Improved purification of hematopoietic stem cells based on their elevated aldehyde dehydrogenase activity

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ABSTRACT

Background and Objectives

Primitive human hematopoietic cells contain higher levels of aldehyde dehydrogenase (ALDH) activity than their terminally differentiating progeny but the particular stages at which ALDH levels change have not been well defined. The objective of this study was to compare ALDH levels among the earliest stages of hematopoietic cell differentiation and to determine whether these could be exploited to obtain improved purity of human cord blood cells with long-term lympho-myeloid repopulating activity *in vivo*.

Design and Methods

ALDEFLUOR-stained human cord blood cells displaying different levels of ALDH activity were first analyzed for co-expression of various surface markers. Subsets of these cells were then isolated by multi-parametric flow cytometry and assessed for short- and long-term repopulating activity in sublethally irradiated immunodeficient mice.

Results

Most short-term myeloid repopulating cells (STRC-M) and all long-term lympho-myeloid repopulating cells (LTRC-ML) stained selectively as ALDH$^+$. Limiting dilution analysis of the frequencies of both STRC-M and LTRC-ML showed that they were similarly and most highly enriched in the 10% top ALDH$^+$ cells. Removal of cells expressing CD2, CD3, CD7, CD14, CD16, CD24, CD36, CD38, CD56, CD66b, or glycophorin A from the ALDH$^+$ low-density fraction of human cord blood cells with low light side-scattering properties yielded a population containing LTRC-ML at a frequency of 1/360.

Interpretation and Conclusion

Elevated ALDH activity is a broadly inclusive property of primitive human cord blood cells that, in combination with other markers, allows easy isolation of the stem cell fraction at unprecedented purities.

Key words: hematopoietic stem and progenitor cells, aldehyde dehydrogenase, NOD/SCID mice.

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Hematopoiesis is sustained throughout adult life by balanced self-renewal and differentiation divisions of multipotent cells. The restriction of these cells to specific lineages takes place in an orderly sequence that spans several cell generations, thus creating hierarchies of daughter cells with distinct properties. Hematopoietic stem cells (HSC) represent the cells that head up this hierarchy and are thus able to regenerate and sustain the long term production of all blood cell types when transplanted at low numbers into suitable hosts. Quantification of HSC is achieved by coupling the end-points of donor-derived lymphoid and myeloid cell output to long term limiting dilution transplantation assays.[1] In mice, phenotypically distinct cells with different repopulating activities *in vivo* have been described.[2-7] Some short-term repopulating cells (STRC) lack the extensive self-renewal ability of HSC but retain a broad lympho-myeloid differentiation repertoire. Others also show partial restriction to either the lymphoid[8] or myeloid lineages.[9]

Several decades of clinical experience with allogeneic transplants have established the existence in humans of HSC with functional properties similar to those defined for murine HSC. Evidence of human STRC has also been obtained from transplantation experiments using highly immunodeficient mouse strains such as non-obese diabetic-*scid/scid* (NOD/SCID) mice that have been manipulated either genetically or immunologically to further reduce their residual natural killer (NK) cell activity, thereby allowing superior human cell engraftment.[10-14] Three phenotypically separable types of human STRC have been identified. One has a $CD34^+CD38^+$ phenotype and produces exclusively myeloid progeny rapidly post-transplant but only for 3 to 5 weeks. This cell type has therefore been referred to as a myeloid-restricted STRC (STRC-M). The second is $CD34^+CD38^-$ and produces large numbers of B-lymphoid as well as myeloid progeny after an initial 3 to 4 week delay, but only for the first 2-4 months post-transplant. Accordingly, this second type of human STRC has been called a STRC-ML. Both types of human STRC engraft non-obese diabetic-*scid/scid-β2microglobulin*$^{-/-}$ (NOD/SCID-$\beta2m^{-/-}$) mice or NOD/SCID mice treated with anti-NK-cell antibodies much more efficiently than NOD/SCID mice. This property distinguishes both types of primitive human cells from the $CD34^+CD38^-$ HSC that produce both lymphoid and myeloid cells for periods in excess of 5 months with an efficiency that is unaffected by further reduction of the NK activity of the NOD/SCID hosts. These latter cells also durably engraft sheep transplanted *in utero*.[12-15] More recently, evidence of a lymphoid-restricted human cell with short-term repopulating activity (STRC-L) has also been obtained.[16,17]

Common strategies to obtain populations enriched for primitive hematopoietic cells include first removing the more prevalent and mature cells that express lineage (lin)-specific markers followed by the positive selection of cells expressing markers more specific to primitive cells. The latter include expression of the *MDR-1/ABCB1*-encoded P-glycoprotein that enables cells to efflux Rhodamine-123[18-20] and the *BCRP1/ABCG2*-encoded transporter that enables cells to efflux Hoechst 33342.[3,21-23]

Primitive human hematopoietic cells are known to be resistant to alkylating agents because of a high aldehyde dehydrogenase (ALDH) activity.[23] These cells thus become selectively fluorescent when exposed to BODIPY-Fl1-labeled amino-acetaldehyde (BAAA). BAAA diffuses freely into cells and is then converted by ALDH into BODIPY-Fl1-aminoacetate (BAA) which is retained intracellularly unless effluxed by certain ABC transporters, including those expressed in primitive hematopoietic cells. Thus the detection of BAAA-stained primitive hematopoietic cells by their ALDH-dependent, acquired fluorescence necessitates that these cells also be incubated in the presence of an appropriate transporter inhibitor.[24-26] This approach has been exploited to demonstrate that isolation of the $ALDH^+$ fraction of low-density human hematopoietic cells from several sources yields a population that is enriched in STRC and HSC.[24-27] Here we present the results of studies designed to analyze the extent to which different phenotypically and functionally defined subsets of primitive human cord blood cells differ in their range of ALDH activities. The studies show that BAAA-staining can be used in conjunction with known markers of HSC to isolate HSC at very high purities.

Design and Methods

Cells

Cord blood cells obtained from consenting mothers undergoing Cesarean delivery of normal, full-term infants were anonymized, pooled and the low-density (<1.077 g/mL) cells isolated. In some cases, the cells were then cryopreserved until required.

Cell staining and flow cytometry

Suspensions enriched in $CD34^+$ cells were obtained either by immunomagnetic removal of cells expressing CD2, CD3, CD14, CD16, CD19, CD24, CD56, CD66b and glycophorin A on a column, or by positive selection of iron-labeled $CD34^+$ cells using a magnet as described by the supplier (StemCell Technologies Inc., Vancouver, BC, Canada). Low-density, lin^- or $CD34^+$-selected cells were stained with BAAA (ALDEFLUOR, Aldagen Inc., Durham, NC, USA) by adding 5 μL of BAAA to each mL of cells suspended at 10^6 cells/mL in ice-cold ALDEFLUOR buffer (Aldagen) with removal of a small aliquot (0.1-0.5 mL) to which 10 μL/mL of diethylaminobenzaldehyde (DEAB, Aldagen) was also added. Both the test and the control (+DEAB) cells were incubated for 30 minutes at 37°C, then cooled to 4°C, centrifuged and resuspended in ice-cold ALDEFLUOR buffer. For flow cytometry and cell sorting, cells were first incubated with 10% human serum and an anti-mouse FcR antibody (2.4G2, American Type Culture Collection, Rockville, MD, USA) for 10 minutes at 4°C and then for 30 minutes at 4°C with antibodies against one or

more of the following human antigens: CD133 (Miltenyi Biotech, Bergisch Gladbach, Germany), human Flt-3 (Immunotech, Marseille, France), CD7 (Caltag, Burlingame, CA, USA), CD15 (Immunotech), c-mpl, c-kit, CD19, CD20, CD36, CD38, CD45, CD66b (from Becton Dickinson, San José, CA, USA), and CD34 (8G12), CD71 (OKT9) and glycophorin A (10F7MN) (*from P. Lansdorp, Terry Fox Laboratory, Vancouver, BC, Canada*). The cells were then washed twice in ALDEFLUOR buffer with 0.1% propidium iodide (PI, Sigma Chemicals, St. Louis, MO, USA) added to the final wash. Analyses were performed on a FACSCalibur and cells were isolated using either a FACSVantage or a FACSAria (Becton Dickinson) with gates chosen to exclude at least 99.95% of cells incubated with isotype control antibodies labeled with the same fluorochromes.

Progenitor assays

Assays of colony-forming cells (CFC) and 6-week long-term culture-initiating cells (LTC-IC) using mouse feeders engineered to produce human interleukin-3 (IL-3), granulocyte colony-stimulating factor (G-CSF) and Steel factor (SF) were performed as previously described.[28] To calculate LTC-IC numbers in the cells harvested from bulk cultures, a 6-week output of 28 CFC per LTC-IC was assumed.[28]

Assays of in vivo repopulating cell activity

All animal studies were performed in accordance with institutional guidelines. Test cells were injected together with 10^6 irradiated (15 Gy) human bone marrow cells into 6-12 week-old NOD/SCID or NOD/SCID-$\beta_2m^{-/-}$ mice within 24 hours after administration of 350 cGy ^{137}Cs γ-rays. From 3 to 20 weeks later, bone marrow cell aspirates were obtained.[29] After lysis of the red cells with ammonium chloride, the leukocytes were stained with the same antibodies described above and 20,000 cells analyzed by flow cytometry for each assessment made. Mice were considered to be repopulated with human myeloid cells if in 20,000 propidium iodide-negative (PI^-) bone marrow cells analyzed, five or more cells were positive for human glycophorin A and/or human CD41 and/or human CD45 plus human CD15 and/or human CD66b. Mice were considered to be repopulated with human lymphoid cells if in 20,000 PI^- bone marrow cells analyzed, five or more cells were negative for human CD34 and positive for human CD19 and/or human CD20. STRC-M were defined as the cells that produced exclusively myeloid cells in NOD/SCID-$\beta_2m^{-/-}$ mice for 3 weeks. STRC-ML were defined as the cells that produced both myeloid and B-lymphoid cells in NOD/SCID-$\beta_2m^{-/-}$ mice for 6-12 weeks.[12,30] HSC were defined as the cells that produced both lymphoid and myeloid cells in either NOD/SCID or NOD/SCID-$\beta_2m^{-/-}$ mice for at least 20 weeks, hence also termed LTRC-ML. Mice that did not meet these repopulation end-points at 3, 6-12 and ≥20 weeks post-transplant were considered negative, respectively, for (i.e., had not received any) STRC-M, STRC-ML and LTRC-ML. The frequency of each of these cells in the subset of cord blood cells transplanted was then calculated from the corresponding proportions of mice that were negative at the relevant time post-transplant using Poisson statistics and the method of maximum likelihood with L-calc software (StemCell).

Results

Phenotypic heterogeneity of low-density ALDH$^+$ human cord blood cells

In this study, we defined $ALDH^+$ cells as BAAA-stained cells whose fluorescence was greater than that exhibited by 99.95% of the cells simultaneously exposed to DEAB, a specific ALDH inhibitor (compare Figure 1A and B, and 1C and D). This strategy consistently identified a small (~1%) but distinct population of $ALDH^+$ cells within the low-density fraction of human cord blood cells with low light side-scattering (SSC^{lo}) properties (a well-established feature of primitive hematopoietic cells).[31] Prior removal of the lin^+ cells gave a marked enrichment of the $ALDH^+$ population (16±2% of the total low-density lin^- fraction, Figure 1C), as expected.[24,26] Notably, this was true even when we excluded the weakly fluorescent cells indicated by the dotted box in Figure 1C, which are typically included when the DEAB control is not applied.[26,27]

Co-staining experiments revealed that >80% of $ALDH^+$ cord blood cells are $CD34^+$ and approximately a third have a $CD133^+$ or a $CD34^+CD38^-$ phenotype. In addition, the modal ALDH activity of the $CD34^+CD38^-$ subset was approximately 2-fold higher than that of the $CD34^+CD38^+$ population (Figure 1E). The frequency of $ALDH^+$ cells that expressed Flt3, c-kit or c-mpl (receptors for growth factors reported to activate human HSC) was also increased 4- to 30-fold above the corresponding frequencies in the starting low-density cells (Table 1). The reverse analysis showed that 71% of the low-density $CD34^+$ cord blood cells and approximately one third of the $CD133^+$ or $CD34^+CD38^-$ cells were not $ALDH^+$ (Table 2). These findings confirm that ALDH activity is present in cord blood cells that display other features of primitive cells but does not correlate perfectly with any known primitive phenotype.

Functional heterogeneity of low-density ALDH$^+$ cord blood cells

In vitro assays of the growth and proliferative potential of the $ALDH^+$ and $ALDH^-$ subsets of low-density cord blood cells showed that a substantial proportion of LTC-IC were not captured in the $ALDH^+$ subset even though approximately 40% of the CFC were $ALDH^+$ (Table 2). To determine whether different types of repopulating cells might also vary in their ALDH activity, we isolated the $ALDH^+$ and $ALDH^-$ subsets of low-density SSC^{lo} cord blood cells and then transplanted varying numbers of these cells into NOD/SCID and NOD/SCID-$\beta_2m^{-/-}$ mice. In all three experiments, the $ALDH^+$ cells contained most of the short and long-term repopulating activity (see Figure 2 for a represen-

Table 1. Phenotype analysis of human cord blood cells with different ALDH activities.

Phenotype	*Viable (PI⁻)*	*CD34⁺*	*CD34⁺CD38⁺*	*CD34⁺CD38⁻*	*CD133⁺*	*Flt-3⁺*	*c-mpl⁺*	*c-kit⁺*
LD ALDH⁺	99±1	86±7	57±8	35±8	30±13	1±0.9	7±3	3±0.4
LD ALDH⁻	65±7	7±2	7±3	0.3±0.1	0.2±.1	0.01±0.01	1±0.3	0.4±0.1
Total LD	65±7	7±2	7±3	0.6±.2	0.6±.2	0.03±.01	1.6±0.1	0.4±0.1
LD lin⁻ ALDH⁺	96±2	87±10	57±17	18±1	23±17	ND	ND	ND
LD lin⁻ ALDH⁻	58±7	41±19	22±17	3±2	8±3	ND	ND	ND
Total LD lin⁻	63±6	50±7	30±14	8±2	15±3	ND	ND	ND

The low-density (LD) and/or low-density lin⁻ cells from 14 different pooled samples of cord blood cells were stained with BAAA and monoclonal antibodies against a variety of surface markers. The proportion of cells that were positive for these markers within the subsets shown in the left-hand column was determined by flow cytometry. Values shown are the mean ± SEM. ND: not done.

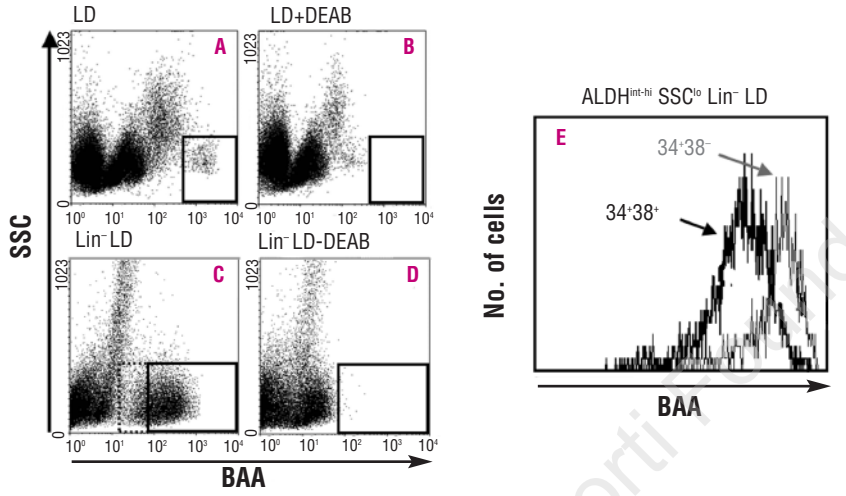

Figure 1. Representative FACS profiles of different subsets of BAAA-stained cord blood cells. (A) Low-density (LD) cells stained with BAAA. (B) Low-density cells stained with BAAA in the presence of DEAB. (C) Low-density lin⁻ cells stained with BAAA. (D) Low-density lin⁻ cells stained with BAAA in the presence of DEAB. The boxes indicated in panels (A) and (C) indicate gates used to identify SSC^lo ALDH⁺ cells based on the exclusion of >99.9% of all SSC^hi cells and >99.9% of the most fluorescent BAAA-stained SSC^lo cells detected in the presence of DEAB as shown in panels (B) and (D). (E) Frequency histograms of CD38⁺ (black line) and CD38⁻ cells (gray line) of increasing BAA fluorescence within the CD34⁺ subset of low-density lin⁻ SSC^lo ALDH⁺ cells isolated from the same low-density cord blood sample shown in panels (C) and (D).

tative experiment). However, ALDH⁻ cells also transiently repopulated some of the recipients particularly when these were NOD/SCID-β2m$^{-/-}$ mice. In this case, the cells produced were exclusively lymphoid and no longer detectable after 12 weeks, thus failing to meet the criteria for inferring their origin from STRC-M, STRC-ML or LTRC-ML and suggesting that STRC-L in 4 independent experiments with different numbers of ALDH⁺ low-density SSC^lo lin⁻ cells isolated from a single large pool of cryopreserved cord blood cells. Thirty-six of the 37 mice transplanted were still repopulated with human cells 20 weeks later and 29 of these mice contained human myeloid as well as human lymphoid cells (Figure 2 top and bottom panel, Figure 3). These results indicate a frequency of 1 LTRC-ML per 4,700 low-density SSC^lo lin⁻ cord blood cells (range defined by ±1 SEM=1/3,100-1/7,400; Figure 3 and Supplementary Table 1). This value is ~4-fold higher than what we previously reported for LTRC-MLs in the low-density SSC^lo lin⁻ CD34⁺ cord blood cells (1 per 16,000 cells)[30] and is consistent with the finding that essentially all human cord blood LTRC-ML are CD34⁺ CD38⁻ and that only 25% of the CD34⁺ cells cord blood cells are ALDH⁺ (Table 2).

To determine whether STRC-M, STRC-ML and LTRC-ML have different ALDH activities, we subdivided the ALDH⁺ population of low-density SSC^lo lin⁻ cord blood cells into the 10% most fluorescent BAA⁺ (ALDH^hi) cells and the remaining 90% (less fluorescent, ALDH^lo) cells. These 2 fractions were then transplanted into NOD/SCID-β2m$^{-/-}$ mice. After 3 weeks, equivalent proportions of the two fractions showed equal (predominantly erythroid/granulopoietic) repopulating activity. This suggested the presence of equal numbers of STRC-M in the 2 ALDH⁺ subsets (Figure 4). This finding was supported by limiting dilution transplant experiments, which showed the frequency of STRC-M to be approximately 8-fold higher in the 10-fold smaller ALDH^hi subset than in the ALDH^lo cells (Table 3).

By 6 weeks post-transplant, a combination of human B-lymphoid and granulopoietic cells had replaced the initial, exclusively myeloid human population in most of the same mice (Figure 4), as expected for transplants that also contained STRC-ML. This pattern was retained out to 20 weeks post-transplant when the progeny of LTRC-ML become dominant. Calculation of STRC-ML and LTRC-ML frequencies in each fraction showed that both were enriched to approximately the same extent as the STRC-M in the ALDH^hi fraction (8- and 6-fold, respectively, Table 3). These results indicate that the STRC-M, STRC-ML and LTRC-ML were equally partitioned by the gates used to separate the top ALDH⁺ (ALDH^hi) subset from the rest of the ALDH⁺ fraction (here called ALDH^lo cells). The ALDH activity of human cord blood cells with both short-term

Table 2. Distribution of particular subsets of cord blood cells between the ALDH⁺ and ALDH⁻ populations.

Subset	*ALDH⁺*	*ALDH⁻*
CD34⁺	24±7	71±7
CD38⁺	0.6±0.1	98±0.4
CD34⁺CD38⁻	62±8	36±7
CD34⁺CD38⁻CD7⁺	24±7	75±7
CD34⁺CD38⁺	25±10	72±10
CD133⁺	63±10	33±10
CFC	41	59
LTC-IC	68	32

Results were obtained on the same 14 pooled samples of low-density cord blood cells analyzed in Table 1, except for the CFC and LTC-IC data which are from a single pool. Using the same staining procedure as for the initial phenotype analysis, cells with different surface marker profiles were backgated for BAA fluorescence intensity. Data are expressed as percent of all low-density cells of the subset measured (mean ± SEM).

and sustained myeloid repopulating potential thus appears quite variable, whether or not lymphopoietic potential is still present.

Purification of HSC within the ALDH⁺ fraction of cord blood cells

The fact that the ALDH⁺ fraction of cord blood cells appears to contain all of the LTRC-ML, as previously reported for the CD34⁺CD38⁻ and CD133⁺ subsets, but do not represent highly overlapping populations with these latter two phenotypes predicted that isolation of the ALDH⁺ subset of either could increase the purity of HSC. To test this possibility, low-density SSC^{lo} lin⁻ CD38⁻ ALDH⁺ cord blood cells were first subdivided into a CD133⁺ and a CD133⁻ fraction. Proportionately matched aliquots of these two fractions were then transplanted into NOD/SCID-$\beta_2m^{-/-}$ mice. As shown in Figure 5, the output of human cells from 3 to 20 weeks later was largely attributable to the CD133⁺ fraction. However, starting at 6 weeks, the similarly prevalent CD133⁻ fraction also produced both lymphoid and myeloid progeny, albeit in much smaller numbers. These findings were confirmed in simultaneous experiments using NOD/SCID hosts, in which the frequencies of LTRC in the CD133⁺ and CD133⁻ subsets of low-density SSC^{lo} lin⁻ CD38⁻ ALDH⁺ cord blood cells were determined to be, respectively: 1 per 1,100 cells (±SEM= 1/650-1/1,800) and 1 per 1,700 cells (±SEM=1/1,000-1/3,100, Supplementary Table 2), as obtained for low-density lin⁻CD34⁺CD38⁻ human cord blood cells.[33,34] Given the approximately equal distribution of cells in the CD133⁺ and CD133⁻ subsets tested here, we can estimate that approximately half of all LTRC-ML may not be CD133⁺, in contrast to data reported by others.[27,35]

We next asked whether increased purity of LTRC-ML could be obtained by isolating the ALDH⁺ subset of low-density SSC^{lo} lin⁻ CD34⁺CD38⁻ cord blood cells. Preliminary experiments showed that the additional removal of CD7⁺ and CD36⁺ cells markedly reduced the size of the ALDH⁺ population (by >10^3-fold relative to the low-density cells and >100-fold relative to the lin⁻ subset of

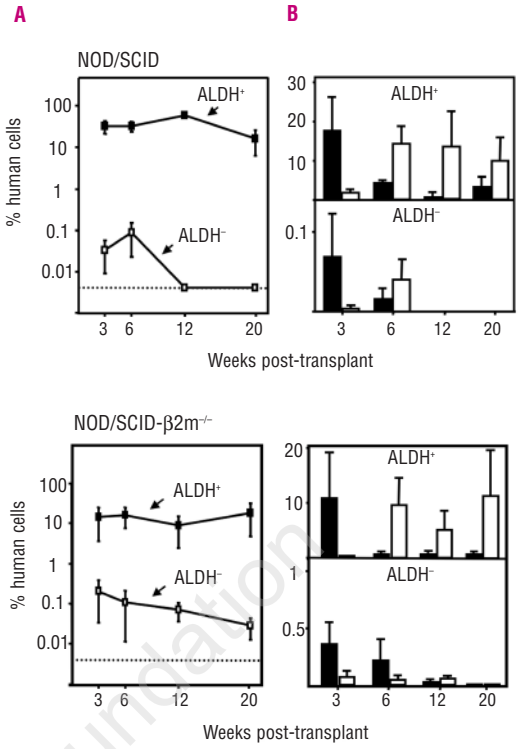

Figure 2. Comparison of the human cell repopulation kinetics of immunodeficient mice transplanted with ALDH⁺ and ALDH⁻ cord blood cells. NOD/SCID mice (top panel) and NOD/SCID-$\beta_2m^{-/-}$ mice (bottom panel) were transplanted with either 2.2×10⁴ ALDH⁺ low-density SSC^{lo} cord blood cells or the corresponding number (1.4×10⁶) of ALDH⁻ low-density SSC^{lo} cells. The marrow of each mouse transplanted was then assessed for the presence of different types of human cells at the times shown. A. Total human hematopoietic (CD45/71⁺) cells. B. Percentages of human cells that were myeloid (solid bars) or B-lymphoid (open bars). Values shown are the mean ± SEM of data from four to six individually analyzed mice in a representative experiment (from a total of three). The dotted line in (A) indicates the threshold of detection of human cells in this experiment as determined from the number of cells per mouse and the number of mice per group analyzed.

Table 3. Limiting dilution analysis of the frequency of STRC-M, STRC-ML and LTRC-ML in the ALDH^{hi} and ALDH^{lo} subsets of low-density lin⁻ human cord blood cells.

Test cells	*No. of cells per mouse*	*Positive mice/total after 3 wks.*	*Frequency of STRC-M (±SEM)*	*Positive mice/total after 6 wks*	*Frequency of STRC-ML (±SEM)*	*Positive mice/ total after 20 wks*	*Frequency of LTRC-ML (± SEM)*
ALDH^{hi}	2,400	6/7	1/1,200 (1/800–1/2,000)	5/7	1/1,900 (1/1,200–1/3,100)	3/4	1/1,700 (1/900–1/3,200)
ALDH^{lo}	20,000	6/7	1/10,000 (1/6,400–1/17,000)	5/7	1/16,000 (1/9,900–1/25,700)	5/6	1/11,000 (1/6,700–1/18,600)

NOD/SCID-$\beta_2m^{-/-}$ mice were injected with the ALDH^{hi} and the ALDH^{lo} fractions of the ALDH⁺ population of low-density lin⁻ human cord blood cells and 3, 6 and 20 weeks later the marrow of the mice was assessed for the presence of human B-lymphoid and human myeloid cells. The frequencies of STRC-M, STRC-ML and LTRC-ML were then determined as described in the Methods. For STRC-M, mice were considered positive only if the only human cells present were myeloid. For STRC-ML and LTRC-ML mice were considered positive only if both human myeloid and human B-lymphoid cells were present.

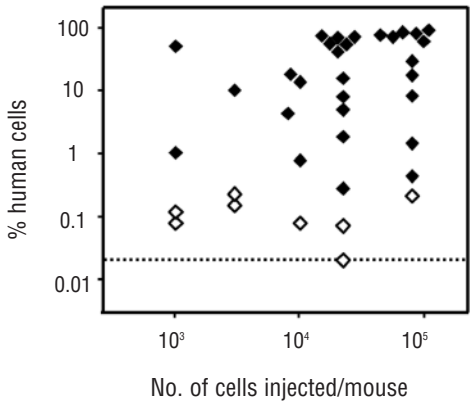

Figure 3. Efficient long-term repopulation of NOD/SCID mice with ALDH$^+$ human cord blood cells. ALDH$^+$ cells were isolated from human low-density lin$^-$ SSC^{lo} cord blood cells and different numbers were then injected into sublethally irradiated NOD/SCID mice. The figure shows the total level of repopulation of the marrow of each transplanted mouse with human hematopoietic cells (CD45$^+$ and/or CD71$^+$) 20 weeks later. Solid symbols indicate those mice that contained human myeloid cells as well as human B-lymphoid cells; open symbols indicate mice that contained only human B-lymphoid cells. No mice contained only human myeloid cells. The dashed line indicates the threshold of human cell detection, which was calculated based on the number of cells per mouse and the number of mice per group analyzed.

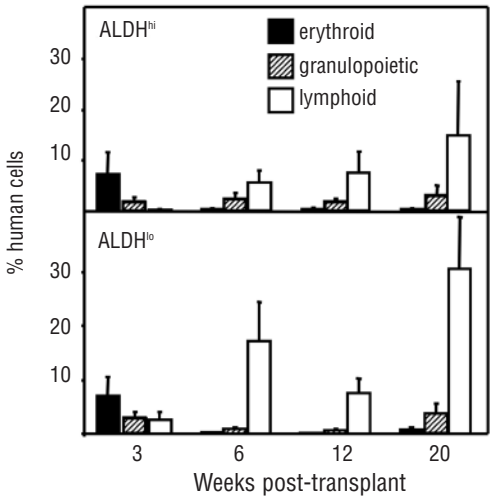

Figure 4. STRC-M, STRC-ML and HSC are not discriminated by different ALDH activities. Mice were transplanted with either the 10% most fluorescent (ALDH^{high}) or the rest of the ALDH$^+$ (ALDH^{hi}) cells isolated from a large pool of low-density lin$^-$ SSC^{lo} cord blood cells. Three, 6, 12 and 20 weeks later, bone marrow cells from the mice were analyzed for the presence of human erythroid (solid bars), granulopoietic (hatched bars) and B-lymphoid cells (open bars). Values shown are the percentages of cells in the marrow that were represented by these different human cell types (mean ± SEM of data from 16 mice). The total numbers of human (CD45/71$^+$) cells in the recipients of the ALDH^{hi} and ALDH^{lo} cells were comparable at all time points (11% vs 18% at week 3, 16% vs 19% at week 6, 15% and 14% at week 12, and 27% vs 44% at week 20).

these). Since both CD7 and CD36 are absent on LTRC-ML,[36,37] antibodies to these two antigens were added to the lin$^-$ cocktail. Varying numbers of low-density SSC^{lo} lin$^-$ CD7$^-$ CD36$^-$ CD38$^-$ ALDH$^+$ cord blood cells were then transplanted into NOD/SCID mice and 20 weeks later, the numbers of mice containing both human lymphoid and human myeloid cells was assessed. The derived frequency of LTRC-ML in this rare subset was found to be 1/360 cells (±SEM=1/270–1/490, Supplementary Table 3). This value is significantly higher ($p<0.05$) than that obtained for either the entire lin$^-$ ALDH$^+$ population (1/4,700, Supplementary Table 1) or the CD133$^+$ or CD38$^-$ subsets of the ALDH$^+$ cells (1/1,100 and 1/1,700, Supplementary Table 2).

Discussion

The data presented here indicate that different subsets of human cord blood cells with *in vitro* or *in vivo* myeloid differentiation potential display broad and largely overlapping ranges of ALDH activity. However, this property is not completely redundant with known surface marker phenotypes. Consequently, as shown here, the purity of the most primitive cells can be increased several-fold when appropriate combinations of these features is exploited. Recently, much attention has been focused on understanding the cellular basis of the heterogeneous patterns of repopulation that are obtained when different types of immunodeficient mice are transplanted with similar sources of human hematopoietic cells.[11,12,14,38,39] In mice, there is now strong evidence of pre-existing biological heterogeneity in the types of hematopoietic cells that can engraft the marrow of lethally irradiated hosts and produce detectable numbers of mature blood cells over defined periods of time.[5-9,40-42] Accumulating evidence suggests that the pattern of human hematopoiesis regenerated in immunodeficient mice transplanted with unfractionated cells also reflects the activity of a spectrum of biologically distinct cells that vary in their relative prevalence in different human hematopoietic tissues as well as in their sensitivity to residual NK activity in the recipient.[12,14,43,44]

ALDH activity has been associated with primitive hematopoietic cells for several decades[45,46] and our development of a reagent that allows ALDH activity to be detected in viable cells[24,47] is now facilitating studies of this property in primitive subsets of human hematopoietic cells. Use of this reagent has confirmed that CFC have less ALDH activity than LTC-IC,[24] also shown here. Similarly, it has been demonstrated that isolation of ALDH$^+$ cells enriches for all types of repopulating cells[16] and can be a useful predictive[25] as well as preparative procedure.[27] In the present study, we provide the first analysis of the distribution of different types of human repopulating cells within the broad range of cells with objectively quantified ALDH activity. In addition, we have exploited ALDH activity of human HSC to isolate these cells at very high purity (0.3%). This value appears slightly lower than the frequency of 1 per 30 Rhodamine-123 dull CD34$^+$CD38$^-$ cells recently reported,[48] but the criteria used to define the repopulating cells in the latter study would have also included the more prevalent STRC-ML.[12,49] Neutrophil recoveries after clinical autologous stem cell transplantation have been shown to correlate with the number of

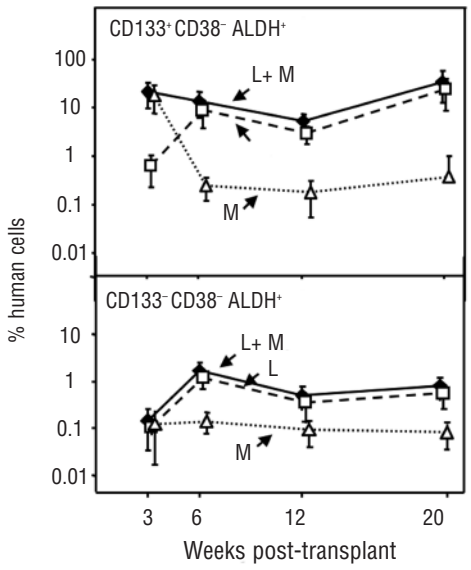

Figure 5. Both CD133⁺ and CD133⁻ subsets of CD38⁻ ALDH⁺ cord blood cells contain HSC. NOD/SCID mice were injected with equivalent numbers of CD133⁺ (1,950 cells/mouse, top panel) or CD133⁻ cells (1,580 cells/mouse, lower panel) within the CD38⁻ ALDH⁺ population of low-density cord blood cells and the presence of human B-lymphoid cells (open squares, dashed lines), human myeloid cells (open triangles, dotted lines) and total human hematopoietic cells (solid diamonds, solid lines) in sequential marrow samples was determined. Values shown are the mean ± SEM of data pooled from two experiments in each of which there were five to eight mice per group.

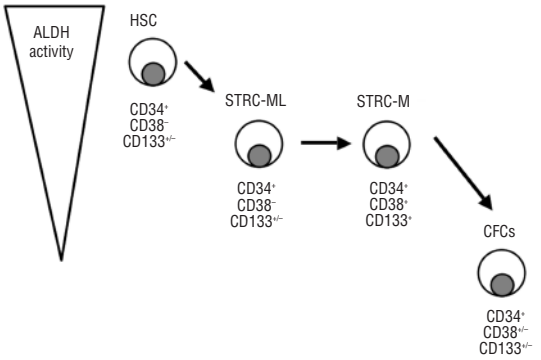

Figure 6. Proposed model of changes in ALDH activity and hematopoietic differentiation. All LTRC-ML (HSC) appear to be ALDH⁺ cells, STRCs include some ALDH⁻ cells.

ALDH⁺ cells present in the original graft.[25] The present results, showing that human cord blood HSC and most STRC-M as well as STRC-ML are also ALDH⁺, suggest that selection of ALDH⁺ cells for clinical transplants will not necessarily have an adverse effect on myeloid recovery. The higher enrichment of HSC in ALDH⁺ vs CD34⁺ cord blood cells (1 HSC in 4,700 vs 16,000 cells, respectively) also suggests that BAAA staining may be a more specific assessment or enrichment tool than staining for CD34⁺ cells. Similarly, our demonstration here that most STRC-M and possibly half of all STRC-ML as well as the LTRC-ML in human cord blood are CD133⁻ suggests that this marker may also be less useful for monitoring or selecting primitive human cells than previously appreciated.

In summary, we have shown that a spectrum of repopulating cell types in human cord blood can be differentially isolated by FACS in a single step based on their elevated ALDH activity. We show that this property can be coupled with other markers to achieve very high purities of LTRC-ML and that a small proportion of STRC are ALDH (Figure 6).

Authors' contributions

OC designed and performed most of the experiments, analyzed most of the data and drafted the manuscript. KL, SI, KL and MH assisted in different ways to each of these efforts; AE and CS contributed to the design of experiments and data interpretation and helped revise the manuscript; CE conceived the project, provided a critique of the experimental designs and assisted in data interpretation as well as undertaking the final revision of the manuscript. The order of the authors was arranged accordingly.

Conflict of Interest

OC declares that he held a research fellowship from Aldagen, Inc., the manufacturers of the ALDEFLUOR assay kit; CS declares that he is a founder and consultant at Aldagen, Inc.; all other authors declare that they have no potential conflict of interest relevant to this paper.

The authors confirm that this work has not been previously published elsewhere and has not been simultaneously submitted for publication to any other scientific journal.

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