Systematic delineation of optimal cytokine concentrations to expand hematopoietic stem/progenitor cells in co-culture with mesenchymal stem cells

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The major obstacle to the widespread use of umbilical cord blood (UCB) in hematopoietic stem/progenitor (HSC) cell therapy is the low cell dose available. A cytokine cocktail for the *ex vivo* expansion of UCB HSC, in co-culture with a bone marrow (BM) mesenchymal stem cells (MSC)-derived stromal layer was optimized using an experimental design approach. Proliferation of total cells (TNC), stem/progenitor cells ($CD34^+$) and colony-forming units (CFU) was assessed after 7 days in culture, while sole and interactive effects of each cytokine on HSC expansion were statistically determined using a two-level Face-Centered Cube Design. The optimal cytokine cocktail obtained for HSC-MSC co-cultures was composed by SCF, Flt-3L and TPO (60, 55 and 50 ng mL^{-1}, respectively), resulting in 33-fold expansion in TNC, 17-fold in $CD34^+$ cells, 3-fold in $CD34^+CD90^+$ cells and 21-fold in CFU-MIX. More importantly, these short-term expanded cells preserved their telomere length and extensively generated cobblestone area-forming cells (CAFCs) *in vitro*. The statistical tools used herein contributed for the rational delineation of the cytokine concentration range, in a cost-effective way, while systematically addressing complex cytokine-to-cytokine interactions, for the efficient HSC expansion towards the generation of clinically significant cell numbers for transplantation.

Introduction

The efficient *ex vivo* expansion of hematopoietic stem/progenitor cells (HSC), especially those from the umbilical cord blood (UCB), which contains a limited numbers of bone marrow repopulating cells per unit, would clearly widespread their clinical applications to adult patients.[1] The generation of clinical significant cell numbers for a HSC transplant, while maintaining their multilineage engraftment capability, would potentially reduce the hematopoietic reconstitution time and/or increase the engraftment success rate.[2] The delineation of protocols for expanding long-term engrafting $CD34^+$ cells would be of direct clinical utility, since most of the transplants performed today with enriched hematopoietic progenitor cells use CD34-enriched cells.[3]

In fact, a very recent study demonstrated that after a 10-day *ex vivo* expansion, UCB $CD34^+$ cells showed multilineage repopulation after long-term and secondary engraftment in sublethally irradiated NOD/SCID/IL-$2R^{-/-}$ mouse, comparable to non-expanded HSC.[4] Within the $CD34^+$ cell fraction, $CD34^+CD90^+$ cells, in particular, demonstrated to be efficient in achieving rapid and sustained cell engraftment;[5] indeed, CD90 (Thy-1) expression has been recognized as superior to CD38 (*i.e.* $CD34^+CD38^-$) and other markers as predictor of the repopulating activity of $CD34^+$ cells.[6]

Several *ex vivo* culture systems have been used with different rates of success namely by testing different cytokine cocktails in stroma-containing or stroma-free cultures with serum or in serum-free conditions.[7–11] Indeed, most cytokine combinations tested include SCF, Flt-3L and TPO presumed to promote extensive cell self-renewal and to limit levels of apoptosis.[9,12] Nevertheless, numerous other molecules have been tested in *ex vivo* HSC cultures: Zhang and co-workers obtained a 20-fold increase in SCID repopulating cells when IGF-binding protein 2 and angiopoietin-like 5 were added to a cytokine cocktail composed by SCF, FGF and TPO;[13] Araki and collaborators tested the chromatin modifying agents 5-aza-2′-deoxycytidine D (5-AzaD) and trichostatin (TSA) with encouraging HSC expansion levels,[14] whereas Peled and co-workers reported significant enhancement of HSC proliferation with lower levels of differentiation using the copper-chelator agent, TEPA.[15] In addition, in combination with other cytokines, *ex vivo* expanded UCB $CD133^+$ cells using TEPA have been used in a phase I/II clinical trial, with high engraftment rates.[16]

In our laboratory, we have previously established a serum-free culture system using human BM MSC-derived feeder layers, supplemented with SCF, Flt-3, bFGF and LIF, which allowed an efficient expansion/maintenance of HSC from BM and UCB.[3,17] In particular for UCB, HSC-MSC cellular interactions were shown to be crucial for the extensive expansion of $CD34^+$, $CD34^+CD38^-$ cells and CFU-MIX *in vitro*.[18]

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An effective *ex vivo* expansion protocol that would maximize the cell output for a clinical application requires multidimensional optimization, featuring proliferation/differentiation, feeding regimen, product yield, quality and operational costs.[19,20] To this end, experimental designs have been implemented, particularly regarding the optimization of cytokine cocktails for the efficient generation of megakaryocytes,[21] hematopoietic repopulating cells[22] and UCB $CD34^+$ cells[23] in liquid cultures. Nevertheless, to our knowledge, no studies focusing the systematic optimization of the cytokine cocktail for such a complex HSC-MSC co-culture system are found in the literature. Furthermore, particular cytokine interactive effects have been reported in the literature, especially focusing on SCF use with other hematopoietic cytokines,[21,22,24] highlighting the importance of depicting epigenetic events occurring in such a complex system.[14]

Here we used an experimental design approach to: (i) optimize our previously established cytokine cocktail,[3] which was anticipated to exert its effect through a BM MSC-derived stromal layer, for instance, by including TPO, while providing a rational basis on the concentrations typically used; and (ii) to determine the synergistic and sole effects of the cytokines used towards the efficient generation of clinically relevant, quality-controlled, cell numbers in a short-term culture period.

Materials and methods

Human donor cell preparation

The umbilical cord blood (UCB) samples were kindly provided by Crioestaminal—Saúde e Tecnologia, SA, and were collected after maternal donor consent. Low density UCB mononuclear cells (MNC) were separated by a Ficoll density gradient (1.077 g ml^{-1}) (GE Healthcare) and then washed twice in Iscove's Modified Dulbecco's Medium (IMDM) (GibcoBRL). UCB MNC were kept cryopreserved in liquid nitrogen until further use.

Human bone marrow mesenchymal stem cell cultures

Bone marrow (BM) aspirates were harvested after informed consent. BM MSC were isolated as previously described[3,17] and kept in liquid nitrogen. Upon thawing, BM MSC were expanded for 3–5 passages (3000 cells cm^{-2}), using Dulbecco's Modified Essential Medium (DMEM) containing 10% Fetal Bovine Serum (FBS) MSC-qualified (GibcoBRL), supplemented with streptomycin (0.025 μg ml^{-1}) and penicillin (0.025 U/ml) (GibcoBRL), at 37 °C and 5% CO_2 in a humidified atmosphere. Medium was changed twice a week. Near cell confluence (80–90%), cells were washed with phosphate saline buffer (PBS, GibcoBRL) and detached from the flask by adding Accutase (Sigma), for 7 min at 37 °C. In order to be used as stromal feeder layers, cells were seeded in 24-well plates (3000 cells cm^{-2}) and grown until confluency. Before performing co-cultures with UCB HSC, BM MSC were treated with Mitomycin C (Sigma) (0.5 μg ml^{-1} solution prepared in IMDM + 10% FBS) to prevent stromal over-growth. The plates were incubated at 37 °C for 2.5 h. Next, cell monolayers were washed twice with PBS for 5 min and fresh DMEM 10% FBS was added. Co-cultures were established within 24–48 h.

Ex vivo expansion of human $CD34^+$-enriched cells

Upon thawing, UCB MNC were $CD34^+$-enriched using magnetic cell sorting (MACS, Miltenyi Biotec), and then seeded in 24-well plates (3×10^4 cells mL^{-1}) (on top of confluent growth-inactivated BM MSC-derived stromal cell layers) in QBSF-60 serum-free medium (Quality Biological, Inc), at 37 °C and 5% CO_2 humidified air, for 7–14 days (cultures half-fed at day 7 and 10, depending on the experiment).

The cytokines used to supplement culture medium were SCF, Flt-3L, bFGF and TPO (Peprotech) and LIF (Chemicon), tested in different combinations and/or concentrations. For the validation studies (14 days cultures), two controls were performed: (i) without exogenously added cytokines, with a stromal layer (*No Cyt*) and (ii) without stromal layer, using the most successful cytokine cocktail optimized by the experimental design approach (*No Stroma*).

The cytokine-related costs of expanding TNC, $CD34^+$ and $CD34^+CD90^+$ cells (€/cells generated) in our co-culture conditions were determined. For that we used the on-line available pricelist for 2010 of the suppliers of human recombinant SCF, Flt-3L, TPO and LIF. The % reduction was calculated by dividing the amount saved by using Cocktail X (Y-X) per the culture costs using cocktail Y.

Proliferative and phenotypic analysis

Both suspension and adherent fractions of hematopoietic cells (detached by addition of Accutase, 6 min, 37 °C) were determined by counting cell numbers using the Trypan Blue (GibcoBRL) exclusion method for each different culture condition. Fold increase (FI) in total nucleated cells (TNC) was calculated by dividing the number of cells of each day by the number of cells on day 0. Cells were also analyzed by flow cytometry (FACSCalibur equipment, Becton Dickinson) using a panel of monoclonal antibodies (FITC-, or PE-conjugated): CD34 and CD90 for stem/progenitor cells; CD14, CD15 and CD33 for myeloid lineage, CD7 (marker for early lymphopoiesis) (Becton Dickinson Immunocytometry Systems) and CD41a (for megakaryocytes). Isotype controls were also prepared for every experiment. A minimum of 10000 events was collected for each sample.

Clonogenic assays and cobblestone area-forming cells

Both fresh and expanded UCB $CD34^+$ cells were characterized in terms of clonogenic potential. The clonogenic assays were performed in triplicate in MethoCult GF H4434 (Stem Cell Technologies, Inc.). Cultures were maintained at 37 °C and 5% CO_2 humidified air. After 14 days, colonies were counted and evaluated accordingly to manufacturer's instructions. Colony-forming unit-granulocyte, macrophage (CFU-GM), colony-forming unit-granulocyte, erythroid, macrophage, megakaryocyte (CFU-Mix) and burst-forming unit-erythroid (BFU-E) progenitors were identified. For the assessment of cobblestone area-forming cells, expanded cells (2000 cells mL^{-1}) were cultured on top of a confluent (growth-inactivated)

monolayer of MS-5 murine fibroblast cell line in long-term culture medium (Myelocult™, StemCell Technologies) supplemented with 10^{-6} M hydrocortisone (Sigma) in 24-well plates. Cultures were half-fed once a week and cobblestone areas of more than 5 tightly packed cells underneath the stromal layer were scored 2 weeks after seeding.[25]

Telomere length analysis

Relative telomere length (RTL) of fresh and expanded $CD34^+$-enriched cells was determined by telomere fluorescence *in situ* hybridization and flow cytometry (flow FISH) using a telomere-specific peptide nucleic acid (PNA) probe (Dako), as described in ref. 26, using the 1301 cell line (Instituto Nazionale per la Ricerca sul Cancro c/o CBA, Genova, Italy) as control cells. Telomere length measurements were normalized to day 0 values.

Experimental design

A two-level face-centered cube design (FC-CD) was performed in order to optimize the concentrations of 4 factors: SCF, Flt-3L, TPO and LIF, tested either at 0 ng mL^{-1} (low level, −1) or 100, 100, 50 and 10 ng mL^{-1} (high level, +1), respectively. The concentration of bFGF was kept constant (5 ng mL^{-1}), since this growth factor was included in the original cocktail basically to assure the maintenance of the stromal feeder layers in the absence of serum.[3] This FC-CD was composed by 27 runs: 16 factorial points, 3 replicated center points (that provides an estimation of the experimental error[27]) and 8 axial points. A second order model was obtained by fitting the experimental data to eqn (1).

$$y = K + \beta_1(x_1) + \beta_2(x_2) + \beta_3(x_3) + \beta_4(x_4) + \beta_{1,2}(x_{1,2}) + \beta_{1,3}(x_{1,3}) + \beta_{1,4}(x_{1,4}) + \beta_{2,3}(x_{2,3}) + \beta_{2,4}(x_{2,4}) + \beta_{3,4}(x_{3,4}) + \beta_{1,1}(x_1)^2 + \beta_{2,2}(x_2)^2 + \beta_{3,3}(x_3)^2 + \beta_{4,4}(x_4)^2 \quad (1)$$

where y is the response measured (fold increase in TNC, $CD34^+$ cells, CFU-MIX, CFU-GM at day 7), β_i the regression coefficients corresponding to the main effects, $\beta_{i,j}$ the coefficients for the second order interactions and $\beta_{i,i}$ the quadratic coefficients.

Statistical analysis

The determination of the regression coefficients followed a sequential backward elimination procedure, where the least significant terms ($p > 0.05$) of the eqn (1), in each step, were eliminated and absorbed into the error. Nevertheless, in the case of significant second order interactions or quadratic coefficients, the eliminated terms corresponding to the main effects were reintroduced in the model (as described in ref. 21, but only if *Lack of Fit* test remained non-significant ($p > 0.05$)).

Validation results are presented as mean ± standard error of mean (SEM). Comparisons between experimental results were determined by Mann-Whitney test for independent samples, when appropriate. A p-value less than 0.05 was considered statistically significant.

Results

We have previously established a BM MSC-derived stromal-based system for the successful expansion of hematopoietic stem/progenitor cell *ex vivo* using a serum-free culture medium supplemented with SCF, Flt-3L, bFGF and LIF.[3] Here our goal is to rationally delineate the optimal cytokine concentrations for the successful expansion of UCB HSC, in a stromal-based system. In particular, we aim to further improve the rate of expansion of UCB HSC by altering the cytokine cocktail previously studied, not only in terms of the concentrations used (ng mL^{-1}), but also by adding TPO to the initial cocktail. Indeed, in order to maintain the stem cell pool and prevent apoptosis *in vitro*, cytokine cocktails typically composed by SCF, Flt-3, TPO among others, such as LIF and/or IL-6 have been reported.[3,9,12,17,22]

FC-CD for the optimization of hematopoietic stem/progenitor cell expansion

UCB $CD34^+$ enriched (94.1 ± 0.8%) hematopoietic stem/progenitor cells (initial cell density: 3×10^4 cells mL^{-1}) were cultured on top of confluent BM MSC-derived stromal feeder layers ($2.8 \pm 0.6 \times 10^3$ cells/well), for 7 days, using the cytokine cocktails presented in Table 1, according to a two-level Face Centered Cube Design.[27] Based on our previous knowledge,[3,17,18] as well as cocktails reported in the literature, we established the range of the study between 0 and 10 ng ml^{-1} of LIF/50 ng mL^{-1} of TPO/100 ng mL^{-1} of SCF and Flt-3L.

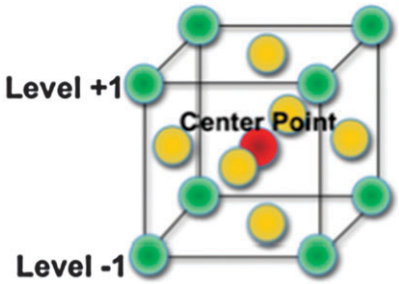
This strategy allowed the statistical determination of the main effects of SCF, Flt-3L, LIF and TPO on hematopoietic stem/progenitor cell expansion, as well as all second-order interactions, present in eqn (1), for each of the 4 responses measured: fold increase in TNC, $CD34^+$ cells, CFU-GM and CFU-MIX. The regression coefficients, their p-value and the summary of fitting (R^2 and *Lack of Fit* test) for each of these responses are presented in Table 2.

The second order polynomials generated for each response described a significant percentage of the experimental data ($0.87 > R^2 > 0.74$), with no *Lack Of Fit* associated ($0.38 > p > 0.12$) (Table 2).

Table 2 presents the statistically significant parameter values of eqn (1), as well as the summary of the fit for each model. The statistical analysis of our data showed that LIF does not have a significant impact in any of the responses (both main and interactive effects)—$p > 0.05$. In addition, it is possible to observe that Flt-3L main effect is not significant for any of the responses ($p > 0.163$), while SCF was non-significant only for the expansion of CFU-MIX ($p = 0.291$). Nevertheless, for both cases, since the quadratic terms ($Flt\text{-}3L^2$ and SCF^2) were significant ($p < 0.01$), the first order coefficients were reintroduced into the model. Interestingly, for all of the responses measured, the second order terms SCF^2 and $Flt\text{-}3L^2$ were negative indicating a downward concavity of the models, suggesting the existence of a maximum value in the cytokine concentration range in study (Fig. 1).

As a result of the models established, the optimal cytokine concentrations for each response were graphically determined and are presented in Table 3. The predicted SCF concentrations that maximize the responses range from 52 to 68 ng mL^{-1},

Table 1 Experimental design—Face centered composite design: A—Design matrix for the optimization of the cytokine cocktail: SCF, Flt-3L, LIF and TPO. B—Cube representation of the design used in the present studies. SCF, Flt-3L, TPO and LIF were tested either at 0 ng mL^{-1} (low level, −1) or 100, 100, 50 and 10 ng mL^{-1} (high level, +1), respectively. Mid level (0) = 50 ng mL^{-1} for SCF and Flt-3L, 25 ng mL^{-1} for TPO and 5 ng mL^{-1} for LIF



SCF	Flt-3L	LIF	TPO
+	+	+	+
+	+	+	—
+	+	—	+
+	+	—	—
+	—	+	+
+	—	+	—
+	—	—	+
+	—	—	—
—	+	+	+
—	+	+	—
—	+	—	+
—	+	—	—
—	—	+	+
—	—	+	—
—	—	—	+
—	—	—	—
0	0	0	0
0	0	0	0
0	0	0	0
0	0	+	0
0	0	—	0
0	0	0	+
0	0	0	—
0	+	0	0
0	—	0	0
+	0	0	0
—	0	0	0

while Flt-3L varies from 52 to 55 ng mL^{-1}. Since TPO had a positive main effect (in the absence of a significant second order term), the maximum concentration tested (50 ng mL^{-1}) also maximizes the expansion of hematopoietic stem/progenitor cells, although the optimum value for this cytokine should be located outside of the range tested (> 50 ng mL^{-1}). Interestingly, all the optimized cocktails are roughly similar, which clearly indicates that the responses measured are highly correlated.

Validation

The determined optimized cocktail for the expansion of $CD34^+$ cells (Z9) was then validated against three other conditions, as presented in Table 4: the previous cytokine cocktail used in our lab (CR5)[3] and two controls—in the absence of cytokines (*No Cyt*) and without stroma (*No Stroma*).

The HSC-MSC co-cultures were performed in a 14-day period, being analyzed at days 3, 7, 10 and 14 in terms of TNC, the hematopoietic stem/progenitor phenotypes $CD34^+$ and $CD34^+CD90^+$, and for the more committed progenitors from the early lymphoid ($CD7^+$), myeloid ($CD14^+$, $CD15^+$ and $CD33^+$) and megakaryocytic lineages ($CD41a^+$ cells) (Fig. 2 and Table 5). In addition, the clonogenic potential of both fresh and expanded cells was assessed by CFU-MIX and CFU-GM assays (Fig. 3a and b), as well as their ability to form cobblestone areas (CAFC) and cell relative telomere length (RTL) (Fig. 4a and b).

In agreement with the model predictions, Z9 cocktail provided the highest fold increases in TNC (26±2-fold) and $CD34^+$ cells (16 ± 1-fold) after 7 days in culture (Fig. 2a and b) ($p < 0.05$). Most importantly, Z9 combination led to the highest expansion of the most primitive $CD34^+CD90^+$ cells (3.8 ± 0.7-fold) and CAFC (26.4 ± 4.3) ($p < 0.05$), while retaining their relative telomere length (97 ± 3%) (Fig. 4a and b) after one-week culture period. When compared with our previous cytokine cocktail (CR5),[3] Z9 provided significantly higher stem/progenitor cell expansion levels ($p < 0.05$), representing an optimization factor of 1.7 for TNC and $CD34^+$ cell expansion, and a 2.7 factor for $CD34^+CD90^+$ cell expansion. In addition, the lower levels of cytokines required in Z9 along with higher HSC productivities, led to

Table 2 Parameter values of eqn (1) and summary of fit for the fold increase in TNC, $CD34^+$ cells, CFU-GM and CFU-MIX. Only statistically significant terms ($p < 0.05$) were considered for the model. Nevertheless, non-significant main effects were reintroduced into the model when the second order terms were significant. All models did not present *Lack of Fit* ($p > 0.05$)

	FI (TNC)		FI ($CD34^+$ cells)		FI (CFU-GM)		FI (CFU-MIX)	
Term	Value	*P* value	Value	*P* value	Value	*P* value	Value	*P* value
K	−1.037	<0.01	−0.045	<0.01	−0.395	<0.01	−0.846	<0.01
β_{SCF}	0.613	<0.01	0.394	<0.01	0.219	<0.01	0.317	0.291
$\beta_{Flt\text{-}3L}$	0.405	0.163	0.223	0.200	0.146	0.165	0.260	0.465
β_{TPO}	0.097	0.039	0.069	0.046	0.056	0.031	0.004	0.095
$\beta_{SCFxSCF}$	−0.005	<0.01	−0.003	<0.01	−0.002	<0.01	−0.003	<0.01
$\beta_{Flt\text{-}3LxFlt\text{-}3L}$	−0.004	<0.01	−0.002	<0.01	−0.001	<0.01	−0.002	<0.01
R^2	0.87		0.82		0.74		0.87	
RMSE[a]	4.7		3.5		2.9		2.7	
Lack of fit test *p*-value	0.12		0.38		0.31		0.19	

[a] RMSE—Root mean square error.

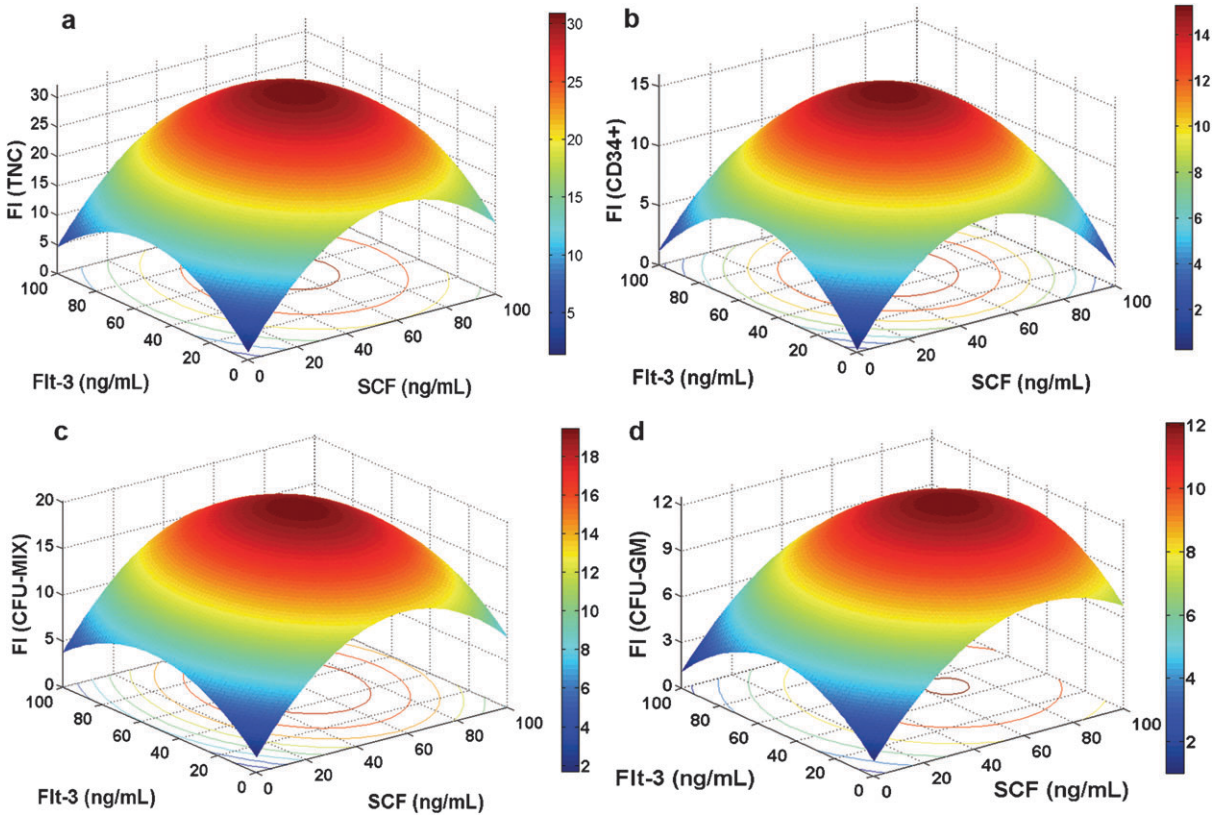


Fig. 1 3-D representation of the Face-centered composite design (FCCD) as a function of Flt-3L and SCF. (a): Fold increase in TNC. (b): Fold increase in $CD34^+$ cells. (c): Fold increase in CFU-MIX. (d): Fold increase in CFU-GM. TPO was set at 50 ng mL^{-1}.

Table 3 Optimized cytokine concentrations for the expansion of TNC, $CD34^+$ cells, CFU-MIX and CFU-GM and respective maximum expansion reached. The cytokine cocktails that maximized each response were determined graphically and code-named. For TNC and $CD34^+$ cell expansion, Z9—60 ng mL^{-1} of SCF, 55 ng mL^{-1} of Flt-3L and 50 ng mL^{-1} of TPO; for the CFU-MIX, M1—52 ng mL^{-1} of SCF and Flt-3L and 50 ng mL^{-1} of TPO; for CFU-GM, G1—68 ng mL^{-1} of SCF, 54 ng mL^{-1} of Flt-3L and 50 ng mL^{-1} of TPO

Response	Code name	SCF/ng mL^{-1}	Flt-3L/ng mL^{-1}	TPO/ng mL^{-1}	Optimized FI
$CD34^+$ cells	Z9	60	55	50	21
TNC	Z9	60	55	50	33
CFU-MIX	M1	52	52	50	17
CFU-GM	G1	68	54	50	13

Table 4 Validation of the model predictions. Four conditions were tested: the optimized cocktail for the expansion of TNC and $CD34^+$ cells (Z9), the previously used cocktail (CR5) and two controls—in the absence of cytokines (*No Cyt*) and using Z9 cocktail in the absence of stroma (*No Stroma*)

Code name	SCF/ng mL^{-1}	Flt-3L/ng mL^{-1}	TPO/ng mL^{-1}	LIF/ng mL^{-1}
Z9	60	55	50	—
CR5	100	100	0	0.1
No Cyt	—	—	—	—
No Stroma (Z9)	60	55	50	—

a reduction of 47, 55 and 62% in cytokine-related culture costs for the generation of TNC, $CD34^+$ and $CD34^+CD90^+$ cells, respectively, in a 7-day period.

As expected, hematopoietic cells cultured either in the absence of the stromal feeder layer (*No Stroma*) or without cytokines (*No Cyt*) did not present any significant expansion of TNC (when compared to any of the other conditions—$p < 0.05$), being roughly similar at day 14 (16-fold and 12-fold, respectively). Interestingly, the *No Cyt* condition presented higher CAFCs expansion levels (although non-significant; $p > 0.05$), when compared to the *No Stroma* condition, especially at day 14 (2.7 ± 1.4 and 0.2 ± 0.1, respectively) (Fig. 4a).

The differentiative potential of the expanded cells was studied during time in culture, testing for the early lymphocytic

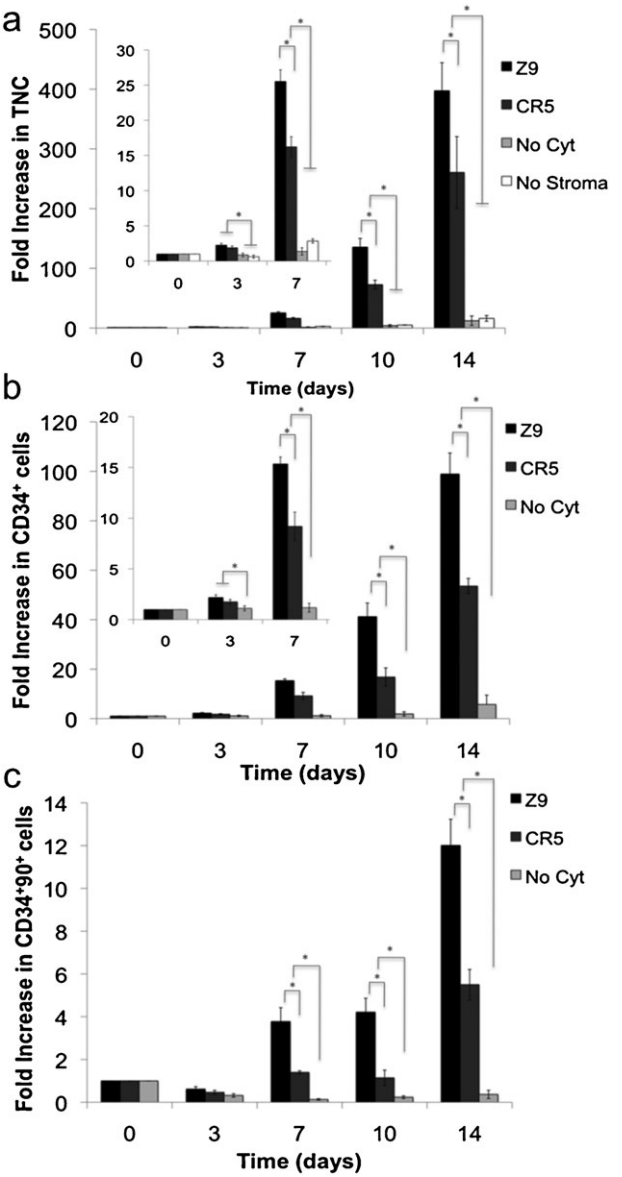


Fig. 2 Validation of the model predictions. Fold increase in (a) TNC ($n = 6$), (b) $CD34^+$ ($n = 6$) and (c) $CD34^+CD90^+$ ($n = 6$) cells after two weeks in HSC-MSC co-culture supplemented with the cytokine cocktails present in Table 4. No phenotypic analyses were performed for the *No Stroma* condition due to the low amounts of cells generated. Results are presented as Mean ± SEM. * $p < 0.05$.

($CD7^+$ cells), myeloid ($CD14^+$, $CD15^+$ and $CD33^+$ cells) and megakaryocytic ($CD41a^+$ cells) lineages (Table 5). Z9 and CR5 conditions were able to successfully maintain/expand these progenitors in agreement with our previous results.[3,17] As expected by the absence of TPO, CR5 cocktail resulted in significantly lower levels of the megakaryocytic progenitor cells $CD41a^+$ (0.8 ± 0.1% and 1.3 ± 0.1% at days 7 and 14) when compared to Z9 (2.3 ± 0.8 and 1.3 ± 0.1% at days 7 and 14, respectively) ($p < 0.05$).

In terms of clonogenic potential, Z9 presented higher expansion rates of CFU-MIX (106 ± 10-fold at day 14) and CFU-GM (488 ± 9-fold) when compared to CR5 (49 ± 4 and 389 ± 17, respectively; $p < 0.05$), especially later in culture, consistent with the observed expansion of TNC and $CD34^+$ cells (Fig. 2a and b), as well as with the differentiative potential of the expanded cells shifted mainly towards the myeloid lineage, as assessed by the immunophenotypic analysis (Table 5).

Discussion

We have previously reported that the expansion/maintenance of HSC from both BM and UCB is highly favored in the presence of BM MSC-derived feeder layer from human origin in a cytokine supplemented (SCF + Flt-3L + LIF + bFGF) serum-free culture system.[3,17] Particularly for UCB HSC, we showed that the *ex vivo* expansion is not only dependent on the presence of the feeder layer but also on the cellular interactions established in the co-culture, using a cytokine cocktail which was anticipated to exert their effect through stromal or accessory cells.[3,28]

In the present study, our goal was to optimize our previously established cytokine cocktail, while providing a rational basis on the concentrations to be used, by using an experimental design. We focused on the generation of clinically significant cell numbers in a short-term (1 week) period, as the optimal time for an efficient static liquid culture should be limited to 7–14 days.[19] To our best knowledge, no systematic optimization on the cytokine cocktail composition was performed in a HSC-MSC co-culture system. In particular, we tested if we could further improve the rate of expansion of UCB stem/progenitor cells in our stromal-based culture system by including to the early acting SCF and Flt-3L, TPO, a cytokine described as efficient at promoting the viability of the more primitive cells, while suppressing apoptosis.[12,29]

A FC-CD was performed in which 4 factors (SCF, Flt-3L, TPO and LIF) in two different levels were correlated with the fold expansion of both TNC, $CD34^+$ cells, CFU-MIX and CFU-GM after 7 days in co-culture with BM MSC. bFGF concentration was not included as a target of our optimization strategy, since this factor was included basically for the maintenance of the stromal feeder layers in the absence of serum.[3]

Consistent with other reports, SCF, Flt-3L and TPO exhibited statistically significant positive effects on the expansion of HSC.[12] Interestingly, for all the responses measured, the second order terms SCF^2 and $Flt\text{-}3L^2$ were found to be negative and highly significant, leading to the determination of a local maximum in the range of our study (0–100 ng mL^{-1}), due to the downward concavity of the resulting models. Biologically, these negative second order terms probably mean a significant inhibition of hematopoietic stem/progenitor cell growth when large amounts of SCF and/or Flt-3 are added to the cultures, suggesting a substrate inhibition-like effect, typically found in biological reactions. In addition, our design allowed the estimation of interactive cytokine effects, which have been reported in the literature, especially involving SCF with other cytokines.[14,21,22,30] Although none of the interactions determined were statistically significant, for any of the responses measured, there is an improvement in the response by using increasing concentrations of SCF and Flt-3L, resulting in an optimized cytokine cocktail composed by SCF (60 ng mL^{-1}), Flt-3L (55 ng mL^{-1}) and TPO (50 ng mL^{-1})—Z9, for the

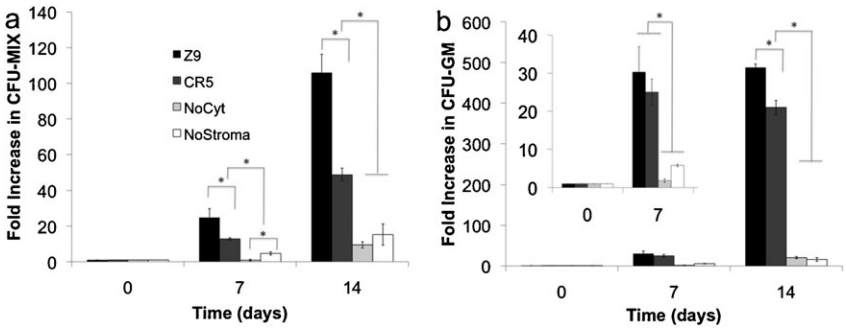


Fig. 3 Clonogenic Potential of expanded cells supplemented with the cytokine cocktails presented in Table 4: the optimal cytokine cocktail obtained in this study (Z9), our previous cytokine cocktail (CR5) and two controls, without cytokines (*No Cyt*) and in the absence of stroma, using Z9 cocktail (*No Stroma*). Fold increase in CFU-MIX (a) and CFU-GM (b), during time in culture for each condition, are presented as values ± SEM ($n = 4$; $*p < 0.05$).

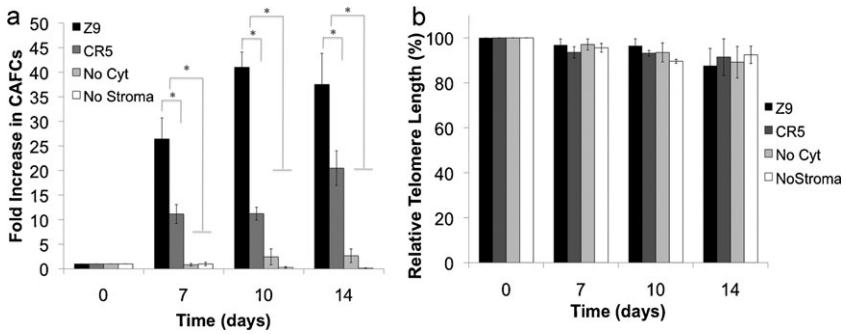


Fig. 4 Expansion of umbilical cord blood hematopoietic cells in co-culture with BM MSC using different cytokine combinations, present in Table 4. The fold increase in cobblestone area-forming cells (CAFCs) (a) and the relative telomere length (b) were determined during time in culture for Z9, CR5, *No Cyt* and *No Stroma* conditions. Results are presented as Mean ± SEM ($n = 4$; $*p < 0.05$).

expansion of TNC, and $CD34^+$ cells, after 7 days in co-culture. These model-predicted values were validated in another set of experiments and represent a 2-fold higher cell productivity with significant reduction in culture costs (50–65%) due to the substantial reduction in cytokine concentrations, when compared with our previously used cytokine cocktail.[3] In addition, Z9 combination provided a 4-fold expansion of the more primitive $CD34^+CD90^+$ cells[14] after 7 days in culture, while retaining their telomere length and the ability to form cobblestone areas *in vitro*. Telomere length and telomerase activity constitute strong evidence of the quality of the expanded graft.[31] Consistent with our results, Gammaitoni and co-workers reported telomere length maintenance and up-regulation of telomerase activity in long-term cultures of UCB, BM and mobilized peripheral blood (MPB) $CD34^+$ cells, with a 20-fold increase in CAFCs after 4 weeks in co-culture with OP9 cells.[32] Interestingly, in our studies, hematopoietic cells cultured in the presence of stroma, non cytokine-supplemented, provided consistently higher expansion levels of CAFCs, when compared with cells grown without feeder layer, supplemented with our optimized cytokine cocktail (Z9). These results suggest a strong effect of the BM-derived stroma on the maintenance/expansion of CAFCs,[33] described as a measure of SCID repopulating ability.[34] We reason that BM MSC-based feeder cell layer, even in the absence of exogenously added cytokines, is able to produce growth factors such as SCF, LIF and Flt-3L.[35] The produced growth factors, although at a basal level, together with the cell-to-cell interactions provided by the feeder cells,[28] are able to preserve a subset of the more primitive long-term repopulating cells, here assessed by the CAFC content, during the two-week culture period.[36]

Table 5 Differentiative potential of expanded cells with the cytokine cocktails Z9 and CR5. Results are presented as percentages ± SEM ($n = 4$). $*p < 0.05$. Non-adherent cells were harvested periodically and analyzed by flow cytometry with CD7 FITC, CD14 PE, CD15 FITC, CD33 PE and CD41a PE antibodies. Isotype control antibodies were used to determine the level of non-specific binding. Due to the limiting cell number, no differentiative potential was attained both for the *No Stroma* and *No Cyt* conditions

	% $CD7^+$		% $CD14^+$		% $CD15^+$		% $CD33^+$		% $CD41a^+$	
Time (days)	Z9	CR5	Z9	CR5	Z9	CR5	Z9	CR5	Z9	CR5
0	8.5 ± 0.9		7.3 ± 2.5		11.5 ± 1.8		88.4 ± 3.7		1.4 ± 0.2	
10	40.0 ± 4.0	38.2 ± 6.4	24.1 ± 4.3	16.7 ± 2.4	46.2 ± 3.9	46.7 ± 7.9	87.7 ± 3.6	92.6 ± 1.3	2.3 ± 0.8 (*)	0.8 ± 0.1 (*)
14	46.6 ± 3.1	48.9 ± 3.0	23.5 ± 4.3	25.1 ± 2.1	52.5 ± 4.6	49.4 ± 4.8	93.8 ± 1.4	89.2 ± 1.9	5.8 ± 0.7 (*)	1.3 ± 0.1 (*)

In the present FC-CD studies (7 days), LIF presented a positive main effect on HSC-MSC co-cultures, though without statistical significance. Nevertheless, since previous results by our group suggest that LIF might have a more pronounced effect on progenitor expansion, especially later in culture,[18,28] ongoing studies are currently being performed in our lab to depict the precise mechanism of action of LIF in the present co-culture system.

The differentiative potential of the expanded cells using the different cocktails was primarily shifted towards the myeloid lineage, as assessed by immunophenotypic analysis and clonogenic potential studies, as previously reported, while maintaining a significant percentage of cells with an early lymphocytic potential ($CD7^+$ cells).[3,37] As expected by the absence of TPO, the CR5 cocktail[3] yielded the lowest levels of the megakaryocytic progenitor cells $CD41a^+$ when compared to Z9 (with TPO). Indeed, it has been widely reported in the literature the classical strong effect of TPO on thrombopoiesis.[38]

In summary, we have successfully optimized a cytokine cocktail for the *ex vivo* expansion of UCB HSC in co-culture with BM MSC, in a one-week time period, using an experimental design approach. In addition, our results contribute for the rational delineation of the concentration range of the cytokines to be used in a HSC expansion protocol, in a systematic and cost-effective way, while quantitatively addressing the complex interactions among cytokines/growth factors. More importantly, the expanded cells maintained their telomere length and extensively originated cobblestones *in vitro*. This work offers important clues to better understand the cellular determinants underlying *ex vivo* expansion of HSC, providing the basis for the establishment of efficient and controlled culture systems for the generation of clinically significant cell numbers in the settings of BM transplantation using UCB expanded cells.

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