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A Stro-1⁺ human universal stromal feeder layer to expand/maintain human bone marrow hematopoietic stem/progenitor cells in a serum-free culture system

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Objective. To compare the ability of allogeneic versus autologous purified human Stro-1⁺ mesenchymal stem cell (MSC) populations from different human donors to support the ex vivo expansion and maintenance of human hematopoietic stem/progenitor cells (HSCs). Furthermore, we compared the results obtained with MSC as a feeder layer to traditional allogeneic stromal layers grown in long-term bone marrow culture media (LT-ST).

Methods. Adult human bone marrow CD34⁺-enriched cells were cultured in serum-free medium for 2 to 3 weeks over the respective MSC-irradiated feeder layers or over traditional allogeneic LT-ST stromal layers in the presence of stem cell factor, basic fibroblast growth factor, leukemia inhibitory factor, and Flt-3 and analyzed every 2 to 4 days for expansion, phenotype, and clonogenic ability.

Results. There was a progressive expansion of total numbers of cells in all the experimental groups; however, allogeneic MSCs were more efficient at expanding CD34⁺CD38⁻ cells and showed a higher clonogenic potential than both allogeneic LT-ST and autologous MSCs. The differentiative potential of cells cultured on both MSC and LT-ST was primarily shifted toward myeloid lineage; however, only MSCs were able to maintain/expand a CD7⁺ population with lymphocytic potential. Importantly, transplantation into preimmune fetal sheep demonstrated that the HSCs cultured over MSCs retained their engraftment capability.

Conclusion. These results indicate that purified Stro-1⁺ MSCs may be used as a universal and reproducible stromal feeder layer to efficiently expand and maintain human bone marrow HSCs ex vivo. © 2006 International Society for Experimental Hematology. Published by Elsevier Inc.

Effective ex vivo expansion of hematopoietic stem/progenitor cells (HSCs) has thus far been an unachievable major goal in hematology, leading to a limitation in the development and application of cell-based therapies to human clinical trials [1–4]. A major issue in HSC expansion still consists in the delineation of in vitro culture systems and combinations of growth factors that would allow the maintenance of hematopoietic cells with the ability to provide short- and long-term engraftment in an in vivo model [1,4]. In this context, some of the most promising results with the ex vivo expansion of

human HSCs have been obtained using culture systems in which different types of feeder layers were combined with cocktails of specific cytokines [5–9].

Hematopoiesis depends upon a complex interaction of growth and regulatory factors within the bone marrow (BM) microenvironment, where specific cellular interactions between primitive hematopoietic progenitors and mesenchymal stromal tissue of nonhematopoietic origin take place [10–12]. Thus, the intimate contact between stromal cells and HSCs results in the modulation of HSCs' final behavior such as quiescence, proliferation, maturation, or even apoptosis [13]. Contributing to the stromal cell layer are different populations of cells, some of hematopoietic origin, derived from HSCs, such as macrophages and others derived from the so called mesenchymal stem cells (MSCs) such as

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fibroblasts, smooth muscle cells, adipocytes, reticular endothelial, and osteogenic precursor cells [11,12,14,15]. Long-term culture (LTC) of BM cells was first described by Dexter et al. and since then has been used as an *in vitro* model of hematopoiesis to study interactions between early progenitors and stromal regulating factors [10–12,16]. Furthermore, several murine and human cell lines such as M2-10B4, MS5, and 14F1.1, mostly of embryonic origin, are also reported to be able to support growth of hematopoietic cells *in vitro* [17–20]. Because concerns exist regarding the safety of products intended for patient use, due to the potential for contamination of infectious agents when these products are produced in cultures containing animal-derived components or transformed cell lines, the ability to expand human HSCs under serum-free conditions using stromal layers of human origin would have significant clinical applications [1,3,21]. We and others reported that BM-derived MSCs have the ability to support and expand *in vitro* hematopoiesis due to their capacity to produce cytokines and growth factors that regulate the proliferation, differentiation, and maintenance of human HSCs [3,12,14,22].

In our previous studies, we used a human stromal-based serum-free culture system, in which a cocktail of cytokines was anticipated to exert its effect through either stromal and/or accessory cells, leading to the *ex vivo* expansion of BM HSCs without exhausting the more primitive stem cells [3]. Since many patients who could benefit from an HSC expansion protocol for autologous transplant may have suffered damage to their BM stroma due to chemo- and/or radiotherapy, we wanted to establish the feasibility of using a donor-independent, human allogeneic mesenchymal stem cell-based serum-free culture system that consistently would allow the efficient expansion of human BM-derived HSCs. Thus, we hypothesized that the use of purified MSC populations based on the phenotype Stro-1⁺, CD45⁻, Gly A⁻ would minimize the variability between different stromal layers. Furthermore, we sought to compare autologous versus allogeneic sources of MSC stroma as well as perform a side-by-side comparison with other well-established HSC culture systems such as LTC. Our results show that allogeneic Stro-1⁺, CD45⁻, Gly A⁻ MSCs are a reliable, consistent, and superior source of stromal layer cells able to efficiently expand human HSC not only toward the myeloid lineage but also to maintain/expand a population with lymphocytic potential. Importantly, human HSCs expanded over allogeneic MSC layers retained their engraftment capability *in vivo*, demonstrating the potential of these cells to be used as a universal stromal feeder layer to efficiently expand and maintain human BM HSCs *ex vivo*.

Materials and methods

Preparation of human donor CD34⁺ cells

Heparinized human BM was obtained from healthy donors after informed consent. Low density BM mononuclear cells (MNCs)

were separated by a Ficoll density gradient (1.077 g/mL; Sigma, St. Louis, MO, USA) and washed twice in Iscove's modified Dulbecco's media (IMDM; Gibco Laboratories, Grand Island, NY, USA). BM MNCs from each donor were then enriched for CD34⁺ cells using magnetic cell sorting (Miltenyi Biotec Inc., Auburn, CA, USA). For autologous studies, both feeder layers and CD34⁺ cells were isolated from the same donor, while in allogeneic studies, feeder layers and CD34⁺ cells were obtained from different donors.

Establishment of human bone marrow feeder layers

Two types of stromal feeder layers were obtained from BM MNCs: the traditional Dexter's long-term BM stroma (LT-ST) and an MSC-based stroma obtained by purification of Stro-1⁺, CD45⁻, Gly A⁻ cells.

To obtain LT-ST stroma, human adult BM MNCs were cultured until confluence in gelatin-coated T₂₅ flasks (5 mL) in IMDM with 12.5% fetal bovine serum (FBS) (Hyclone, Logan, UT, USA), 12.5% horse serum (Sigma), 10⁻⁶ M hydrocortisone (Sigma), and 10⁻⁴ M 2-mercaptoethanol. MSCs were isolated from human adult BM MNCs by magnetic cell sorting for Stro-1 positivity (R&D Systems Inc., Minneapolis MN, USA) and CD45 and Gly-A negativity (Miltenyi Biotec) and then cultured until confluence in gelatin-coated T₂₅ flasks (5 mL) with mesenchymal stem cell growth medium (MSCGM; Poietics, BioWhittaker, Baltimore, MD, USA).

Confluence of LT-ST and MSC feeder layers were obtained after culture for 10 to 14 days. Feeder layers were then γ -irradiated (1400 rads) and maintained at 37°C under 5% CO₂ humidified air and used within 1 to 5 days after irradiation. Irradiation of stromal layers at 1400 cGy proved to be efficient to prevent stromal feeder overgrowth while maintaining optimal viability and growth factor production.

Ex vivo expansion of BM CD34⁺-enriched cells

BM CD34⁺-enriched cells were cultured in T₂₅ flasks (5 mL) for 2 to 3 weeks in QBSF-60 serum-free medium (Quality Biological, Gaithersburg, MD, USA) over the respective feeder layers (autologous or allogeneic) in the presence of stem cell factor (SCF) (100 ng/ml), basic fibroblast growth factor (bFGF) (5 ng/ml), leukemia inhibitory factor (LIF) (10 U/ml), and Flt-3 (100 ng/ml; Peprotech, Rocky Hill, NJ, USA). Cultures were half-fed every 2 to 4 days with half of the cultures being harvested and used for analysis and the same volume being replaced with fresh medium. At each time point *x*, the number of viable cells was calculated using the Trypan Blue exclusion method; the number of cells obtained was then multiplied by a 2^{*n*} factor accounting for the half-feeding procedure, *n* being the number of medium renewals performed before day *x*.

Assessment of proliferation and phenotypic analysis

The *ex vivo* expansion of CD34⁺-enriched population was determined at each time point by counting the content of hematopoietic cells in each culture flask using Trypan blue stain 0.4% solution (GibcoBRL, Carlsbad, CA, USA) and phenotypical analysis was performed by flow cytometry using monoclonal antibodies to various clusters of differentiation (CD): CD7, CD14, CD15, CD33, CD34, and CD38 (Becton Dickinson Immunocytometry Systems [BDIS], San Jose, CA, USA) as previously described [3]. Isotype controls were used in every experiment to

exclude the possibility of nonspecific binding of antibodies to Fc receptors. A minimum of 10000 events was collected for each sample.

Evaluation of clonogenic potential

Assays for clonogenic progenitors were performed in duplicate in MethoCult GF H4434 (Stem Cell Technologies Inc., Vancouver, British Columbia, Canada) with freshly purified and expanded CD34⁺ enriched cells. Cells 1×10^3 (day 0) and 1×10^4 (days 7 and 14) were plated and cultures were incubated in a fully humidified incubator at 37°C in 5% CO₂ in air. After 14 days, colony-forming units-mix (CFU-Mix), burst-forming units-erythroid (BFU-E), and colony-forming unit-granulocyte, macrophage (CFU-GM) colonies were counted and categorized according to standard criteria [1]. CFU numbers were calculated by dividing the number of colonies present at day 14 by the number of cells plated; this was then multiplied by the total number of cells in culture for the day of harvest. The total clonogenic potential refers to the accumulated output of colonies generated in culture at days 7 and 14. Total fold increase in clonogenic potential was calculated by dividing the accumulated output of colonies (days 7 and 14) by the CFU numbers at day 0.

In vivo evaluation of expanded cells

These studies were performed in 10 fetal sheep at 55 to 65 days of gestation following the transplantation procedure as described previously [1]. Briefly, after 2 weeks of culture, expanded cells cocultured with either allogeneic MSCs or LT-ST were harvested and transplanted at the same concentration of 1.18×10^6 cells (post-culture)/fetus into six and four preimmune fetal sheep, respectively. The transplanted sheep were then analyzed for human donor cell engraftment at 2 months posttransplant.

Assessment of human donor cell engraftment in fetal sheep

The presence of donor cells in hematopoietic tissues of the recipients (peripheral blood [PB] and BM) was determined by flow cytometric analysis performed on a FACScan (BDIS). Monoclonal antibodies directly conjugated with fluorescein isothiocyanate (FITC) or phycoerythrin (PE) were used. These included: CD45, CD34, CD20, CD33, CD3, CD7, CD10, CD13, HLA-DR (BDIS), and glycophorin A (Immunotech, Miami, FL, USA).

Statistics and data analysis

The experimental results are presented as the mean plus/minus the standard error of the mean (SEM). The two-sided Wilcoxon rank sum test for independent samples was used to perform statistical analysis and a *p* value <0.05 was considered significant.

Results

Comparison of ex vivo expansion of BM CD34⁺-enriched cells over allogeneic MSC or LT-ST feeder layers

To compare the ability of purified populations of MSC or LT-ST, to support the expansion/maintenance of human HSCs, we evaluated the ex vivo expansion of human BM CD34⁺-enriched cells in serum-free medium supplemented with SCF, bFGF, LIF, and Flt-3 over allogeneic MSC and

LT-ST stromal layers. The ability to support ex vivo expansion of human BM CD34⁺ was evaluated by the fold increase in total number of cells plated as well as proliferation of more primitive populations, such as CD34⁺ and CD34⁺CD38⁻ cells.

Both MSC and LT-ST feeder layers were efficient in expanding the total number of cells in culture (starting population: $1.0\text{--}4.0 \times 10^6$ total cells), until the 19th day of culture, with a median fold increase of 159 and 190 for MSC and LT-ST, respectively, with no significant differences between the two culture systems (*p* < 0.05).

Figure 1A and B show the fold increase in CD34⁺ (starting population: $0.3\text{--}1.5 \times 10^6$ CD34⁺ cells) and CD34⁺CD38⁻ cells (starting population: $1.1\text{--}4.3 \times 10^4$ CD34⁺CD38⁻ cells), respectively. As can be seen in Figure 1A, MSC feeder layers supported more efficient expansion of CD34⁺ cells, with a significant difference in the

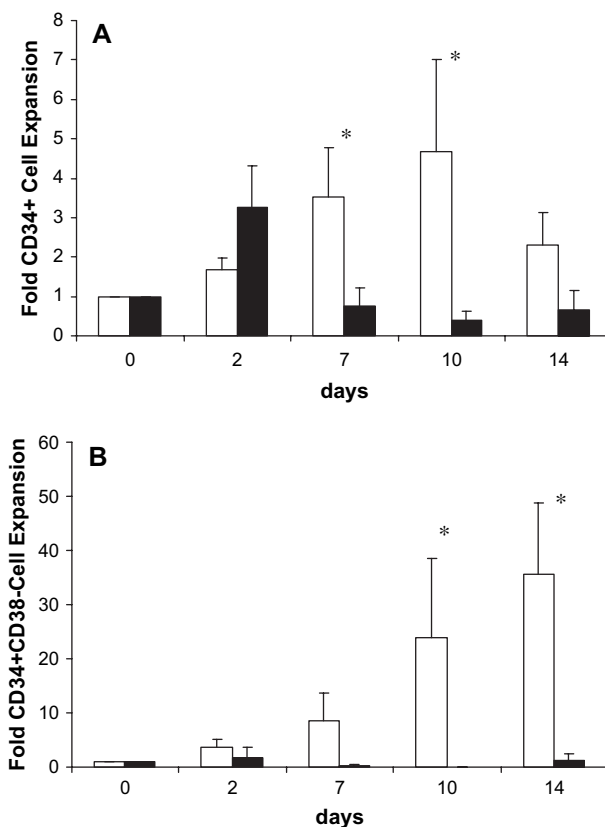


Figure 1. Expansion of bone marrow (BM) CD34⁺-enriched cells cultured over mesenchymal stem cell-based (MSC) and Dexter's long-term bone marrow (LT-ST) stromal layers, both of allogeneic origin. (A): CD34⁺ cells. (B): CD34⁺CD38⁻ cells. BM CD34⁺-enriched cells were cultured over MSCs (white bars) and LT-ST (black bars) feeder layers for 2 to 3 weeks and differences in the proliferation were assessed in terms of fold increase in total number of CD34⁺ cells and CD34⁺CD38⁻ cells. Nonadherent cells in culture were harvested periodically, enumerated, and stained with CD34 FITC and CD38 PE antibodies. Results represent mean of fold expansion \pm SEM. In both A and B, n = 6 for allogeneic MSCs; n = 4 for allogeneic LT-ST. **p* < 0.05.

output of CD34⁺ cells. At days 7 and 10, a fold increase in CD34⁺ cells of 3.5 ± 1.3 and 4.7 ± 2.3 was seen, respectively, in cocultures with MSC, and LT-ST layers provided fold increases of 0.8 ± 0.4 at day 7 and 0.4 ± 0.2 at day 10 ($p < 0.05$). This difference was even more significant for cells with a more primitive CD34⁺CD38⁻ phenotype; once more, MSC feeder layers allowed a significant expansion of these primitive cells that reached an average increase of 36-fold at day 14 of culture, and LT-ST stromas provided only a 1.3-fold increase in CD34⁺CD38⁻ cells ($p < 0.05$).

The total clonogenic potential of the expanded cells was also evaluated. The clonogenic potential before culture (day 0) was: CFU-Mix (1.2 ± 0.8) $\times 10^4$; BFU-E (3.0 ± 1.0) $\times 10^3$; CFU-GM (9.8 ± 4.9) $\times 10^4$. In cells expanded over allogeneic MSCs, the total CFU-Mix, BFU-E, and CFU-GM were, respectively, (8.7 ± 5.9) $\times 10^4$, (7.0 ± 3.0) $\times 10^3$, and (103 ± 63) $\times 10^4$, corresponding to a fold increase of clonogenic potential of 7.3 in CFU-Mix, 2.3 in BFU-E, and 10.5 in CFU-GM. Cells expanded over allogeneic LT-ST layers had a significantly lower clonogenic potential with total CFU-Mix, BFU-E, and CFU-GM being (3.1 ± 1.2) $\times 10^4$, (3.0 ± 2.0) $\times 10^3$, (57 ± 19) $\times 10^4$, respectively, corresponding to a fold increase of 2.6 for CFU-Mix and a 5.8 increase for the CFU-GM; no increase in BFU-E was noted.

Phenotypic analysis of the expanded BM CD34⁺-enriched cells over MSC and LT-ST allogeneic feeder layers

To evaluate the effect of the culture conditions on the differentiative potential of the expanded cells, cultures were analyzed periodically by flow cytometry for the expression of CD7, CD14, CD15, and CD33 antigens (Table 1). In both culture systems, using either MSC or LT-ST feeder layers, lymphoid cells positive for CD3 and/or CD19 present at day 0 of culture, which remained after CD34 enrichment, decreased progressively until disappearing by days 6 to 8 (data not shown).

As expected, the differentiative potential of the CD34⁺-enriched cells cultured on both MSCs and LT-ST was

primarily shifted toward the myeloid lineage with cells generated expressing CD15, CD33, and CD14 markers (Table 1). Of note is the observation that only the cultures expanded over MSC feeder layers were able to expand/maintain a distinct CD7⁺ population that reached 48 to 64% by day 14 of culture, while LT-ST-based cultures were not.

Because at day 0 the purity of the CD34⁺-enriched populations used in these studies after MiniMACS sorting was 60 to 70% and $19.1\% \pm 4.9\%$ of the cells expressed CD7, we evaluated the fold increase of CD7⁺ cells during time in culture. As can be seen in Figure 2 the ability to efficiently maintain/expand this CD7⁺ population was unique to the MSC feeder layers (increase of 265 ± 116 fold at day 14).

Allogeneic MSC feeder layers are better supporters of ex vivo expansion of BM CD34⁺ cells than autologous MSCs

Although the previous data suggested that allogeneic MSCs were a suitable feeder layer to expand adult BM CD34⁺ cells, we further investigated whether the MSC culture system could be improved by using autologous MSC feeder layers. Therefore, we compared autologous and allogeneic MSCs in their ability to support the maintenance and expansion of human BM HSCs.

No significant differences were found between autologous and allogeneic culture systems, regarding fold increase in total number of cells with the time in culture. The fold increase over autologous and allogeneic MSC feeder layers was 156 ± 54 for autologous and 159 ± 88 for allogeneic stromas (starting populations: $1.0\text{--}2.0 \times 10^6$ and $1.0\text{--}4.0 \times 10^6$ cells for cultures with autologous and allogeneic MSC layers, respectively) after 19 days of culture.

However, expansion of cells with a CD34⁺ phenotype (starting populations of $0.3\text{--}1.0 \times 10^6$ and $0.3\text{--}1.5 \times 10^6$ CD34⁺ cells with autologous and allogeneic MSC, respectively) was more efficient over the allogeneic MSC stromal layers (Fig. 3A), with a fold increase of 3.5 ± 1.3 after 7 days and 4.7 ± 2.3 after 10 days in culture when compared with the autologous ones that supported

Table 1. Differentiative potential of the bone marrow (BM) CD34⁺-enriched cells expanded on both allogeneic mesenchymal stem cell-based (MSC) and Dexter's long-term bone marrow (LT-ST) feeder layers

Days	%CD15 ⁺		%CD33 ⁺		%CD7 ⁺		%CD14 ⁺	
	MSC	LT-ST	MSC	LT-ST	MSC	LT-ST	MSC	LT-ST
0	20.6 \pm 4.3		31.2 \pm 6.9		19.1 \pm 4.9		3.1 \pm 0.5	
7	53.3 \pm 8.2	50.6 \pm 8.1	67.3 \pm 19.7	65.2 \pm 18.0	42.3 \pm 5.4	5.6 \pm 1.4	12.7 \pm 0.3	33.9 \pm 7.5
14	54.0 \pm 10.4	53.4 \pm 7.9	69.2 \pm 9.4	30.0 \pm 0.90	55.7 \pm 8.1	2.4 \pm 0.4	18.6 \pm 2.8	22.8 \pm 7.9

At day 0 the purity of the CD34⁺-enriched populations after MiniMACS sorting was 60 to 70%. Nonadherent cells were harvested periodically and analyzed by flow cytometry with CD7 FITC, CD14 PE, CD15 FITC, and CD33 PE antibodies. Isotype control antibodies were used to determine the level of non-specific binding. The results displayed represent the expression (%) of each antigen and, because some cells expressed more than one antigen, the sum of the expression percentage can be higher than 100%. Results presented as mean \pm SEM (n = 6 for allogeneic MSCs; n = 2 for allogeneic LT-ST).

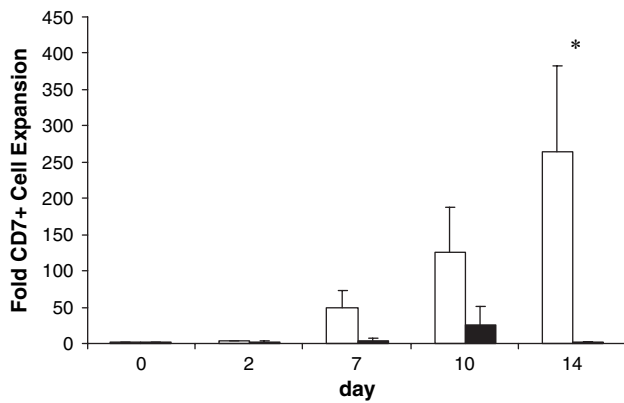


Figure 2. Expansion of bone marrow (BM) CD7⁺ cells cultured over mesenchymal stem cell-based (MSC) and Dexter's long-term bone marrow (LT-ST) stromal layers, both from allogeneic origin. Nonadherent cells in culture over MSC (white bars) and LT-ST (black bars) feeder layers were harvested periodically, enumerated, and stained with CD7 FITC antibody. Each bar represents mean fold CD7⁺ cell expansion \pm SEM (n = 6 for allogeneic MSCs; n = 4 for allogeneic LT-ST). **p* < 0.05.

a fold increase of only 2.1 ± 0.2 at day 7 and 1.7 ± 0.2 at day 10. Overall allogeneic MSC layers gave consistently higher levels of CD34⁺ progenitors expansion throughout the 2-week culture period.

The same outcome could also be seen in terms of expansion of more primitive populations such as CD34⁺CD38⁻ (Fig. 3B), where the MSC feeder layer of allogeneic origin was more efficient at expanding this phenotype (fold increase values of 24.0 ± 14.5 and 35.7 ± 13.1 , at days 10 and 14, respectively) compared with its autologous counterpart (fold increase values of 7.1 ± 5.4 and 12.8 ± 0.17 , at days 10 and 14, respectively; $1.0\text{--}2.0 \times 10^4$ and $1.0\text{--}4.0 \times 10^4$ CD34⁺CD38⁻ initial cells numbers with autologous and allogeneic MSCs).

Total clonogenic output also showed that allogeneic MSC stroma was more efficient in supporting hematopoiesis than its autologous counterpart. Once more, the clonogenic potential before culture (day 0) was: CFU-Mix ($1.2 \pm 0.8 \times 10^4$); BFU-E ($3.0 \pm 1.0 \times 10^3$); CFU-GM ($9.8 \pm 4.9 \times 10^4$). In cells expanded over allogeneic MSCs the total CFU-Mix, BFU-E, and CFU-GM were respectively ($8.7 \pm 5.9 \times 10^4$), ($7.0 \pm 3.0 \times 10^3$), and ($103 \pm 63 \times 10^4$), corresponding to fold increases of 7.3, 2.3, and 10.5 for CFU-Mix, BFU-E, and CFU-GM, respectively. In cells expanded over autologous MSC layers, the clonogenic potential was lower with total CFU-Mix, BFU-E, and CFU-GM being 9×10^3 , 0, and 39×10^4 , respectively.

Engraftment capability of the expanded BM CD34⁺-enriched cells grown over MSC and LT-ST feeder layers

Next, we used the preimmune fetal sheep model to compare the in vivo engraftment capability of the BM CD34⁺-enriched cells that had been expanded ex vivo over MSC and LT-ST feeder layers.

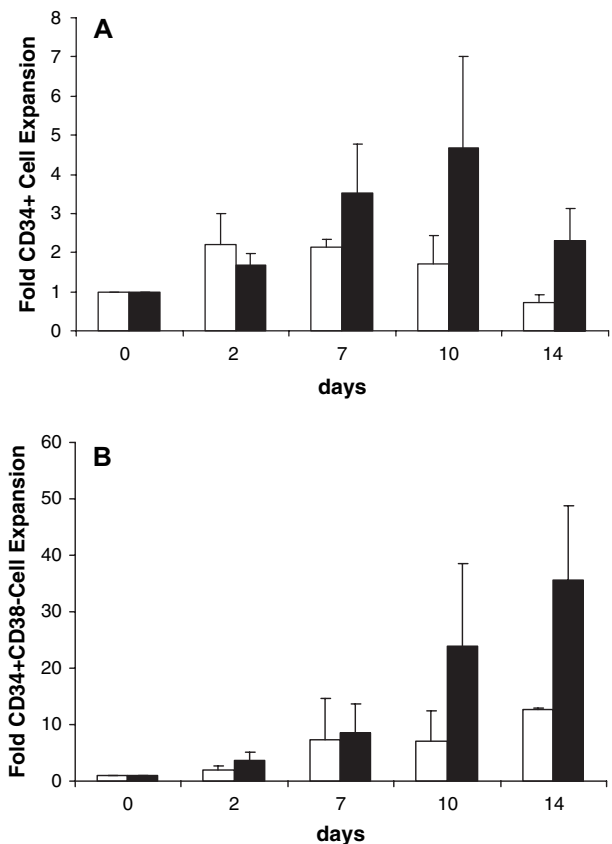


Figure 3. Expansion of bone marrow (BM) CD34⁺-enriched cells cultured over autologous and allogeneic mesenchymal stem cell-based (MSC) feeder layers. (A): CD34⁺ cells. (B): CD34⁺CD38⁻ cells. BM CD34⁺-enriched cells were cultured over autologous (white bars) and allogeneic MSC (black bars) feeder layers for 2 to 3 weeks and differences in the proliferation were assessed in terms of fold increase in total number of viable cells, CD34⁺ cells, and CD34⁺CD38⁻ cells. Nonadherent cells in culture were harvested periodically, enumerated, and stained with CD34 FITC and CD38 PE antibodies. Results represent mean of fold expansion \pm SEM (n = 2 for autologous MSCs; n = 6 for allogeneic MSCs).

To this end, 10 fetal sheep were each transplanted with 1.18×10^6 human BM CD34⁺-enriched cells expanded for 2 weeks over MSC (n = 6) or LT-ST (n = 4) stromal layers, and these recipients were evaluated at 2 months posttransplant.

In animals transplanted with HSCs expanded over LT-ST, only three of the four animals exhibited human hematopoietic chimerism, with the levels of human CD45⁺ cells in PB and BM ranging from 0.24 to 1.87% and 0.56 to 0.65%, respectively.

In contrast, all six fetuses transplanted with CD34⁺-enriched cells expanded over MSC feeder layers engrafted successfully, displaying the presence of human CD45⁺ cells in PB and BM at levels of 1.2 to 5.65% and 0.34 to 0.82%, respectively. This data demonstrates that human BM CD34⁺-enriched cells expanded over MSC layers engrafted more efficiently and gave rise to higher levels of human donor cells in circulation after transplant than

their LT-ST counterparts. Furthermore, the levels of hematopoietic engraftment in the BM and levels of human cells in the PB of animals transplanted with MSC expanded cells were similar to the levels seen in the BM and PB of animals transplanted with comparable numbers of fresh human CD34⁺ cells [1,23].

Discussion

We have previously reported that human allogeneic MSC layers in a serum-free culture system enabled the *ex vivo* expansion/maintenance of human HSCs [3]. The advantage of this culture system resided in not only the use of pre-established human stromal layers, overcoming the limitations of using xenogeneic and/or transformed and immortalized human stromal cell lines, but also in its serum-free conditions. The ability to expand human HSCs in serum-free conditions increases the reproducibility of the cultures and avoids the possible infectious potential present in serum [1,3].

Thus, we hypothesized that using a serum-free culture system, identical to the one previously described but this time using a well-characterized and homogeneous layer of MSCs derived from Stro-1⁺ cells, would improve the reliability and reproducibility of the stromal cell layer support function [3]. Furthermore, we compared autologous versus allogeneic sources of MSC stroma and performed a side-by-side comparison with other stromal layers more heterogeneous in cell composition, using as an example the traditional stromal layers grown in long-term bone marrow culture media and used in long-term HSC culture assays [10].

Our results show that allogeneic MSC feeder layers were more effective in expansion/maintenance of the more primitive hematopoietic progenitors such as CD34⁺ and CD34⁺CD38⁻ cells when compared with allogeneic LT-ST, despite both feeder layers being able to equally promote successful expansion in terms of total cell number. These results are in agreement with reports by other investigators who showed that by using a transformed Stro-1⁺ feeder layer, it was possible to obtain a total cell expansion of 270-fold for BM-enriched cells [24]. Other groups, using a 10% FBS-containing system plus exogenous cytokines, reported a 5.4-fold increase in BM-derived CD34⁺ cells after a 7-day culture period over human brain endothelial cells [9]. Also using animal serum, Gammaitoni et al. described successful *ex vivo* expansion of BM and PB HSCs for up to 10 weeks under stroma-free conditions [25]. On the other hand, Shimakura et al. [26] were also able to establish a xenogeneic coculture system using the murine stromal cell line HESS-5 that was capable of effectively expanding umbilical cord blood (CB), BM, and PB. However, to make this coculture system relevant to clinical applications, the authors had to consider the hypothesis of using a membrane insert separating hemato-

poietic progenitors from the murine feeder layer to harvest the expanded cells without contamination with HESS-5 cells [26,27].

Here, in our serum-free culture system, we describe that using human allogeneic MSC stromal layers, after 7 days in culture, it was possible to obtain on average a 3.5-fold increase in CD34⁺ cells, although this increase was much lower using LT-ST layers (0.8-fold). This increase in more primitive cells in cultures over MSC layers was also noted by the higher CFU output in MSC cocultures. Another advantage of the culture system using purified MSC stromal layers was the ability to maintain and expand a CD7⁺ cell population with lymphocytic potential [3,28]. In fact, after 2 weeks in culture, 56% of hematopoietic cells expanded over allogeneic MSC were CD7⁺, contrasting to 2.4% of CD7⁺ cells in the LT-ST system. To investigate whether the culture system using MSC feeder layers could be improved by the use of autologous MSC and/or whether the use of MSC layers to expand HSCs was truly donor-independent, we compared autologous and allogeneic MSC feeder layers in their ability to expand/maintain HSCs. Surprisingly, although both allogeneic and autologous were equally effective in expanding total numbers of cells, allogeneic MSC feeder layers were more efficient in expanding the more primitive phenotypes such as CD34⁺ and CD34⁺CD38⁻ cells than the autologous MSCs. This result was confirmed by the higher clonogenic potential seen with HSCs expanded over allogeneic MSCs.

Several other efforts have been made to develop stroma- and serum-free culture system to expand *ex vivo* human CB HSC, as CB contains a finite number of primitive cells, seldom enough to successfully transplant larger recipients [3,29,30]. Nevertheless, a recent study by McNiece et al. suggests that stroma-free conditions may not maintain nor expand long-term repopulating cells, as had previously been hypothesized for the *ex vivo* expansion of human CB cells [31]. Yao et al. reported a successful stroma- and serum-free system for *ex vivo* expansion of human HSCs from CB cells; however, a combination of nine cytokines were used, including high proliferation-inducing cytokines like interleukin-3, which could hinder the engraftment ability of the expanded cells in an *in vivo* model [29,31].

To evaluate whether our expanded HSCs still maintained *in vivo* engraftment potential, we transplanted fetal sheep recipients with an identical number of cells expanded over allogeneic MSC and LT-ST feeder layers. HSCs expanded over MSC feeder layers led to the highest percentage of engrafted animals. Furthermore, the levels of human (donor) cell engraftment in PB of the animals transplanted with hematopoietic cells expanded over MSC feeder layers was higher than with HSCs expanded over LT-ST, although levels of human cells in the BM was similar in both groups. Because identical cell numbers expanded over MSC and LT-ST feeder layers were

transplanted, our results suggest that HSCs cocultured over MSCs had enhanced engraftment ability when compared with HSCs grown over LT-ST. Importantly, our results indicate that HSCs expanded over MSC feeder layers preserve their *in vivo* engraftment capability.

In conclusion, our results show that allogeneic Stro-1⁺, CD45⁻, Gly A⁻ MSCs are a reliable, consistent, and readily-available source of stromal cells that have the potential to be used as a universal feeder layer to efficiently expand and maintain human BM HSCs *ex vivo*.

Acknowledgments

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