

RESEARCH ARTICLE

Low O₂ concentrations enhance the positive effect of IL-17 on the maintenance of erythroid progenitors during co-culture of CD34⁺ and mesenchymal stem cells

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ABSTRACT. Co-culture of haematopoietic cells with a stromal cell layer does not mimic the physiological, micro-environmental niche, whose major feature is a low oxygen (O₂) concentration. Thus, in order to study the effects of IL-17 in a context which better approximates the physiological state, we investigated its effects on cell expansion, colony-forming ability, and the phenotypical profile of normal, human blood CD34⁺ cells co-cultured for five days with MSC layers at various O₂ concentrations (20%, 12.5% and 3% O₂). We demonstrated that IL-17 enhances CD34⁺ and total CFC production during the five days of MSC/CD34⁺ co-culture. This effect depends upon the O₂ concentration, reaching its maximum at 3% O₂, and is more pronounced on erythroid progenitors (BFU-E). In addition, the stimulation of IL-6 production by IL-17 in MSC cultures and co-cultures is enhanced by low O₂ concentration. The expression of some differentiation markers (CD34, CD13 and CD41) on haematopoietic cells in co-cultures also depends upon the oxygen concentration. Our results strengthen the concept that physiological levels of O₂ (mistakenly called hypoxia), should be considered as an important environmental factor that significantly influences cytokine activity.

Keywords: hematopoietic progenitors, CD34⁺, IL-17, oxygen, hypoxia

Haematopoiesis in adult bone marrow is governed by complex interactions between haematopoietic stem cells (HSCs) and progenitors with their environment. Mesenchymal stem cells (MSCs) [1] are part of the bone marrow hematopoietic niche. They participate in the maintenance of normal haematopoiesis as demonstrated in co-culture systems that mimic some features of the physiological niche. Bone marrow MSCs improve *ex vivo* expansion of human CD34⁺ progenitors [2-4] through cell-to-cell and cell-to-matrix interactions, as well as through secretion of haematopoietic cytokines. They also contribute to the regulation of HSC self-renewal, proliferation, differentiation and settlement [5].

Physiological O₂ concentrations in bone marrow range from < 0.1% in subendosteal areas, where primitive HSCs reside, to 7% in perivascular areas, mostly populated with committed progenitors and precursors [6, 7]. Several publications support the fundamental role of these low O₂ concentrations in the regulation of stem cell homeostasis: *i)* whereas culture of HSC at 20% O₂

leads to the rapid exhaustion of their stem cell potential, almost anoxic conditions (0.1% O₂) promote the maintenance and return of HSCs to quiescence in G₀ [8]; *ii)* cultures at higher O₂ levels (1, 1.5 and 3%) maintain cell proliferation, allow better preservation and favor self-renewal of primitive murine and human HSCs [9-16]. Other articles have shown that the number and size of BFU-E-derived colonies [17, 18] and CFC expansion in liquid culture were enhanced at 5% O₂ [19, 20]. Furthermore, the effects of cytokines on haematopoietic progenitors at physiologically relevant, low O₂ concentrations are different from those at the non-physiological, 21% O₂ concentration commonly used for cultures [14, 16, 21, 22]. This should be taken into consideration when defining the physiological activities of cytokines. Interleukin (IL)-17 is the “founding member” of a new cytokine family. IL-17, which is produced exclusively by a newly defined Th cell subset termed Th17 [23], exhibits multiple biological activities on numerous cell types due to the ubiquitous expression of its receptor [24]. Particu-

larly important is its role in immune responses to infection and in the pathogenesis of inflammatory diseases [25]. As an important regulator of host defense, IL-17 stimulates granulopoiesis, neutrophil trafficking [26-28], and erythropoiesis [29, 30]. Most of the effects of IL-17 on haematopoietic cells are indirect via induction of secretion of various cytokines, (GM-CSF, G-CSF, IL-6, and chemokines) by stromal cells [31, 32].

The effects of IL-17 on haematopoietic cells in culture were assessed at 20% O₂, while its action at low, physiologically relevant concentrations, remained unexplored. In the present work, we investigated its effects on cell expansion, colony-forming ability, and the phenotypical profile of human normal blood CD34⁺ cells co-cultured for five days with MSC layers at various O₂ concentrations (20%, 12.5% and 3% O₂).

METHODS AND MATERIALS

Cells and cell culture

Isolation of CD34⁺ cells

Human CD34⁺ cells from peripheral blood leukodepletion filters (Leucoflex, Macopharma, Tourcoing, France) were purified as described elsewhere [33] using the immunomagnetic MACS CD34 isolation kit (Miltenyi Biotec, Paris, France). CD34⁺ cell enrichment, evaluated by flow cytometry analysis using a phycoerythrin-anti-human CD34 antibody (Becton Dickinson, Le Pont de Claix, France), was > 90% in all cases.

Preparation of mesenchymal stem cell (MSC) adherent layers

Human bone marrow MSCs were isolated by culture of bone marrow of healthy donors obtained from filters used during the preparation of allogeneic haematopoietic grafts. Bone marrow cells were cultivated at a concentration of 5 x 10⁴ cells/cm² in minimum essential medium, alpha modification (MEM- α) (Invitrogen, Cergy-Pontoise, France) supplemented with 10% foetal calf serum (FCS) (Perbio Hyclone, France) and ciprofloxacin (10 μ g/mL; Bayer, Puteaux, France) (referred to as complete MEM- α medium). After 72 h at 37°C, 5% CO₂, non-adherent cells were removed and medium was changed. Cultures were fed every three-four days for 21 days or until confluence. Adherent cells, mainly MSCs, were then trypsinized, harvested and cultured at a concentration of 10³/cm² for one-three weeks. MSCs were cryopreserved or not before usage. For this study, thawed MSCs were plated in 24-well plates at 1 x 10⁴/cm² in MEM- α medium (BioWhittaker, Lonza, Verviers, Belgium), containing 10% FCS, and 2% L-glutamine-penicillin-streptomycin mixture (Cambrex BioWhittaker, Walkersville, MD, USA). Cells were cultured (37°C, humidified atmosphere, 95% air, 5% CO₂) until monolayer confluence, with twice-weekly changes of culture medium.

Co-culture of MSCs and CD34⁺ cells

CD34⁺ cells were then purified, seeded (1x10⁵/well) on MSCs monolayers in 24-well plates and co-cultured for

five days in Stem alpha A medium (Stem Alpha, St. Clement les Places, France) supplemented with 10% FCS and 2% L-glutamine-penicillin-streptomycin mixture in the presence or absence of increasing concentrations of rhIL-17 (5, 50, 100 ng/mL) (R&D Systems, Minneapolis, USA). The co-cultures were maintained at 37°C either at 5% CO₂ and 3 or 12.5% O₂ (Proox Culture Chamber with O₂ and CO₂ regulators BioSpherix, Ltd., Redfield, NY, USA) or at 5% CO₂ and 20% O₂ (Incubator Igo 150 Cell Life, Jouan, St. Herblain, France). At day five, non-adherent cells were carefully harvested and washed. Viable cells were enumerated (by trypan blue exclusion) and processed for further analyses as described below. The experiments were performed with five unrelated MSC-CD34⁺ donor combinations.

Colony-forming cell (CFC) assay

Freshly isolated CD34⁺ or cultured non-adherent cells harvested at day five were cultured in a cytokine-supplemented methylcellulose kit (Stem Alpha ID; Stem Alpha, Saint Clement les Places, France) to enumerate CFCs (colony-forming units-granulocyte macrophage (CFU-GM), burst-forming units-erythroid (BFU-E), and colony-forming units-mix [CFU-Mix]). Freshly isolated CD34⁺ and cultured cells were respectively plated at concentrations of 250 or 1 200 cells/mL and incubated for 14 days at 37°C in a humidified atmosphere with 20% O₂ and 5% CO₂ before the colonies were scored using an inverted microscope.

Detection of cell differentiation antigens by flow cytometry

After five days of co-culture with MSCs in the presence or absence of IL-17 at different O₂ concentrations, non-adherent cells were washed in phosphate-buffered saline with 1% human serum albumin, labeled (25 minutes in dark) with anti-CD34, -CD33, -CD13, and -CD41 monoclonal antibodies coupled to PE and with anti-CD14 and -CD61 coupled to FITC (Beckman Coulter, Immunotech, Marseille, France). They were then washed and resuspended. The phenotype of hematopoietic cells was analyzed by flow cytometry. Fluorochrome-conjugated isotype antibodies were used to determine the level of nonspecific binding.

ELISA dosage of IL-6 in culture supernatants

Cell-free supernatants from 48 h cultures (at 3%, 12.5% or 20% O₂ ; with and without 100 ng/mL IL-17) of MSC alone and of MSC with CD34⁺ cells (1 x 10⁵) (both cultures and co-cultures were performed with the same medium) were collected and stored at -70°C until testing. IL-6 concentration was measured using an ELISA kit (R&D Systems, Minneapolis, USA) according to the manufacturer's instructions. Detection limit for IL-6 reported by the manufacturer was 0.7 pg/mL.

Statistical analysis

Mean values (\pm SEM) were calculated with data from five independent experiments. The significance of differences was assessed using Student's t test.

RESULTS

Combined effects of hypoxia and IL-17 on the total and CD34⁺ cell number in MSC/CD34⁺ co-cultures

The number of non-adherent cells harvested after five days of incubation without IL-17 at 12.5% (arterial blood O₂ concentration) and 3% O₂ (mean O₂ concentration in bone marrow) was significantly lower than at 20% O₂ (figure 1A). Addition of IL-17 induced a dose-dependent increase in the total cell number at 3% O₂ that was significant only at 100 ng/mL (figure 1A). The differences observed between co-cultures at 3% and 12.5% O₂ supplemented with 100 ng/mL IL-17 were also significant in paired comparisons.

As shown in figure 1B, the maintenance of CD34⁺ cells after five days of co-culture was significantly improved only at the lowest 3% O₂ concentration and with 50 ng/mL IL-17, reaching 60% preservation instead of approximately 20% in all co-cultures without IL-17.

Physiologically low O₂ concentration improves the haematopoietic-supporting activity of IL-17 in MSC/CD34⁺ co-cultures

Addition of IL-17 (50 and 100 ng/mL) induced a significant improvement in the maintenance of BFU-E at all O₂ concentrations tested in comparison with corresponding controls cultured without IL-17 (figure 2A). It should be mentioned that addition of IL-17 to co-cultures at 3% O₂,

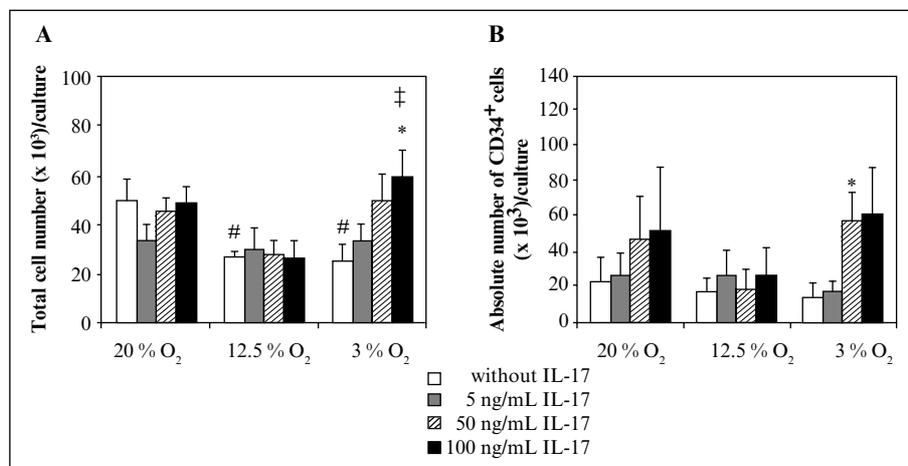


Figure 1

Expansion trends of: **A)** total nucleated cells, and **B)** CD34⁺ cells in IL-17-stimulated MSC/CD34⁺ co-cultures at different O₂ concentrations. CD34⁺ cells were co-cultured with MSCs in the presence or absence of IL-17 (5, 50, 100 ng/mL) at 20%, 12.5% and 3% O₂. After five days, the non-adherent cells harvested from co-cultures were enumerated for viable cells and analyzed by flow cytometry for CD34 expression. The data points represent means \pm SEM of five independent experiments. Significant differences were obtained using Student's t-test: *p < 0.05 for IL-17-stimulated versus non-stimulated co-cultures with corresponding O₂ levels; # p < 0.05 for co-cultures at 3% or 12.5% O₂ versus 20% O₂; ‡ p < 0.05 for co-cultures at 3% O₂ versus 12.5% O₂.

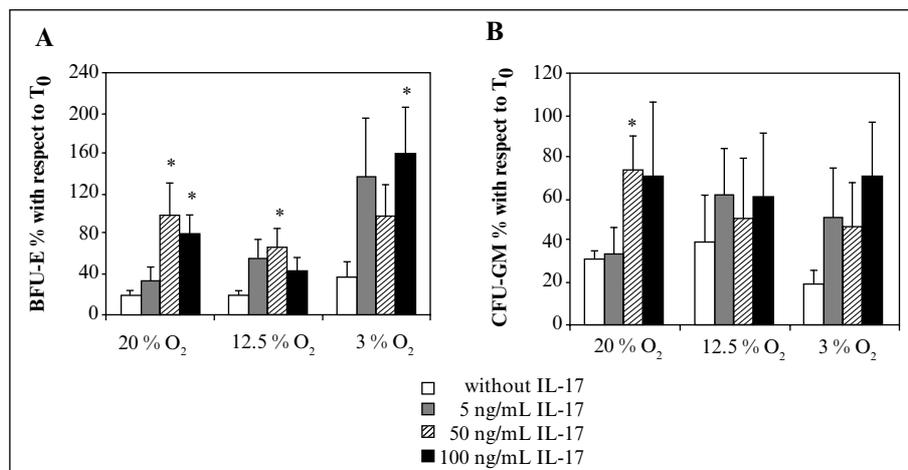


Figure 2

Expansion trends of: **A)** BFU-E and **B)** CFU-GM in IL-17-stimulated MSC/CD34⁺ co-cultures at different O₂ concentrations. CD34⁺ cells co-cultured with MSCs in the presence or absence of IL-17 (5, 50, 100 ng/mL) at 20%, 12.5% and 3% O₂ were harvested after five days. The results of CFCs analyses are presented as the percentages of clonogenic progenitors with respect to their numbers obtained at day 0 (T₀). The starting CD34⁺ cell fraction seeded (1 x 10⁵ cells) at T₀ contained 11 800 \pm 1 580 and 5 933 \pm 1641 of BFU-E and CFU-GM, respectively. The data points represent means \pm SEM of five independent experiments. Significant differences were obtained using Student's t-test: * p < 0.05 for IL-17-stimulated versus non-stimulated co-cultures with corresponding O₂ levels.

at a dose of 100 ng/mL, even induced an expansion of BFU-Es to 160% of their number at day 0. These results show that low O₂ concentrations (below 12.5%) and IL-17 (> 5 ng/mL) have a synergistic effect on the maintenance/expansion of BFU-Es in short-term, liquid culture. Although the CFU-GM number in all cultures supplemented with IL-17 was 1.5 to 3 times higher compared to that without the IL-17, its beneficial effect on their survival was significant only in co-cultures at 20% O₂ with 50 ng/ml IL-17. However, it barely enabled maintenance of their day 0 numbers (*figure 2B*). At low O₂ concentrations however, the positive effect of IL-17 on CFU-GM was not statistically significant, and their maintenance varied from 45 to 75% with respect to day 0 values.

No significant alterations were induced by IL-17 at any O₂ concentration within the CFU-Mix compartment (data not shown). IL-17 improves the maintenance of total committed progenitors at all O₂ concentrations, but this was most pronounced at 3% O₂ (*table 1*).

Influence of IL-17 on the phenotype of haematopoietic cells with respect to O₂ concentration

Non-adherent cells harvested at day five of co-culture were analyzed for the expression of haematopoietic differentiation antigens CD34, CD33, CD13, CD14, CD41 and CD61.

The percentage of CD34⁺ cells decreased from about 90% (day 0 cell fraction) to 30-35% (day five of co-culture) without IL-17 whatever the O₂ concentration (*figure 3A*). Addition of IL-17 enhanced the percentage of cells maintaining their CD34 expression at all O₂ con-

Table 1
Absolute numbers of total CFC in IL-17-stimulated MSC/CD34⁺ co-cultures at different O₂ concentrations

Total CFC	20% O ₂	12.5% O ₂	3% O ₂
Without IL-17	4 293 ± 526	4 022 ± 1 108	5 355 ± 1 925
IL-17 5 ng/mL	6 072 ± 2 480	8 978 ± 3 070	18 473 ± 9 519
IL-17 50 ng/mL	15 217 ± 4 036*	10 048 ± 2 438*	13 187 ± 3 346*
IL-17 100 ng/mL	12 764 ± 4 596	8 194 ± 3 217	22 822 ± 7 220* [‡]

CD34⁺ cells were co-cultured with MSCs in the presence or absence of IL-17 (5, 50, 100 ng/mL) at 20%, 12.5% and 3% O₂. At day 5 of co-culture, the non-adherent cells were harvested and assayed for CFCs. Progenitor cell numbers presented were calculated per total number of cells in cultures. CD34⁺ cell fraction seeded (1 × 10⁵ cells) starting at day 0 (T₀) contained 18 400 ± 16 68 of total CFC. The data points represent means ± SEM of five independent experiments. Significant differences (t-test): * p < 0.05 for IL-17-stimulated versus non-stimulated co-cultures for corresponding O₂ level; [‡] p < 0.05 for co-cultures at 3% O₂ versus 12.5% O₂.

centrations tested. However, this effect was significant only for co-cultures performed at 3% O₂ and 50 ng/mL IL-17. Concerning CD13 expression, this was also evident only at 3% O₂, but was significant for all IL-17 doses (*figure 3B*). IL-17 also increased (from 50 to 80%) the percentage of cells expressing CD41⁺, but only in co-cultures at 12.5% O₂ (*figure 3B*).

No significant variation in the percentage of CD14, CD33 and CD61-expressing cells was observed, whatever the culture conditions (data not shown).

Hypoxia potentiates the stimulating effect of IL-17 on IL-6 secretion by MSCs

Since IL-17 induces the production of IL-6 by the S17 murine bone marrow stromal cell line [34], we examined whether the effect of IL-17 on haematopoietic progenitors in MSC/CD34⁺ co-cultures at different O₂ concentra-

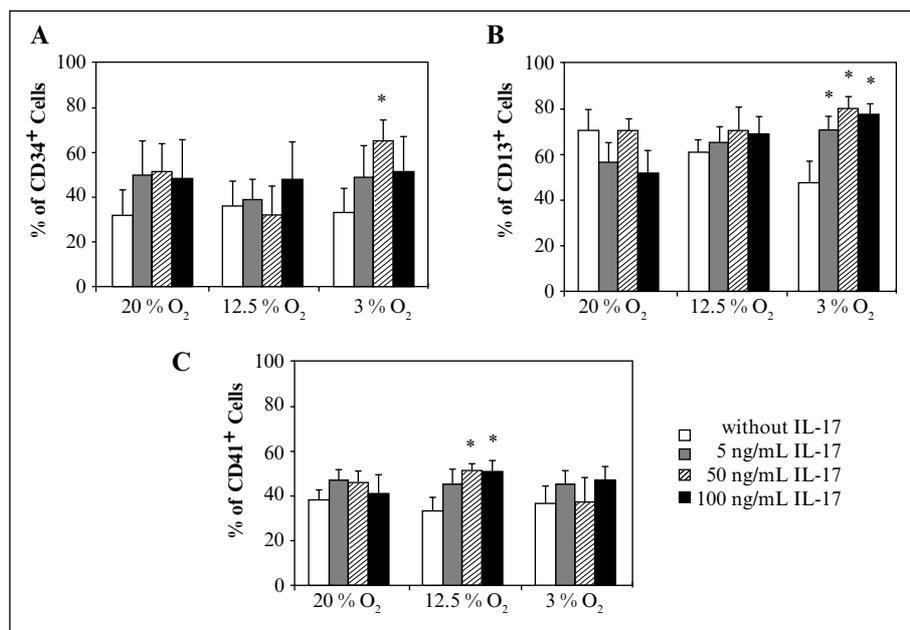


Figure 3

Percentages of cells expressing specific phenotypes in IL-17-stimulated MSC/CD34⁺ co-cultures at different O₂ concentrations. CD34⁺ cells were co-cultured with MSCs in the presence or absence of IL-17 (5, 50, 100 ng/mL) at 20%, 12.5% and 3% O₂. After five days of co-culture, the non-adherent cells were recovered, stained and analyzed by flow cytometry for CD34 (A), CD13 (B), and CD41 (C) expression. Fluorochrome-conjugated isotype antibodies were used as control to determine the level of nonspecific binding. The results are presented as the percentages of cells expressing specific phenotype per culture. The data points represent means ± SEM of five independent experiments. Significant differences were obtained using Student's t-test: * p < 0.05 for IL-17-stimulated versus non-stimulated co-cultures with corresponding O₂ levels.

tions could be mediated by IL-6. For this purpose, we measured IL-6 concentrations in supernatants from MSCs and MSC/CD34⁺ after 48 H of culture at 20, 12.5 and 3% O₂, without and with 100 ng/mL IL-17.

figure 4 shows that IL-17 significantly increased IL-6 secretion both by MSCs and MSC/CD34⁺ co-cultures whatever the O₂ concentration. In addition, comparison of IL-6 release by MSC and MSC/CD34⁺ showed that it was higher in co-cultures whatever the O₂ concentration. A slight increase was detected at 3% O₂ when compared to 12.5 and 20%.

DISCUSSION

We have demonstrated that IL-17 enhances CD34⁺ and total CFC production over five days of MSC/CD34⁺ co-culture at 20% O₂. These results extend those obtained by Fossiez *et al.* [31] with human cord blood CD34⁺ cells/fibroblast co-cultures at 20% O₂. IL-17 increased the number of BFU-E in MSC/CD34⁺ co-cultures. This effect depends on the O₂ concentration reaching its maximum at 3% O₂ (figure 2A). These results extend to human cells, and the low O₂ concentration in our previous *in vivo* and *in vitro* findings with murine bone marrow showing that IL-17 stimulates BFU-E production [29, 30].

Our present results at 20% O₂ seem to confirm both *in vitro* and *in vivo* results showing IL-17-stimulation of granulopoiesis, accompanied by an expansion of CFU-GM [29, 31, 32]. However, by using a system that better mimics the physiological bone marrow environment (MSC, adult CD34⁺ cells and 3% O₂, a concentration present in bone marrow areas where progenitors reside [6]), we could not provide firm evidence that IL-17-enhanced CFU-GM expansion is increased at 3% O₂ since the apparently positive effects of IL-17 on CFU-GM were not statistically significant and their number did not reach the day 0 values (figure 2B). Alternatively,

the enhanced percentage of CD13⁺ cells (when the low O₂ concentration (3%) was associated with IL-17), suggests an increased differentiation into the precursors of a myelo-monocytic lineage. In that respect, the absence of numerical expansion of the CFU-GM compartment could result, rather from their decrease due to differentiation (stimulated by the IL-17) than from the absence of supporting activity of IL-17.

We also explored the effects of IL-17 on the differentiation of CD34⁺ cells after five days in culture by studying several differentiation markers. Interestingly, the percentage of CD13⁺ cells increased only at 3% O₂ and that of CD41⁺ cells only at 12.5% O₂. Our group has already described O₂ concentration-dependent effects on differentiation i.e. on the expression of differentiation markers (CD117, CD34 and CD133; [22], as well as the expression of the gene *cd34* [35]. Also, Mostafa *et al.* [36] demonstrated the regulatory role of O₂ concentrations in megakaryocyte gene expression, differentiation and maturation. Altogether, these results suggest that: *i*) the *in vivo* effects of IL-17 on the differentiation of haematopoietic progenitors is partly driven by the O₂ levels surrounding them in their bone marrow niches; *ii*) the haematopoiesis both in the steady state and under stress conditions depends upon adequate combinations of cytokines and O₂ concentrations in the various bone marrow niches.

MSCs regulate haematopoiesis through their production of growth factors and cytokines such as SCF, IL-6, GM-CSF [5]. It was recently demonstrated that IL-17 is a growth factor for human and murine MSCs [37]. IL-17 triggers IL-6 production in different cell types [38] including the murine stromal cell line S17 [34]. Our study provides the first evidence that IL-17 enhances the secretion of IL-6 by MSCs, and suggests that this constitutive and IL-17-stimulated IL-6 secretion is increased at 3% O₂. Since the bone marrow environment O₂ concentration is regularly low, the increase in chemokine receptor and angiogenic factor expression in MSCs [39, 40] and increased IL-6 production (this paper) at low O₂ concentration is rather a steady state hallmark of the microenvironmental niche (0.1 to 5% O₂). Hence, the effects of IL-17 on BFU-E described here could be considered in the context of a physiological, regulatory network, involving at least the stromal cells as a part of the bone marrow microenvironment.

We found higher concentrations of IL-6 in MSC/CD34⁺ co-cultures than in cultures of MSC alone, whatever the IL-17 and O₂ concentrations. Since it was shown that CD34⁺ cells provide signals that induce IL-6 up-regulation by bone marrow stroma [41, 42], a similar mechanism could explain a higher IL-6 production by MSC/CD34⁺ co-cultures than by MSCs alone. It is also possible that IL-17 induced CD34⁺ cells to produce IL-6, as demonstrated for IL-3 [43]. Although it is possible that the CD13⁺ cells (enhanced at day five of culture) produce the IL-6 upon IL-17 stimulation, it is not likely that they were generated in significant numbers during the 48 H of culture (IL-6 dosage was performed after 48 h).

IL-6 stimulates proliferation and differentiation of numerous haematopoietic cell types including CFU-GM and

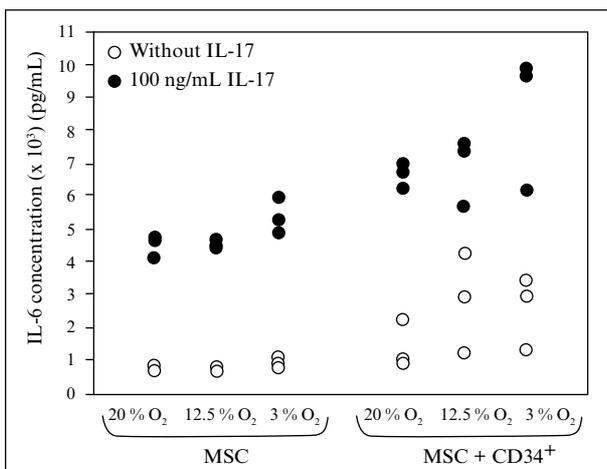


Figure 4

IL-6 release in IL-17-stimulated MSC and MSC/CD34⁺ cultures at different O₂ concentrations. MSCs were cultured, either alone or with CD34⁺ cells, in the absence (○) or presence of 100 ng/mL IL-17 (●) at 20%, 12.5% and 3% O₂. After 48 h, the cell-free supernatants were collected and assayed for IL-6 by ELISA. The values represent means ± SEM of three independent experiments.

BFU-E [44, 45] and participates in supporting haematopoiesis by bone marrow stromal cells [46]. Thus, one can suggest that in our co-cultures, the effect of IL-17 on BFU-E was indirect, due to the induction of IL-6 synthesis by MSCs. Although this mechanism was already supported in our previous reports demonstrating IL-17 stimulation of BFU-E in mice [29, 30], it could not be confirmed by the present, indirect data.

Few publications demonstrate that O₂ concentrations similar to physiological ones in bone marrow modify the responses of haematopoietic cells to cytokines when compared to those at 20% O₂ [8, 16, 21, 47]. Our results provide one more demonstration of this fact and strengthen the concept that physiological levels of O₂ (misleadingly called hypoxia) should now be considered as an important environmental factor that significantly influences cytokine activity. Accordingly, *ex vivo* studies exploring the action of cytokines on haematopoietic cells at physiological O₂ concentrations, may improve our knowledge of the *in vivo* regulation of haematopoiesis.

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