

**Original Research Article****Combination of low O<sub>2</sub> concentration and Mesenchymal Stromal Cells during culture of Cord Blood CD34<sup>+</sup> Cells Improves the Maintenance and Proliferative Capacity of Hematopoietic Stem Cells**

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**Running head:** Low oxygenation and MSC maintain HSCs

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**Abstract**

The physiological approach suggests that an environment associating the Mesenchymal Stromal Cells (MSC) and low O<sub>2</sub> concentration would be most favorable for the maintenance of Hematopoietic Stem Cells (HSC) in course of ex vivo expansion of hematopoietic grafts. To test this hypothesis, we performed a co-culture of cord blood CD34<sup>+</sup> cells with or without MSC in presence of cytokines for 10 days at 20, 5 and 1.5% O<sub>2</sub> and assessed the impact on total cells, CD34<sup>+</sup> cells, committed progenitors (Colony-Forming Cells - CFC) and stem cells activity (pre-CFC and Scid Repopulating Cells -SRC). Not surprisingly, the expansion of total cells, CD34<sup>+</sup> cells and CFC was higher in co-culture and at 20% O<sub>2</sub> compared to simple culture and low O<sub>2</sub> concentrations respectively. However, co-culture at low O<sub>2</sub> concentrations provided CD34<sup>+</sup> cell and CFC amplification similar to classical culture at 20% O<sub>2</sub>. Interestingly, low O<sub>2</sub> concentrations ensured a better pre-CFC and SRC preservation/expansion in co-culture. Indeed, SRC activity in co-culture at 1.5% O<sub>2</sub> was higher than in freshly isolated CD34<sup>+</sup> cells. Interleukin-6 production by MSC at physiologically low O<sub>2</sub> concentrations might be one of the factors mediating this effect.

Our data demonstrate that association of co-culture and low O<sub>2</sub> concentration not only induces sufficient expansion of committed progenitors (with respect to the classical culture), but also ensures a better maintenance/expansion of hematopoietic stem cells, pointing to the oxygenation as a physiological regulatory factor but also as a cell engineering tool.

**Key words: hematopoietic stem cells, pre-CFC, oxygen, hypoxia, mesenchymal stromal cells.**

## Introduction

Starting from the beginning of the nineties, a lot of data accumulated showing the importance of low oxygen concentration for the functional maintenance of stem cells. Hematopoietic stem cell, as a paradigmatic case, was cultured at low O<sub>2</sub> concentrations that were mainly called “hypoxia”. This is a confusing term since 1 to 7 % represent physiological oxygen concentrations of the tissues providing hematopoietic stem cell environment (Winkler et al., 2010; Guitart et al., 2010; Chow et al., 2001; Shima et al., 2009). These physiologically low O<sub>2</sub> concentrations protect the primitive hematopoietic cells against oxidative stress *in vivo* (Parmar et al., 2007; Jang and Sharkis, 2007) and *ex vivo* (Fan et al., 2007; 2008). On the other hand, the ROS (Reactive Oxygen Species) molecules, exhibiting a harmful effect on primitive hematopoietic cells in terms of survival and function, are strongly generated in the culture at 20% O<sub>2</sub> (Yahata et al., 2011; Ito et al., 2006). Most of these papers (Eliasson et al., 2010; Cipolleschi et al., 1993; Koller et al., 1992a,b; Danet et al., 2003; Shima et al., 2009; Csete, 2005), including those of our group (Ivanovic et al., 2004; 2002; Hermitte et al., 2006) refer to experiments performed in cytokine-supplemented liquid cultures. All these data allowed not only a better understanding of self renewal, which is an elementary property of a stem cell, but also a vision concerning the differentiation and the stem cell persistence during development, as a mirror of the evolution : our concept called “oxygen stem cell paradigm” (Ivanovic, 2009). This concept was already employed in the cell engineering enabling new advances in several fields. As illustrative examples, *ex vivo* expansion of hematopoietic stem and progenitor cells (Reviewed in: Ivanovic & Boiron 2009; Ivanovic et al., 2011) and *ex vivo* RBC (Red Blood Cells) production could be cited (Vlaski et al., 2009).

The liquid culture however, does not approximate at best in vivo stem cell environment. Some other culture models as the co-culture of hematopoietic cells with stromal cells could certainly be considered to better mimic the complexity of in vivo hematopoietic environment – in line with the “stem cell niche” concept (Schofield, 1978). As a matter of fact, the invention of “long term cultures” (Dexter et al., 1977; Winkler et al., 2010) was based on this approach. Recently, lots of papers were published (Li et al., 2007; Walenda et al., 2010; Magin et al., 2009) evaluating the potential advantages of these co-cultures with respect to liquid cultures in terms of ex vivo expansion of progenitor and stem cells (Zhang et al., 2004; Wang et al., 2004; Huang et al., 2007; Robinson et al., 2006; McNiece et al., 2004; Fei et al., 2007; Chivu et al., 2004; Van Overstraeten-Schlögel et al., 2006; Yamaguchi et al., 2002). The results mainly agreed that the presence of stromal cells provides a better maintenance of stem cell potential. On the contrary, this type of culture is more complex than liquid culture and hence, difficult to be transferred at clinical grade level. Therefore, only one clinical trial using hematopoietic cells produced in co-cultures has been published so far (Robinson et al., 2011).

It is interesting that low oxygen concentrations (in the above mentioned range) also influence proliferative capacity (Carrancio et al., 2008; Lavrentieva et al., 2010; Grayson et al., 2007), viability (Lavrentieva et al., 2010), motility (Rosová et al., 2008), differentiation potential (Fehrer et al., 2007; Grayson et al., 2006) and proliferation kinetics (Dos Santos et al., 2010) of Mesenchymal Stem Cells (MSC) also enhancing their life span (Jin et al., 2010; Tsai et al., 2011) and modifying their cytokine secretion (Potier et al., 2007; Annabi et al., 2003; Li et al., 2010).

Since there is an obvious interest, both from scientific and technologic viewpoint to optimize ex vivo expansion cultures, we tried to combine the presence of mesenchymal stromal cells with low O<sub>2</sub> concentrations, supposed to be physiological for hematopoietic stem and progenitor cells. In addition, the main factor of cellular response to low O<sub>2</sub> concentration, HIF-1 $\alpha$ , is getting stabilized

at O<sub>2</sub> concentrations  $\leq$  5% (Kirito et al., 2009; Jewell et al., 2001; Danet et al., 2003; Jiang et al., 1996). Thus, our hypothesis that the proliferative capacity of hematopoietic stem cells should be better maintained if they were cultured together with mesenchymal stromal cells at appropriately low O<sub>2</sub> concentrations is tested by exposing to 5 and 1.5 % O<sub>2</sub> cord (placental) blood (CB) CD34<sup>+</sup> cell cultures on the layer of bone MSC.

## **Materials and Methods**

### **Preparation and culture of cells**

#### ***Full-term delivery CB samples***

Samples were collected (with the mother's informed consent) in sterile bags containing anticoagulant and delivered to the Cell Therapy Unit of the (Etablissement Français du Sang Aquitaine-Limousin, Site de Bordeaux). Only samples not appropriate for banking were used in our experiments.

#### ***CD34<sup>+</sup> cell purification***

Mononuclear cells were isolated on Ficoll (d = 1077 g/l, Lymphoprep Nyegaard, Oslo, Norway). The CD34<sup>+</sup> fraction was isolated with one or two runs of immunomagnetic selection on MiniMACS columns (Miltenyi Biotec, Gladbach, Germany and Miltenyi Biotec GmbH, Paris, respectively) according to the manufacturer's instructions. Flow cytometry controls showed >90% pure CD34<sup>+</sup> cell populations. CD34<sup>+</sup> cells were also selected at the end of primary culture/co-culture (C1) by the same procedure above described to analyze their CD133 expression and ALDH activity.

### ***Preparation of Mesenchymal Stromal Cell (MSC) adherent layers***

MSCs were isolated by culture from bone marrow of healthy donors obtained from filters used for allogeneic hematopoietic grafts preparation. After red blood cell lysis in ammonium chloride (8.2 mg/ml; Sigma-Aldrich, France), bone marrow cells were cultured in  $\alpha$ -MEM medium (Minimum Essential Medium Alpha Modification) (BioWhittaker, Lonza, Verviers, Belgium) supplemented with 10% fetal calf serum (FCS) (HyClone Perbio, France), penicillin (100 units/ml), streptomycin (100  $\mu$ g/ml) (Cambrex BioWhittaker, Walkersville, MD, USA), glutamine (2 mM) (Sigma-Aldrich, France) and FGF2 (1 ng/ml) (PeproTech, Levallois Perret, France) (referred to as “complete  $\alpha$ -MEM”). After 3 to 4 days at 37°C, 5% CO<sub>2</sub>, non-adherent cells were removed and medium was changed. Adherent cells, mainly MSCs, were trypsinized upon reaching confluence, harvested and cultured at a concentration of 10<sup>3</sup>/cm<sup>2</sup> for 1-3 weeks. MSCs were cryo-preserved or not before usage. For this study, thawed MSCs ( $\leq$  5th passage) were incubated in 12-well plates (2.5x10<sup>4</sup>/ml/well) in complete  $\alpha$ -MEM at 37°C, water-saturated atmosphere, 95% air and 5% CO<sub>2</sub> until monolayer confluence 2 or 3 days later.

### ***Co-culture of MSCs and CD34<sup>+</sup> cells***

Purified CD34<sup>+</sup> cells were seeded at 2x10<sup>4</sup>/ml/well with or without MSCs monolayers in 12-well plates (Nunc, Denmark) and cultured for 10 days in Stem Alpha A serum free medium (Stem Alpha SA, St. Genis l'Argentière, France) supplemented with human cytokines [Stem Cell Factor (SCF) and Granulocyte Colony-Stimulating Factor (G-CSF) (Amgen-Roche, neupogen, 100 ng/ml each), Thrombopoietin (TPO) (PeproTech; 20 ng/ml) and Interleukin (IL)-3 (Recombinant Human-rHu ; Pepro Tech, London; 0.5 ng/ml)].

The cultures were incubated at 37°C either at 5% CO<sub>2</sub> and 20 (Incubator Igo 150 Cell Life, Jouan, St. Herblain, France), 5 or 1.5% O<sub>2</sub> (Proox Culture Chamber with O<sub>2</sub> and CO<sub>2</sub> regulators

BioSpherix, Ltd., Redfield, NY, USA) in a water-saturated atmosphere. At Day-10, non-adherent cells were carefully harvested and washed, the viable cells enumerated (trypan blue exclusion test) and processed for further analyses as described below.

### **Flow cytometry analysis:**

#### ***Membrane antigens expression:***

After 10 days of culture with or without MSCs at different O<sub>2</sub> concentrations, non-adherent cells were washed in Basal Iscove medium (Biochrom AG, Berlin, Germany) and suspended in phosphate buffer saline, labeled (20 minutes in dark) with anti-CD33 and -CD13 (myeloid markers), -CD19 (lymphoid marker), -CD41 and -CD61 (megakaryocytic markers) and -CD34 monoclonal antibodies in order to observe lineage differentiation. The anti-CD45 (pan-hematopoietic marker) was used to exclude the non-hematopoietic cells (MSC cells) from analysis. For analysis of undifferentiated cells, anti-CD133 (Hess et al., 2006; Isidori et al., 2007) was used in combination with CD34 expression and ALDH activity (see below). All these antibodies were conjugated either with FITC, PE, PercP or APC fluorochromes as mentioned in table 1. After labelling, cells were washed and re-suspended and their phenotype was analyzed by flow cytometry (FACSCalibur, Becton Dickenson). Fluorochrome-conjugated isotype antibodies were used to determine the level of non-specific binding.

***Aldehyde dehydrogenase (ALDH) activity:***

The high activity of ALDH is known as a marker of freshly isolated primitive hematopoietic progenitors and stem cells (Hess et al., 2004; 2006).

ALDH labelling was performed using Aldefluor reagent (StemCell Technologies, Grenoble, France) according to the manufacturer's instructions. Briefly, activated Aldefluor substrate (1  $\mu$ l) was added to  $0.1 \times 10^6$  CD34<sup>+</sup> cells (purified at Day-0 and after culture (Day-10)) suspended in 100  $\mu$ l Aldefluor assay buffer and incubated for 30 to 60 minutes at 37°C to allow the conversion of Aldefluor substrate, a green fluorescent product retained within the cell (Storms RW et al, 1999). As negative control, an aliquot of Aldefluor-stained cells was incubated with 1  $\mu$ L of a specific ALDH inhibitor (DEAB). Cells were co-stained with human CD34-PE and CD133-APC antibodies.

**Colony-Forming Cell (CFC) Assay**

To detect the committed hematopoietic progenitors, freshly isolated CD34<sup>+</sup> or cultured cells harvested at Day-10 were seeded in a cytokine-supplemented methylcellulose medium Stem Alpha ID (Stem Alpha SA) in a Petri dish or 24-well plates (Nunc, Denmark) respectively, at concentration of 250 cells/ml or volume of 0.5  $\mu$ l / 250  $\mu$ l, respectively (Ivanovic et al., 2004). After 14 days of incubation at 37°C in water-saturated atmosphere, 20% O<sub>2</sub> and 5% CO<sub>2</sub>, the CFCs [Colony-Forming Units-Granulocyte Macrophage (CFU-GM), Burst-Forming Units-Erythroid (BFU-E), and Colony-Forming Units-Mix (CFU-Mix)] (>50 cells) were enumerated using an inverted microscope.

### **Pre-CFCs (primitive progenitors) Assay**

The production of committed progenitors during a long-term secondary culture (C2) reflects the presence and quantity of a primitive population situated between progenitors and stem cells (pre-CFC) in C1 (Ivanovic et al., 2000; Ivanovic et al., 2002; Kovacević-Filipović et al., 2007). C1 cells (total Day-10 progeny of 20,000 CD34<sup>+</sup> cells plated at Day-0 in 1-ml cultures) were washed and resuspended in 1 ml of medium. The C2 was carried out with 200 µl of C1 cell suspension (in each condition) and the volume was adjusted to 1 ml of cytokine-supplemented (IL-3, SCF, G-CSF and TPO, as C1 culture concentrations) serum-free medium (Stem Alpha A), and cultured for 8 weeks at 20% O<sub>2</sub> with a weekly demi-depopulation and addition of fresh medium that was taken into account for normalization of total CFC contents in C2. Every week of C2, the cells were plated in methylcellulose to detect committed progenitors as mentioned above.

### **Scid-Repopulating Cells (SRC) Assay**

Stem cell activity was evaluated by in vivo assay – transplantation of human cells to immunodeficient (NOG/Scid) mice. After 10 days, 1000 cells sorted on Day-0 (and stored at -80°C), or their progeny obtained under different C1 conditions were injected to 6- to 10-week-old NOG/Scid mice conditioned by intraperitoneal injections of Busulfan 25 mg/kg (Busilvex®, Pierre Fabre, Boulogne, France) at D-2 and D-1 (according to the previously established protocol) (Robert-Richard et al., 2006) (central animal-keeping facility of University of Bordeaux 2). Positive control (mice injected with 15000- 20000 CD34<sup>+</sup> purified at D0) and negative control (non-injected mice) were included. After 8 weeks, the animals were euthanized, their femora were isolated and the BM was flushed with 1 ml of RPMI 1640 complemented with human albumin (0.4%). Cells were washed [phosphate-buffered saline, EDTA 5 mM, human albumin (0.4%)] and

incubated with a FITC-coupled anti-human CD45, PE-coupled anti-human CD19 and APC-coupled anti-human CD33 antibodies for 20 minutes at 4°C in the dark.

Washed cells were analyzed on a FACSCalibur (Becton, Dickinson) to detect and quantify human chimerism. To avoid false-positive results due to control isotype, we used non engrafted mice as controls.

To detect the progenitors (CFC) of human origin in murine bone marrow, 15 and 30µl of femoral cell suspension (see above) were seeded per 250 µl of methylcellulose (in duplicate) (Stem Alpha-1; Stem Alpha SA) supplemented with 10% human plasma, 25 ng/mL (rHu IL-3) (Pepro Tech, London), 25 ng/mL rHu GM-CSF) (R&D Systems Europe, Lille, France), 50 ng/mL rHu SCF (Amgen-Roche, Neupogen) and 3 U/mL rHu Erythropoietin (EPO) (Tebu- Bio, Le Perray, France) (Robert-Richard et al., 2006).

After 14 days of incubation, the human CFCs (>50 cells) were enumerated using an inverted microscope.

### **Cytokine production**

Quantitative studies of cytokine production during C1 (primary culture/co-culture) and the culture of MSC alone (at 20 and 5% O<sub>2</sub>) were carried out by the multiplexed bead immunoassay combined technique. The supernatants were collected and stored at -20 and – 80°C. Multiplex analysis was performed using the kits purchased from Biosources- Invitrogen [human IL-1β (interleukine-1β), human IL-10 (interleukine 10), human IL-6 (interleukine 6) and human GM-CSF] according to the manufacturer's protocol. To generate a standard curve, serial dilutions of appropriate standards provided by the manufacturer were prepared and used. The limits of detection for growth factors were as follows: IL-1β (<15 pg/ ml), IL-10 (<5 pg/ ml), IL-6 (<3 pg/ ml), GM-CSF (<15 pg/ ml). The fluorescent signature was detected by Luminex 100 Analyzer.

### Statistical analysis

The t-test was used to evaluate the significance of difference between the experimental conditions.

In the experiences with mice, Mann-Whitney test was used.

### Results

#### **Impact of oxygen concentration on amplification of total cells, CD34<sup>+</sup> cells and clonogenic progenitors (CFC) after 10 days of C1.**

In culture conditions used here, the decrease in O<sub>2</sub> concentration (from 20, 5 and 1.5 %) provokes a relative decrease in fold expansion of total cells both in culture and co-culture. In both cases, at 1.5 % O<sub>2</sub>, this decrease was close to half the value found at 20 %. There is a trend to enhance the fold expansion in co-culture comparing to culture, but it was not statistically significant (Fig.1A).

This enhancement in co-culture is however evident for CD34<sup>+</sup> cells: 2 to 3 times higher fold expansion was evidenced in co-culture with respect to the cultures (comparing the same O<sub>2</sub> concentrations). Again, the decrease of fold expansion was paralleled by decrease in O<sub>2</sub> concentration becoming statistically significant at 1.5 % O<sub>2</sub> vs. 20 % O<sub>2</sub> in co-culture (Fig.1B).

When the fold expansion of committed progenitors (CFU-GM, BFU-E and CFU mix) was analyzed, the situation was similar to the one of CD34<sup>+</sup> cells (Fig. 1C). This fold expansion at 1.5 and 5% O<sub>2</sub> was lower than at 20% O<sub>2</sub> after 10 days in co-cultures. However, co-culture at low O<sub>2</sub> (1.5 and 5%) assured similar and even higher fold expansion than classical culture at 20% O<sub>2</sub>.

### **Effect of low O<sub>2</sub> concentrations and co-culture on membrane markers expression and ALDH activity**

We did not find a significant difference in terms of the expression of myeloid markers (CD33 and CD13) or megakaryocytic markers (CD41 and CD61) in our conditions. The CD19 (lymphoid marker) was not detectable.

However, the decrease in O<sub>2</sub> concentration results in decrease in percentage of CD34<sup>+</sup> cells expressing CD133. This decrease is statistically significant in 1.5 % of O<sub>2</sub> (Fig. 2A). Moreover, both 5 % and 1.5% O<sub>2</sub> concentrations resulted in a marked decrease (one half) of CD34<sup>+</sup> cells expressing ALDH<sup>+</sup> activity (Fig. 2B).

### **Low O<sub>2</sub> concentrations better maintain primitive progenitors (pre-CFC) in co-culture**

Committed progenitors production in secondary liquid cultures initiated with the cells recovered from primary cultures or co-cultures demonstrated that:

- a. This sub-population of primitive progenitors and stem cells was not better preserved at low O<sub>2</sub> concentration with respect to 20% O<sub>2</sub> in a simple culture since the small observed differences were not statistically significant (Fig. 3A).
- b. Production of committed progenitors by the cells issued from co-culture at 1.5 and 5 percent is significantly higher with respect to 20%. This difference is more pronounced at Week-2 of secondary culture (3 fold higher at 1.5 and 5 % with respect to 20 % of O<sub>2</sub>) (Fig. 3B inset).

### **Low O<sub>2</sub> concentrations increase Scid Repopulating Cells (SRC) activity in co-culture**

When 1000 Day-0 CD34<sup>+</sup> cells or their Day-10 progeny were injected to NOG/Scid mice, human cells were detected in some mice but were not in others 7 to 8 weeks later, demonstrating that the

dose of 1000 cells falls within the linear portion of a sigmoid dose/response (transplanted/cell engraftment) curve, i.e. this cell number represents a limiting dilution dose. Based on human CD45 chimerism in NOG/Scid mice (SRC-<sub>CD</sub>), it is evident that the co-culture better preserves this sub-population of stem cells than simple culture (Fig. 4A). In addition, this effect is enhanced with decrease in O<sub>2</sub> concentration (Fig. 4A). Although the proportion of positive mice (the human CD45<sup>+</sup> cells detected in their bone marrow) increases in parallel with a decrease in O<sub>2</sub> concentration, even in simple culture, this increase reaches its peak in co-culture, starting from 5% O<sub>2</sub> (Fig. 4B). So, at 1.5 and 5% O<sub>2</sub>, the co-cultures seem to maintain a full SRC-<sub>CD</sub> Day-0 number. When analyzing the percentage of CD45<sup>+</sup> cells per femur of engrafted mice, this low O<sub>2</sub> concentration-dependent maintenance of stem cells became even more pronounced. The most pronounced effect, however, remains a combination of co-culture with 1.5% O<sub>2</sub> (almost 6 times higher values compared to Day-0 cells) (Fig. 4C). Having in mind that the frequency of SRC<sub>CD</sub> at Day-0 and at Day-10 in co-cultures at 5 and 1.5% O<sub>2</sub> seems to be similar (as shown in Fig. 4B), this result suggests that an appropriately low O<sub>2</sub> concentration, associated with the presence of stromal cells enhanced the individual proliferative capacity of SRC. An analysis of human CD19 and CD33<sup>+</sup> cells in bone marrow of the same mice revealed that low O<sub>2</sub> concentrations combined with co-culture maintained the differentiation balance between lymphoid and myeloid line similar to that of Day-0 cells (Fig. 4D).

If the analysis was based on human progenitors (CFC), generated during 7 to 8 weeks after injection of cells to NOG/Scid mice, it is obvious that the simple cultures maintained better this stem cell sub-population (SRC-<sub>CFC</sub>) activity at lower O<sub>2</sub> concentrations (5 and 1.5%) (Fig. 5A, inset). The co-culture by itself has a positive effect on the SRC-<sub>CFC</sub> maintenance, even at 20 %. In general, co-culture ensures maintenance of proliferative capacity of SRC-<sub>CFC</sub> at a level similar to Day-0 (Fig. 5A). However, if the differentiation ratio (CFU-GM and BFU-E) was analyzed, only

co-culture at 1.5 % O<sub>2</sub> maintained the Day-0 ratio while in all other conditions of culture or co-cultures, this ratio shifted in favour of CFU-GM. This result suggests that a low O<sub>2</sub> concentration is necessary to maintain not only the proliferative capacity of stem cells, but also their differentiation potential.

### **Impact of O<sub>2</sub> concentration on cytokine secretion**

Production of IL-6 is higher in co-culture at 5% with respect to 20% O<sub>2</sub> until Day-5, but at Day-7 this trend is not statistically significant (Fig. 6A). This “stagnation” in IL-6 production was not detected in simple MSC culture: it increases exponentially, tripling at Day-7 with respect to Day-5 (Fig. 6B). We have not noted a secretion of IL-6 in simple CD34<sup>+</sup> culture. These data implying the consumption of IL-6 by hematopoietic cells amplified in co-culture. The IL-1beta, IL-10 and GM-CSF were not detected in our conditions.

### **Discussion**

The most interesting result of this study is that association of low O<sub>2</sub> concentration (1.5 % O<sub>2</sub>) and co-culture was the condition able not only to maintain the frequency and full differentiation capacity of stem cells (SRC), but also to enhance their proliferative capacity. The last point is suggested by more than 5 times higher human chimerism in engrafted murine bone marrow, in situation when SRC frequency did not change with respect to Day-0 (the same proportion of “positive” mice).

Being heavily stimulated by cytokines (very high concentrations with respect to the physiological ones) the hematopoietic stem and progenitor cells in ex vivo expansion cultures represent rather a paradigm of regenerating hematopoiesis than of steady state one (Ivanovic and Boiron, 2009). It is

evident that in these circumstances a high differentiation pressure on stem cells exists (analogous to the model of Loeffler et al., 1989). The above mentioned results confirm the major role of micro-environment (mimicked by stromal cells and low O<sub>2</sub> concentrations in this case) for stem cell maintenance. On the basis of the analysis of the results concerning co-cultures presented here, it is evident that the better maintenance of stem cells is paralleled with the lower numerical expansion of committed progenitors: the lowest number of CFC in co-culture was found at 1.5% of O<sub>2</sub> where the best preservation of SRC functional capacity was detected. It should be stressed, however, that this number of committed progenitors corresponds to their maximal expansion in simple culture at 20 % O<sub>2</sub>. Thus, co-culture combined with appropriately low O<sub>2</sub> concentration (1.5 %) could provide a tool to get a big number of committed progenitors, in parallel with complete preservation or even enhancing of stem cells functional capacities. These data stress the paramount importance of respecting physiological conditions in ex vivo cell engineering. In this regard, it was demonstrated that “hyperoxia” (20%) favors a high production of ROS inducing important negative effects in different cell types (Yahata et al., 2011; Ito et al., 2006; Fan et al., 2007; 2008; Kubo et al., 2008; Carrera et al., 2010). In line with the SRC data are the results concerning pre-CFC. Again, only the low O<sub>2</sub> concentration conditions combined with co-culture were able to preserve a high capacity of repopulation of secondary culture with committed progenitors (pre-CFC activity). However, unlike SRC<sub>CD</sub>, where 1.5% O<sub>2</sub> concentration was most effective, both 5 and 1.5% were effective for pre-CFC. This underlines functional difference between two stem cell populations, a schedule completely in line with all the data related to the heterogeneity of stem and progenitor cells sub-populations with respect to their demands for O<sub>2</sub> published so far (Cipolleschi et al., 1993, Simsek et al., 2010., Reviewed in Ivanovic et al 2009; Parmar et al., 2007).

In our conditions, CD34<sup>+</sup>/CD133<sup>+</sup> cells, as well as the degree of ALDH<sup>+</sup> activity in CD34<sup>+</sup> cell population behaved with respect to O<sub>2</sub> concentrations similarly as committed progenitor's number:

decreasing in parallel with O<sub>2</sub> concentrations. Even in steady state, most of CD34<sup>+</sup>/CD133<sup>+</sup> cells and CD34<sup>+</sup>/ALDH<sup>+</sup> cells are, in fact, the committed progenitors. So, this finding is perfectly coherent, having in mind a low frequency of stem cells in these phenotypically/metabolically-characterized populations. The relative enhancing of stem cells (SRC and pre-CFC) frequency at low O<sub>2</sub> concentrations could not be evidenced in overwhelming progenitor population, having opposite numerical trend.

Although several studies concerning low O<sub>2</sub> concentration (5%) for co-cultures of hematopoietic and stromal cells were published (Koller et al., 1992b; Song et al., 2009; Zhambalova et al., 2009), our study is first performed in serum-free conditions, comparing 1.5 and 5% O<sub>2</sub> and using in vivo approach (SRC) for stem cell detection. These studies could not be directly compared due to differences in conditions: Zhambalova et al studied the culture of Mononuclear Cells (MNC) on stromal cells without adding cytokines, Koller et al did not employ SRC assay, Song et al employed the osteoblasts as stromal cells... Nevertheless, the common feature in all studies, a better maintenance of primitive cells in co-culture at low O<sub>2</sub> concentrations, is confirmed here with the “gold standard” assay for human stem cell detection – in vivo SRC assay. Furthermore, our study introduces the notion of enhancing of proliferative activity of SRC co-cultured with stromal cells.

The detailed examination of experiments done with IL-3 imply that its pro-differentiation effect on stem cells is related i) to the presence of serum in medium and ii) to a high dose employed (Ivanovic, 2004). So, IL-3 in a low dose in a serum-free culture showed some synergistic benefic effect with low O<sub>2</sub> concentration on stem cell maintenance and, simultaneously, on expansion of committed progenitors (Ivanovic et al., 2004). So, the serum-free condition employed here enabled us to add a low dose of IL-3 to cytokine cocktail.

Positive effect of low O<sub>2</sub> concentration associated to MSC on HSC maintenance could result from numerous factors whose production is affected by O<sub>2</sub> concentration (HGF, VEGF, IL1, THF, IL-10...) (Potier et al., 2007; Annabi et al., 2003; Li et al., 2010, Tamama et al., 2011). The list of genes coding cytokines, growth factor and their receptors, as well as other molecules exhibiting the “HIF-1-binding sequence” - HRE (Hypoxia Response Element), is long (reviewed in: Semenza 2007). Our results suggest that at least in part, this effect could be operated by IL-6 (Rodríguez et al., 2004; Ulich et al., 1991) whose production by MSC is stimulated at low O<sub>2</sub> concentration. This viewpoint is in line with recent literature data, demonstrating a decrease of expansion of hematopoietic cells cultivated with bone marrow MNC supernatants treated with an IL-6 inhibitor. This effect was only observed with the supernatant from low O<sub>2</sub> concentration culture (Li et al., 2011). Similarly, the positive effects on committed progenitors in co-cultures at low O<sub>2</sub> concentration upon IL-17 stimulation seem to be associated with an increased IL-6 secretion (Krstic et al., 2009). Furthermore, this scenario is supported by data obtained with murine bone marrow cells: the exogenous IL-6 synergizes with the low O<sub>2</sub> concentration (1%) to functionally maintain the pre-CFCs subpopulation, an effect undetectable at 20% O<sub>2</sub> (Kovacevic-Filipovic et al., 2007).

In conclusion, the data presented in this paper firmly support the thesis that low O<sub>2</sub> concentration is a physiological regulator of hematopoiesis. In fact, it would be better to say that the data obtained in cultures at low O<sub>2</sub> concentrations approximate better the physiological reality and therefore, some data obtained at 20% O<sub>2</sub> might be rather considered as an artifact than as a “standard” control (Ivanovic, 2009).

In addition, from a biological viewpoint (excluding safety and technology considerations), a co-culture with mesenchymal stromal cells seems to be an interesting start-point for development of clinical grade ex vivo expansion procedure.

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## LEGENDS TO THE FIGURES

Figure 1. **Impact of oxygen concentrations and MSC on hematopoietic cells amplification in a Day-10 culture.** Purified CD34<sup>+</sup> cells were cultured without (white bars, simple cultures) or with (black bars, co-cultures) mesenchymal stromal cells. A. Total cells, B. CD34<sup>+</sup> cells, C. Colony Forming Cells (CFC). Results are expressed as mean  $\pm$  SE; n= 8 to 9; \* p<0.05; \*\* p<0.01.

Figure 2. **Analysis of CD34<sup>+</sup> cells for CD133 expression and ALDH activity.** At the end of co-culture, CD34<sup>+</sup> cells were purified and analyzed by flow cytometry for A. Expression of CD133. B. ALDH activity. Results are expressed as mean  $\pm$  SE ; n = 7; \* p<0.05; \*\* p<0.01.

Figure 3. **Impact of low O<sub>2</sub> concentrations and MSC on pre-CFC activity.** The curves represent generation of CFC during 8 weeks of secondary cultures (C2) initiated with the cellular products obtained after 10 days of primary culture/co-culture (C1) (20.000 CD34<sup>+</sup> cells at Day-0 (D0)). A. Time course of CFC in C2 initiated with the cells issued from simple cultures. B. Time course of CFC in C2 initiated with the cells issued from co-cultures. Inset: zoom on situation in C2 after 2 weeks. Black lozenges/bars: 20% O<sub>2</sub>; white squares/bars: 5% O<sub>2</sub>; gray triangles/bars: 1.5% O<sub>2</sub>. Results are expressed as mean + SE; n = 5 to 9; \* p <0.05.

Figure 4. **Impact of low O<sub>2</sub> concentrations and MSC on SRC activity (SRC-CD).** The activity of Scid Repopulating Cells was detected on the basis of human CD45<sup>+</sup> chimerism in bone marrow of NOG/Scid mice (SRC-CD) 7 and 8 weeks after injections with 1.000 steady state cord blood cells (D0) or their progeny after 10 days of cultures without (simple cultures) or with (co-cultures) MSC under 20%, 5% or 1.5% O<sub>2</sub>. A. Frequency of positive mice for human engraftment (human CD45<sup>+</sup> chimerism >0.5 %). The cultures, co-cultures and Day-0 point (D0) are represented by white, black and grey bars, respectively. The number (N) of positive per number of analyzed mice is presented inside bars for each condition separately. Note that the percentage of engraftment with respect to Day-0 was reduced in cultures but fully maintained in co-cultures at low O<sub>2</sub> concentrations (5 and 1.5%) (p<0.05). B. Individual mice bone marrow labeling with human CD45<sup>+</sup> antibody; white,

black and grey circles represent cultures, co-cultures and D0, respectively. C. Median human CD45 chimerism in positive mice. D. Relative proportion of B lymphoid (CD19<sup>+</sup>, white bars) and myeloid (CD33<sup>+</sup>, black bars) cells within human CD45<sup>+</sup> cells. \* p<0.05; \*\* p<0.01; \*\*\* p<0.001. The number of “positive” mice (N) for each corresponding condition in (C) and (D) is stated inside the bars in (A) and individually visible in (B). Cumulative results from 4 independent experiments presented.

**Figure 5. Impact of oxygen concentrations and MSC on SRC activity (SRC-CFC).** The mice were sacrificed 7 to 8 weeks after injection of 1.000 steady state cord blood cells (D0) or their Day-10 (D10) cultures/co-culture progeny, and their bone marrows were harvested and analyzed for human CFC content. A. Individual mice human CFC content per femur. Inset: median CFC content per femur (only mice positive for human CD45 antigen were considered – the number of mice analyzed is given in corresponding bars of plot B). The cultures, co-cultures and D0 are represented by white, black and grey circles/bars, respectively. B. Relative proportion of granulomonocytic (white bars) and erythroid progenitors (black bars) of human origin in murine bone marrow. Only mice positive for human CD45 antigen were considered; the number of mice (n) is given in the bars. \* p<0.05; \*\* p<0.01. Cumulative results from 4 independent experiments presented.

**Figure 6. Time-course of IL-6 production in co-cultures and MSC cultures.** A. Co-cultures of MSC with hematopoietic CD34<sup>+</sup> cells. B. MSC cultures. White bars=20%O<sub>2</sub>; Black bars= 5%O<sub>2</sub>; n=4 to 8; \*- p<0.05.

TABLE 1. **Summary of the monoclonal antibodies used for cell labeling.**

BD Pharmingen, Pont-de-Claix, France	CD33 PE, CD41 PE, CD90 FITC, CD19 PE
BD, Bioscience, San Jose, CA, USA	CD34 PE, CD13 PE, CD61 FITC, CD45 PerCep, CD33 APC, CD45 FITC
Miltenyi Biotec, Gladbach, Germany	CD133 APC

Figure 1

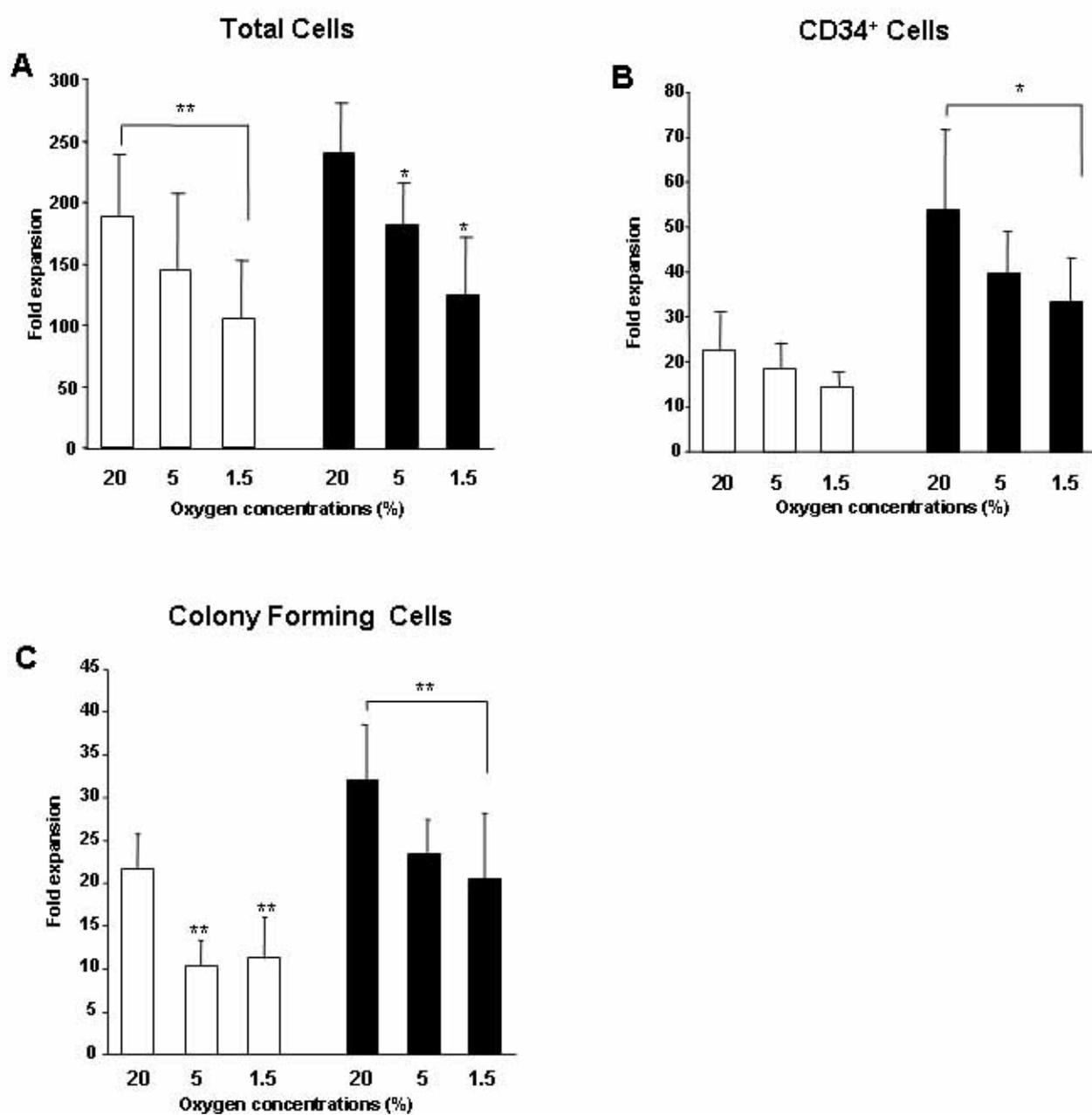


Figure 2

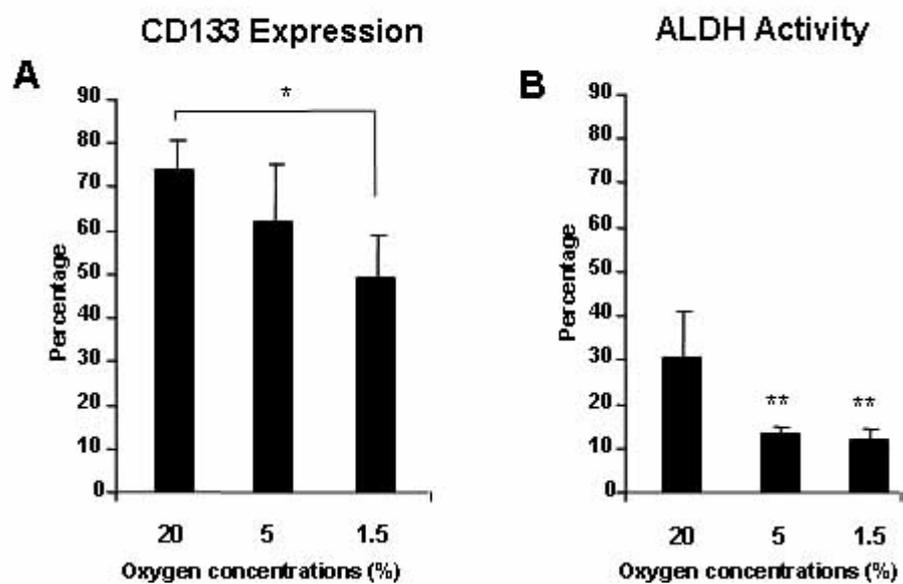


Figure 3

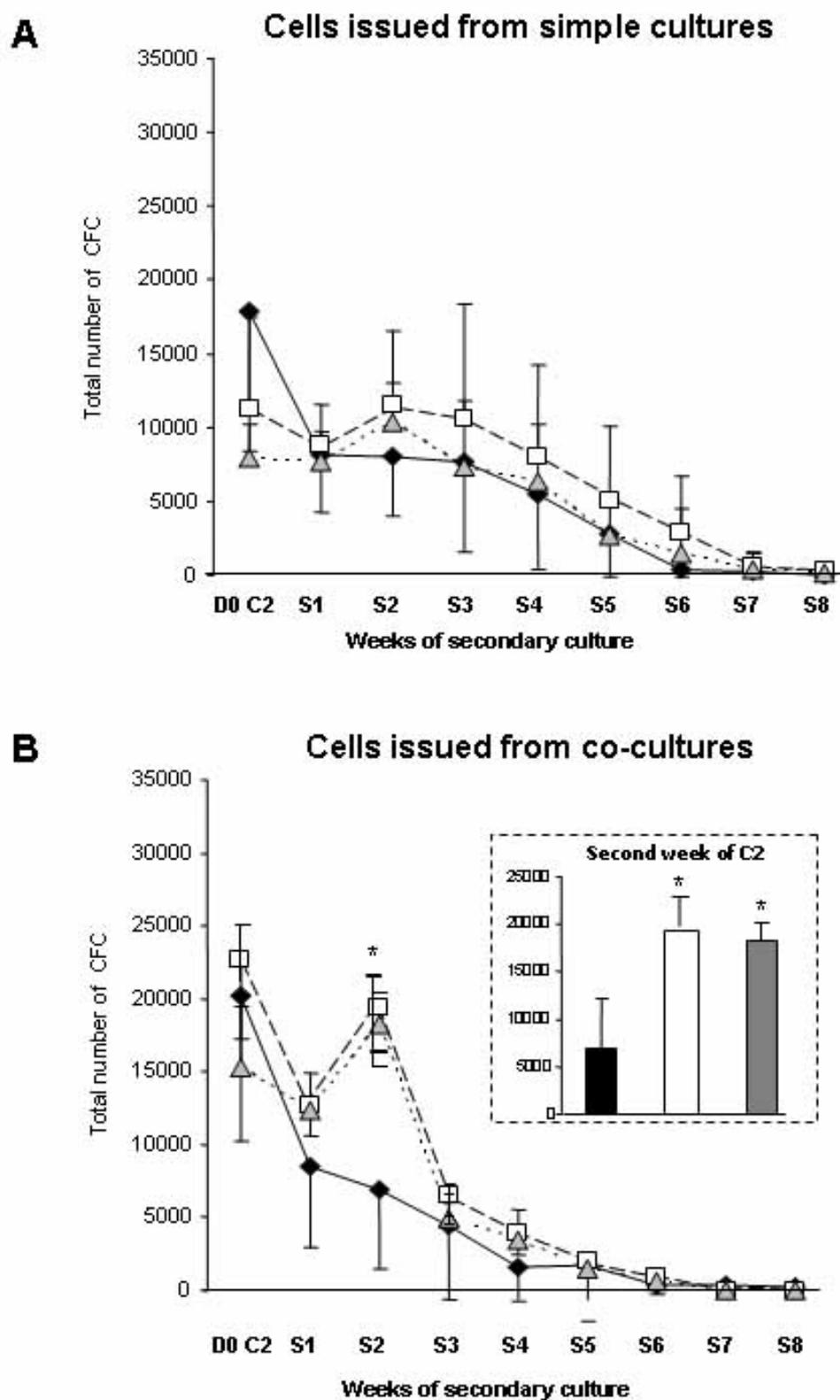


Figure 4

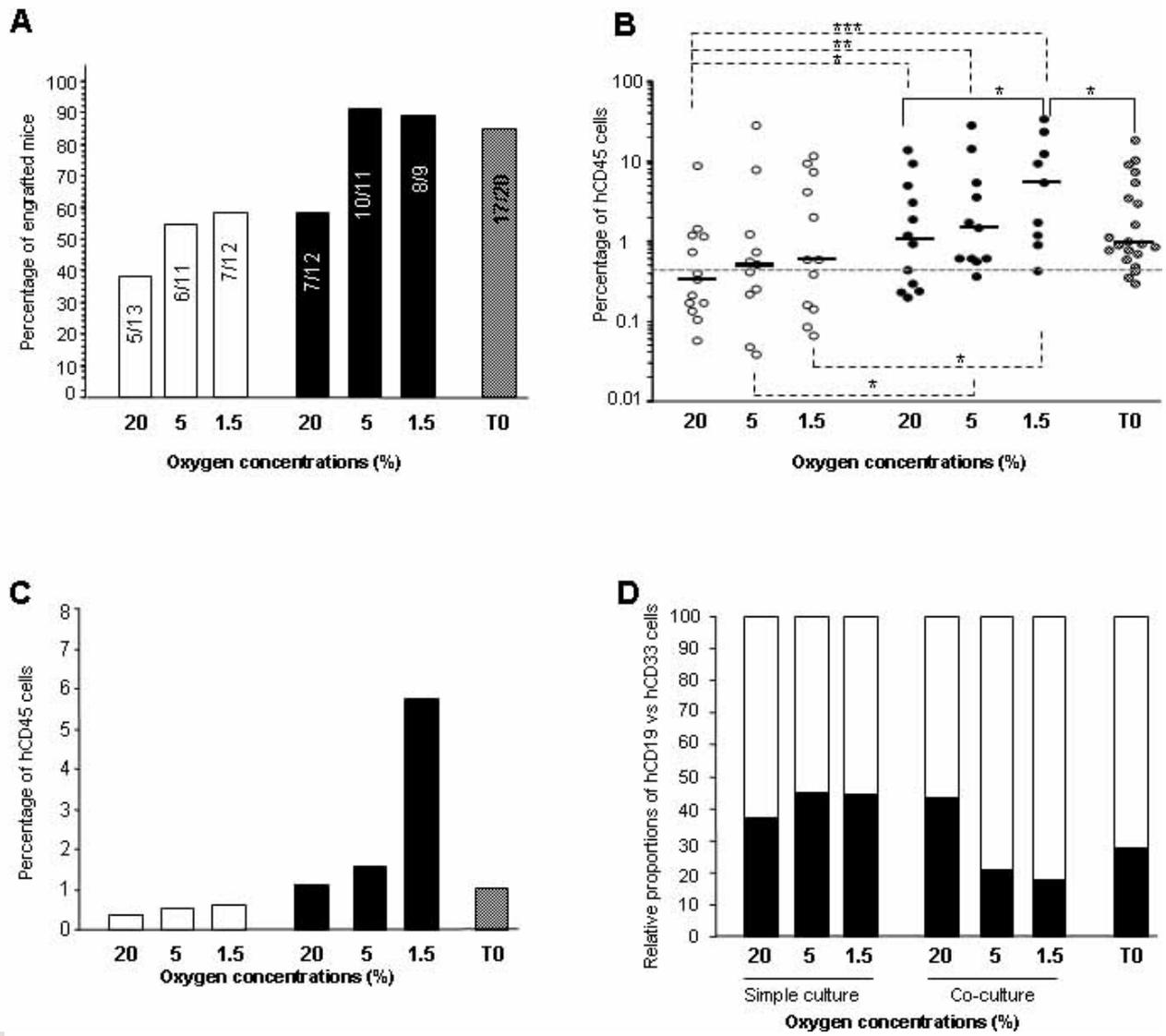


Figure 5

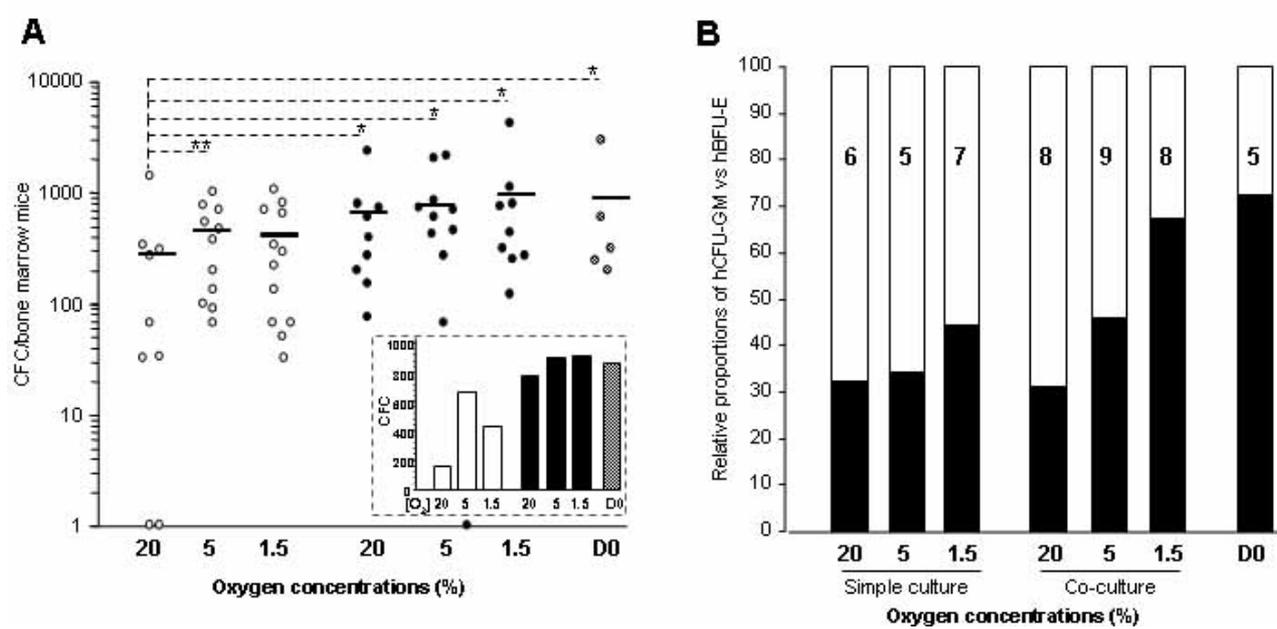


Figure 6

