**Ex-vivo Expansion of Umbilical Cord Blood Hematopoietic Stem Cells**

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The major obstacle to the widespread use of umbilical cord blood (UCB) in hematopoietic stem (HSC) cell therapy is the low cell dose available, which often leads to graft failure. The engrafting capability of a UCB unit can be enhanced by ex-vivo expansion by a rational combination of many factors such as initial stem/progenitor cell enrichments and oxygen tension, culture duration, cytokine cocktails, blocking in vitro differentiation of early progenitor cells and co-culture with stromal feeder layers. Interactions of vascular/stromal cells with HSCs are known to be of great importance for their maintenance, thus making stromal cells ideal to mimic HSCs adult niche – the bone marrow (BM) – in in vitro conditions. With the objective of expanding Mesenchymal Stem/Stromal Cells (MSCs) on a xenogeneic (xeno)-free manner, chemically defined medium (StemPro®), and Human platelet lysate (HPL)-supplemented media were used to expand BM MSCs. HPL-supplemented media was able to expand and establish BM MSC-derived feeder layers capable of supporting HSC expansion, indicative that HSC/MSC co-culture can be done in a fully xeno-free manner throughout all steps of the process. To surpass availability issues associated with BM MSCs, HPL-supplemented media was also able to expand MSCs from alternative sources, usually regarded as biological waste, such as adipose tissue (AT) and umbilical cord matrix (UCM), and establish functional feeder layers. Additionally, a two-level face-centred cube design (FC-CD) approach was used for the optimization of a cytokine cocktail to supplement StemSpan SFEM II expansion medium, in the presence or absence of MSC-derived stromal feeder layers.

**Keywords:** Cord Blood; Cell Therapy; Hematopoietic Stem Cells; Mesenchymal Stem Cells; Ex-vivo Expansion

**Introduction**

The hematopoietic system supplies our body with >100 billion mature blood cells every day that carry out functions such as oxygen transport, immunity, and tissue remodelling. Hematopoietic stem cells (HSCs), located at the top of the hematopoietic hierarchy, are responsible for replenishing our pool of blood cells throughout life.⁸

The entire hematopoietic system can be repopulated following infusion of HSCs, meaning their transplantation can be used to treat/cure a range of haematological diseases. Nowadays, more than 30,000 patients with haematological malignancies every year receive high-dose chemotherapy followed by HSC transplantation from Bone marrow, G-CSF mobilized Peripheral Blood (mPB), and Umbilical Cord Blood (UCB).⁹,¹⁰ HSC transplantation faces two major immunological challenges: i) The recipient’s immune system may reject the transplant, which leads to graft failure; ii) The occurrence of graft-versus-host disease (GvHD), in which T-lymphocytes within the graft attack the recipient’s skin, liver, and gastrointestinal tract, causing damage that, if left unchecked, can be fatal.¹¹,¹²

Umbilical Cord Blood was first used in clinical practice, in 1988 in a 5-year old patient with severe aplastic anemia.¹³,¹⁴ The first signs of engraftment appeared after 22 days with no GVHD signs, and more than 20 years after the UCB transplant, the patient remains healthy with complete long-term haematological and immunological donor reconstitution.¹³,¹⁵ These encouraging results suggested that: i) a single cord contained enough HSC to reconstitute haematopoiesis; ii) UCB could safely be collected at birth; and iii) UCB HSPC could be cryopreserved and thawed without negatively affecting repopulating ability.

Since then, a boom in allogeneic UCB transplantation has been observed. In fact, UCB transplantations surpassed the number of bone marrow transplants in 2009.¹⁶ Much of this success is due to the creation of worldwide network of cord blood banks, allowing the collection, cryopreservation, and distribution of over 600,000 UCB.¹⁷ Many advantageous characteristics turn UCB an ideal HSC source for transplantation¹⁰: i)
Increased availability of banked samples – UCB is donated, quality-tested and banked in advance, turning it ideal in acute settings and abolishing the long delay inherent to the use of bone marrow; ii) Immaturity of the immune cells present in UCB is far less likely to cause GvHD than bone marrow or mPB; iii) Contrary to bone marrow and mPB, perfect HLA-matching is not necessary between donor and recipient for a UCB transplant to be successful; iv) UCB is less likely to transmit viruses when compared to bone marrow or mPB; However, UCB transplantation also poses some drawbacks, such as higher rates of engraftment failure due to low volume collection, and delayed engraftment of neutrophils and platelets (time to neutrophil recovery is a major indicator of post-transplant mortality), which lengthen hospital stays and increases the risk of serious complications. A single UCB unit has sufficient cells to repopulate new-borns or small children, but not larger weight adolescents and adults, limiting the use of UCB to paediatric cases. One can enhance the engrafting capability of a UCB unit by ex-vivo expansion, accomplishing not only increased cell numbers with haematopoietic reconstitution potential, but also providing a selective expansion of short-term engrafting HSCs. If one takes into account that neutrophil recovery depends on early-engrafting cells, and not LT-HSCs, the ability to expand these progenitors becomes critical. Expansion of UCB units can augment the numbers of colony forming unit granulocyte-macrophages (CFU-GMs), which are higher in UCB compared with mPB or BM. Moreover, the proliferative ability of UCB CD34+CD38− is higher than their BM counterparts. Even though ex vivo expansion leads to earlier initial haematopoietic recovery, it ends up with later graft failure, due to the loss of long-term repopulating activity. Ultimately, ex-vivo expanded grafts provide a clinical advantage, especially in combination with ‘un-manipulated’ fractions of the same cell source unit, where the former would provide faster initial haematopoietic reconstitution, and the latter long-term sustainable haematopoiesis. Many procedures have been attempted improve the expansion the HSCs pool, by a rational combination of many factors such as initial stem/progenitor cell enrichments and oxygen tension, culture duration, cytokine cocktails, blocking in vitro differentiation of early progenitor cells and co-culture with stromal feeder layers. Most traditional cytokine/supplement combinations rely on early-acting cytokines SCF and Flt-3L, and some also on TPO and members of the interleukin (IL) family. When comparing liquid-suspension cultures with stromal co-culture, it is to notice that CD34+ expansion is higher in the latter. Furthermore, although cytokine leads to an increase in Total nucleated cells (TNCs) after UCB expansion, the rapidity of engraftment did not improve in clinical trials relying solely on cytokines. Rather than long-term HSC, this is probably due to the expansion of early acting multipotent progenitors (MPPs) and cytokine-induced differentiation of more committed progenitors (CD34+ CD38− or CD34+ Lin−) into cells of committed lineage (CD34+ Lin+) that have poor BM homing capabilities. Furthermore, intercellular signalling between cells from distinct lineages and stages of differentiation, or within the same lineage, is able to disturb stem/progenitor cell expansion. This inhibition can be done through competition for cytokines and nutrients, or by secreting factors that alter the HSC cell cycle rate and promote differentiation and/or apoptosis. In adults, HSCs and their primitive progeny are located within the Bone Marrow (BM), contiguously to the endosteal surface of trabecular bone, and interactions of vascular/stromal cells with HSCs are known to be of great importance for their maintenance, thus making BM MSCs cells ideal to mimic their natural niche when culturing HSCs in vitro. Consistently, it was reported that adherent stromal feeder layers are able to promote ex vivo expansion/maintenance of human HSCs cultured on top. This system also preserves the ability of expanded UCB cells to engraft in an in vivo model. However, it is still not know if these positive effects are solely due to soluble factors, or if direct cell-cell contact is required. Nonetheless, direct contact of
haematopoietic cells with stromal cells has been associated with an improvement in stem/progenitor cell expansion.\textsuperscript{6,36} Increased numbers of more lineage-committed cells\textsuperscript{37}, and CD7\textsuperscript{+} early lymphoid progenitors were also reported in these type of cultures.\textsuperscript{6,36} Distinct co-culture systems have been tested for the expansion of HSCs. Although most of them use bone marrow mesenchymal stem/stromal cells (MSC)-derived as feeder layers, some systems use different stromal sources, different isolation protocols of stromal cells, and diverse cytokine combinations. Consequently, divergent results have been reported and thus the role of stroma in hematopoietic cell co-cultures remains a very controversial issue.\textsuperscript{6}

Materials and Methods

Human Samples

Bone marrow aspirates, Umbilical Cord, and Adipose tissue samples were obtained from IPO Lisboa, Hospital São Francisco Xavier, and Clínica de Todos-Os-Santos, respectively. All the samples were obtained after informed consent of the patients, and their harvesting and collection performed in accordance with the protocols of the respective institutions.

Processing Umbilical Cord Blood

Low-density mononuclear cells (MNCs) were isolated from UCB samples by Ficoll density gradient centrifugation, followed by a red blood cell lysis with NH4Cl (10 minutes at room temperature). The lysis was stopped by adding fetal bovine serum (FBS, HyClone). MNCs were quickly stored at -80°C in cell culture freezing medium (Recovery\textsuperscript{TM}; Gibco\textsuperscript{®} Life Technologies\textsuperscript{TM}, 50 x 10^6 cells/vial). The samples were banked in liquid nitrogen at -180°C the day after.

Purification of CD34\textsuperscript{+}-enriched cells.

To obtain a suspension of purified Hematopoietic Stem and Progenitor Cells (HSCs), UCB MNCs were picked from the cryopreserved samples and thawed in Dulbecco's Modified Eagle Medium (DMEM, Gibco\textsuperscript{®} Life Technologies\textsuperscript{TM}) with 20%FBS supplemented with DNase I (10µg/mL) to avoid clump formation. A maximum of 250 x 10\textsuperscript{6} cells (5 vials) were thawed per 50 mL of thawing medium. UCB MNCs were then enriched for CD34\textsuperscript{+} cells trough magnetic activated cell sorting (MACS\textsuperscript{®}, Miltenyi Biotech and EasySep\textsuperscript{®}, StemCell Technologies\textsuperscript{TM}), following manufacturer’s instructions.

MSC cell culture

Thawing

Frozen MSC samples were thawed in DMEM 20% FBS or StemGro\textsuperscript{®} hMSC Medium (Corning\textsuperscript{®}) in case xeno-free conditions were required, counted and plated as mentioned in the following sections.

Ex-vivo expansion in static conditions

Adherent cultures of MSCs were plated with a cell density between 3 x 10\textsuperscript{3} cells/cm\textsuperscript{2} and 6 x 10\textsuperscript{3} cells/cm\textsuperscript{2}, in polystyrene T-Flasks or flat bottom multiwell-plates (Corning\textsuperscript{®}).

Culture Media: To grow MSCs, serum-containing (SC) or xeno-free (XF) media can be used. The SC medium consists in DMEM supplemented with 10% (v/v) Fetal Bovine Serum, MSC qualified (FBS, Life Technologies\textsuperscript{TM}). MSCs expanded in DMEM 10%FBS will be hereinafter referred to as SC MSCs. The XF medium used was either StemPro\textsuperscript{®} MSC SFM (Life Technologies), supplemented with GlutaMax\textsuperscript{TM} (Life Technologies\textsuperscript{TM}), or DMEM supplemented with 5% xeno-free Human Platelet Lysate (HPL) (BSSub\textsuperscript{TM}-XF, AventaCell BioMedical Co., Ltd.). MSCs expanded in StemPro\textsuperscript{®} and BSSub\textsuperscript{TM}-XF will be hereinafter referred to as XF MSCs and HPL-XF MSCs, respectively. 1% (v/v) of antibiotic was added to all culture media to avoid contaminations. In the specific case of XF MSCs expansion, a pre-coating with CELLstart\textsuperscript{TM} (Invitrogen\textsuperscript{®}, Carlsbad, CA) is needed before plating the cells (1 hour at 37°C, 5% CO\textsubscript{2}). CELLstart\textsuperscript{TM} is a fully-defined, xenogeneic-free humanized substrate for stem cell culture, produced under cGMP\textsuperscript{39}, and compensates for the lack of adhesion molecules in StemPro\textsuperscript{®}, which are necessary for the MSCs to be cultured in an adherent fashion.

Passaging: If a given MSC population is to be maintained in culture for long periods, adherent MSCs must be harvested upon reaching 70%-80% confluency.
from the recipient with proteolytic enzymes, and re-plated into a new recipient. The media in which cells are cultured are determinant in the choice of which enzyme solutions to use. Accutase® (Sigma) was used in the case of SC MSCs, while TrypLE™ Select CTS™ (1X) was used for XF and HPL-XF MSCs. Washing media was added in a 1:3 proportion to dilute the enzymatic agent, DMEM+10% FBS (HyClone™) for SC MSCs and StemGro® hMSC Medium (Corning®) for XF MSCs.

**HSC/MSC Expansion Systems.**

A HSC/MSC co-culture system was used to expand fresh CD34⁺ cells.

- **Establishment of MSC-derived feeder layers.**

  To establish a MSC-derived stromal feeder layer capable of supporting HSPC expansion, 95-100% and 60-70% confluent BM MSCs and AT/UCM MSC, respectively, had their growth inactivated by replacing the culture media with DMEM-10%FBS, supplemented with mitomycin C (0.5 µg/mL), for 2.5 hours, at 37°C, 5% CO₂.

- **HSC expansion media.**

  Two expansion media were used. i) Quality Biological Serum-Free medium (QBSF-60; Quality Biological, Inc.), supplemented with a specific cytokine cocktail, hereinafter referred as Z9: SCF (60 ng/ml), Flt-3L (55 ng/ml), TPO (50ng/ml) and bFGF (5 ng/ml); ii) StemSpan™ SFEM II (Stem Cell Technologies™) + Z9.

- **Expansion of CD34⁺-enriched cells in Static conditions.**

  Suspensions of CD34⁺-enriched cells were cultured (30-50 × 10⁶ cells/mL) on top of a previously inactivated human MSC-derived feeder layer, for 7 days without media change, at 37°C, 5%CO₂. CD34⁺-enriched cells were also cultured in the absence of stromal feeder layer (noStr) as a control condition.

- **In vitro assays.**

  Fresh CD34⁺-cells (day-0) were harvested for CAFCs assay (2 x 10³ cells per condition), for CFU-C assay (1 x 10³ cells per condition), and for Flow Cytometry analysis. At the end of the co-culture (day-7), the number of total nucleated cells (TNC) after expansion was determined, harvested for CAFCs assay (2 x 10³ cells per condition), for CFU-C assay (5 x 10³ cells per condition), and for Flow Cytometry analysis.

- **Proliferative analysis.**

  Total hematopoietic expansion is evaluated by assessing the fold-increase in total cell number. This is calculated by dividing the number of cells at the end of the culture period (day-7) by the number of cells at the beginning of the culture (day-0).

- **Phenotypic analysis.**

  Both fresh CD34⁺-enriched cells and expanded CD34⁺-enriched cells were analysed by flow cytometry (FACScalibur, Becton Dickinson), using a panel of monoclonal antibodies (FITC-, or PE-conjugated) against: CD90 for HSCs; CD34 and CD133 for stem/progenitor cells; CD41 for megakaryocyte lineage, and CD14 for monocytic lineage; CD15 and CD33 for myeloid lineage; CD7 for early lymphoid cells. A minimum of 3 x 10⁴ cells/tube were incubated with these monoclonal antibodies for 15 min in the dark at room temperature. Cells were washed afterwards with PBS and fixed with 1% paraformaldehyde (Sigma). Appropriate isotype controls were also prepared for every experiment to exclude the possibility of non-specific binding of antibodies to Fc receptors. A minimum of 10 000 events was collected for each sample. Analysis was performed using FlowJo software.

- **Clonogenic Potential assay.**

  Colony forming unit in culture (CFU-C) assays are short-term, semi-solid colony assays. Three different scores were attributed to the colonies according to their composition, size, and color: Colony-forming unit–granulocyte macrophage (CFU–GM), colony-forming unit–granulocyte, erythroid, macrophage, megakaryocyte (CFU-Mix) and burst forming unit–erythroid (BFU–E).⁴⁰ Both fresh (day-0) and expanded (day-7) UCB CD34⁺-enriched cells are analysed to evaluate their clonogenic potential. Fold-increase in clonogenic potential from day-0 to day-7 is measured for each of the three scores. The clonogenic assays are performed by plating 1 x 10³ fresh cells at day-0, and 5 x 10³ expanded cells at day-7, in MethoCult GF H4434 (Stem Cell Technologies).
The assay is done on 4-well plates with 2cm² each. Three of the wells were loaded with the cell sample, the fourth was loaded with purified water to provide a steady source of humidity throughout the assay. The clonogenic cultures were maintained at 37°C and 5% CO₂. After 14 days, the colonies were counted and categorized. Total CFU numbers were calculated by dividing the number of counted colonies for day-0 or day-7, by the number of cells plated for day-0 or day-7, and this value then multiplied by the total number of cells (TNC) in culture for the day of harvest (day-0 or day-7).

**Results and Discussion**

**Establishment of xenogeneic (xeno)-free MSC-derived feeder layers for HSC expansion.**

Efforts are being made in the direction of using human origin, clinical-grade MSCs to support HSCs – either in the form of feeder layer-assisted expansion⁵,⁶, or co-transplantation to improve engraftment⁴¹ – prepared under good manufacturing practice (GMP)-compliant conditions, to overcome possible contamination risks from compounds of animal origin such as Fetal Bovine Serum (FBS). In our laboratory, although we have successfully established a serum-free, MSCs/HSCs co-culture expansion system⁶, the MSC-derived feeder layers are still established using serum-supplemented medium (DMEM 10%FBS). To prepare MSCs under GMP-compliant conditions is the next step regarding their usage in clinical practice. In this study, two xeno-free alternatives to FBS were tested for their ability to expand BM MSCs, with the objective of establishing a stromal feeder layer capable of supporting hematopoietic expansion of UCB HSCs. Additionally, alternative sources of MSCs were tested.

![Representative image of a senescent XF MSC-derived feeder layer](image)

Chemically defined medium – GIBCO® StemPro® MSC SFM (Life Technologies™)

StemPro® MSC SFM XenoFree (StemPro) has been developed for the expansion of human MSCs under completely serum-free and xeno-free conditions. Using this medium, human MSCs can be expanded for multiple passages while maintaining their multipotent phenotype (i.e. ability to differentiate into osteogenic, chondrogenic and adipogenic lineages). With our current system, as to establish a BM MSC-derived stromal feeder layer capable of supporting HSPC expansion, 95-100% confluent BM MSCs must have its growth inactivated by replacing the culture media with DMEM-10%FBS + 0.5 μg/mL mitomycin-C. After the inactivation step, MSCs will not spend resources on growth metabolism, but rather on the metabolism responsible for supporting HSCs expansion in culture. However, using the same approach with BM MSCs expanded in StemPro did not allow for a viable feeder layer (Figure 1).

This occurrence impaired the possibility of establishing a MSC/HSC co-culture expansion system and it was hypothesized that XF MSCs may differ from SS MSCs in terms of sensibility to mitomycin C treatment. Thus, an array of mitomycin-C concentrations was tested to treat XF MSCs, in different inactivation media. In this way, confluent XF MSCs were treated with either 0.5, 5, or 50 μg.mL⁻¹ of mitomycin-C and cultured in QBSF+Z9 expansion media for seven days.
Furthermore, with the goal of maintaining a fully GMP-compliant inactivation protocol, mitomycin-C was also diluted in StemPro medium, as opposed to the standard serum-supplemented inactivation medium. SC MSC-derived feeder layers were included in the test as viability controls as no senescence episodes were observed in those cultures. Only the culture where the inactivation step was performed using 0.5 ug.mL^{-1} of mitomycin-C diluted in DMEM 10%FBS had its number of cells maintained from day 0 to day 7 (Figure 2).

**Figure 2 - StemPro® MSC SFM XenoFree treated with 0.5 ug.mL-1 of mitomycin-C**

This culture had 76% of cell viability, which is sub-optimal when compared with our current system, which is always above 90%. Feeder-layer senescence is a serious hurdle to the establishment of a functional HSC/MSC co-culture system, as it does not allow for the MSCs to support HSCs growth. Most importantly, feeder layer death and detachment ends up contaminating the final cellular product – the expanded HSCs – with cellular debris that need to be removed prior to transplantation. Because of the mentioned limitations, GIBCO® StemPro® MSC SFM (Life Technologies™) does not seem to be a viable alternative to establish MSC-derived feeder layers. This may be due to lack of sufficient adhesion molecules to withstand 7 days in a confluent state. In fact, the need for a pre-coating with CELLstart™ for the expansion of XF MSCs and the exacerbated senescence when using StemPro® in the inactivation protocol support this hypothesis.

HPL supplemented medium has been used with a variety of cells of primary origin and established cell lines with non-xenogeneic formulation, due to absence of bovine or other animal-derived proteins. In collaboration with AventaCell BioMedical Co., Ltd., we exploited the use of BSSubTM-XF (hereinafter referred to as HPL-XF)-supplemented medium (DMEM 5%HPL) for the establishment of MSC XF-derived feeder layers capable of supporting HSC expansion. In the same way we did for the feeder layers grown with StemPro, we wanted to see how MSCs cultivated in DMEM 5%HPL reacted to the inactivation step with mitomycin-C. Taking a step further, BSSub™-XF-supplemented medium was also used to expand UCM MSCs and AT MSCs, with the objective of establishing MSC-derived feeder layers. Even though they are both usually regarded as biological waste, adipose tissue was found to provide an alternative HSC niche to the bone marrow and about 500 times more MSCs can be isolated from fat tissue than from the same amount of BM. On the other hand, primitive MSCs present in UCM increases its potential in therapeutic applications. Furthermore, UCM is collected alongside UCB, allowing for quick harvesting of both components of the co-culture expansion system, from the same donor (UCB-HSCs and UCM-MSCs). Thus, is of great interest to study both these sources on their capability of supporting hematopoiesis. Although BM is the most common source of MSCs, umbilical cords and lipoaspirates are routinely discarded after birth and lipoaspirations, respectively, in clinics and hospitals, making these easily accessible and non-invasive sources of MSCs, obviating ethical concerns.

For what we could observe in culture, MSCs grow faster when cultured with HPL-supplemented medium when compared with FBS-supplemented medium. Thus, 60-70% MSCs from three different sources (BM, AT, UCM) were inactivated with either 0.5, 5, or 50 ug.mL-1 of Human Platelet Lysate (HPL) – BSSub™-XF (AventaCell BioMedical Co., Ltd.)
mitomycin-C, and maintained in co-culture media for 7 days. Their total number of cells and viability was assessed, and results compared with non-inactivated samples. Additionally, since the inactivation step can be done up to 48h before starting MSC/HSC co-cultures it was hypothesized that additional 48h in the presence of MSC expansion medium would give time for the cells to recover after the harsh inactivation step. Therefore, an additional condition was performed for BM and UCM MSCs, with mitomycin-C treated cultures left in DMEM 5%HPL-XF for 48h after mitomycin-C growth inactivation, before starting the 7-day culture in StemSpan + bFGF. All sources were shown to be capable of establishing xeno-free feeder layers, with efficient growth arrest and high viabilities at the end of the culture (Figure 3). BM MSCs and UCM MSCs were shown to have optimal results after a 5 ug.mL-1 mitomycin-c treatment, while AT MSCs only required a 0.5 ug.mL-1 concentration.

Taking a step further, these HPL-XF MSC-derived feeder layers had their hematopoietic supportive capacity evaluated and compared with SC MSC-derived feeder layers. A pool of UCB HSPC (3 x 10^4 cells/mL) was expanded in StemSpan + Z9 in co-culture with a single donor of BM MSCs expanded either in DMEM 5%HPL-XF or DMEM 10%FBS, and compared with cultures absent of stroma (noSTR). After 7 days, the fold increase in total number of cells and their clonogenic potential was assessed (Figure 4). Even tough HPL-XF MSCs were not able to support expansion as much as SC MSCs did, this condition still improved the ex-vivo expansion of UCB HSPCs when compared with the no stroma condition (Figure 4a). Additionally, a similar clonogenic potential was obtained (Figure 4b). This indicates that HPL is an efficient xeno-free alternative to our current system, which is based on DMEM 10%FBS-expanded MSCs. Of notice, even though it had lower fold increase in TNC, HPL-XF MSCs expanded higher numbers of CD15 and CD7 populations (Figure 4c).

Further studies with more BM MSCs donors should take this data into account, because CD15 is expressed in mature neutrophils46, whose number is a critical parameter in UCB transplantation, which usually suffers
from delayed engraftment of neutrophils and platelets.\textsuperscript{18} On the other hand, CD7 surface marker is expressed on the earliest, immature thymocytes that express neither CD4 nor CD8. Because of their immaturity, this does not increase the chance of GvHD. However, it is also expressed on mature T cells, which, if present, will cause GvHD upon transplantation. Thus, further studies should account for this possibility.

To the author’s knowledge, even though several MSC sources have already been expanded with HPL,\textsuperscript{47} or with other serum-free alternatives\textsuperscript{48}, this is the first study that shows that is feasible to perform HSC/MSC coculture without using any compounds from animal origin in all steps of the process, using BM MSCs to support the expansion of HSCs in a fully xeno-free fashion. Still, some studies have already been performed on the characteristics of MSCs from AT and UCM sources, such proliferation and differentiation potential,\textsuperscript{47,49} and immunomodulatory activity\textsuperscript{50-52}, but none of them reported the derivation of stromal feeder-layer from alternative sources of MSCs with the objective of supporting HSPCs’ expansion in a fully xeno-free fashion. Further studies should include a side-by-side comparison of BM, AT, and UCM sources for their ability to support ex vivo expansion of UCB HSCs, as well as their bone marrow homing capabilities of the expanded cells, short-term and long-term engraftment.

**Systematic delineation of optimal cytokine concentrations through a two-level face-centered cube design (FC-CD).**

DoE allows for a systematic way of changing process inputs and analysing the resulting process outputs in order to quantify the cause and effect relationship between factors, as well as the random variability of the process in a minimum number of runs. Previously at our laboratory, a cytokine cocktail was successfully optimized using an experimental design approach, for the ex vivo expansion of UCB HSC in co-culture with human BM MSC-derived feeder layers in a serum-free culture medium (QBSF-60) supplemented with SCF, Flt-3L, TPO and bFGF for 7 days\textsuperscript{3}. In the last years, new media have been developed for the expansion of HSPCs, such as StemSpan SFEM II from Stem Cell Technologies, that can efficiently supports the expansion of HSCs without the presence of a stromal feeder layer.\textsuperscript{53} Therefore, in this study, we wanted to optimize the cytokine cocktail for this new medium as well as explore the differences in terms of cytokine concentrations in the presence and absence of a stromal feeder layer.

**Table 1 - Design matrix for the optimization of the cytokine cocktail, and cube representation of the design used in the present studies:** SCF, Flt-3L, TPO were tested either at 0 ng mL\textsuperscript{-1} (low level, -1) or 100 mL\textsuperscript{-1} (high level, +1), respectively. Mid level (0) = 50 ng mL\textsuperscript{-1}

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<th>Runs</th>
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Thus, a two-level face-centered cube design (FC-CD) was performed in order to optimize the concentrations of 3 factors in StemSpan SFEM II, either in the presence or absence of a stromal feeder layer: SCF, Flt-3L, and TPO, tested either at 0 ng mL\textsuperscript{-1} (low level, -1) or 100 ng mL\textsuperscript{-1} (high level, +1) (Table 1). An additional factor, bFGF, was kept at a constant concentration (5 ng mL\textsuperscript{-1}), since this growth factor assures the maintenance of the stromal feeder layers in the absence of serum.\textsuperscript{5} The experimental design was composed by 17 runs: 8 factorial points, 6 axial points, 3 replicated center points (which provide an estimation of the experimental error). A second order model is obtained by fitting the experimental data to Equation 1.
Equation 1

\[ y = \beta_1(X_1) + \beta_2(X_2) + \beta_3(X_3) + \beta_{1,2}(X_1,X_2) + \beta_{1,3}(X_1,X_3) + \beta_{2,3}(X_2,X_3) + \beta_1X_1^2 + \beta_2X_2^2 + \beta_3X_3^2 + \epsilon \]

where \( y \) is the response measured (i.e. in this case fold increase in TNC, CD34+ cells, BFU-E, CFU-GM, CFU-Mix), \( \beta \) the regression coefficients corresponding to the main effects, \( \beta_{ij} \) the coefficients for the second order interactions and \( \beta_{ii} \) the quadratic coefficients. To determine the regression coefficients, a sequential backward elimination procedure was followed, where the least significant terms (\( p > 0.05 \)) of the Equation 1, in each step, were eliminated and absorbed into the error.

A pool of UCB HSPC (initial cell density: \( 3 \times 10^4 \) cells.mL\(^{-1} \)) was cultured on StemSpan for 7 days, using the cytokine cocktails presented in Table 1, either on the presence or absence of a stromal feeder layer. Then, using the Pareto chart of the effects, the magnitude and the importance of a given factor on the process outputs were determined. Both stroma and no stroma models only retrieved significant values for the FI TNC response variable. Following the aforementioned sequential backward elimination procedure for the FI TNC variable, the least significant terms were eliminated pooled into the error, and a new Pareto chart was plotted for both models (Figure 5). Fitted response surface plots were used with the purpose of interpreting the model. TPO had a straightforward analysis, with a predicted concentration of 70-90 ng.mL\(^{-1} \) for both models. Of notice, this cytokine had been predicted to have its optimum value outside of the range tested at the time by our group (50 ng.mL\(^{-1} \)).

On the other hand, SCF optimal concentration is not independent of the presence of stroma, since it is already secreted by MSCs. Expectedly, SCF optimal value for FI TNC maximization for the no stroma model had an higher optimum values (Figure 4, 80-120ng.mL\(^{-1} \)) than for the stroma model (Figure 4, 90-110 ng.mL\(^{-1} \)). Similarly, the stroma model was predicted to have lower optimum concentrations of FLT3-L than the no stroma model (Figure 4). In fact, for the no stroma model, since FLT3-L had a positive main effect (in the absence of a significant second order term), the maximum concentration tested also maximizes the expansion of hematopoietic stem/progenitor cells, raising the question whether the optimum value for this cytokine should be located outside of the range tested. Altogether, both models seem to need a readjustment in the maximum levels of the factors being tested, since SCF and FLT3-L were either predicted to be located near or beyond the maximum concentrations tested, for the FI TNC response variable. Concerning the remaining response variables, which were all non-significant, the model assumed that differences in the process outputs were a result of random variability and not due to the influence of different factor concentrations. Replicates of the experiments should be done to gather more information until obtaining statistically significant values. Each new measurement should be taken under consistent experimental conditions, but replicates will be done in different days. To account for this, one must introduce data into the model as blocks. Blocks are categorical variables that explain variation in the response variable not caused by the factors, but due to incidental differences between days, thus allowing to minimize bias and variance of the error because of nuisance factors.
Conclusions and Future Perspectives

Traditional methods of UCB ex-vivo expansion solely using cytokines were disappointing. On the other hand, expanding UCB HSCs in co-culture with MSCs led to sizeable increase in graft content and improved engraftment, highlighting the importance of optimizing the cocktail cytokines in the presence of stroma. However, MSCs are still today retrospectively isolated from primary human BM samples based on their high adherence to plastic. This leads to highly heterogeneous populations, which leads to reproducibility issues if one wants to translate the HSC/MSC into clinical practice. This heterogeneity of the MSC population can be one of the causes compromising the maintenance of the most primitive HSC with long-term multilineage engraftment capacity.

To address this issue, future studies should rely on specific surface marker selection of MSC populations. Furthermore, alternative MSCs sources such as AT and UCM are still to be studied on their capability to expand UCB cells effectively and improve engraftment in in vivo studies. Other methods used HSC-differentiation blockers, such as nicotinamide analogues, copper chelators, inducing constitutive Notch signalling, or an aryl hydrocarbon receptor antagonist (StemReginin1).

Many of these methods lead to substantial expansions of total nucleated cells and CD34+ cells, and significantly improved time to neutrophil or platelet engraftment in patients transplanted with the expanded products, when compared to the recipients of unmanipulated UCB transplantation. Thus, it would be of great interest to combine it some of the aforementioned approaches, and perform in vivo transplantation assays, which can undoubtedly demonstrate the cells ability to repopulate all blood lineages, constituting the ultimate proof of HSC activity. On that topic, an efficient approach to enhance engraftment focuses on increasing the homing capacity of UCB cells to the bone marrow, trough modulation of membrane lipid rafts, modulation of homing molecules, enhancing metabolic response to homing stimuli, or bioavailability enhancement of chemotractants. Finally, after weighing all options, performing these approaches in a bioreactor culture system should be taken into account, as to surpass cell productivity limitations and limited monitoring typical of static cell culture.

Figure 6 - Fitted response surface plots for SCF, FLT3-L and TPO in function of FI TNC. TOP) No stroma model; Bottom) Stromal model
References


