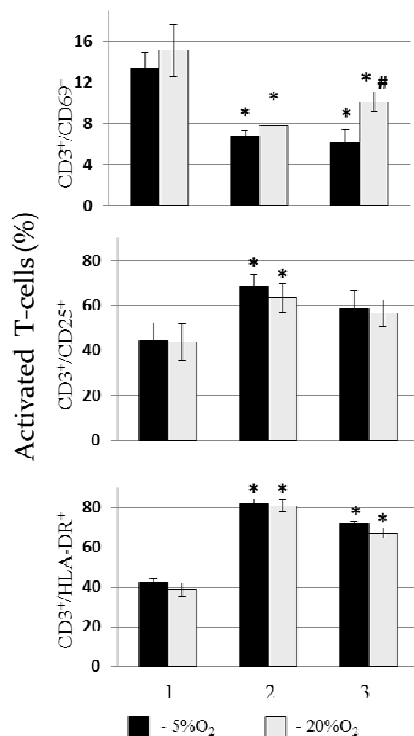
Fig. 1 T-cell activation in different culture condition; * - significant difference from T-cells monoculture (p<0,05); # - significant difference from value at 20% O₂ (p<0,05); 1 – T-cell monoculture, 2 – "Monolayer", 3 – "Transwell"
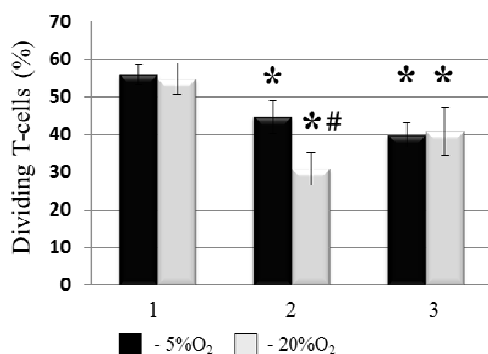

Fig. 2 The proliferative activity of T-cells * - significant difference compared with the T-cell monoculture (p<0,05); # - significant difference compared with the value at 20% O₂ (p<0,05); 1 – T-cell monoculture, 2 – "Monolayer", 3 – "Transwell"

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Was detected a significant anti-inflammatory shift of the cytokine profile. Thus, the levels of proinflammatory TNF-alpha and IL-8 significantly were decreased. In the case of IL-8 effect was not dependent on the O₂ concentration in "monolayer". In the "transwell', the drop was less pronounced. Production of TNF-alpha was reduced in the presence/absence of cell contact, in "transwell" this decrease was more evident (p <0,05) (Fig. 4).

Anti-inflammatory IL-10 significantly increased. In "monolayer" the IL-10 production was more elevated at the 20% O₂, the absence of cell contacts weakened the effect and it was the same as at 20 and at 5% O₂ (Fig. 4).

IV. DISCUSSION

The suppression of lymphocytes activation and subsequent proliferation is considered as qualitative indicator of the immunosuppressive effect of MSCs [4], [16], [17]. The special interest is the impact of MSCs into inhibition of HLA-DR late activation marker bearing T-cells. The presence of this antigen press immune cells to involvement in the "graft versus host" response. There was previously shown that at 20% O₂, MSCs were able to suppress effectively the late T-cell activation [4]. We found that under tissue-related O₂ (5%) the ability of MSCs to suppress the late activation of PHA-stimulated T-lymphocytes has been maintained. However, direct cell-to-cell contact was required for a more pronounced effect. In "transwell" the inhibition at 5% O₂ was less obvious.

The suppressive effects of MSCs on early activation were manifested not in all cases. Thus, along with a decrease of CD3⁺/CD25⁺ and CD3⁺/CD69 ⁺ share after MSC-lymphocytes interaction at 20% O₂ [16], [17], the absence of changes [18], [4] as well as additional stimulation of activation [19] was described. According our data MSCs were able to cause an increase of early T-cell activation at atmospheric/reduced O₂. However, the absence of direct cell-to-cell contact could significantly weakened this effect.

It is known that MSCs possess the pronounced anti-proliferative effect on lymphocytes [4], [5], [20]-[22]. As one of the reason the cell cycle arrest in phase G₀ have been discussed [3].

We have shown that low O₂ has potentiated the suppressive effect of MSCs. The similar effect of MSCs with CD3⁺/CD8⁺ cells was demonstrated in 1% hypoxia [23]. In both studies the enhancing effect of hypoxia was observed only in direct interaction. We assume that in this case the hypoxic impact has been realized through mechanisms which are involved cell interaction. It is necessary to note, that proliferative activity of T-cells in monoculture did not depend on the O₂ level in culture medium that supports the data of others under similar experimental conditions (5% O₂, stimulation with PHA) [11].

The production of pro-and anti-inflammatory cytokines is directly related to the activation of lymphocytes. It is not surprise, that MSCs have an influence on this parameter too. Anti-inflammatory cytokine profile shift that has been demonstrated in our study is consistent with the results of others [4], [24]-[26]. In particular, after interaction with MSCs the reduced TNF-α production [4] and, increased secretion of anti-inflammatory IL-10 [26]-[28] were shown. However, this has been demonstrated at atmospheric O₂ concentration only.
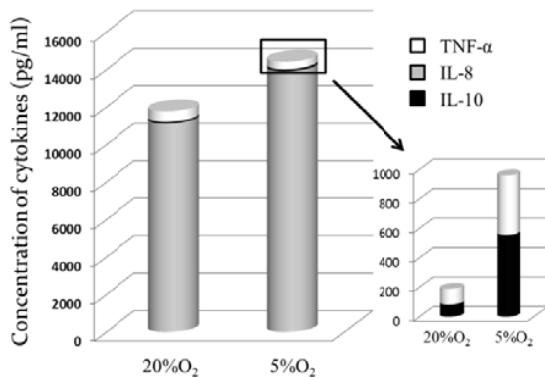
Fig. 3 The cytokine profile of T-cells in monoculture at ambient and tissue-related O_2
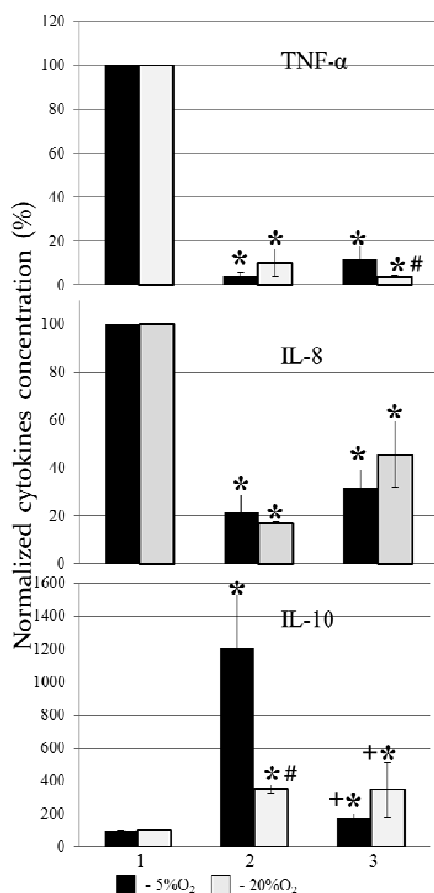

Fig. 4 Change production of cytokines by T-cells after interaction with MSCs; * - Significant difference from monoculture of T-cells (p<0,05); # - Significant difference from the value at 20% O_2 (p<0,05); ✣ - Significant difference from the value in cell contact (p<0,05); 1 – T-cell monoculture, 2 – "Monolayer", 3 – "Transwell"

It is known that the O_2 level can affect the production of soluble factors by different cell types, including T-cells and MSCs. Thus, under hypoxic conditions (2-5% O_2) it was shown the increased secretion of IFN-γ, IL-2, IL-4 and decreased production of IL-1β by stimulated lymphocytes

[11], [12]. Our data also confirmed the increase of TNF-alpha and IL-10 in hypoxic milieu. The shift in cytokine profile of lymphocytes may be associated with the accumulation of extracellular adenosine at low O_2 concentration and increased activity of HIF-1 and HIF-2, which are known to induce the expression of many genes [29].

Under reduced O_2 tension MSC immunosuppressive effects were retained, but their extent might vary depending on the presence/absence of cell-to-cell contacts.

It is important that having pronounced immunosuppressive effect, MSCs have no negative impact on T-cells viability. In "transwell" culture the share of living T-cells remained at the monoculture level. Under direct cellular contacts, the proportion of living T-cells even increased. This impact is probably due to that MSCs secrete factors such as HGF, VEGF, TGF-β, bFGF, GM-CSF which are possessing anti-apoptotic effects [13]. Presumably, for "switching-on" of this activity direct cellular contacts are required in.

According to our data reduced O_2 did not affect the viability of T-cells. The references on the effect of low O_2 on the viability of T-lymphocytes are rather contradictory. Thus, the viability of the activated lymphocytes increased at 5% O_2 [11], did not change at 2% [29], while decreased at 1% O_2 [30]. It can be assumed that the effect of hypoxia on the viability of MNCs is determined by its severity. Perhaps, due to the tissue-related O_2 level implicated in our study we did not observed hampered effect of O_2 concentration on lymphocytes.

Thus, microenvironmental hypoxia is able to potentiate the immunosuppressive effects of MSCs. The shift in the soluble mediator production may be one of a path involved in the realization of immunomodulatory effects. So, under reduced O_2 the production of HGF, TGF-β [13], IL-1β and IL-10 [14] increased. These mediators are involved in the inhibiting of stimulated T-cell proliferation [31]-[34]. Their important role in the suppression of cell division has been validated in experiments with the use of neutralizing antibodies against TGF-β and HGF. The presence of these antibodies restores the proliferative response of T-cells in co-culture with MSCs, but in the absence of antibody the suppression of the proliferation was occured [31]. The stimulatory effect of hypoxia on the production IL-10 can also be a case of immunosuppression potentiating. This interleukin having an anti-inflammatory action can also induce the expression of other immunosuppressive factor - HLA-G [3], [35], [36].

V. CONCLUSION

Thus, MSC immunomodulatory properties are modulated under low O_2 in the local milieu. Wherein some parameters of the immune response (proliferation, cytokine production) in hypoxia are affected significantly, the others change minimally. The absence of direct intercellular contacts attenuates some effects of hypoxia. In this regard, the evaluation of mechanisms of such "selective" hypoxic impact in MSC immunomodulation is of particular interest. These studies are of demand due to the implicated reduced O_2 concentration is tissue related (the expected place of MSCs and lymphocytes interaction), and the results obtained *in vitro*,

with certainty may be extrapolated to the situation *in vivo*.

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