Brief Report: A Differential Transcriptomic Profile of Ex Vivo Expanded Adult Human Hematopoietic Stem Cells Empowers Them for Engraftment Better than Their Surface Phenotype

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Key Words. Hematopoietic stem cells • Adult hematopoietic stem cells expansion • Stemregenin1 • UM171

ABSTRACT

Transplantation of small cord blood (CB) units, or of autologous ex vivo-genetically modified adult hematopoietic stem cells (HSC), face the common challenge of suboptimal HSC doses for infusion and impaired engraftment of the transplanted cells. Ex vivo expansion of HSCs, using either cell-based coculture approaches or especially small molecules have been successfully tested mainly in CB and in prolonged cultures. Here, we explored whether innovative combinations of small molecules can sufficiently, after short culture, expand adult HSCs while retaining their functionality in vivo. We found that 5-day cultured cells, in the presence of the small molecule combinations tested, achieved higher engraftment levels in NSG mice than both their uncultured and their cytokine only-cultured counterparts. Surprisingly, the engraftment levels were neither concordant to the numbers of phenotypically similar HSCs expanded under different small molecule combinations, nor explained by their distinct companion cells present. Transcriptomic comparative analysis of sorted, phenotypically similar, ex vivo generated HSCs transplanted in equal numbers, suggested that HSCs generated under expansion conditions that maintain low expression of the Rap1/Ras/P3K-AKT pathway exhibit a superior functional profile in vivo.

SIGNIFICANCE STATEMENT

Here, we documented that the engraftment potential of ex vivo expanded phenotypically similar hematopoietic stem cells (HSCs) is dictated by the specific expansion conditions rather than their surface phenotype. The results of our study have important implications for all investigators studying the ex vivo expansion of adult HSCs.

INTRODUCTION

Successful hematopoietic stem cells (HSC) transplantation protocols have been associated with high numbers of donor cells. The need for large numbers of engraftable HSCs becomes particularly challenging in case of cord blood (CB)-transplantation because of the size of suitable grafts, and in adult HSC-gene therapy protocols, where the culture conditions may lead to impaired engraftment potential [1]. Even though long-term HSC cultures in cytokine supplemented medium can dramatically increase the numbers of immunophenotypically identified HSCs [2, 3], the numbers of engraftable HSCs under these conditions can at best be maintained, and their functionality is largely compromised [2]. Methods to improve maintenance as well as expansion of the genetically modified cells, would enhance hematologic recovery in myeloablated patients and increase long-term in vivo engraftment of the modified graft, greatly improving the short-term safety and long-term efficacy of such protocols [4].

Recently, high throughput screening of large chemical libraries identified a number of small molecules as potential new tools for HSC expansion. Some of the most promising molecules described, include the aryl hydrocarbon receptor (AHR) antagonist StemRegenin1 (SR1) [5], the p38-MAPK14 inhibitor LY2228820 (Ly) [6], and the pyrimidoindole derivative UM171 [7]. Expansion efforts using these molecules, however, have been focused on CB-HSCs, while their effects on adult HSCs have been less explored. In the present study, we compared these promising conditions, alone and in combinations, for expanding ex vivo, engraftable adult HSCs. To avoid loss of stemness of the expanded cells with prolonged cultures, we...
tested the different protocols in a short 5-day culture, which
would likely be beneficial in a gene therapy setting.

MATERIALS AND METHODS

Mobilized peripheral blood CD34+ cells from healthy donors
were cultured for 5 days in serum free medium, supplemented
with Fms related tyrosine kinase 3 (Flt-3), stem cell factor (SCF),
and thrombopoietin (TPO) and in the presence of the small mole-
cules SR1, Ly, and UM171, alone or in combinations; SR1 + Ly,
SR1 + Ly + UM171. Hematopoietic stem and progenitor cell (HSPC)
expansion, evaluated by flow cytometry and clonogenic assays
was compared to either the input HSPCs (Day 0) or the expansion
culture without small molecules. For the in vivo experiments,
NSG/IL2γ null mice were transplanted with the uncultured inocu-
lum or the total cell output after culture, unless otherwise stated.
For more details, see supplemental Methods.

RESULTS AND DISCUSSION

To optimize ex vivo expansion of adult HSCs we tested three
modalities (SR1, Ly, and UM171) alone or in certain combinations,
but only for a short time in culture to preserve stemness. UM171
alone yielded more phenotypically characterized HSCs compared
to the other two small molecules tested alone (Fig. 1A, 1B). Then,
we subsequently combined SR1 and Ly hoping to uncover syner-
gistic and/or additive effects, as inhibition of p38/MAPK is also
AHR dependent [8]. This combination achieved similar expansion
of HSCs to that of UM171 alone and combination of all three mol-
ecules appeared to have an additive effect.

Of interest, under the different protocols apart from the
effects on HSC numbers, distinct downstream lineages were
enhanced. In UM171 cultures, mainly progenitors with a myeloid
bias were enhanced (Fig. 2A). By contrast, a high number of mega-
karyocytic progenitors (Fig. 2B, 2C), was present in all SR1-
containing cultures along with more mature megakaryocytes,
even in the absence of TPO and these were completely absent
from the UM171 cultures (Fig. 2D–2F). Although aryl hydrocarbon
pathway repression has been previously shown to promote mega-
karyocytic specification [9, 10], the mechanism through which
both the megakaryocytic and erythroid differentiation is sup-
pressed by UM171, requires further studies.

Consistent with the phenotypic and CFU culture data (Fig. 2A–
2F), transcriptomic analysis of cultured cells also uncovered high
expression of granulocytic/monocytic genes, and suppression
Figure 2. Distinct lineage enhancement within the SR1+Ly and UM171 cultures. Fold expansion of total numbers of BFU-E and CFU-GM (A) and CFU-Mk (B), over the day 0-uncultured cell inoculum. (C): Representative morphology of CFU-Mk, derived from UM171 (left) and SR1+Ly (right) cultured cells. Colonies from UM171 cultures were smaller in size and consisted of a few scattered cells. (×40 objective) (D): Morphologically recognizable mature megakaryocytes present in SR1-containing suspension cultures, and absent from the UM171-cultures. Preparations were stained with Giemsa and pictures were taken with a ×40 objective. (E): Frequency of CD41+/CD42+ cells among the different conditions, and contour plot for CD41+/CD42+ cells in SR1+Ly cultures. (F): Sorted CD41+/CD42+ cells were cytospun and stained with Giemsa or plated in Megacult medium to test production of proplatelets. (×80 magnification). (G): Z-scored expression of megakaryocytic, erythroid, and granulocytic marker genes from bulk cultures. Control: 5-day cultured cells w/o small molecules [H] Biplot of the 1,000 top variable genes. Cells cultured under UM171 have a diametrically opposite profile to the cells cultured with SR1+Ly cells. Cells cultured under the SR1+Ly+UM171 combination exhibited a comparable transcriptomic profile to that of the SR1+Ly-cells, implying that the SR1+Ly combination is dominant over UM171. Genes are represented as dots and treatments as the loadings vectors. Colors represent K-means clusters (K = 5) and the most significant (p < .05) gene-ontology term of the corresponding cluster is annotated. Error bars represent SEM from three independent experiments. *, p < .05 versus all other conditions. Abbreviations: BFU-E, burst forming units-erythroid; CFU-GM, colony forming units-granulocytic/monocytic; CFU-Mk, colony forming units-megakaryocytic.
of megakaryocytic/erythroid marker genes in the UM171 cultures; in contrast, cells cultured in the presence of SR1+Ly displayed enhanced erythroid/megakaryocytic but suppressed granulocytic/monocytic gene signature (Fig. 2G). Overall, populations expanded under UM171 or SR1+Ly seem to be distinguished by diametrically opposed expression profiles (Fig. 2H).

The in vivo functional potential of HSCs expanded under different small molecule combinations (UM171, SR1+Ly, and SR1+Ly+UM171), was tested after transplantation in NSG mice. These mice were transplanted with the entire cultured output generated from the same starting inoculum of CD34+ cells. Higher levels of human chimerism were observed in the peripheral blood of mice receiving small molecule-expanded cells compared to the uncultured cells (Fig. 3A), 4–16 weeks post transplantation. Cells cultured for the same time period only with cytokines, achieved lower engraftment compared to the uncultured cells (Supporting Information Fig. S1), as previously reported [11]. However, cells cultured in the presence of SR1+Ly exhibited superior engraftment compared to similar total cell numbers of UM171-cells transplanted (Figs. 1A, 3A). SR1+Ly-cells also yielded higher platelet numbers in peripheral blood than all other conditions, 8-weeks post-transplantation, possibly due to the higher numbers of mature megakaryocytes in the inoculum (Fig. 3B), a feature that could potentially be associated with faster platelet recovery in a transplantation setting. The SR1+Ly+UM171 combination, did not further improve the engraftment of the SR1+Ly graft (Fig. 3A), even though contained the highest number of HSCs (Fig. 1A). Bone marrow studies at week 16, confirmed the robust engraftment of human cells in the SR1+Ly (±UM171)-recipients (Fig. 3C). Multilineage engraftment was detected in every condition. Of note, a significantly lower percentage of CD34+ and CD41+ cells was detected in the UM171-recipients (Fig. 3D). Furthermore, the engraftment difference between SR1+Ly and UM171 was maintained in secondary recipients (Fig. 3E).

Our in vivo data so far show that in contrast to expectations the number of ex vivo expanded phenotypic-HSCs under different expansion conditions is not predictive of the engraftment outcome. To explain the different engraftment outcomes, we hypothesized that there either are intrinsic differences among the expanded HSCs, or that the non-HSC, companion cells are responsible. For example, megakaryocytes, that are abound in the SR1+Ly cultures, have been implicated in affecting HSC engraftment potential as well as
Figure 4. Functional differences and transcriptomic profile of ex vivo expanded adult HSCs. (A): Equal numbers of FACS sorted CD34⁺/CD38⁻/CD90⁺ cells, from SR1-Ly or UM171 cultures at day 5, were transplanted in NSG mice. (B): Equal numbers of FACS sorted CD34⁺/CD38⁻/CD90⁺ cells from SR1-Ly or UM171 cultures, were transplanted in NSG mice, along with the same number of their alternate non-HSC companion cells. (C): Engraftment (human CD45⁺ cells) was assessed in PB and BM 4–16 weeks post transplantation. (D): Human cell engraftment in PB and BM 4–16 weeks post transplantation. The companion cells enhanced the overall engraftment of both HSC populations, however without changing the outcome. (E): For the in vivo homing experiments, 5 × 10⁵ sorted CD34⁺/CD38⁻/CD90⁺ cells from SR1-Ly or UM171 cultures, were transplanted in NSG recipients. The mice were sacrificed 48 hours post transplantation. (F): Total human and CD34⁺ cells in vivo expansion, 12 days post transplantation. 5 × 10⁵ sorted CD34⁺/CD38⁻/CD90⁺ cells from SR1-Ly or UM171 cultures, were transplanted. (G): MA plot of the shrunken values of the differentially expressed genes (1.5-fold change, p < .05) in SR1-Ly versus UM171 treated CD34⁺/CD38⁻/CD90⁺ sorted cells and gene set enrichment analysis within the SR1-Ly downregulated genes showing the top five ranking KEGG pathway terms. Error bars represent SEM from three independent experiments, with 4 mice per group in each experiment. *, p < .05 versus all other groups, ns: not significant. Abbreviations: BM, bone marrow; IBD, inflammatory bowel disease; FACS, fluorescence-activated cell sorting; HSC, hematopoietic stem cells; LTM, leukocyte transendothelial migration; PB, peripheral blood.
To explore the aforementioned possibilities, we designed a new set of in vivo experiments. We transplanted the same number of sorted CD34+/CD38–/CD90+ cells, expanded under SR1+Ly or UM171, either alone (Fig. 4A) or in combination with their switched-over non-HSC companion cells (Fig. 4B). Once again, the SR1+Ly-isolated HSCs achieved significantly higher engraftment levels than their numerically and phenotypically similar counterparts under UM171 (Fig. 4C). Upon addition of alternate companion cells, engraftment differences were maintained suggesting HSC-intrinsic rather than extrinsic effects (Fig. 4D), although, the theoretical possibility of an indirect effect exerted by the companion cells, shaping intrinsic properties of the HSCs during the 5-day culture, cannot be excluded.

To account for engraftment differences of sorted CD34+/CD38–/CD90+ expanded cells, we explored the homing potential of these donor populations. Surface molecule expression revealed no significant differences in any of the adhesion molecules tested (Supporting Information Fig. S2). Furthermore, in vivo homing experiments could not uncover a superior homing potential of the SR1+Ly HSCs (Fig. 4E). Instead, a faster and superior in vivo bone marrow expansion potential was documented 12 days post transplantation (Fig. 4F).

To further explore whether the in vivo functional advantage of SR1+Ly versus UM171 expanded HSCs could be associated with their intrinsic transcriptional profile, we performed RNA sequencing of sorted populations (CD34+/CD38–/CD90+) expanded under the two different protocols. Surveying the differentially expressed genes, we found that genes involved in the Rap1, Ras, and PI3K-Akt pathways, were significantly downregulated in SR1+Ly versus UM171 HSCs (Fig. 4F; Supporting Information Figs. S3–S5). These three closely regulated pathways play central role in regulating proliferation, apoptosis, differentiation, cell adhesion and metabolism [15–18] with both complementary and antagonistic cell-context dependent effects in hematopoiesis [19]. Highly active Rap1 and Ras pathways trigger PI3K and activated PI3K-Akt signaling has been associated with HSC exhaustion and depletion [20–22] as well as thrombocytopenia [20], whereas silencing enhances cell engraftment [23]. This latter outcome is consistent with our results with SR1+Ly versus UM171 reflecting the enhanced engraftment, of the former.

**CONCLUSION**

In summary, our data show that adult HSPCs cultured for 5 days in the presence of SR1+ly, achieve post transplantation significantly higher and long-term sustained in vivo engraftment levels compared to cells cultured in the presence of UM171-alone. The transcriptomic profile of ex vivo expanded phenotypically similar HSCs, under different small molecule combinations, uncovers specific functional differences that likely impact their engraftment potential.

**ACKNOWLEDGMENTS**

We thank Andrew Nishida, Daniel Bates, and Douglass Dunn from the Altius Institute for Biomedical Sciences for their help with RNA-seq data preprocessing, RNA library preparation and sequencing, and RNA isolation respectively, and the student Demetri Dalas for providing technical assistance. This work was supported by NIH Grant R01-DK101328–01A1.

**AUTHOR CONTRIBUTIONS**

N.P. collection and assembly of data, data analysis and interpretation, manuscript writing; G.G. collection and assembly of data, data analysis and interpretation; S.P. experiment conduction; T.P. conception and design, data interpretation, manuscript writing and final approval of manuscript.

**DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST**

The authors indicated no potential conflicts of interest.

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