Expansion of LTC-ICs and Maintenance of p21 and BCL-2 Expression in Cord Blood CD34⁺/CD38⁻ Early Progenitors Cultured over Human MSCs as a Feeder Layer

SUZANNE KADEREIT,[a] LINDA S. DEEDS,[a] STEPHEN E. HAYNESWORTH,[b] OMER N. KOC,[a,c] MARGARET M. KOZIK,[a] EMESE SZEKELY,[c] KATHLEEN DAUM-WOODS,[c] GLENN W. GOETCHIUS,[a] PINGFU FU,[d] LISBETH A. WELNIAK,[e] WILLIAM J. MURPHY,[e] MARY J. LAUGHLIN[a]

[a]Department of Medicine, [b]Department of Biology, [c]NCI Comprehensive Cancer Center, [d]Department of Epidemiology and Biostatistics, Case Western Reserve University/University Hospitals of Cleveland, Cleveland, Ohio, USA; [e]SAIC Frederick, NCI at Frederick, Frederick, Maryland, USA

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ABSTRACT

Allogeneic transplantation with umbilical cord blood (UCB) is limited in adult recipients by a low CD34⁺ cell dose. Clinical trials incorporating cytokine-based UCB in vitro expansion have not demonstrated significant shortening of hematologic recovery despite substantial increases in CD34⁺ cell dose, suggesting loss of stem cell function. To sustain stem cell function during cytokine-based in vitro expansion, a feeder layer of human mesenchymal stem cells (MSCs) was incorporated in an attempt to mimic the stem cell niche in the marrow microenvironment. UCB expansion on MSCs resulted in a 7.7-fold increase in total LTC-IC output and a 3.8-fold increase of total early CD34⁺ progenitors (CD38⁻/*HLA-DR*⁻). Importantly, early CD34⁺/CD38⁻/*HLA-DR*⁻ progenitors from cultures expanded on MSCs demonstrated higher cytoplasmic expression of the cell-cycle inhibitor, $p21^{cip1/waf1}$, and the antiapoptotic protein, BCL-2, compared with UCB expanded in cytokines alone, suggesting improved maintenance of stem cell function in the presence of MSCs. Moreover, the presence of MSCs did not elicit UCB lymphocyte activation. Taken together, these results strongly suggest that the addition of MSCs as a feeder layer provides improved conditions for expansion of early UCB CD34⁺/CD38⁻/*HLA-DR*⁻ hematopoietic progenitors and may serve to inhibit their differentiation and rates of apoptosis during short-term in vitro expansion.

INTRODUCTION

Umbilical cord blood (UCB) from related and unrelated donors has emerged as a novel source of stem cells for patients requiring allogeneic transplantation [1-4]. Although UCB contains hematopoietic progenitor cells at a higher frequency and with a higher proliferative capac-

Correspondence: Mary J. Laughlin, M.D., Director, Allogeneic Transplant Program, Case Western Reserve University, University Hospitals Ireland Comprehensive Cancer Center, 11100 Euclid Avenue, Cleveland, Ohio 44106-5061, USA. Telephone: 216-844-4766; Fax: 216-844-3616; e-mail: mjl13@po.cwru.edu *Received May 22, 2002; accepted for publication July 22, 2002.*

ity than adult-derived bone marrow, the low number of total hematopoietic stem cells (HSCs) contained in one UCB graft limits the potential for rapid hematological recovery in adult patients [5, 6].

In an attempt to shorten the time interval to attain donor-derived hematopoietic recovery after UCB transplantation and allow transplantation of adult recipients, phase I clinical trials have been undertaken to expand UCB in cytokines in vitro prior to infusion [7-9]. However, these clinical trials have failed to demonstrate more rapid hematopoietic recovery in UCB recipients, suggesting that cytokine-based expansion may result in differentiation of early self-replicative stem cells. These observations corroborate recent advances in the cellular and molecular mechanisms underlying the regulation of stem cell differentiation, indicating that microenvironment or stem cell "niche" is crucial for maintenance of self-renewal capacity of stem cells [10-14].

Mesenchymal stem cells (MSCs) are a nonhematopoietic, well-characterized homogeneous population of adherent skeletal and connective tissue progenitor cells within the bone marrow stroma [15-17]. Importantly, MSCs provide a rich environment of signals, including cytokines, extracellular matrix proteins, adhesion molecules, and cell-cell interactions, controlling the proliferation, survival, and differentiation of early lymphohematopoietic stem cells [18, 19]. Studies have shown that MSCs secrete, at baseline, interleukin-6 (IL-6), IL-7, IL-8, IL-11, IL-12, IL-14, and IL-15, macrophage-colony-stimulating factor (M-CSF), flt-3 ligand (FL), and stem cell factor (SCF), similar to the cytokines and growth factors expressed by marrow-derived stromal cells [16]. Accordingly, MSCs have been shown, similar to Dexter-type stromal cells, to support long-term culture initiating cell (LTC-IC) colonies during prolonged in vitro bone marrow culture [19]. Importantly, however, IL-1α-stimulated MSCs, but not bone marrow-derived stromal cells, secrete leukemia inhibitory factor (LIF), which has been shown to inhibit embryonic stem cell differentiation [16, 20, 21].

For these reasons, we hypothesized that the presence of a feeder layer of human MSCs during short-term UCB cytokine-based expansion may provide a "niche-like" milieu for hematopoietic stem cells and may, thus, inhibit cytokine-driven differentiation of early $CD34^+$ hematopoietic progenitor cells. We, therefore, compared expansion of UCB mononuclear cells (MNCs) during short-term culture in a combination of early-acting cytokines alone with expansion over an MSC feeder layer with added cytokines. We showed that the presence of MSCs improved expansion of early $CD34^+/CD38^-/$*HLA-DR*$^-$ progenitors and LTC-ICs compared with cytokines alone, while maintaining p21 and BCL-2 protein expression.

Materials and Methods

Cells

UCB was obtained according to institutional guidelines, and MNCs were prepared as described [22] and cryopreserved until expansion. Prior to expansion, monocytes were removed by adherence. For analyses of unexpanded cells, aliquots of the same original UCB were thawed and assayed for comparative baseline studies.

MSCs from bone marrow of adult healthy donors, collected according to institutional guidelines, were isolated by Percoll density gradient centrifugation and culture-expanded as previously described [18]. Second passage MSCs were seeded at 3×10^3 cells/cm^2, grown to confluency in Dulbecco's modified Eagle's medium with 10% fetal bovine serum (FBS) (GIBCO Life Technologies; Gaithersburg, MD; http://www.lifetech.com), and irradiated (15 Gy, ^{137}Cs irradiation) prior to co-culture with UCB, to prevent MSC overgrowth.

UCB Expansion Cultures

MNCs (2×10^6 cells/ml) from UCB were plated in StemPro™ supplemented with 10% FBS, L-glutamine, penicillin, streptomycin, and amphotericin B (GIBCO Life Technologies). Cells were either expanded for 12 days in cytokines, as previously described [23], or in parallel in the same cytokines over a monolayer of MSCs. Cytokines included: IL-3 (20 ng/ml), IL-6 (20 ng/ml), FL (100 ng/ml) (R&D Systems; Minneapolis, MN; http://www.rnd systems.com), SCF (100 ng/ml), G-CSF (20 ng/ml), and erythropoeitin ([EPO] 0.1 U/ml) (Amgen Inc.; Thousand Oaks, CA; http://www.amgen.com). Every third day, half-media changes were performed to replenish cytokines, and the total volume was adjusted to maintain the cells at 2×10^6 cells/ml. After 12 days, cultures were harvested by trypsinization to include cobblestone-forming cells embedded in the MSC layer.

Flow Cytometry

For CD34 analysis, 1×10^6 cells were blocked with mouse IgG and stained with monoclonal antibodies against CD34, CD14, CD38 (Pharmingen; San Diego, CA; http://www.bdbiosciences.com/pharmingen), and *HLA-DR* (Becton Dickinson; San Jose, CA; http://www.bd.com). One hundred thousand events were acquired on an ELITE ESP flow cytometer (Beckman Coulter Inc.; Miami, FL; http://www.coulter.com), and data were analyzed with WinList (Verity Software House Inc.; Topsham, ME). $CD34^+$ percentages were calculated as percentages of lymphocyte gate (set on $CD45^+$, low side scatter cells of the unexpanded UCB, and maintained throughout analysis), excluding $CD14^+$ cells. Propidium iodide was included to assess day 12 UCB viability. For intracellular staining, cells were surface stained as

above, washed, permeabilized with FACS Permeabilizing Solution (Becton Dickinson), and stained with monoclonal antibody against BCL-2 (Pharmingen) or p21$^{cip1/waf1}$ (Biosource International, Inc.; Camarillo, CA; http://www.biosource.com). The same gating strategy as above was applied.

Colony-Forming Unit (CFU) and LTC-IC Assays

MNCs (1×10^5) were grown in duplicate in methylcellulose (Stem Cell Technologies; Vancouver, Canada; http://www.stemcell.com) containing 10 ng/ml IL-3, 3 U/ml EPO, 50 ng/ml SCF, and 10 ng/ml GM-CSF. Hemin (0.1 mM; Sigma Chemicals; St. Louis, MO; http://www.sigma aldrich.com) was added, and cells were incubated at 37°C and 5% CO_2. After 12-14 days, colonies greater than 50 cells were enumerated and expressed as total CFUs, as previously described [24].

For LTC-IC analysis, dilutions of 500, 1,000, 2,000, 4,000, and 8,000 cells/well were plated at 10 wells each dilution in myeloCult H5100™ medium (Stem Cell Technologies) supplemented with 10^{-6} M hydrocortisone into 96-well plates over a near confluent monolayer of irradiated allogeneic human bone marrow stroma and maintained at 37°C and 5% CO_2. Weekly half-media changes were performed, and after 5 weeks, both nonadherent and adherent cells were transferred from the 96-well plates on a well/well basis into fresh 96-well plates with complete methylcellulose and cultured as described above for CFUs. A well that generated at least one progenitor colony was considered positive for limiting dilution calculations of LTC-IC frequency. LTC-IC frequency was estimated using Poisson statistics [25]. Absolute LTC-IC counts were calculated by multiplying the total nucleated cell number measured at day 0 or day 12 with LTC-IC frequency measured at these time points, to yield absolute LTC-IC counts.

Mixed Lymphocyte Cultures

Day 12 expanded cells were washed thoroughly and mixed with irradiated (30 Gy, ^{137}Cs irradiation) pooled peripheral blood lymphocytes (PBLs) from five normal adult donors (third party antigen) or with irradiated PBLs from the MSC donor, as stimulators. Responder expanded UCB cells and stimulator cells were mixed at final concentrations of 0.15×10^6 cells/ml and 0.05×10^6 cells/ml, respectively, plated into 96-well plates (Costar; Corning, NY) containing RPMI with 10% FBS, 1 mmol/l sodium pyruvate, 10 mmol/l HEPES (GIBCO Life Technologies), and 58 μmol/l 2-mercaptoethanol (Sigma), and incubated for up to 144 hours. For the last 12 hours of each culture time point, 1 μCi/well of ^{3}H-thymidine was added. Plates were harvested at 12, 24, 48, 72, 96, 120, and 144 hours, and ^{3}H-thymidine incorporation (counts per minute, cpm) was measured on a scintillation counter (Wallac MicroBeta; Gaithersburg, MD).

Statistical Analyses

Values are the mean ± standard error (SE). Statistical significance was determined between the indicated values by Kruskal-Wallis tests and Wilcoxon signed rank tests.

Results

Cellular Expansion and Increase in Proportions of $CD34^+/CD38^-/HLA\text{-}DR^-$ and $CD34^+$ Progenitors in the Presence of MSCs

Monocyte-depleted MNCs from UCB were cultured for 12 days in either cytokines (IL-3, IL-6, FL, G-CSF, SCF, and EPO) [23], the same cytokines over a feeder layer of MSCs, or without additional cytokines on MSCs alone. While culture of UCB MNCs on MSCs without added cytokines resulted in the loss of cells during 12 days of culture (data not shown), a significant improvement in cellular output, on average a 4.6-fold increase, was observed for UCB expanded on MSCs in the presence of added cytokines. Nucleated cell numbers, however, remained significantly ($p = 0.005$, $n = 13$) below that obtained after expansion in the presence of cytokines alone, yielding a 10.5-fold increase in nucleated cells (Table 1). Thus, the presence of MSCs resulted in a lower overall nucleated cellular expansion by early-acting

Table 1. Improved expansion of CD34$^+$ cells in the presence of MSCs

	Nucleated cells	CD34$^+$/CD38$^-$/*HLA-DR*$^-$		CD 34$^+$CD38$^+$/*HLA-DR*$^+$	
	Fold expansion	**%**	**Fold expansion**	**%**	**Fold expansion**
Unexpanded	— —	1.01 (± 0.15)	— —	2.23 (± 0.40)	— —
Cytokines	10.5* (± 1.61)	2.09 (± 0.26)	4.33 (± 0.93)	3.29 (± 0.47)	2.76 (± 0.53)
Cytokines + MSCs	4.6 (± 0.67)	2.84* (± 0.29)	3.75 (± 0.99)	4.61* (± 0.40)	2.83 (± 0.88)

MNCs from UCB were cultured in cytokines alone or in cytokines on MSCs. On day 12, total viable cell counts were taken and expressed as fold expansion over culture input. Data are expressed as mean (± SE, $n = 13$). CD34$^+$ cells were expressed as percentages of lymphocyte gate or as fold expansion by multiplying with the cellular output. *$p < 0.05$.

cytokines by more than twofold, indicative of a growth-inhibitory effect exerted by the MSC feeder layer.

Four-color flow cytometric analyses of CD34$^+$ populations demonstrated that, when UCB was expanded in cytokines over MSCs, a higher proportion of cells was maintained within a lymphocyte gate drawn on unexpanded CD45$^+$ UCB cells (Fig. 1, left light scatter histograms) compared with UCB cells cultured in cytokines alone. Furthermore, proportions of early CD34$^+$ progenitors (CD38$^-$/*HLA-DR*$^-$), as well as total CD34$^+$ progenitors, were significantly higher after cytokine expansion on MSCs, compared with cytokines-alone cultures (Table 1). After 12 days of culture, early CD34$^+$ progenitors were, on average, 2.84% (± 0.29%) compared with 2.09% (± 0.26%, $p = 0.045$), and total CD34$^+$ progenitors were noted to be, on average, 4.61% (± 0.40%) compared with 3.29% (± 0.47%, $p = 0.015$) in MSC-based versus cytokine-alone cultures, respectively. When comparing total output of early CD34$^+$/CD38$^-$/*HLA-DR*$^-$ progenitors and total CD34$^+$ progenitors between the two expansion conditions on day 12, there was no statistically significant difference in fold increase in absolute numbers of progenitors, despite a twofold lesser cellular expansion in the presence of MSCs. Expansion on MSCs resulted in a 3.75-fold (± 0.99-fold) expansion in absolute numbers of CD34$^+$/CD38$^-$/*HLA-DR*$^-$ progenitors, compared with a 4.33-fold (± 0.93-fold) expansion in the presence of cytokines alone ($p = 0.37$). Absolute numbers of

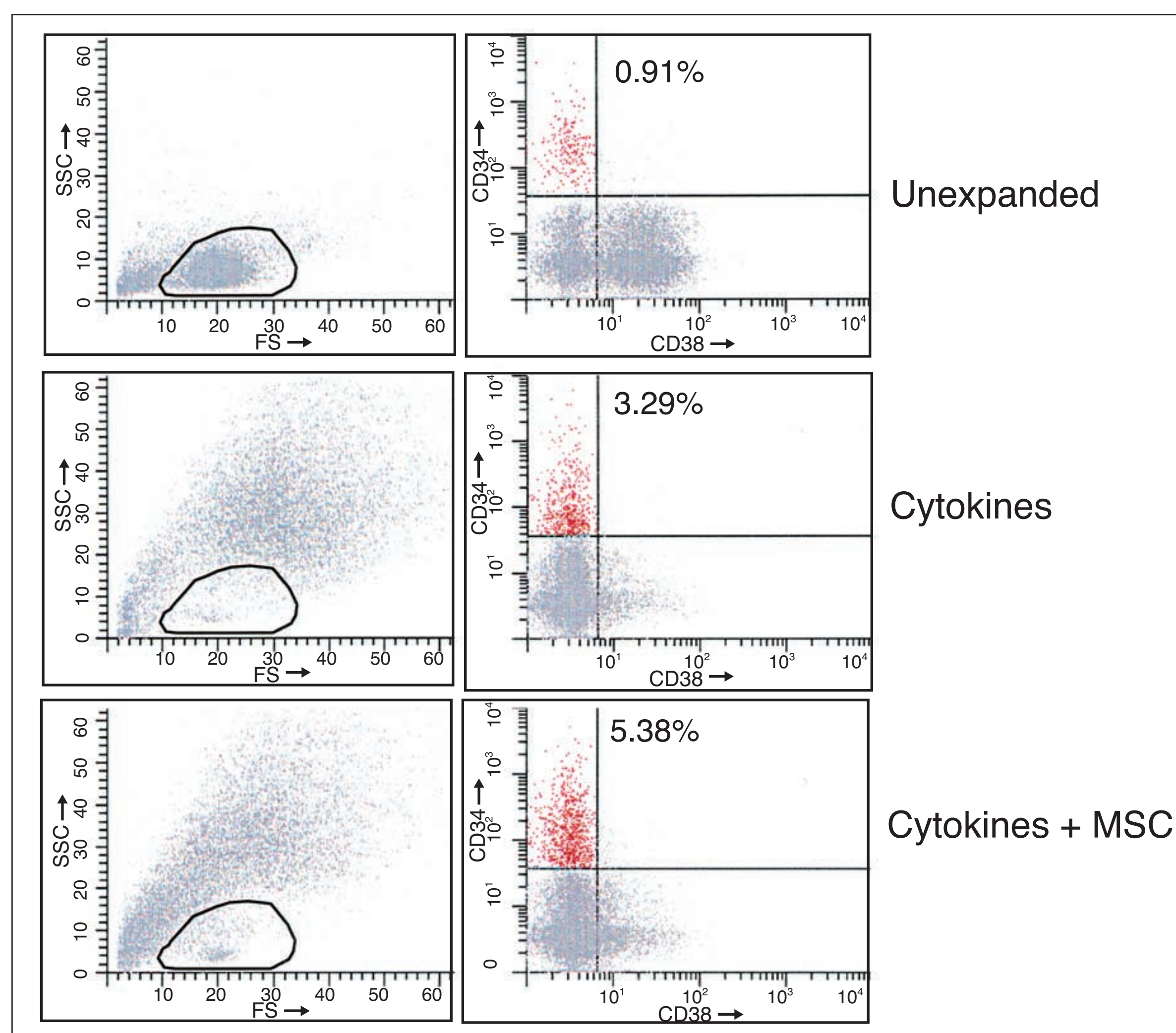
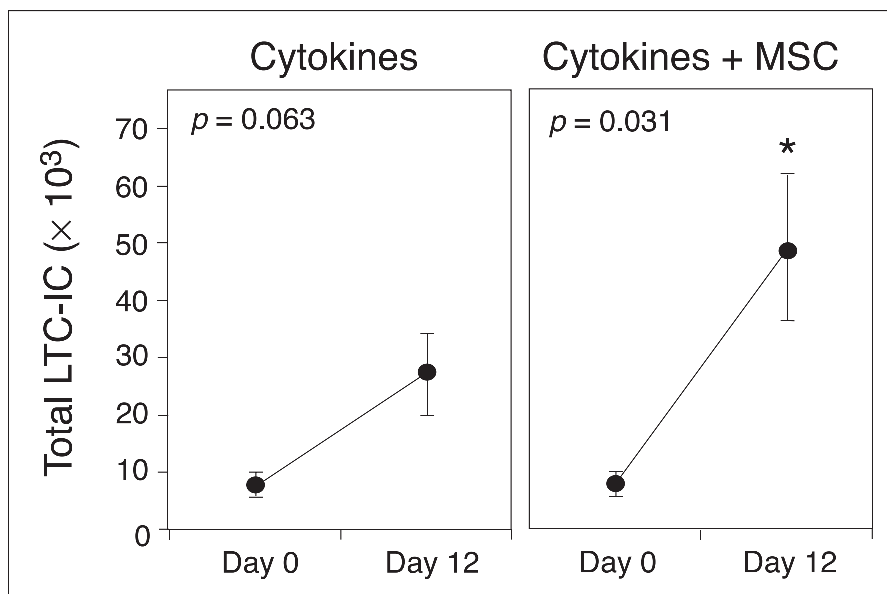


Figure 1. Analysis of CD34$^+$ populations after 12 days of expansion culture. *MNCs from UCB were cultured in cytokines or in cytokines on MSCs and analyzed at day 12 of expansion. A lymphocyte gate, based on CD45$^+$ cells in unexpanded UCB, was drawn on a light scatter histogram and maintained throughout analyses (left histograms). CD14$^+$ and* HLA-DR$^+$ *cells in the gate were excluded, and CD34-specific fluorescence was plotted against CD38-specific fluorescence to quantify percentages of early CD34$^+$ progenitor cells (CD34$^+$/CD38$^-$/*HLA-DR$^-$*) within the lymphocyte gate. Representative* (n = *13) histograms are shown. Left: light scatter showing the morphology of the expanded cells and the applied lymphocyte gate. Right: numbers are percentages of CD34$^+$/CD38$^-$/*HLA-DR$^-$ *cells within the lymphocyte gate (red events).*

*Figure 2. Increase in total LTC-ICs in presence of MSC feeder layer. Unexpanded (day 0) and expanded (day 12) cells were analyzed for LTC-IC frequency. LTC-IC frequency was multiplied by the cellular yield and expressed as total LTC-IC yield. Values are the mean ± SE (n = 6.). * = significant difference compared with unexpanded UCB.*



total CD34⁺ progenitors were also similar after expansion in both conditions, with a 2.83-fold (± 0.88-fold) expansion on MSCs compared with a 2.76-fold (± 0.53-fold) expansion on cytokines alone ($p = 0.63$) (Table 1). Thus, the presence of an MSC feeder layer during cytokine expansion resulted in a significantly greater CD34⁺ progenitor cell pool, to output levels comparable with cytokines-alone expanded cells.

Significant Increase in LTC-ICs in the Presence of MSCs

Despite an only 4.6-fold increase in cellular yield in the MSCs plus cytokines cultures, total numbers of LTC-ICs were significantly increased, rising from 8,025 (± 2,323) in unexpanded cord blood to 49,511 (± 13,018) after 12 days of culture, attaining on average a 7.7-fold (± 2.1-fold, $n = 6$) increase over unexpanded cells ($p = 0.031$) (Fig. 2). In cytokines alone, LTC-IC yields were lower, and were noted to expand from 7,019 (± 2,498) at the onset of culture to 27,285 (± 7,005) after culture, on average, a 5.7-fold (± 1.4-fold, $n = 6$) increase over unexpanded UCB. Comparison of LTC-IC expansion at day 12 in cytokines-alone conditions with day 0 cell input did not attain statistical significance ($p = 0.063$). CFU yield was similar between the two culture conditions, with an average of a 6.5-fold (± 2.4-fold) increase of total CFUs in cultures incorporating cytokines and MSCs, compared with a 10.9-fold (± 4.4-fold) increase in cytokines alone ($p = 0.57$, $n = 7$).

Early UCB Progenitors Maintain a More Immature Phenotype During Expansion on MSCs

Further studies were conducted to characterize potential mechanisms underlying improved LTC-IC output after expansion on MSCs. In light scatter, unexpanded early CD34⁺/CD38⁻/*HLA-DR*⁻ progenitors (red events) fell, on average, 93.4% (± 0.7%) within the left lower corner of the light scatter, displaying low granularity and size, indicative of relative quiescence ($n = 13$). In MSC-expanded cultures, early CD34⁺ progenitor cells showed preservation of lower size and granularity when compared with cells expanded in cytokines alone, with 68.6% (± 3.7%) compared with 57.0% (± 4.8%), respectively ($p = 0.022$), falling within the left lower corner of the light scatter (Fig. 3A).

Expressions of the cell-cycle inhibitor, p21$^{cip1/waf1}$, and the antiapoptotic protein, BCL-2, have been associated with HSC pool maintenance [26, 27]. After expansion in cytokines alone, p21$^{cip1/waf1}$ protein expression decreased in early CD34⁺ progenitors, while it was maintained in MSC-based expansion conditions (Fig. 3B and Table 2), resulting in higher expression levels of p21$^{cip1/waf1}$ protein in early CD34⁺ progenitors expanded in the presence of MSCs ($p = 0.012$, $n = 5$). Moreover, although there was a loss of BCL-2 protein expression intensity as well as percentages of expressing cells during culture in both expansion conditions, early CD34⁺ progenitors from MSC-expanded UCB consistently expressed higher levels of BCL-2 protein, compared with UCB expanded in cytokines alone (Fig. 3C and Table 3, $p = 0.02$, $n = 10$). Percentages of bright BCL-2-expressing cells, 81.2% (± 7.4%) in unexpanded UCB, were also significantly ($p = 0.011$) higher in the early CD34⁺ progenitor pool of MSC-expanded cells compared with the cytokines-alone expanded cells, with 41.3% (± 3.8%) versus 26.7% (± 3.1%), respectively. Thus, UCB early progenitors maintained a smaller size and lower granularity and expressed higher protein levels of p21$^{cip1/waf1}$ and BCL-2 when expanded on MSCs plus cytokines compared with cytokines alone.

Cellular Expansion in Presence of MSCs Does not Result in Acquisition of Antigen Reactivity

MSCs express HLA class I antigens and do not express HLA class II nor a full complement of costimulatory adhesion molecules under normal conditions [28]. MSCs could, thus, exert a tolerizing effect on UCB graft lymphocytes during

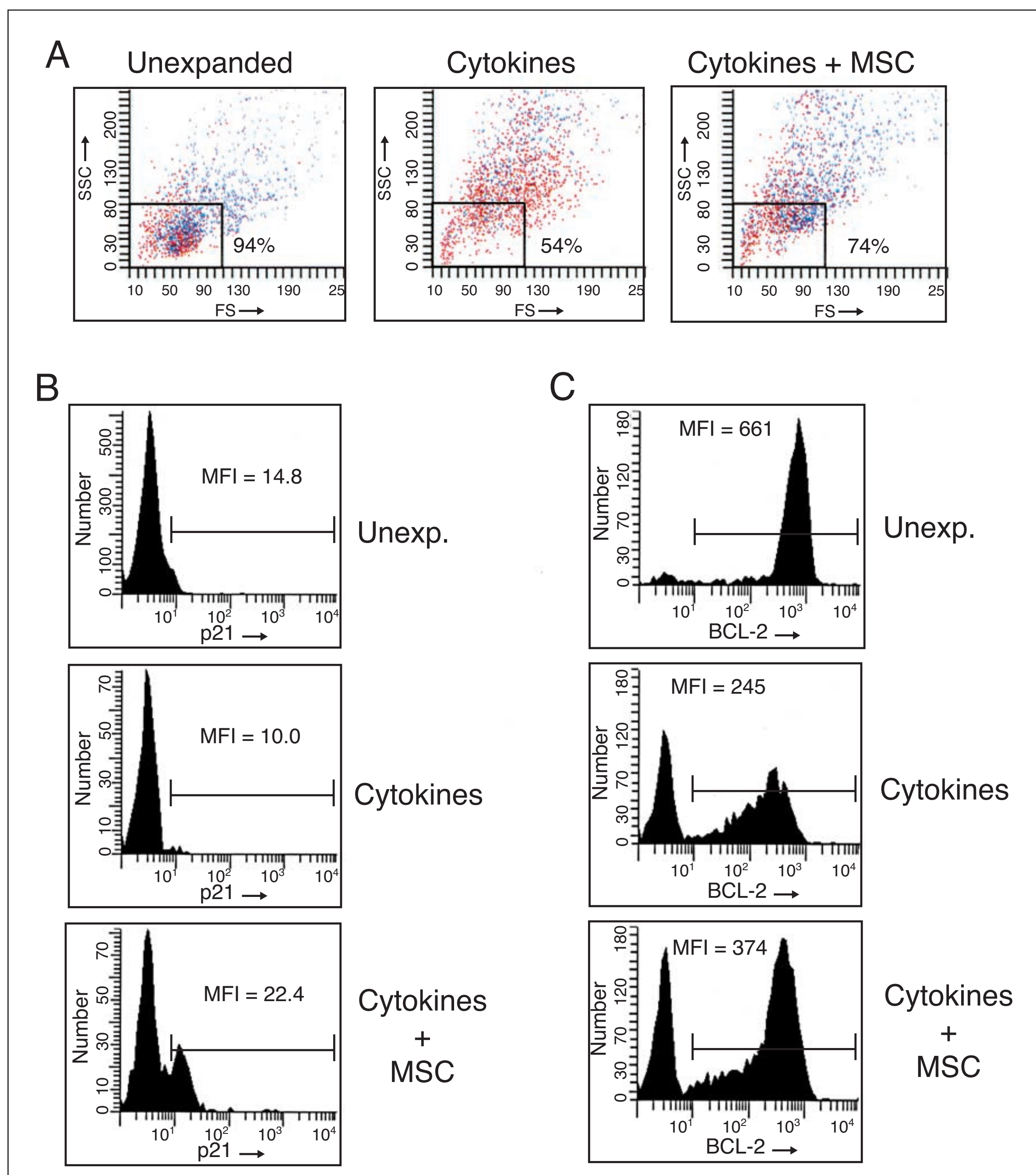


Figure 3. Characterization of early CD34⁺ progenitors after expansion on MSCs. *CD34⁺ cells were analyzed for size and granularity, and their expression levels of p21*$^{cip1/waf1}$ *and BCL-2 were assessed by flow cytometry. Representative histograms are shown. A) Only CD34⁺ cells (after exclusion of CD14⁺ cells) were plotted in light scatter and enhanced graphically by representing very early CD34⁺ progenitors (CD38⁻/*HLA-DR⁻*) as red events and late progenitors (CD34⁺/CD38⁺/*HLA-DR⁺*) as blue events. Numbers are percentages of CD34⁺/CD38⁻/*HLA-DR⁻ *cells falling within the left lower region (boxed). Shown are the results of one (of 13) representative experiment. B) Intracellular p21*$^{cip1/waf1}$ *staining was measured in gated early CD34⁺ progenitors (CD38⁻/*HLA-DR⁻*) and plotted as p21*$^{cip1/waf1}$*-specific fluorescence intensity. Numbers are mean fluorescence intensity (MFI),* n = *5. C) Intracellular BCL-2 staining was measured in gated early CD34⁺ progenitors (CD38⁻/*HLA-DR⁻*) as in (B). Numbers reflect MFI,* n = *10.*

expansion, possibly resulting in MSC-antigen-specific tolerance. This could allow expansion of UCB on MSCs derived from the prospective recipient and, thus, to tolerize the graft to its recipient, potentially reducing graft-versus-host disease after transplantation. Conversely, the presence of cytokines, such as FL, IL-3, and IL-6, could result in highly

Table 2. Expression of p21$^{cip1/waf1}$ protein in gated CD38$^-$/*HLA-DR*$^-$ early CD34$^+$ progenitors							
***n* =**	**1**	**2**	**3**	**4**	**5**	**Mean**	**SE**
Unexpanded	14.8	37.4	20.8	13.3	9.4	19.1	4.90
Cytokines	10.0	14.1	12.9	9.9	12.3	11.8	0.82
Cytokines + MSCs	22.4	28.7	14.9	22.8	14.1	20.6*	2.72

Numbers represent mean fluorescence intensity of intracellular staining. *$p = 0.012$, comparing the two expansion conditions.

Table 3. Expression of BCL-2 protein in gated CD38$^-$/*HLA-DR*$^-$ early CD34$^+$ progenitors												
***n* =**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**Mean**	**SE**
Unexpanded	273	221	481	661	280	334	261	420	ND	304	359.4	46.5
Cytokines	133	73	160	245	88	72	77	62	105	94	110.9	17.7
Cytokines + MSCs	183	87	206	375	125	109	254	116	178	175	180.8*	26.9

Numbers represent mean fluorescence intensity of intracellular staining. *$p = 0.02$ comparing the two expansion conditions. ND = not determined.

activated UCB graft lymphocytes, potentially resulting in severe graft-versus-host reactions after infusion of the expanded cells. To address these concerns, at outcome of expansion (day 12), cells from both culture conditions and unexpanded cells were washed thoroughly and set up in mixed lymphocyte reaction (MLR) cultures with stimulators including: irradiated MSC-donor PBLs or irradiated third-party PBLs (pooled from five adults) and media alone (autoproliferation). During the first 24-48 hours, higher autoproliferative rates (media alone) were observed in the cells from both culture types, which was noted to decrease over time (Fig. 4A). We did not, however, observe any distinct difference in the rate of UCB lymphocyte proliferation against MSC-donor lymphocytes, when compared with the proliferation against third-party PBLs (Fig. 4B and 4C). There was no MSC-donor-specific tolerization of the UCB lymphocytes expanded in the presence of MSCs nor was there an MSC-donor-specific memory response during the first 48 hours of MLR. Moreover, there was no distinct difference in proliferation rates when comparing cells expanded on MSCs with cells expanded in cytokines alone. Importantly, however, neither cytokine-expanded nor MSC-expanded UCB lymphocytes acquired reactivity against alloantigens, as there was no significant proliferation above autoproliferation (Fig. 4A and 4C). Rather, there was a loss

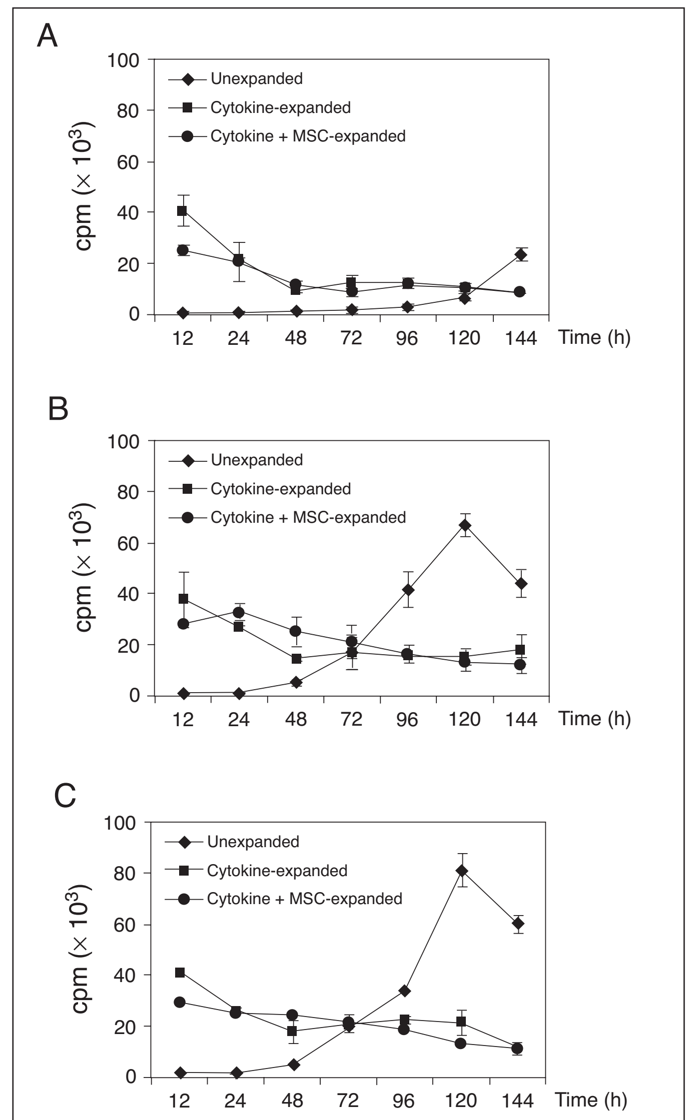


Figure 4. MSC-expanded UCB does not acquire antigen-specific reactivity. *After expansion in cytokines or in cytokines on MSCs, cells were washed and replated for culture for up to 144 hours without stimulation in medium alone (A), in the presence of irradiated PBLs from the MSC donor (B), or in the presence of irradiated PBLs from a pool of five adults (C) and proliferation was measured by ^{3}H-thymidine incorporation. Shown is one representative experiment of three similar ones.*

of reactivity in expanded cells when compared with unexpanded UCB lymphocytes from the same source (Fig. 4B and 4C). This reduced lymphocyte proliferation may be attributable in part to the loss of up to 90% of the UCB lymphocytes in both expansion conditions compared with unexpanded UCB (data not shown).

Discussion

Recent reports have shown that extrinsic signals, such as soluble factors and adhesion/matrix proteins provided by the stem cell microenvironment or stem cell "niche," play a crucial role in the asymmetric division of stem cells and maintenance of the stem cell pool [10-14, 29, 30]. Accordingly, several groups have reported expansion of hematopoietic progenitors in the presence of stromal elements and other types of feeder layers, with varying outcomes [31-33].

Human MSCs not only provide a rich environment of cytokines, extracellular matrix proteins, and adhesion molecules, but also, unlike stromal cells, produce LIF, a cytokine that has been shown to maintain embryonic stem cells in a self-replicative state [20, 21]. Moreover, it has been shown that hematopoietic progenitor cells cultured on MSCs form cobblestone areas reaching deep into the MSC layer, suggestive of establishment of close contact and a niche-like environment [16]. For these reasons, we studied human MSCs as a feeder layer during standard cytokine-based in vitro UCB expansion conditions, and showed here a clear benefit of MSCs, with increased $CD34^+$, CFU, and LTC-IC counts, above unexpanded cells, as well as greater expression of regulatory proteins critical to HSC survival and maintenance of self-replicative capacity, compared with cells expanded in cytokines alone.

Both $p21^{cip1/waf1}$ and BCL-2 have been shown to play a critical role in regulation of HSC differentiation [34]. $p21^{cip1/waf1}$, an inhibitor of cyclin-dependent kinases, mediates cell cycle arrest in G_1 [35]. $p21^{cip1/waf1}$-deficient mice revealed a dichotomy in function of $p21^{cip1/waf1}$ in the HSC/progenitor compartment. In HSCs, self-renewal is impaired, and there is greater proliferation under normal homeostatic conditions, resulting in exhaustion of the stem cell pool. These results suggest that $p21^{cip1/waf1}$ may be a molecular switch governing the entry of HSCs into the cell cycle, and that, in its absence, greater HSC cell cycling leads to stem cell exhaustion [26]. In hematopoietic progenitor cells, however, $p21^{cip1/waf1}$ appears to play a pro-proliferative role. Lower numbers of progenitors in $p21^{cip1/waf1}$-deficient mice, as assessed by colony formation assays [36], can be increased significantly by retroviral overexpression of $p21^{cip1/waf1}$ in bone marrow cells of $p21^{cip1/waf1}$-deficient mice [37]. Thus, preservation of $p21^{cip1/waf1}$ protein expression in the presence of MSCs suggests maintenance of early progenitor potential despite cytokine-driven cellular expansion, as underscored by the significant (7.7-fold) increase in LTC-ICs seen in this study. Conversely, our observation of loss of $p21^{cip1/waf1}$ expression during expansion with cytokines alone suggests that early $CD34^+$ progenitors obtained in those conditions may be differentiated, thereby potentially altering their engraftment potential. Accordingly, LTC-IC yield in cytokines-alone cultures was not significantly increased over unexpanded cells.

A direct role for the antiapoptotic protein BCL-2 in regulation of HSC fate has been suggested by results from BCL-2-overexpressing transgenic mice, which have greater numbers of HSCs in their bone marrow with less HSCs in the $S/G_2/M$ phase. Moreover, HSCs from *bcl-2* transgenic mice cycle more slowly, have greater plating capacity, and demonstrate an advantage following competitive reconstitution [27]. These findings suggest that overexpression of BCL-2 may maintain HSCs in a more immature stage. Thus, our observation of higher BCL-2 protein levels in early $CD34^+/CD38^-/$*HLA-DR*$^-$ progenitors in the presence of MSCs, when compared with cytokines-alone conditions, suggests that the MSC feeder layer may serve to inhibit differentiation and apoptosis of early $CD34^+$ progenitors, as corroborated by higher LTC-IC yields in the presence of MSCs.

Interestingly, the presence of MSCs during expansion with cytokines, including FL, IL-3, and IL-6, did not result in the activation of UCB lymphocytes nor the acquisition of memory against MSC-donor-specific antigens. Unfortunately, it did not result, either, in the acquisition of MSC-donor-antigen-specific tolerance. The latter would potentially allow specific tolerization of the graft to the prospective recipient by deriving MSCs from the recipient prior to transplantation, thereby potentially reducing graft lymphocyte reactivity to the patient. The observed general lack of reactivity against antigens in our studies may be attributable in part to the low percentage (5%-10%) of remaining T lymphocytes after 12 days of culture. The UCB stem cell product after expansion in the presence of MSCs would, therefore, not be expected to generate a strong graft-versus-host reaction in vivo and may, therefore, be suitable for clinical application.

These studies, designed to compare in vitro readouts of UCB cultured for 12 days in the presence of cytokines with or without MSCs as a feeder layer, support the hypothesis that MSCs may preserve early $CD34^+$ progenitor potential by reducing differentiation-induced cell-cycle progress and apoptosis, and may, thus, contribute to the preservation/expansion of this early progenitor cell pool.

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