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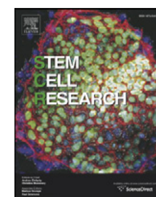

Targeting the aryl hydrocarbon receptor nuclear translocator complex with DMOG and Stemregenin 1 improves primitive hematopoietic stem cell expansion

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ABSTRACT

Culture conditions used for the expansion of hematopoietic stem and progenitor cells (HSCs and HPCs, collectively HSPCs) should ideally favor the self renewal of long-term HSCs. At 20% O_2, the synthesis of HIF-1α is balanced by its hydroxylation and proteasomal degradation. This favors HSPC differentiation, but can be prevented by culturing CD34+ cord blood cells in the presence of dimethyloxaloylglycine (DMOG). This differentiation may also be reduced by culturing the cells in the presence of Stemregenin 1, an antagonist of the aryl hydrocarbon receptor (AhR). The objective of this study was to investigate how hypoxia, DMOG and Stemregenin 1 might affect the expansion of HSPCs with the aim of identifying optimal conditions for expansion in culture. It was found that DMOG decreased proliferation but was effective in preserving the number of cells in the primitive hematopoietic sub-populations *in vitro*. The effect of DMOG was similar to hypoxia, although differences were observed with regard to the side population and CD34+ sub-populations. Stemregenin 1 on the other hand increased the size of the primitive as well as the other HSC sub-populations. The use of Stemregenin 1 with DMOG increased the proportion of primitive HSCs to 3.54% compared to 2.61% for Stemregenin 1 alone. *In vivo* engraftment studies confirmed these findings and showed that fewer cells (3710) are required for long-term engraftment when HSCs are grown in Stemregenin 1 together with hypoxia than in Stemregenin 1 under conditions of normoxia (13430).

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1. Introduction

The identification of culture conditions that promote the expansion of primitive hematopoietic stem and progenitor cells (HSCs and HPCs, collectively referred to as HSPCs) has the potential to significantly improve hematopoietic stem cell transplantation. The culturing of HSPCs generally leads to differentiation as well as the loss of stem cell characteristics after 2 to 3 weeks because the culture conditions are very different from the conditions the cells experience *in vivo* (Chaurasia et al., 2014; Cheng et al., 2000; Mikkola and Orkin, 2006). One of the challenges of culturing HSPCs related to use in transplantation is to maintain them in a primitive state. Mimicking the bone marrow niche has been shown to be effective in improving *in vitro* culture conditions by limiting differentiation and promoting quiescence (Cheng et al., 2000; Guitart et al., 2010). Long-term hematopoietic stem cells (LT-HSCs), which reside in a niche in the bone marrow which is close to arterioles where the oxygen concentration is below 3%, can either be in a quiescent (G0) or in a cycling state (G1/S/G2/M phase) (Takubo, 2012; Kunisaki et al., 2013). LT-HSCs can be stimulated to cycle and migrate to the central vein to differentiate. However, various factors present in the bone marrow contribute to maintaining the cells in a state of quiescence. These include cytokines, components of the hematopoietic niche and hypoxia (Cheng et al., 2000; Cipolleschi et al., 1993; Hermitte et al., 2006; Wilson and Trumpp, 2006). Conditions that promote the expansion of HSPCs have been identified but conditions that favor the cycling state of LT-HSCs without causing differentiation need to be improved (Huang et al., 2012; Mikkola and Orkin, 2006).

Under conditions of hypoxia, hypoxia-inducible factor (HIF) forms a complex with Aryl hydrocarbon receptor nuclear translocator (ARNT) which binds to the hypoxia response elements (HRE) in the promoter

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regions of various genes (Takubo, 2012). Genes containing HRE elements express proteins which contribute significantly to maintaining cells in a quiescent state (Takubo, 2012). ARNT can complex with the aryl hydrocarbon receptor (AhR) to induce genes that promote differentiation (Boitano et al., 2010). ARNT/AhR protein complexes bind to dioxin response elements (DRE) to directly regulate genes including HES-1, c-Myc and C/EBP, which promote cell differentiation (Lindsey and Papoutsakis, 2012). PU.1-, β-catenin-, CXCR4-, and Stat5-dependent hematopoietic processes are also affected by AhR regulation (Singh et al., 2009). There are strong indications that the regulation mediated by AhR is cell and differentiation-stage specific, which makes it difficult to characterize (Dever and Opanashuk, 2012). ARNT thus plays a key role in the regulation of hematopoiesis and needs to be further investigated as a possible drug target. The binding of AhR to ARNT can be suppressed with Stemregenin 1 (SR1), an antagonist of AhR which limits differentiation (Mulero-Navarro et al., 2006). It has been shown that SR1 induces cord blood-derived CD34+ cell expansion and CFU formation and also improves the engraftment capacity of these cells (Boitano et al., 2010). A clinical trial performed by Wagner et al. demonstrated that engraftment was more rapid in 100% of patients that received cord blood HSCs that were expanded in the presence of SR1, in contrast to cord blood alone which is characterized by slow engraftment (Wagner et al., 2016).

The functional effects of SR1, conditions that increase HIF-1 α and a combination of the two were explored in cultured CD34+ cells using SR1 and/or DMOG in various assays. CD34 and CD133 cell surface molecules, that diminish with differentiation, were used to quantify a sub-population of HSPCs that includes further sub-populations at various stages of differentiation (Shimba et al., 2003; Takubo et al., 2010). The colony forming cell (CFC) assay was used to directly quantify cells in the HSPC population of primary cultures that are committed progenitors. This assay is also used to indirectly quantify the maintenance/expansion of pre-CFCs during primary cultures that are not committed but give rise to committed progenitor cells during secondary cultures (Gothot et al., 1998). Side population (SP) cells have been identified as a minor HSC subset due to their capacity to efflux drugs such as Hoechst 33342 or Vybrant® DyeCycle™ Violet (VDC) more efficiently than other cells (Arai and Suda, 2008). Transplantation of expanded CD34+ cells into NOD.Cg-Prkdc^{scid}Il2rg^{tm1Wjl}/SzJ (NSG) mice was used to determine the short- and long-term engraftment capacity of the primitive HSC population.

Our objective was to determine how the culturing of CD34+ cells under conditions that influence ARNT complex formation might affect the expansion of HSPCs. We hypothesized that the use of culture conditions that stabilize HIF-1α and antagonize the AhR protein would promote the expansion of the primitive HSC population and thereby enhance the engraftment capacity of the LT-HSC population.

2. Materials and methods

2.1. Isolation of CD34+ cells

Umbilical cord blood units (<24 h old) were obtained from the Etablissement Français du Sang (EFS) in Bordeaux (France), or the Steve Biko Academic Hospital and Netcare Femina Hospital, both in Pretoria (South Africa). CD34$^+$ cells were isolated using the indirect CD34$^+$ MicroBead kit (Miltenyi Biotech, #130-046-703) according to the manufacturer's instructions. The cells were passed twice through the isolation columns (#130-042-401) to obtain >80% purity. Ethics approval was obtained from the human ethics committees of the University of Pretoria, the University of Bordeaux and Netcare.

2.2. Primary liquid culture

CD34+ cells were seeded at a density of $2,3 \times 10^5$ cells/ml and cultured under normoxic (5% CO_2; 20% O_2) or hypoxic conditions (5% CO_2; 1% O_2) in Dulbecco's Modified Eagle Medium (DMEM) containing 1% penicillin/streptomycin (PS), 10% fetal bovine serum (FBS) or the serum free expansion medium Stemline™ II (Supplementary Fig. 1). The cytokines used were either 10 ng/ml hIL-3 or a mixture of hIL-3 (3 ng/ml), hSCF (100 ng/ml), hG-CSF (100 ng/ml), and TPO (20 ng/ml). It has been reported that G-CSF promotes HSC proliferation and engraftment (Schuettpelz et al., 2014). Dimethyl sulfoxide (DMSO), DMOG, YC-1 and/or SR1 were also added to the culture medium where appropriate. DMSO was used as a vehicle control in the 1% O_2 and 20% O_2 conditions. DMOG (Sigma, USA, #D3695) was used at concentrations ranging from 0.1 to 2.5 mM to determine the concentration that efficiently arrests cells in G0 with low toxicity. The concentration selected for further experiments was 0.1 mM. The same was done for YC-1, which is an inhibitor of HIF-1α (Sigma, USA, #Y102), with concentrations ranging from 0.25 to 20 μM; 0.5 μM was selected for further experiments. A concentration of 0.75 μM SR1 (Biovision, USA, #1967-1) was tested for toxicity and deemed to be non-toxic, and was used at this concentration for further experiments as previously described (Boitano et al., 2010).

2.3. Cell viability and cell cycle analysis

Cell viability was assessed on days 0, 2, and 7 using flow cytometry. Samples were stained with 7-Aminoactinomycin (7-AAD) and absolute counts were obtained through the addition of Flowcount™ Fluorospheres (Beckman Coulter, Miami, USA). Live cells, counted on day 7, were used to indicate the rate of cell proliferation that occurred during culture. Cells were stained with anti-CD34 (PC7-conjugated mouse antibody, Ref. #21691, Beckman Coulter, Miami, USA) and anti-CD133 (PE-conjugated mouse antibody, Beckman Coulter, Miami, USA) antibodies. Cell cycle analysis was done on days 0, 2, and 7 of culture. Approximately 100,000 cells were fixed with 0.5% paraformaldehyde (PFA), permeabilized with 0.05% Saponin (Sigma, USA), and stained with an anti-human Ki67 antibody (PE-conjugated mouse antibody, Ref: 556027, BD Biosciences, New Jersey, USA) and the DNA stain, TOPRO III. The cells were analyzed using a Canto II flow cytometer (BD Biosciences) (Supplementary Fig. 2).

2.4. Western blot assays

Proteins were isolated from 1×10^6 cells for each treatment condition on day 7 using cell extraction buffer (FNN0011, Life Technologies, Invitrogen) and quantified using the Bradford assay (B6916, Sigma Aldrich, St. Louis, USA). The Invitrogen iBlot® system was used according to the manufacturer's instructions. Twenty micrograms of protein from each sample were separated on a NuPAGE® Novex® 4–12% Bis-Tris Gel (Life Technologies, Invitrogen) and transferred to Nitrocellulose membranes using the iBlot® Gel Transfer Device and iBlot® Gel Transfer stacks (Life Technologies, Invitrogen). HIF-1α, AhR, β-actin and GAPDH proteins were identified using anti-HIF-1α (NB100-479, Novus Biologicals®, Littleton, USA), anti-AhR (NB100-2289, Novus Biologicals®, Littleton, USA) and anti-β-actin (A2066, Invitrogen®) antibodies respectively, generated in a rabbit host. The iBlot® Western Detection Chromogenic Kit, which uses an anti-rabbit Alk-Phosphatase secondary antibody (Life Technologies, Invitrogen), was used to visualize protein bands.

2.5. Colony forming assays

Colony assays were used to analyze the colony forming unit (CFU) capacity of each treated sample. One hundred cells from each sample on days 0 and 7 were plated in 250 μl Stem-α1D (Stem Alpha, St. Genis L'Argentière, France, http://www.stemalpha.fr) and colonies were counted 12–16 days after the cells were plated. The number of colonies counted was divided by 100 to obtain the proportion of cells capable of forming CFUs. The total number of CFUs in each sample was

calculated by multiplying the proportion of cells capable of forming CFUs by the total number of cells in the sample.

2.6. Side population analysis

Side population analysis was done on days 0 and 7. The cells (3×10^5 cells/tube) were divided into two tubes, centrifuged at 200g, and the supernatant was removed. The cells in the first tube were resuspended in 150 μl DMEM+ (Dulbecco's modified Eagle medium, Invitrogen), 2% (v/v) FBS, 10 mM HEPES (Sigma, USA) and 5 μM VDC Violet stain (Life Technologies, Invitrogen). The cells in the second tube were resuspended in DMEM+ containing 50 μM verapamil (#B7431, Life technologies, California, USA). Both tubes were incubated at 37 °C for 2 h after which the cells were placed on ice and washed with 2 ml cold HBSS+ (Hank's balanced salt solution, Invitrogen, California, USA), 2% (v/v) FBS and 10 mM HEPES (Sigma) and stained with anti-CD34 and anti-CD38 (PC5-conjugated mouse antibody, Beckman Coulter) antibodies together with 7-AAD or annexin V-FITC kit (Beckman Coulter, Miami, USA). The cells were then washed with 1 ml HBSS+ and analyzed by flow cytometry (Gallios flow cytometer, Beckman Coulter, USA). The cells were kept below 4 °C after incubation. Live cell selection was based on 7-AAD negative staining and the side population was measured using a FL9 (620/30BP) *versus* FL10 (550/40 BP) scatter plot. The region that selects for cells that efflux VDC violet dye was set up according to the histogram generated from the untreated verapamil control. After the initial setup, the same gate was maintained for all treatments to allow for comparison of the data (Supplementary Fig. 2).

2.7. Secondary liquid culture

After the cells had been cultured for 7 days (primary liquid culture), each sample was washed with PBS and $2{,}3 \times 10^5$ cells were plated in 1 ml secondary liquid culture medium (Supplementary Fig. 1) composed of DMEM, 10% FBS, 1% Penicillin/Streptomycin, hSCF (100 ng/ml) (Life Technologies, Invitrogen), hIL-3 (3 ng/ml) (Life Technologies, Invitrogen), hTPO (20 ng/ml) (Life Technologies, Invitrogen) and hG-CSF (100 ng/ml) (Life Technologies, Invitrogen). Every second day, cells were resuspended to obtain a homogenous culture and 50 μl cell suspension was removed for the purposes of determining cell count and measuring the proliferative capacity of the treated cells. On days 6, 13 and 19 the cells were washed with PBS and replated at 10% of the total cell count in 1 ml secondary liquid culture medium. To quantify the number of CFU in each treated sample, 100 cells from days 2, 4, and 6, 400 cells from days 8, 11, and 13 and 800 cells from every second day after day 13 were plated in 250 μl Stem-α1D and colonies were counted 12 to 16 days after the cells were plated (Supplementary Fig. 1).

2.8. Engraftment assays

NOD.Cg-Prkdc^{scid}Il2rg^{tm1Wjl}/SzJ (NSG) mice obtained from Jackson laboratories (Bar Harbor, Maine, USA) were used in this study. Mice were maintained in Techniplast 1284L individually ventilated cages under specific-pathogen-free conditions and fed with irradiated Lab diet (5LG4) and autoclaved reverse osmosis water. Only female mice were used in these experiments. Ethics approval was obtained from the Animal Ethics Committee of the University of Pretoria. Busulfan (50 mg/kg, Sigma Aldrich, Munich, Germany), dissolved in DMSO and diluted with PBS, was administered to 5 to 8 week old female mice by a split intraperitoneal injection with a 24 h delay. Freshly isolated and cultured CD34+ cells were injected 48 h after the initial intraperitoneal Busulfan injection by intravenous tail injection at cell numbers ranging from 300 to 1×10^4 or alternatively, their expansion equivalent was injected. Peripheral blood, bone marrow and spleens were collected at 8 weeks after transplantation of CD34+ cells. Red blood cell lysis was performed on the isolated cells using an ammonium chloride solution and the remaining cells were labeled with specific antibodies and immune phenotyped by flow cytometry (Gallios, Beckman Coulter). Bone marrow cells obtained from these mice (primary engraftment) were injected at 50% of the isolated volume into a second set of mice (secondary engraftment) which were sacrificed at 8 weeks post-transplantation. Limiting dilution analysis was done according to Hu and Smyth (2009).

2.9. Statistical analysis

Data are shown as mean ± S.D. or median of replicate experiments as indicated. Column comparisons are done by using Welch's *t*-test for unpaired data using GraphPad Prism 5.04. Statistical significance is indicated on the graphs as follows: (*) $p < 0.05$, (**) $p < 0.005$, (***) $p < 0.0005$ and (****) $p < 0.00005$.

3. Results

3.1. Establishing culture conditions and drug dosages that maintain > 80% viable cells on day 7

Culturing cells in 20% O_2 and a mixture of cytokines including hIL-3 (3 ng/ml), hSCF (100 ng/ml), hG-CSF (100 ng/ml), and TPO (20 ng/ml) for 7 days resulted in a SP percentage of 1.02 ± 0.30 (n = 7) and a 13.02 fold (n = 7) increase in cell number compared to day 0. Cultures containing only hIL-3 gave a SP percentage of 0.33 ± 0.08 (n = 3) and the cell number on day 7 was 4.92 fold (n = 3) higher than on day 0. Cultures containing hIL-3 (3 ng/ml), hSCF (100 ng/ml) and TPO (20 ng/ml) gave a SP percentage of 1.39 ± 0.40 (n = 4) and a 3.02 fold (n = 4) increase in cell number on day 7 compared to day 0. Cultures expanded in Stemline II™ (which does not contain bovine elements and is thus more appropriate for clinical use) with a mixture of cytokines (hIL-3 (3 ng/ml), hSCF (100 ng/ml), hG-CSF (100 ng/ml), and TPO (20 ng/ml)), resulted in a 65 fold increase in cell number with an SP percentage of 1.32 ± 0.26 (n = 4). The Stemline II™ medium was not used for further experiments as the contents of the medium, which are not disclosed, introduces variables that cannot be monitored or accounted for. All further experiments were performed with the cytokine cocktail (hIL-3 (3 ng/ml), hSCF (100 ng/ml), hG-CSF (100 ng/ml), and TPO (20 ng/ml)) using DMEM. Cell viability was >80% on day 7 in all experimental conditions except where indicated. Western blot revealed that HIF-1α was absent in cells from the 20% O_2 sample (CD34+ exposed to the mixture of cytokines, 20% O_2 and DMSO) and AhR expression was high (Fig. 1). The 1% O_2 sample (mixture of cytokines, 1% O_2 and DMSO) effectively stabilized HIF-1α (Fig. 1) as previously documented (Ivanovic, 2009). A concentration of 0.1 mM DMOG, which is much lower than the commonly used toxic concentration of 1 mM (Asikainen et al., 2005), was selected for further experiments as it gives >80% viable cells and a similar level of HIF-1α and AhR expression as seen in the 1% O_2 sample (Fig. 1). The 1% O_2/YC-1 sample was included because YC-1 caused HIF-1α degradation as previously documented (Fig. 1) (Kim et al., 2006). The concentration selected for YC-1 was 0.5 μM, which gives >80% viable cells. A condition referred to as 1% O_2/DMOG showed that the addition of DMOG to 1% O_2 does not affect the stabilization of HIF-1α (Fig. 1). The expression of HIF-1α and AhR in the 20% O_2/SR1 sample was not affected by SR1 (Fig. 1) (Boitano et al., 2010). A concentration of 0.75 μM was selected for SR1 which gives >80% viable cells. HIF-1α expression was inhibited and AhR and was unchanged in samples containing both DMOG and SR1, referred to as 20% O_2/DMOG/SR1 (Fig. 1).

3.2. Effect of O_2, DMOG, SR-1 and YC-1 on cell viability and cell cycle

Cell proliferation and viability were determined on freshly isolated CD34+ cord blood cells cultured for 7 days under the various conditions selected (Fig. 2A). When grown in the presence of 1% O_2, 20% O_2/DMOG or 20% O_2/DMOG/SR1, proliferation was significantly reduced

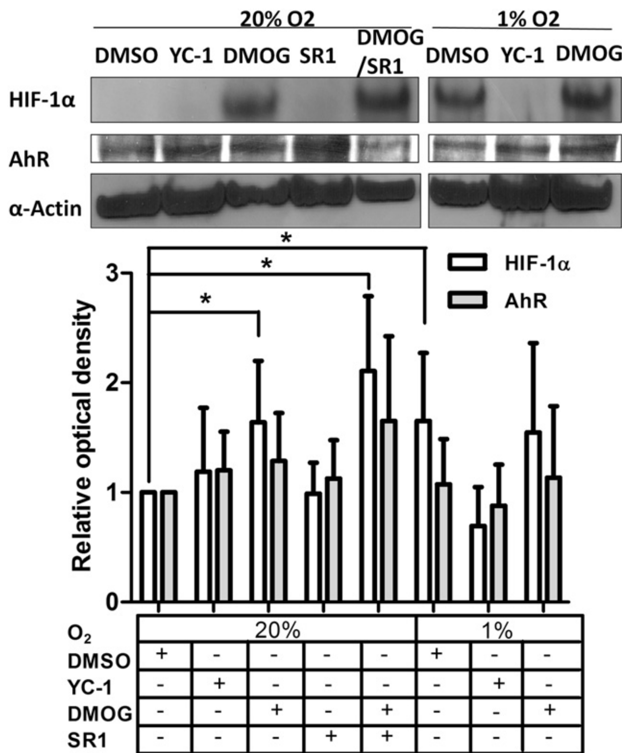

Fig. 1. Protein expression. CD34+ cells were seeded at a density of 2×10^5 cells/ml and cultured under normoxic (5% CO_2; 20% O_2) or hypoxic conditions (5% CO_2; 1% O_2) in DMEM containing 1% PS, 10% FBS, hIL-3 (3 ng/ml), hSCF (100 ng/ml), hG-CSF (100 ng/ml), TPO (20 ng/ml), DMOG (0.1 mM) and SR1 (0.75 μM) as indicated. HIF-1α, AhR and β-Actin proteins visualized by Western blot and densitometric quantification of HIF-1α and AhR proteins (normalized to β-Actin).

when compared to the 20% O_2 control (Fig. 2A). No significant change was observed with cells grown in the presence of 20% O_2/YC-1 or 20% O_2/SR1. However, lower proliferation and a higher mortality were observed with 1% O_2/YC-1 (46.6% dead cells) compared to 1% O_2 (18.6% dead cells) (Fig. 2A). Cell cycle analysis performed of the various culture conditions on days 2 and 7 revealed major variations in proliferation (Fig. 2C and D). The percentage of cells in G_0 observed in each sample on day 2 was inversely correlated with total cell counts. Fig. 2C shows that after 2 days, the population of cells in G_0 decreased rapidly in samples cultured under normoxic conditions, namely 20% O_2, 20% O_2/YC-1 and 20% O_2/SR1. On the other hand, the % of cells in G_0 in samples cultured under hypoxic or hypoxia-mimicking conditions, namely 1% O_2, 20% O_2/DMOG and 20% O_2/SR1, remained significantly higher (Fig. 2C). A smaller G_0 population was observed in the 1% O_2/YC-1 sample when compared to 1% O_2. Similar results were observed on day 7 where the 20% O_2/DMOG and 1% O_2 conditions had significantly higher percentages of cells in the G0 phase ($p < 0.05$, Fig. 2D).

3.3. Effect of O_2, DMOG, SR-1 and YC-1 on progenitor expansion and CD34+ and SP cell phenotype

The proportion of cells in the CD34+ sub-populations of each condition was determined (Fig. 2B) and was used to calculate absolute cell numbers (Fig. 3A–E). Compared to day 0, the proportion of CD34+ cells did not change after 7 days of culture in 1% O_2 while this decreased by 30% in 20% O_2 (Zini et al., 2008). Differentiation that occurred in the 20% O_2 sample is also evident in the small proportional increase of the primitive SP and G_0 populations compared to a significant increase observed in the less primitive CD34, CD34+/CD133+ (Fig. 3A and B) and CFU populations (Fig. 3C). Even with higher levels of differentiation, the total cell number in the primitive populations of the 20% O_2 sample was larger than in the 1% O_2 sample which did not increase from day 0 (Fig. 3D and E).

The 20% O_2/DMOG sample proliferated at a slow rate, similar to the 1% O_2 sample (Fig. 2) and the sizes of the cell sub-populations (Fig. 3A–E) were also comparable. Fig. 3 indicates that the total number of cells in all the sub-populations was greater in the presence of 20% O_2/DMOG than in 1% O_2. The inhibition of HIF-1α by YC-1 in the 1% O_2/YC-1 sample reduced cell quiescence (Fig. 2B) and increased cell differentiation (Fig. 3A–E). The cell numbers in all the sub-populations of the 1% O_2/YC-1 sample were reduced as expected when compared to the 1% O_2 sample (Fig. 3).

The addition of SR1 to cells cultured at 20% O_2 caused no significant change in cell proliferation (Fig. 2A). SR1 did however cause all the cell sub-populations to increase proportionally. The proportion of cells with SP characteristics decreased from day 0 to day 7 in all samples, but the decrease was significantly reduced in the presence of 20% O_2/SR1 and 20% O_2/DMOG/SR1 when compared to 20% O_2 (Fig. 2C). Proliferation thus occurred mainly in the SP (2.3 fold more) (Fig. 3D). Cells cultured in 20% O_2 with DMOG and SR1 gave results similar to treatment with DMOG alone with regard to cell cycle and cell proliferation (Figs. 2C and 3A). However, a significantly larger SP population was observed, which is similar to what was seen with treatment with SR1 alone (Fig. 3E).

3.4. Effect of O_2, DMOG, SR-1 and YC-1 on the proliferation rate of cells capable of forming long term colony forming units

Cells from primary cultures were washed and initiated in secondary cultures and the proliferation rates of cells in secondary cultures are shown in Fig. 4. Proliferation in 20% O_2/DMOG and 20% O_2/DMOG/SR1 samples was similar until approximately day 19, whereafter proliferation in 20% O_2/DMOG/SR1 surpassed proliferation in 20% O_2/DMOG (Fig. 4A). This difference indicates that even though the proliferation rate during primary liquid culture was similar, the cells in the 20% O_2/DMOG/SR1 sample were more primitive as they provided prolonged proliferation. The fact that the total cell proliferation of the 20% O_2/DMOG, 20% O_2/SR1, 20% O_2/DMOG/SR1 and 1% O_2 samples was significantly greater at day 29 when compared to cells grown in the presence of 20% O_2 (Fig. 4A) supports our data above that show that cells used at the start of this assay were more primitive. On the other hand, the 1% O_2/YC-1 sample demonstrated significantly less proliferation compared to the 1% O_2 sample. Cells were removed from secondary culture every second day and placed into semisolid medium to assess colony formation. The number of colonies observed was used to create a pre-CFC profile. The first peak analyzed at day 11 provides indirect information on the "stemness" of the committed progenitors present at day 7 of primary liquid culture. The second peak analyzed at day 23 provides information on the LT-CFUs. The first and second peaks of the 20% O_2/DMOG, 20% O_2/SR1, 20% O_2/DMOG/SR1 and 1% O_2 samples were significantly higher than the 20% O_2 sample, indicating that more committed progenitors and pre-CFCs were present. The absolute CFU cell number obtained by normalizing the pre-CFC data shown in Fig. 4B and C to the proliferation obtained in primary culture (Fig. 2A) reveals the magnitude of the difference in the differentiation capacity of progenitors in the 20% O_2/SR1 sample (Fig. 4B and C). The total LT-CFU cell number obtained for the 20% O_2/SR1 sample was 13.9 fold higher than for the 20% O_2 sample (Fig. 4C).

3.5. Effect of O_2 and SR-1 on cells capable of short-term and long-term engraftment

The short-term engraftment capacity of freshly isolated CD34+ cells and cells expanded for 7 days under various conditions is portrayed by the percentage human CD45+ cells related to mouse CD45+ cells following engraftment into NSG mice (Fig. 5A). The cell numbers transplanted and the expansion equivalent cell numbers are shown in

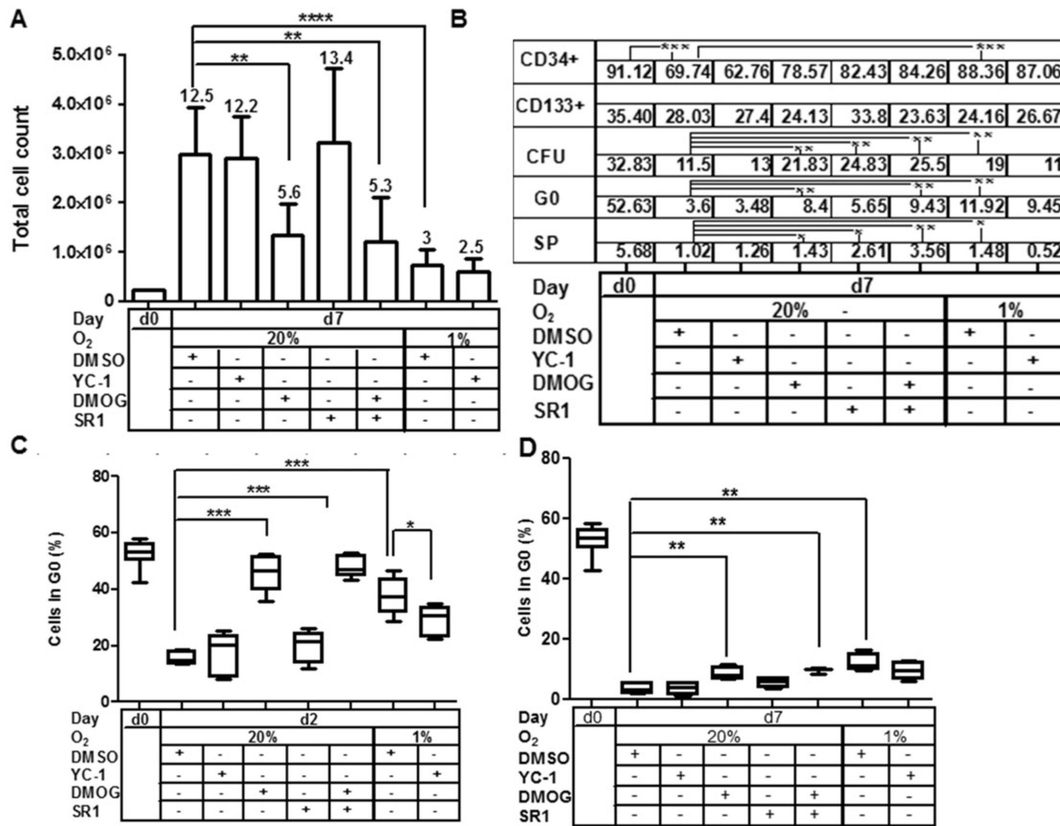

Fig. 2. Proliferation of CD34+ cord blood cells. CD34+ cells were seeded at a density of 2×10^5 cells/ml and cultured under normoxic (5% CO_2; 20% O_2) or hypoxic conditions (5% CO_2; 1% O_2) in DMEM containing 1% PS, 10% FBS, hIL-3 (3 ng/ml), hSCF (100 ng/ml), hG-CSF (100 ng/ml), TPO (20 ng/ml), DMOG (0.1 mM) and SR1 (0.75 μM) as indicated. (A): CD34+ cord blood cells were cultured for 7 days under the various conditions shown. Cells were counted on day 7 and cell viability was determined using Trypan blue. (B): The percentages obtained for the CD34, CD133, CFU, G0 and side population (SP) subpopulations are provided. Eight experiments were performed with a sample number per condition that varied between 3 and 8. (C): The percentage of cells in G0 on day 2 of primary culture. Eight experiments were performed with a sample number per condition that varied between 3 and 8. (D): The percentage of cells in G0 on day 7 of primary culture. Eight experiments were performed with a sample number per condition that varied between 3 and 8.

Table 1. Expansion equivalent refers to the reduction of the number of cells required in order to achieve an equivalent number of cells to the unexpanded samples. An incremental increase in engraftment was observed in mice that received 3×10^2, 1×10^3, 3×10^3 or 10^4 freshly isolated cells. Similar engraftment levels were observed in mice that received the expansion equivalent cell numbers cultured in 20% O_2. Significantly higher engraftment was observed in mice that received an expansion equivalent of 1×10^3 cells expanded in 20% O_2/SR1 and 1% O_2/SR1 compared to those that received an expansion equivalent of 1×10^3 cells expanded in 20% O_2. The mice that received an expansion equivalent of 3×10^2 cells from the 1% O_2/SR1 culture showed significantly higher engraftment compared to the 20% O_2/SR1 culture. The positively engrafted (>0.5%) mice in the primary and secondary engraftment studies are shown in Table 1 and the limiting dilution graphs are shown in Fig. 5B and C. The short-term SCID repopulating cells (SRC) and the long-term SRC frequencies obtained (with the 95% confidence intervals) are shown in Table 2. The long-term SRC frequency increased with the 20% O_2 condition from 1/3771 to 1/2265 while the 1% O_2 condition only increased to 1/3487, whereas the 10%/SR1 condition gave a frequency of 1/890. This is a noteworthy increase in long-term SRCs obtained with a cell dose after expansion of 13,430 cells. The 1% O_2/SR1 condition provided a similar long-term SRC frequency of 1/990 which was however obtained with a dose after expansion of 3770 cells (Table 1). The capacity of the engrafted cells to differentiate into the various hematopoietic lineages is shown in Supplementary Fig. 3A and B. This demonstrates that no significant differences in the differentiation capacity could be observed in the peripheral blood and spleen between the expanded and fresh CD34+ cells (Supplementary Fig. 3A and B).

4. Discussion

Previous studies have found that the ARNT transcription factor plays an important role in the regulation of hematopoiesis (Lindsey and Papoutsakis, 2012; Shimba et al., 2003). In order to further investigate how ARNT complex formation might benefit the expansion of the CD34+ sub-populations, cord blood CD34+ cells were cultured under selected conditions. Hypoxia (Ivan et al., 2001; Takubo et al., 2010) has a dose related effect on promoting cellular quiescence that in turn promotes the maintenance of HSPCs (Cheng et al., 2000; Guitart et al., 2010) by affecting cell proliferation, self renewal and differentiation (Ivanovic, 2009; Ivanovic et al., 2002). However, 1% O_2 is not suitable for the expansion HSCs due to the suppression of cell proliferation which results in a low final number of HSCs after culture.

Similar results were obtained for cultures treated with a low concentration of DMOG (0.1 μM) which is a mimic of 1% O_2. A minimal loss of the CD34 marker in the 20% O_2/DMOG samples suggests that since there is no direct relationship between CD34 expression and maintenance of self renewal, DMOG slows down the differentiation process that occurs at 20% O_2 by stabilizing HIF-1α. The 20% O_2/DMOG sample proliferated at a slow rate, similar to the 1% O_2 sample and the sizes of the cell sub-populations were also comparable. This demonstrates that the addition of 0.1 mM DMOG to culture medium at 20% O_2 can be used to inhibit differentiation in a manner that is almost similar to a hypoxic environment of 1% O_2. The total number of cells in all the sub-populations is greater in the presence of 20% O_2/DMOG than 1% O_2, which makes 20% O_2/DMOG a better condition for expanding HSCs.

Cells cultured under normoxic conditions (20% O_2), where it is known that HIF-1α is degraded and AhR binds ARNT (Boitano et al.,

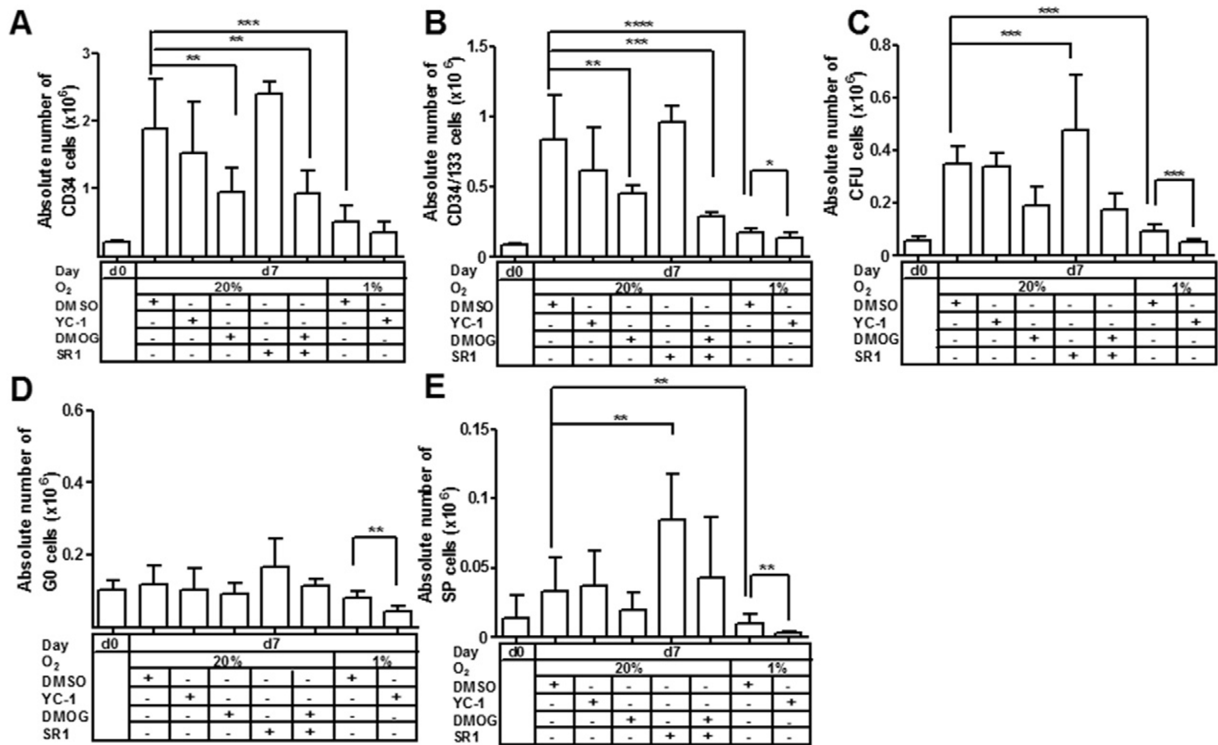

Fig. 3. Cell numbers obtained in the CD34+, CD34+/CD133+, CFU, G0 and SP sub populations. CD34+ cells were seeded at a density of 2×10^5 cells/ml and cultured under normoxic (5% CO_2; 20% O_2) or hypoxic conditions (5% CO_2; 1% O_2) in DMEM containing 1% PS, 10% FBS, hIL-3 (3 ng/ml), hSCF (100 ng/ml), hG-CSF (100 ng/ml), and TPO (20 ng/ml). CD34+ cord blood cells were cultured for 7 days under various conditions as shown. (A): The proportion of CD34 positive cells was multiplied by the total number of cells in solution. The sample number is 8. (B): The proportion of total CD34+/CD133+ cells was multiplied by the total number of cells in solution. The sample number is 3. (C): The proportion of total colony forming units relating to the number of cells plated was multiplied by the total number of cells in solution. The sample number is 8. (D): The proportion of cells in the G_0 phase was multiplied by the total number of cells in solution. The sample number is 7. (E): The proportion of cells with side population characteristics was multiplied by the total number of cells in solution. Eight experiments were performed with a sample number per condition that varied between 3 and 8.

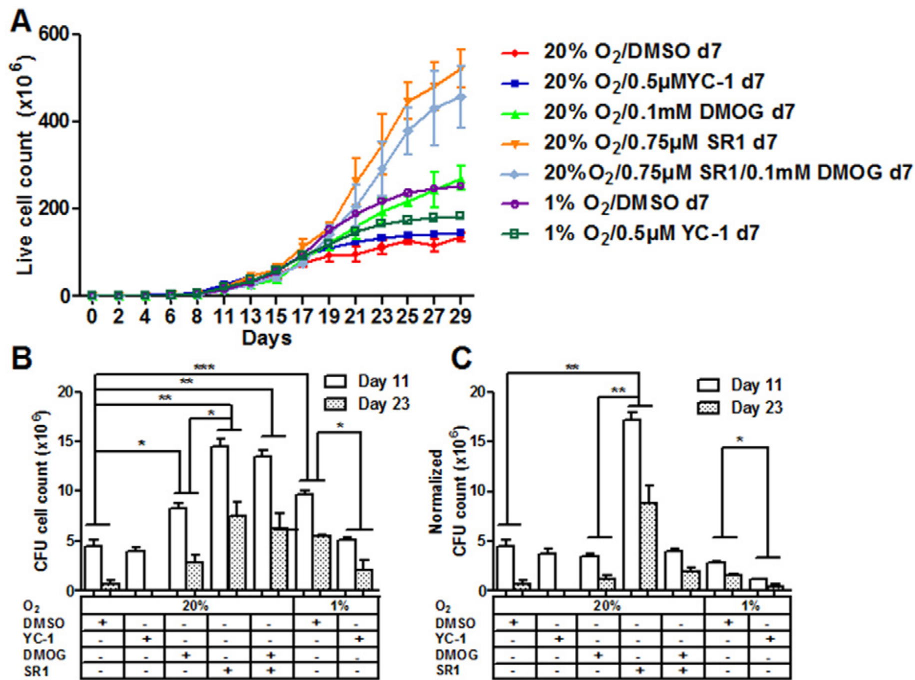

Fig. 4. Pre-CFC assay of secondary cultures. CD34+ cells were seeded at a density of 2×10^5 cells/ml and cultured under normoxic (5% CO_2; 20% O_2) or hypoxic conditions (5% CO_2; 1% O_2) in DMEM containing 1% PS, 10% FBS, hIL-3 (3 ng/ml), hSCF (100 ng/ml), hG-CSF (100 ng/ml), TPO (20 ng/ml), DMOG (0.1 mM) and SR1 (0.75 μM) as indicated. The cells were washed after 7 days and seeded at a density of 2×10^5 cells/ml and cultured under normoxic (5% CO_2; 20% O_2) conditions in DMEM containing 1% PS, 10% FBS, hIL-3 (3 ng/ml), hSCF (100 ng/ml), hG-CSF (100 ng/ml), TPO (20 ng/ml), (A): Proliferation of cells in secondary liquid culture (LC2). The live cell counts of every second day until day 29 are represented on the graph. (B): The number of CFUs on days eleven and twenty three are presented for each sample. (C): The normalized CFU cell number on days eleven and twenty three are presented for each sample. Eight experiments were performed with a sample number per condition that varied between 3 and 6.

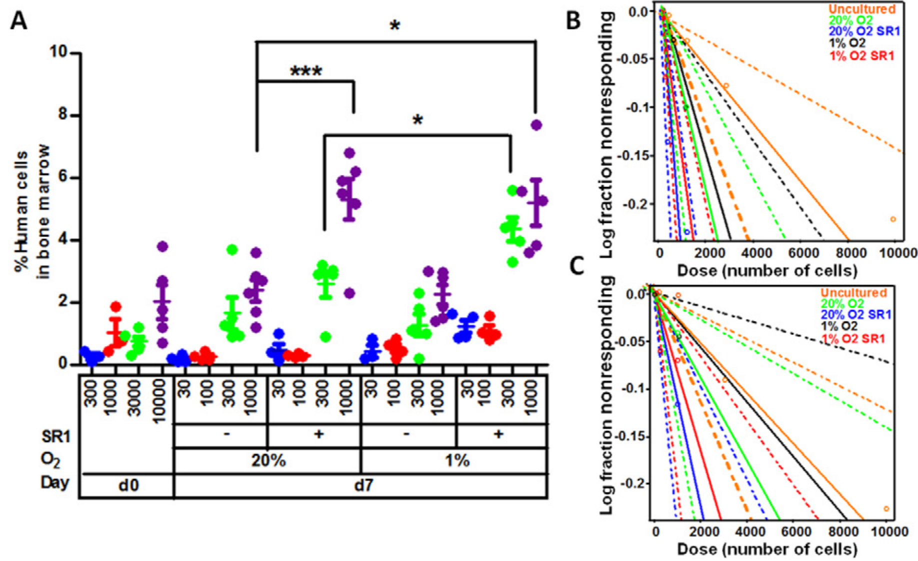

Fig. 5. Engraftment of expanded CD34+ cells. CD34+ cells were seeded at a density of 2×10^5 cells/ml and cultured under normoxic (5% CO_2; 20% O_2) or hypoxic conditions (5% CO_2; 1% O_2) in DMEM containing 1% PS, 10% FBS, hIL-3 (3 ng/ml), hSCF (100 ng/ml), hG-CSF (100 ng/ml), TPO (20 ng/ml) and SR1 (0.75 μM) as indicated. Freshly isolated human CD34+ cells were transplanted at 300, 1000, 3000 and 10,000 cells. The expansion equivalent of 30, 100, 3000 and 10,000 cells was also transplanted. (A): Human CD45+ cell chimerism obtained in NSG mice with primary engrafted CD34+ cells. The percentage human CD45 cells relative to mouse CD45 cells in the femurs of the mice at week 8 is shown. (A): This graph shows a linear dilution analysis of non-responding cells that did not engraft. Uncultured as well as 7 day expanded cells were transplanted separately into NSG mice and analyzed after 8 weeks to reveal the extent of primary engraftment. (B): This graph indicates the non-responding cells that did not engraft when cells, obtained from the femurs of mice of the primary engraftment, were transplanted into NSG mice and analyzed after 8 weeks to reveal the extent of secondary engraftment.

2010; Ivanovic, 2009), proliferate at a high rate and only maintain a small proportion of quiescent cells. However, even though the percentage of primitive and progenitor cells is low, the total/absolute number is significantly higher than in samples cultured under hypoxia.

To determine if the expansion of primitive HSCs can be improved by limiting ARNT/AhR complex formation, CD34+ cord blood cells were cultured with SR1, an antagonist of AhR (Boitano et al., 2010). Isolated CD34+ cells cultured in 20% O_2 with SR1, where it is known that both HIF-1α and AhR are suppressed (Boitano et al., 2010; Ivanovic, 2009), proliferate at a high rate. We demonstrate that this occurs mainly in the SP cell compartment which suggests that the expansion of the primitive HSC population is favored. This was confirmed following engraftment of NSG mice that provided a 4.23 fold increase in LT-SRC frequency obtained with a cell dose after expansion of 13,430 cells. The final number of primitive HSCs obtained after culture was far greater than the other conditions, which indicates that primitive HSC expansion is favored when cells are cultured under normoxic conditions with SR1.

Cells cultured in both DMOG (Ivanovic, 2009) and SR1 (Boitano et al., 2010) proliferate at a similar rate to cultures containing DMOG alone. However, the primitive stem cell population is larger than when cells are cultured in DMOG alone as demonstrated by the increased proportion of cells that have side population characteristics as well as cells with short and long-term CFU abilities. Culturing cells in DMOG or hypoxia with SR1 is useful to obtain a high percentage of primitive progenitor cells. The engraftment data support this finding where the short-term and long-term (LT-SRC frequency of 3.8) engraftment was similar to the condition where HIF-1α was not stabilized and an AhR antagonist was used (LT-SRC frequency of 4.23). This was, however, obtained with a much lower cell dose after expansion of 3770 cells.

Table 1
Primary and secondary engraftment of treated HSCs.

Conditions	Cell dose transplanted		Primary transplant	Secondary transplant
Uncultured	300		0/4	0/4
	1000		1/4	0/4
	3000		3/5	3/5
	10,000		5/5	5/5
Control	Fraction of 7 day culture		Primary transplant	Secondary transplant
	Equivalent starting dose	Dose after expansion		
	30	374.4	0/4	0/4
	100	1248.0	0/4	0/4
	300	3744.0	2/5	1/5
	1000	12,480.0	4/6	2/6
SR1	30	402.9	0/4	0/4
	100	1343.0	0/4	0/4
	300	4029.0	4/5	2/5
	1000	13,430.0	6/6	4/6
1% O_2	30	91.2	0/4	0/4
	100	304.0	0/4	0/4
	300	912.0	1/5	0/5
	1000	3040.0	4/6	2/6
1% O_2 SR1	30	111.3	0/4	0/4
	100	371.0	0/4	0/4
	300	1113.0	3/5	2/5
	1000	3710.0	5/6	3/5

Table 2
Short-term and long-term engraftment of treated HSCs.

Culture condition	ST-SRC frequency per starting cells	95% confidence interval	ST-SRC frequency per total cells
Uncultured	1/3098	1/1430–1/6690	64
20% O_2 DMSO	1/889	1/389–1/2032	3374
20% O_2 SR1	1/284	1/134–1/601	11,355
1% O_2 DMSO	1/1114	1/461–1/2687	1095
1% O_2 SR1	1/537	1/253–1/1138	2274
Culture condition	**LT-SRC frequency per starting cells**	**95% Confidence interval**	**LT-SRC frequency per total cells**
Uncultured	1/3771	1/1749–1/8134	53
20% O_2 DMSO	1/2265	1/724–1/7086	1324
20% O_2 SR1	1/890	1/390–1/2033	3623
1% O_2 DMSO	1/3487	1/886–1/13725	349
1% O_2 SR1	1/992	1/403–1/2444	1237

This indicates that the 1% O_2/SR1 treatment increased the capacity of the cells to engraft and repopulate the bone marrow but did not increase the number of these cells during culturing. The hypothesis that the use of a culturing condition that stabilizes HIF-1α and antagonizes the AhR protein would promote the engraftment capacity of the LT-HSC population, was thus accepted.

5. Conclusion

The use of SR1 at 20% O_2 proved to be the best condition for the expansion of HPSCs into larger cell numbers; however, the use of SR1 at 1% O_2 resulted in an increase in the proportion of primitive HSCs in the sample. It will in the future be necessary to confirm the effectiveness and safety of cells treated with SR1. We have demonstrated the value of comparing the HSC sub-populations of treated samples in terms of proportion and absolute cell number and believe that our results will impact on the translation of *in vitro*/*ex vivo* findings to the clinic.

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Author contribution

Carlo Jackson: Conception and design, execution of experiments, manuscript writing

Chrisna Durandt: Provision of expertise in flow cytometry

Ilse Janse van Rensburg: Provision of expertise in handling of mice

Vincent Praloran: Supervision, financial support, manuscript editing

Philippe Brunet de la Grange: Supervision, training, expertise and manuscript editing

Michael Pepper: Project management, supervision, financial support, manuscript editing

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Disclosure of potential conflicts of interests

The authors indicate that there are no conflicts of interest to disclose.

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