A Feeder-Free and Serum-Free Culture System Supports Expansion of CD34+ AML and CML Stem/Progenitor Cells

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INTRODUCTION

Leukemia is a group of neoplastic blood cell disorders that are caused by malignant transformation and dysregulated growth of hematopoietic stem and progenitor cells (HSPCs). Chronic myeloid leukemia (CML) has served as a model disease for other leukemias, as it is caused by a single genetic translocation between chromosomes 9 and 22, which results in the formation of the BCR-ABL fusion gene and constitutively active BCR-ABL tyrosine kinase in HSPCs. The identification of BCR-ABL has lead to development of tyrosine kinase inhibitors that target BCR-ABL and effectively inhibit malignant cell growth in CML patients. Acute myeloid leukemia (AML) is genetically much more complex, involving multiple chromosomal and genetic abnormalities between patients and between different leukemic clones during disease progression in individual patients. This complexity has made it difficult to identify suitable genetic targets and develop novel antineoplastic drugs that can target AML clones specifically and effectively. Leukemia cells can be cultured ex vivo, but current culture conditions don't support survival of leukemic HSPCs or require co-culture with stromal feeder cells. In this study we developed a feeder-free and serum-free culture system that enables efficient expansion of leukemic stem/progenitor cells from most CML and AML patients. CD34+ cells were immunomagnetically isolated from previously frozen CML and AML samples (n = 6 each) and cultured in serum-free StemSpan™ SFEM II medium plus CD34+ Expansion Supplement with UM171. After 7 and 14 days, the cell number, immunophenotype, aldehyde dehydrogenase (ALDH) activity, and colony-forming potential of the expanded cells were measured. The frequency of CD34+ CML cells after 7 and 14 days was 81% and 45%, respectively, and the frequency of CD34+ AML cells after 7 and 14 days was 81% and 65%, respectively. CD34+ CML cell numbers increased 65-fold and CD34+ AML cell numbers increased 30-fold after 7 days of culture, and both CD34+ CML and AML cell numbers increased 10-fold when cultured for an additional 7 days. Furthermore, expanded cells were able to generate BFU-E- and CFU-GM-derived colonies, and 71 of 74 colonies derived from CML samples (n = 6) were BCR-ABL+ as determined by single colony qRT-PCR. Finally, the expanded CML and AML cells showed similar sensitivity to growth inhibition by imatinib, dasatinib, doxorubicin, and azacitidine compared to the non-expanded CD34+ cells from the same patients. Taken together, these results demonstrate that CD34+ CML and AML cells can be expanded effectively in culture under stroma- and serum-free conditions, and maintain the immunophenotype, clonogenic growth, and drug responsiveness of the CD34+ cells in the original patient sample.

METHODS

Culture Protocol

CD34+ cells from cryopreserved CML or AML peripheral blood mononuclear cells (PBMCs) and bone marrow mononuclear cells (BMMCs) were purified using EasySep™ Human Cord Blood CD34 Positive Selection Kit II. The isolated CD34+ cells were seeded into 12-well plates at 10^3 - 10^4 cells/mL in 1 mL serum-free StemSpan™ SFEM II supplemented with StemSpan™ CD34+ Expansion Supplement with or without the addition of UM171. The cells were incubated at 37°C for 7 days, then 10% of the cells were seeded into fresh culture medium for an additional 7 days of culture. On day 0, day 7, and day 14, cells were analyzed by immunophenotyping for cell surface markers commonly expressed on HSPCs, colony-forming unit (CFU) assays using MethoCult™ H4435 Enriched medium (Catalog #04435), and used to assess drug sensitivity.

Readout of the Expanded CML/AML Cells

Flow Cytometry

Cells were harvested on day 7 and day 14, stained with fluorescently labeled antibodies against CD45, CD34, CD90, CD45RA, and with ALDEFLUOR™ Reagent to measure aldehyde dehydrogenase (ALDH) activity, and analyzed by flow cytometry. Sequential gates were used to determine the percentages and numbers of viable CD45+, CD45+CD34+, and CD45+CD34+CD90+CD45RA- cells, based on fluorescence minus one (FMO) controls, and of CD45+ALDH+ cells, based on DEAB (diethylaminobenzaldehyde) control. Dead cells were excluded by light scatter profile and 7-AAD staining.

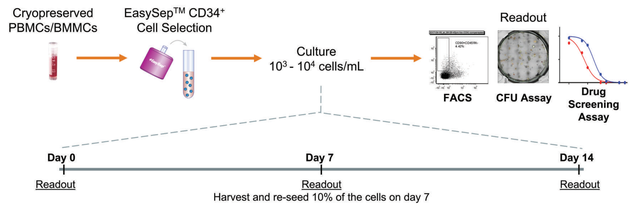


FIGURE 1. Experimental Setup

CFU Assay

CML/AML cells, after CD34+ cell isolation, or after 7 or 14 days of expansion were plated in CFU assays with MethoCult™ H4435 Enriched medium. Day 0 CD34+ cells were seeded at 500 - 1500 cells/mL, day 7 cells were seeded at 750 - 1500 cells/mL, and day 14 cells were seeded at 1500 - 2000 cells/mL. 1.1 mL of cell suspension was plated in each well of a SmartDish™ plate in duplicates. Colonies produced were counted after incubation at 37°C for 14 days and were imaged using STEMvision™. Single-colony qRT-PCR was also performed on CML samples to detect BCR-ABL genes.

Drug Screening Assay

Purified CD34+ CML/AML cells were expanded in StemSpan™ SFEM II supplemented with StemSpan™ CD34+ Expansion Supplement and UM171 (StemSpan™ Leukemic Cell Culture Medium) for 7 days and 14 days. Both non-expanded purified CD34+ cells and expanded CD34+ cells were incubated at 37°C in StemSpan™ Leukemic Cell Culture Medium at a seeding density of 1000 - 2000 cells/200 µL in 96-well plates for 7 days with control (0.04% DMSO) or with test compounds imatinib (IM), dasatinib (DA), doxorubicin (DOX), or azacitidine (AZA). Cells were assessed by flow cytometry with 7-AAD staining at the end of 7-day drug treatments.

RESULTS

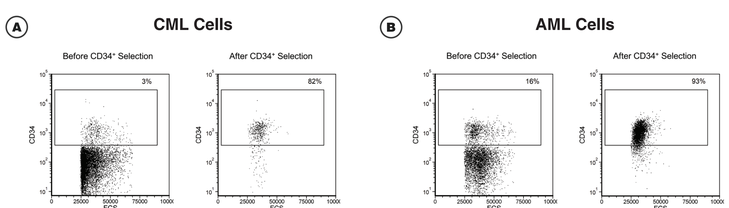


FIGURE 2. Isolation of Leukemic CD34+ Cells
Cryopreserved CML (A) or AML (B) PBMCs and BMMCs were thawed and CD34+ cells were isolated using EasySep™ Human Cord Blood CD34 Positive Selection Kit II. The percentage of CD34+ cells before and after CD34+ cell isolation was measured by flow cytometry. Dead cells were excluded by light scatter profile and viability staining. In this example, the purity of CD34+ cells increased from 3% to 82% (CML) and from 16% to 93% (AML).

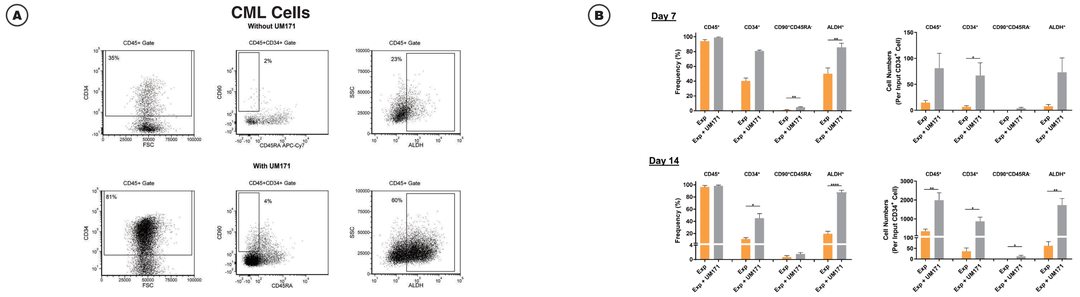


FIGURE 3. Expansion of CD34+ CML Cells
CD34+ CML cells were incubated at 37°C in StemSpan™ SFEM II supplemented with CD34+ Expansion Supplement (Exp) with or without UM171. After 7 and 14 days, the cultured cells were stained with fluorescently labeled antibodies against CD45, CD34, CD90, CD45RA, and with ALDEFLUOR™ to measure ALDH activity, and were analyzed by flow cytometry. **(A)** Representative flow cytometry profiles on Day 7 are shown. **(B)** The frequency and cell number of these subsets per initial input CD34+ cell on Day 7 and Day 14 are shown. StemSpan™ SFEM II supplemented with CD34+ Expansion Supplement supports the expansion of CML cells in culture, and the addition of UM171 further enhances the expansion of all subsets. Data shown are mean ± SEM (n = 6). P-values were calculated using a two-tailed paired Student's t-test (*P < 0.05; **P < 0.01; ***P < 0.0001). All six CML samples tested were able to expand in culture.

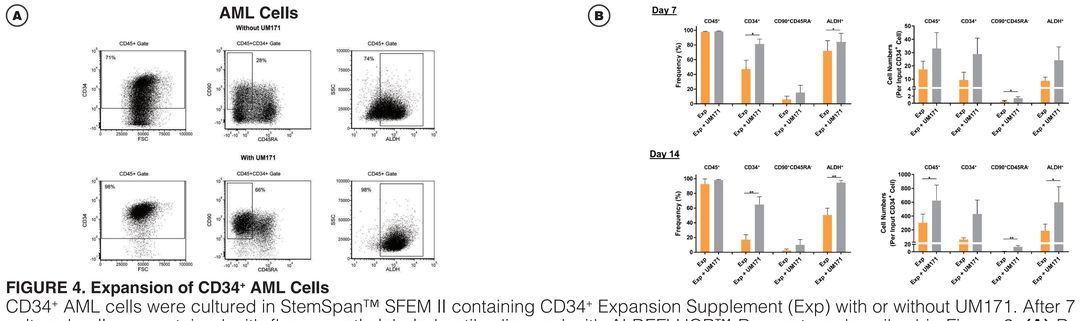
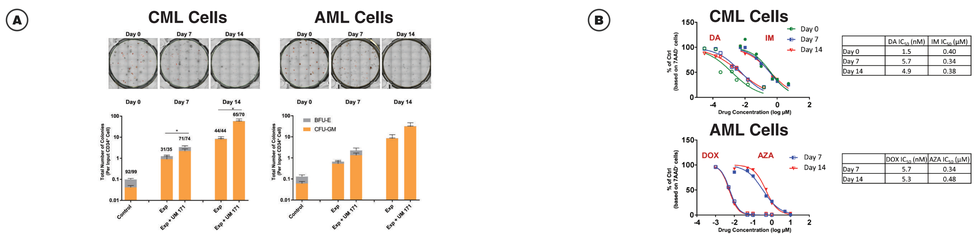


FIGURE 4. Expansion of CD34+ AML Cells
CD34+ AML cells were cultured in StemSpan™ SFEM II containing CD34+ Expansion Supplement (Exp) with or without UM171. After 7 and 14 days, the cultured cells were stained with fluorescently labeled antibodies and with ALDEFLUOR™ Reagent as described in Figure 3. **(A)** Representative flow cytometry profiles on Day 7 are shown. **(B)** The frequency and cell number of these subsets per initial input CD34+ cell on Day 7 and Day 14 are shown. When cultured with UM171, the frequencies of CD34+ cells, CD34+CD90+CD45RA- cells, and ALDH+ cells in AML cultures at Day 7 and Day 14 were similar to those of CML samples (Figure 3); the numbers of CD34+ cells, CD34+CD90+CD45RA- cells, and ALDH+ cells in AML samples were 2.5-fold lower than those of CML samples (Figure 3). Data shown are mean ± SEM (n = 6). P-values were calculated using a two-tailed paired Student's t-test (*P < 0.05; **P < 0.01). Six out of ten AML samples tested were able to expand in culture.



	DA IC_{50} (nM)	IM IC_{50} (µM)
Day 0	1.5	0.40
Day 7	5.7	0.34
Day 14	4.9	0.38

	DOX IC_{50} (nM)	AZA IC_{50} (µM)
Day 7	5.7	0.34
Day 14	5.3	0.48

FIGURE 5. Colony-Forming Potential and Drug Sensitivity of CD34+ CML/AML Cells are Maintained During Culture
(A) Non-expanded CML/AML cells after CD34+ cell isolation (Day 0), or after 7 days and 14 days of expansion with or without UM171 as described in Figure 3, were tested in CFU assays using MethoCult™ H4435 Enriched medium. After incubation at 37°C for 14 days, colonies were imaged using STEMvision™, then counted. CFUs are expressed as the total number of colonies per input CD34+ cell. SFEM II supplemented with CD34+ Expansion Supplement (Exp) supports the expansion of colony-forming progenitor cells in culture, and addition of UM171 further promotes CFU output. Numbers above each of the individual bars indicate the proportion of *BCR-ABL* positive colonies, measured by qRT-PCR on individual plucked colonies across 6 different CML samples (8 - 12 colonies were plucked for each sample per condition). Data shown are mean ± SEM (n = 6). P-values were calculated using a two-tailed paired Student's t-test (*P < 0.05). **(B)** Non-expanded CML/AML cells after CD34+ cell isolation (Day 0), or after 7 days and 14 days of expansion with UM171 were subjected to in vitro drug treatments for 7 days in the same culture system. Dose-response curves of Day 0, Day 7, and Day 14 cells were generated using flow cytometry based on 7-AAD- cells at Day 7 post drug treatment. Cell numbers at each drug concentration were normalized to the cell number in the solvent control (% of Ctrl) and the drug concentrations at which cell proliferation was 50% of the control (IC_{50}) was calculated. CD34+ CML/AML cells respond to drugs in a dose-dependent manner. Drug inhibition profiles and IC_{50} values were similar for non-expanded and expanded cells, demonstrating that culture-expanded AML and CML HSPCs are useful for drug screening purposes.

Summary

- CD34+ CML and AML cells can be maintained and expanded in StemSpan™ SFEM II medium supplemented with CD34+ Expansion Supplement and UM171
- CD34+ CML and AML cells can expand 65-fold and 30-fold, respectively, after 7 days of culture, with ~10-fold additional expansion after 14 days of culture
- The expanded cells display high % CD34+ and ALDH+ cells, have colony-forming potential, and show similar drug sensitivity compared to that of non-expanded cells in culture-based drug testing assays