

Improved *Ex Vivo* Expansion of Functional CD34⁺ Cells Using Stemline™ II Hematopoietic Stem Cell Expansion Medium

Daniel W. Allison,¹ Stacy L. Leugers,¹ Barry J. Pronold,² Gary Van Zant,²
Jenny A. Harrington,³ Ian K. McNiece,³ and Laurel M. Donahue¹

¹Cell Culture R&D, Sigma-Aldrich, St. Louis, MO, United States.

²University of Kentucky, College of Medicine, Markey Cancer Center, Lexington, KY, United States.

³Johns Hopkins University Oncology Center, Division of Hematologic Malignancies, Baltimore, MD, United States.

Abstract

Umbilical cord blood (CB) can serve as an alternate source of a hematopoietic cell graft for patients receiving high dose chemotherapy or transplantation for a number of genetic diseases. CB products have been used for transplantation of a number of patients who lacked a suitably matched donor. Unfortunately, CB products contain lower cell numbers than bone marrow or PBSC products limiting the use of CB products to mainly pediatric patients. *Ex vivo* expansion of CB products may provide increased cell numbers and provide more rapid engraftment of CB products. With these parameters in mind, we have developed Stemline™ II Hematopoietic Stem Cell Expansion Medium. This formulation has led to a significant increase in the number of cells expanded from cord blood CD34⁺ cells, as well as 4-HC purged CD34⁺ cells from peripheral blood (PBSC) products. Flow cytometry shows the surface antigen profiles to be consistent with previously reported profiles for *ex vivo* expanded cells. The expanded cells form all of the appropriate hematopoietic lineages in a colony-forming unit assay (CFU) and demonstrate long term engraftment (primary and secondary recipient) in NOD/SCID mouse models. This serves as an indication that the expanded cells are representative of all of the proper lineages required for a successful transplant. Clinical studies are currently being designed to evaluate the engraftment potential of CB and PBSC cells expanded in Stemline™ II Hematopoietic Stem Cell Expansion Medium.

Introduction

Hematopoietic stem cells (HSC) have the ability to repopulate the hematopoietic system by differentiating into all of the necessary erythroid, lymphoid, and myeloid lineages. Due to this rare ability, HSCs are used as therapeutic agents in the treatment of malignant and benign diseases of the blood forming and immune systems. There have been many advances in the area of clinical HSC research, but the availability of suitable cells for transplantation still remains a major limiting factor.

HSCs can be isolated from three different sources: umbilical cord blood (CB), bone marrow, and mobilized peripheral blood. CB is currently the preferred source because it has been shown to have a lower risk of graft versus host disease (GVHD), presumably due to its immunological naiveté. However, because the volume of CB is limited, each umbilical cord has only enough cells to successfully transplant a small child. In order to transplant an adult, the HSCs from CB must be expanded *ex vivo*. The expansion must be performed in a manner to ensure that the HSCs not only differentiate along appropriate hematopoietic lineages, but also self-renew, leaving undifferentiated stem cells in the expanded culture. The differentiated cells will allow for

short-term engraftment that will reduce the effects of neutropenia and thrombocytopenia in the patient. The undifferentiated cells will allow for long-term engraftment that will establish a new, permanent hematopoietic system for the patient. In order to expand these very specific cell types in the absence of potentially adventitious agents such as fetal bovine serum, an optimized serum-free medium and cytokine cocktail is needed.

To this end, Stemline™ Hematopoietic Stem Cell Expansion Media were developed for the expansion of HSCs. They are serum-free media that allow for expansion of both differentiated and undifferentiated HSCs. Stemline™ and Stemline™ II are both able to expand HSCs from CB, bone marrow, and mobilized peripheral blood. In bench-scale and clinical-scale expansions, both media have shown promising results in expanding a mixed population of cells that remain fully functional. The original medium, Stemline™, expands CD34⁺ cells better than or equal to other commercially available serum-free HSC media. Stemline™ II is a newer version of the medium that has an increased expansion potential for CD34⁺ cells.

MATERIALS & METHODS

Cell Preparation

For all experiments, cryopreserved, human CD34⁺ cells were obtained from independent suppliers (Stemgenix; Amherst, New York; AllCells, LLC; Berkeley, California) and were handled in a manner consistent with the manufacturer's instructions with regard to storage and reconstitution. Cells were counted using either a hemocytometer or Guava Personal Cytometer (Guava Technologies; Hayward, California) to determine cell density and viability.

Serum-Free Expansion Medium Preparation and Bench-Scale Expansion

All materials are from Sigma-Aldrich unless otherwise noted. Stemline™ Hematopoietic Stem Cell Expansion Media, IMDM, X-VIVO 15 (Cambrex; Walkersville, Maryland), HPGM (Cambrex; Walkersville, Maryland), QBSF-60 (Quality Biological, Gaithersburg, Maryland), StemPro-34 (Invitrogen; Carlsbad, California), and StemSpan H3000 (StemCell Technologies; Vancouver, British Columbia) were purchased fresh, aliquoted and stored according to the manufacturer's recommendations. For each experiment, a 10 ml volume of each expansion medium was warmed to 25 °C. One ml of each medium was pipetted in triplicate in 24-well culture plates (Corning/Costar; Corning, New York) to which SCF, TPO and G-CSF were added to a final concentration of 100 ng/ml each. Sterile PBS was added to unused wells to maintain humidity. Plates were incubated at 37 °C and 5% CO₂ for 15 minutes prior to the addition of the revived CD34⁺ cells. Viable recovered CD34⁺ cells were added to each well

at 1.0×10^4 cells/ml and allowed to proliferate in a humidified incubator at 37 °C and 5% CO₂ for 10 days. Following the incubation period, the expanded total nucleated cells were counted.

Flow Cytometry

The direct determination of the absolute count of CD38⁺ and CD38⁻ cells was assessed utilizing the Immunotech Stem Kit CD34⁺ Hematopoietic Progenitor Cell (HPC) Enumeration kit (Beckman-Coulter; Fullerton, CA), CD38-PE, CD34-ECD, and CD45-FITC Antibodies. The processed samples were identified and enumerated using Coulter's flow cytometer (EPICS XL-MCL).

Clinical-Scale Expansion

A 2-step, clinical-scale assay (McNiece, et al., Experimental Hematology 2000. 28: 1181-1186) using Teflon® culture bags (American Fluoroseal, Inc.; Gaithersburg, Maryland) was set up for a comparison study between Stemline™ and Stemline™ II. For clinical-scale studies, CB CD34⁺ cells were cultured for 7 days in 100 ml Teflon® culture bags containing 50 ml of each culture medium plus cytokine concentrations as previously described. Cells were harvested from these bags and a 10 ml aliquot was transferred to a second 100 ml Teflon® bag containing 90 ml of each selected medium plus cytokines and cultured for an additional 7-day culture period. At the end of the culture protocol, cells were harvested, counted by hemocytometer, viability tested, and assayed for functional hematopoietic activity *in vivo* and *in vitro*.

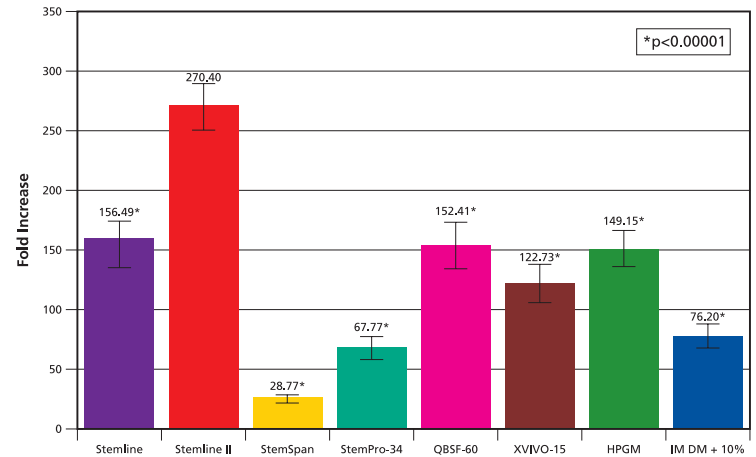
NOD/SCID Studies

Immunodeficient NOD/SCID mice were used as recipients of transplanted human cord blood cells expanded at clinical-scale as described above. NOD/SCID mice were lightly irradiated (3.0 Gy) 3-6 hours prior to infusion of human cells by injection intravenously into the retro-orbital plexus, as described in detail by Szilvassy et al. (in Hematopoietic Stem Cell Protocols, Klug and Jordan eds., Humana Press, 2001, pp 167-187). The mice, prior to and throughout the treatment period, were maintained under pathogen barrier conditions and their drinking water was supplemented with antibiotics. Depending on the experiment, between 500,000 and 6 million cells were transplanted. At various times after transplant, blood was obtained from each recipient by retro-orbital blood sampling. Human cells were distinguished by immunofluorescence and flow cytometry using CD45 antibody (Becton-Dickinson; Franklin Lakes, New Jersey). CD34 was used to distinguish progenitor cells and representation of human cells in the lymphocyte lineage was determined using CD19 and CD20; human myeloid cells were identified by CD15 and CD66b. The same panel of antibodies was used in the analysis of bone marrow at the termination of the experiment, or in preparation for injection into secondary recipients to verify the presence of self-renewing human stem cells in the primary recipients, and ultimately in the cord blood cells expanded in both Stemline™ culture media.

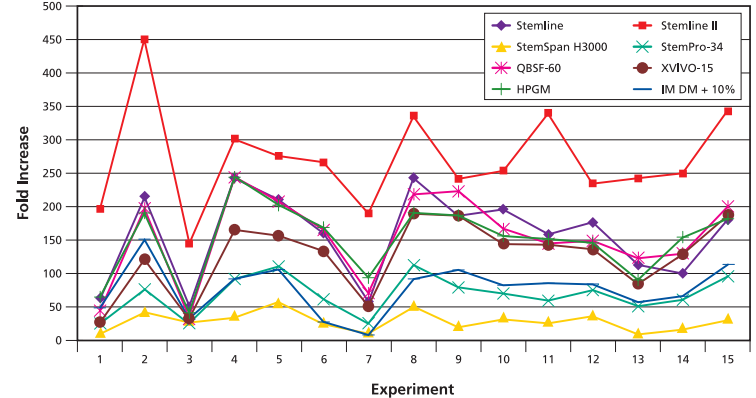
PBSC Expansion

PBSC were obtained from high-yield (>20 x 10⁶ CD34⁺ cells collected/kg) autologous leukapheresis mobilized blood products from cancer patients undergoing chemotherapy at the Johns Hopkins Hospital under an institutional review board-approved protocol. The PBSC mobilization regime was cyclophosphamide (2 g/m²) followed by G-CSF (10 mg/kg/d) for 14 days. PBSC were expanded using a 2-step, clinical-scale expansion protocol as described above. Cultures were assayed for total nucleated cells (TNC), committed progenitors (granulocyte-macrophage-colony forming cells, GM-CFC) and primitive progenitors (high proliferative potential colony forming cells, HPP-CFC). CFU assays were performed using standard procedures. PBSC CD34⁺ cells were then incubated in a water bath at 37 °C with 30 µg/ml mafosfamide (Baxter Oncology, Frankfurt, Germany). After 30 minutes, the mafosfamide reaction was stopped by washing twice with cold Dulbecco's Phosphate-Buffered Saline containing 1% fetal bovine serum. Cells were then cultured as previously described.

Fold Increase of Total Nucleated Cells from CD34⁺ Cord Blood (Mean ± S.E.M.; n = 15 donors)



Fold Increase of Total Nucleated Cells from CD34⁺ Cord Blood (n = 15 donors)



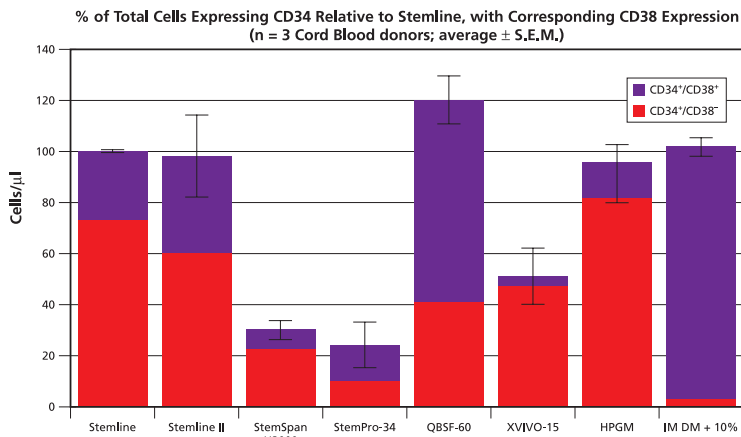


Figure 1: Expansion of CD34⁺ cells from cord blood in Stemline™ and Stemline™ II compared to other commercially available, serum-free, HSC media. Assays were set up in 24-well tissue culture plates with each medium tested in triplicate. 1 ml of the appropriate medium was added to each well, and the plate was inoculated with 10,000 cells/well. The cells were counted on day 10 and the fold increase was determined by $\text{cells}_{\text{final}}/\text{cells}_{\text{initial}}$. In cord blood, both Stemline™ media outperform the other serum-free HSC media. While Stemline™ already performed better than or equal to the other HSC media, Stemline™ II exhibited a significant increase in expansion compared with the other products ($p < 0.00001$). The second graph represents the fold increase for each medium in all 15 donors. This also shows that Stemline II consistently provides the maximum number of TNC. The third graph in this series shows the percentage of CD34⁺ cells/µl expanded from the initial CD34⁺ cord blood cells, normalized to the number expanded in Stemline™. This represents an average ± S.E.M. for 3 donors. This data is further broken down into CD38⁻ and CD38⁺ progenitors as an indication of their degree of differentiation.

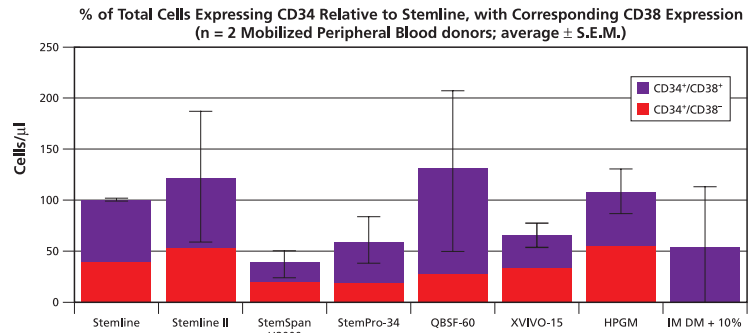
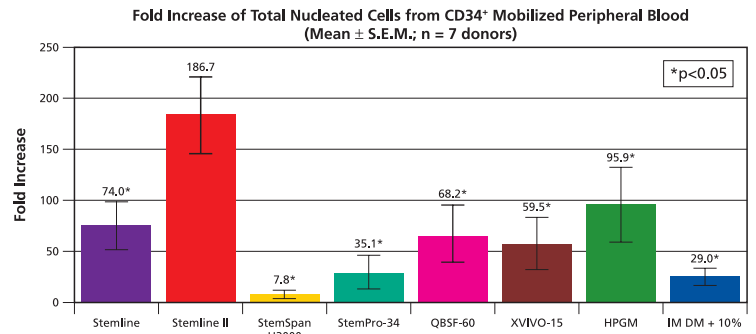


Figure 3: Bench-scale expansion of CD34⁺ cells from mobilized peripheral blood in Stemline™ and Stemline™ II. Assays were set up as in Figure 1. The cells were counted on day 14 and the fold increase was determined by $\text{cells}_{\text{final}}/\text{cells}_{\text{initial}}$. In mobilized peripheral blood, both Stemline™ products consistently exhibit high levels of expansion of total nucleated cells ($n=7$; $p < 0.05$). Flow cytometry on the expanded cells reveals that Stemline™ II also expands a large number of CD34⁺ stem cells (both CD38⁺ and CD38⁻). Cells/µl is normalized to the average number of cells/ml in Stemline™ ± S.E.M. ($n=2$ mobilized peripheral blood donors).

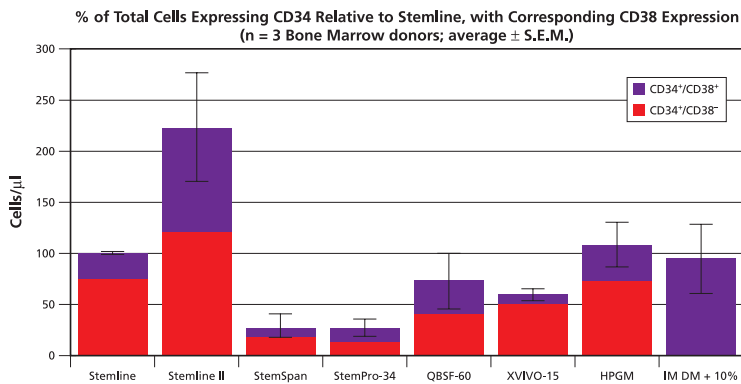
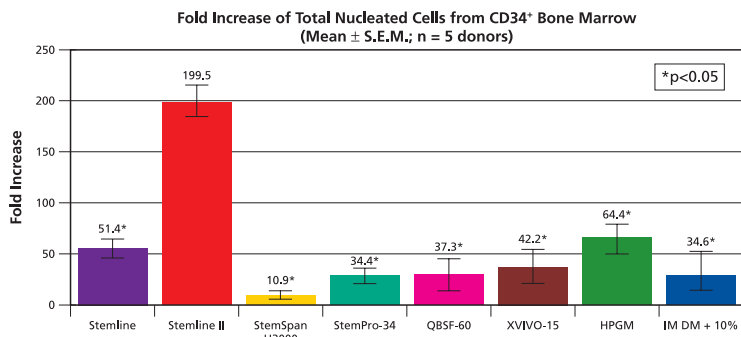


Figure 2: Stemline™ and Stemline™ II bench-scale expansion of CD34⁺ cells from bone marrow compared to other HSC media. Assays were set up as in Figure 1. The cells were counted on day 14 and the fold increase was determined by $\text{cells}_{\text{final}}/\text{cells}_{\text{initial}}$. For the expansion of bone marrow CD34⁺ cells, Stemline™ performed as well as, or better than, the other competitors. However Stemline™ II was vastly superior to the other commercially available serum-free HSC media, giving approximately 5 fold more total nucleated cells ($n=5$ donors; $p < 0.05$). Flow cytometry on the expanded cells reveals that Stemline™ II also expands a greater number of CD34⁺ stem cells (both CD38⁺ and CD38⁻). Cells/µl is normalized to the average number of cells/ml in Stemline™ ± S.E.M. ($n=3$ bone marrow donors).

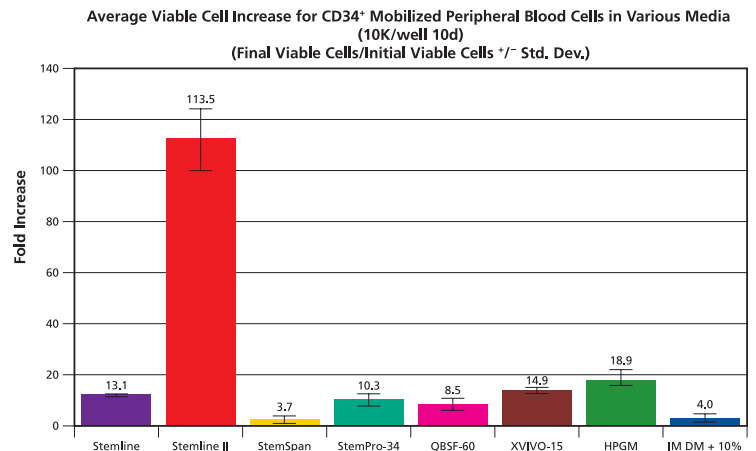
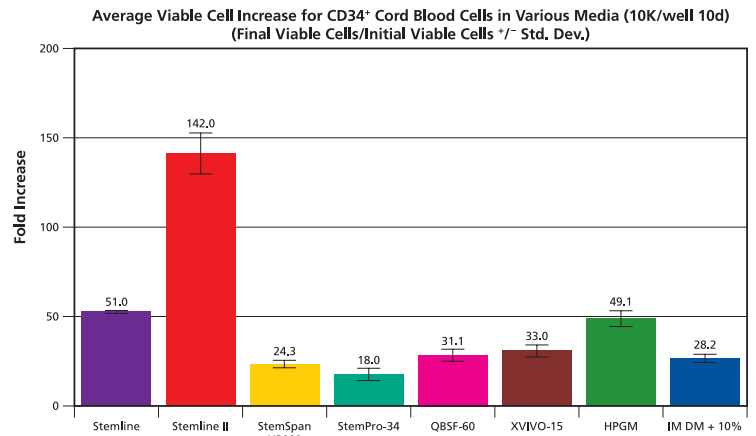


Figure 4: Bench-scale expansion of CD34⁺ cells from "bad" cord blood or mobilized peripheral blood donors in Stemline™ and Stemline™ II. Assays were set up as in Figure 1. The cells were counted on day 10 and the fold increase was determined by $\text{cells}_{\text{final}}/\text{cells}_{\text{initial}}$. In these donors (cord blood in the top graph and mobilized peripheral blood in the bottom graph), Stemline™ II manages to reach relatively high levels of expansion while the other media do not. These so-called "bad donors" are fairly

common and due to the low levels of expansion seen in most media, would not generate enough cells for transplant. Stemline™ II may be able to rescue these so-called “bad donors”.

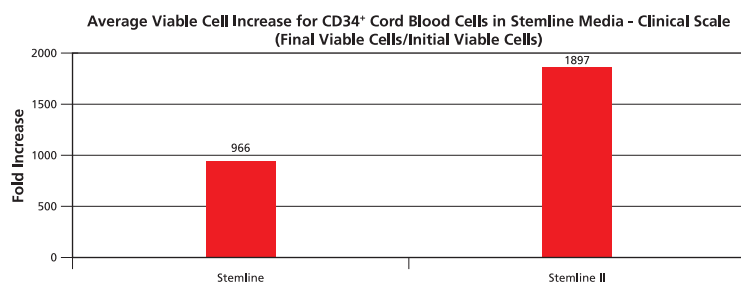


Figure 5: Comparison of Stemline™ II to Stemline™ in a clinical-scale expansion of CD34+ cells from cord blood. A two step clinical-scale expansion was performed to compare cell growth in Stemline™ and Stemline™ II. Briefly, the cells were seeded into 100 ml bags and incubated for 7 days. On day 7, a portion of the expanded cells was inoculated into a fresh 100 ml bag for an additional 7 days. Both media demonstrate increased potential for expanding CD34+ cells from cord blood, supporting excellent growth and high viability (>80%).

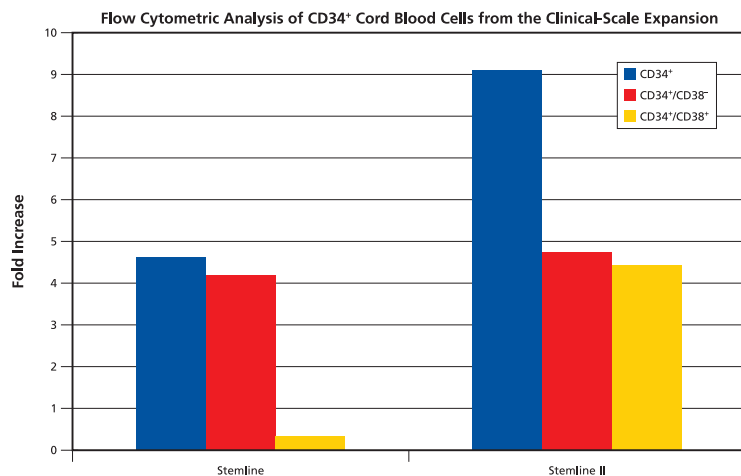
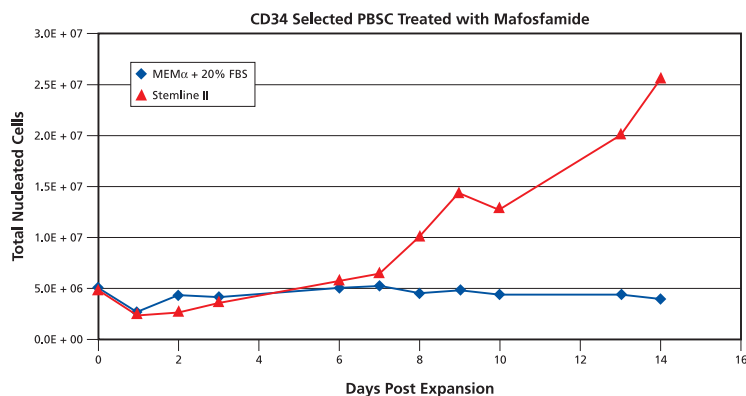


Figure 6: Flow cytometric analysis on CD34+ cord blood cells from clinical-scale expansion. After the two step expansions were complete, a sample of cells from the Stemline™ and Stemline™ II cultures was analyzed by flow cytometry for expression of CD34 and CD38. The majority of the CD34+ cells expanded in Stemline™ remained undifferentiated, early progenitors (CD34+38-), while cells expanded in Stemline™ II contained both early (34+38-) and late progenitor (34+38+) phenotypes. Both media expand high levels of early progenitors, which is important for long term engraftment. Stemline™ II also expands high levels of the late progenitors required for early engraftment and amelioration of the post-transplant nadir in mature myeloid cells.

	Stemline			Stemline II		
	600,000	1,800,000	5,400,000	600,000	1,800,000	5,400,000
Injected Cells	600,000	1,800,000	5,400,000	600,000	1,800,000	5,400,000
Survival Rate	5/10 50%	3/10 30%	3/7 43%	7/10 70%	6/10 60%	6/7 86%
Average % CD45+	0.064± 0.061	0.017± 0.006	0.143± 0.081	0.036± 0.013	0.018± 0.019	0.108± 0.162
Average % CD45+/CD34+	0.000± 0.000	0.003± 0.006	0.007± 0.006	0.011± 0.009	0.002± 0.004	0.010± 0.000

Table 1: Summary data from the transplantation of expanded cells in the NOD/SCID mouse model. After the two step expansions were complete, a sample of cells from the Stemline™ and Stemline™ II cultures were prepared for transplantation into NOD/SCID mice. Three different doses of cells were chosen for injection into the mice. Due to an underestimation of the number of cells required for successful transplantation following *in vitro* expansion, a limiting dilution of cells was not reached. This will need to be repeated with lower doses of cells. However, a high percentage of the mice survived transplantation with cells expanded from both media (higher with Stemline™ II), all of which contained a small number of CD45+ human cells as proof of engraftment (an even smaller number of which were also CD34+). Both media expanded enough functional, early progenitors to achieve long term engraftment. The higher survival rate in Stemline™ II may be explained by the higher levels of the late progenitors required for early engraftment and amelioration of the post-transplant nadir in mature myeloid cells. Further studies with secondary recipients are in process, with survival in excess of 9 weeks.



MEMα + 20% FBS	Day 0	Day 14	Stemline™ II	Day 0	Day 14
GM-CFC (x 10 ⁴)	4.9	41.2	GM-CFC (x 10 ⁴)	4.9	53.6
HPP-CFC (x 10 ⁴)	1.0	8.7	HPP-CFC (x 10 ⁴)	1.0	9.0

Figure 7: Comparison of TNC during ex vivo expansion of mafosfamide treated CD34+ PBSC in Stemline™ II versus MEMα plus 20% FBS. As further proof of the efficacy of Stemline™ II for the expansion of hematopoietic stem cells, peripheral blood stem cells (PBSC) were treated with mafosfamide prior to expansion in either Stemline™ II or the standard MEMα + 20% FBS. For expansion of mafosfamide treated CD34+ PBSC, Stemline™ II provides a significant increase in the TNC as well as a comparable fold increase of GM-CFC and HPP-CFC compared to MEMα plus 20% FBS when using identical cytokine combinations. Other serum-free formulations had not worked well in the past (data not shown). These results have supported moving forward with clinical trials to evaluate the engraftment potential of mafosfamide treated CD34+ PBSC cells cultured in Stemline™ II Hematopoietic Stem Cell Expansion Medium in patients.

Conclusions

Bench-scale expansions

- Stemline™ and Stemline™ II are capable of expanding CD34+ cells from umbilical cord blood, adult bone marrow, and mobilized peripheral blood in bench-scale expansions. Both Stemline™ media expand CD34+ cells from all three sources better than the serum-free commercially available competitors.

Clinical-scale expansions

- Both Stemline™ media were able to expand CD34+ cells from cord blood in a clinical-scale expansion.
- Flow cytometric analysis of the clinical-scale expansions reveals that Stemline™ and Stemline™ II expand comparable numbers of early progenitor cells (CD34+/CD38-).
- Stemline™ II also has the additional benefit of a higher capacity for the expansion of the CD34+/CD38+ late progenitors required for short-term engraftment.

Clinical applications

- Cells expanded in both Stemline™ and Stemline™ II were capable of repopulating NOD/SCID mice using serial passage, demonstrating self-renewal of expanded cells.
- Stemline™ II was able to expand mafosfamide treated PBSC better than the standard serum containing medium and will move to clinical trials soon.

Product Name	Product Number	Package Size	Physical State/ Storage	Required Glutamine Supplementation	Animal Components	Regulatory Items
Stemline™	S 0189	500 ml	Liquid/4 °C	4 mM	Human Serum Albumin	Manufactured cGMP DMF on file
Stemline™ II	S 0192	500 ml	Liquid/4 °C	N/A	Human Serum Albumin	Manufactured cGMP DMF in process

Table 2: Basic characteristics of Stemline™ media.

Acknowledgments

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