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Comprehensive phenotyping of hematopoietic stem and progenitor cells in the human fetal liver

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Abstract

Hematopoietic stem cells (HSCs) reside at the top of the hematopoietic hierarchy and can give rise to all the mature blood cell types in our body, while at the same time maintaining a pool of HSCs through self-renewing divisions. This potential is reflected in their functional definition as cells that are capable of long-term multi-lineage engraftment upon transplantation. While all HSCs meet these criteria, subtle differences exist between developmentally different populations of these cells. Here we present a comprehensive overview of traditional and more recently described markers for phenotyping HSCs and their downstream progeny. To address the need to assess the growing number of surface molecules expressed in various HSC-enriched fractions at different developmental stages, we have developed an extensive multi-parameter spectral flow cytometry panel to phenotype hematopoietic stem and multipotent progenitor cells (HSC/MPPs) throughout development. In this study we then employ this panel to comprehensively profile the HSC compartment in the human fetal liver (FL), which is endowed with superior engraftment potential compared to postnatal sources. Spectral cytometry lends an improved resolution of marker expression to our comprehensive approach, allowing to extract combinatorial expression signatures of several relevant HSC/MPP markers to precisely characterize the HSC/MPP fraction in a variety of tissues.

KEYWORDS
flow cytometry, hematopoiesis, hematopoietic stem cells, immunophenotyping panel, spectral cytometry

1 | INTRODUCTION

HSCs have the capacity to reconstitute the entire hematopoietic system of a host. This remarkable regenerative potential has led to their use in existing and emerging therapies for a variety of disorders. In the clinic, HSCs can be isolated from the bone marrow (BM), from peripheral blood upon mobilization or from cord blood (CB). While less accessible for transplantation purposes, prenatal HSCs such as those residing in the fetal liver (FL) hold valuable information for the optimization of this process. During hematopoietic development, newly emerged HSCs travel to the FL where they expand in number before migrating to their final niche in the bone marrow [1, 2]. Despite undergoing active expansion, FL HSCs display superior engraftment potential compared to CB or BM HSCs [3]. Therefore, studying this prenatal HSC source can provide insights into how to retain or even increase the engraftment potential of postnatal HSCs. The panel described in this report was designed to phenotype the FL HSC fraction as part of a multi-modal profiling effort to establish the molecular signature of engraftment capacity [4]. While it is illustrated here in the context of the FL, the selection of markers included in this panel makes it a valuable resource for the characterization of HSC subsets in other tissues and at various developmental time points.

ISAC CYTOMETRY Journal of Quantitative Cell Science PART A

TABLE 1 Multiparameter spectral cytometry panel used in the current study

Markers	Conjugate	Clone	Reagent notes	Vendor	Cat. #	Reagent dilution	Unmixing control
CD45	BUV395	HI30	Pan-hematopoietic marker	BD	563791	100	Lymphocytes
Livedead	Live Dead Blue	n/a	Live dead discrimination	Thermo Fisher	L34961	800	Live+dead cells
CD66c	BUV661	B1.1/CD66	Granulocyte lineage	BD	741653	50	Beads
CD19	BUV737	SJ25C1	B cells	BD	612757	100	Beads
CD14	BUV805	M5E2	Monocytes	BD	612902	50	Beads
CD90	BV421	5E10	HSC/MPP	Biolegend	328121	50	Lymphocytes
CD235a	BV480	104	Erythroid cells	BD	746358	400	Erythrocytes
CD10	BV510	S7	Lymphocyte precursors	Biolegend	312219	50	CD3 BV510 lymphocytes
CD33	BV570	M17	Myeloid cells	Biolegend	303417	400	Beads
CD38	BV605	HIT2	Lymphocyte/monocyte marker; alternative clone HB-7 failed in CiTE-seq pilot	Biolegend	303532	50	Beads
CD49f	BV650	GoH3	HSC/MPPs	BD	563706	200	Beads
CD45RA	BV711	HI100	Naïve lymphocytes	Biolegend	304137	100	Lymphocytes
CD56	BV786	NCAM16.2	Natural killers; alternative clone HCD56 fails to resolve high and medium positive populations in PBMCs	BD	564058	100	Lymphocytes
CD164	FITC	67D2	HSC/MPPs	Biolegend	324805	50	Beads
CD3	Spark Blue 550	SK7	T cells	Biolegend	344851	100	Beads
CD41	PerCP-Cy55	HIP8	Megakaryocytes/platelets	Biolegend	303719	100	Lymphocytes
GPI-80	PE	3H9	HSC/MPPs	MBL	D087-5	50	Beads
CD133	PE-Dazzle 594	clone 7	HSC/MPPs	Biolegend	372811	100	Beads
CD69	PE-Cy5	FN50	HSC/MPPs or lymphocyte activation	Biolegend	310907	25	Beads
CD34	PE-Cy7	581	HSC/MPPs; this clone is compatible with magnetic isolation of the CD34+ enriched fraction	Biolegend	343515	100	Fetal liver
CD201 (EPCR)	APC	RCR-401	HSC/MPPs	Biolegend	351906	25	CD8 APC lymphocytes
CD42b	Alexa 700	HIP1	Megakaryocytes/platelets	Biolegend	303927	25	Lymphocytes

TABLE 2 Surface expression profiles for markers used to identify HSC and PBMC cells in the current study

	CD45	CD66c	CD19	CD14	CD90	CD235a	CD10	CD33	CD38	CD49f	CD45RA	CD56	CD164	CD3	CD41	GPI-80	CD133	CD69	CD34	CD201	CD42b
T cells	+	−	−	−	+/−	−		−	+/−	+/−	+/−	+/−	−	+	−	−	−	+/−	−	−	−
B cells	+	−	+	−	−	−		−	+/−	−	+	−	−	−	−	−	+/−	−	−	−	−
Monocytes	+	−	−	+	−	−		+	+/−	+/−	+/−	−	+	−	+/−	+/−	−	−	−	−	−
Natural killers	+	−	−	−	−	−		−	+/−	−	+	+/++	−	−	−	−	−	+/−	−	−	−
Erythroid cells	−	−	−	−	−	+		−	−	−	−	−	−	−	−	−	−	−	−	−	−
Granulocytes	+	+	−	−	+/−	−		−	−	−	−	−	−	−	−	+	−	−	−	−	−
Lymphocyte precursors	+	−	−	−	−	−	+	−	+/−	−	+/−	−	−	−	−	−	−	+/−	−	−	−
Megakaryocytes	−	−	−	−	−	−		−	−	+	−	−	+/−	−	+	−	−	+/−	−	+	+
Platelets	−	−	−	−	−	−		−	−	+	−	−	+/−	−	+	−	−	+/−	−	+/−	+
HSC/MPPs	−	+/−	−	−	+/−	−	−	+/−	+/−	+/−	+/−	−	+/−	−	−	+/−	+/−	+/−	+/−	+/−	−

2 | COMPREHENSIVE MARKER PALETTE FOR BROAD HSC CHARACTERIZATION

CD34 is traditionally used as a positive marker to denote the HSC population and is central to most HSC phenotyping and enrichment strategies as its expression marks the vast majority of HSC/MPPs. The CD34+ hematopoietic fraction, however, is heterogeneous and represents a broad collection of hematopoietic stem and progenitor cells including cells that are not imbued with engraftment potential. Further distinction between HSC/MPPs and more committed hematopoietic progenitors is guided by the expression of markers such as CD38, CD45RA and markers specific to the different mature blood cell lineages (lin). In general, there exists a consensus that the most primitive hematopoietic progenitors can be found in the CD34+ [5], lin-[6, 7], CD38− [8], CD45RA− [9], CD90+ [7] fraction [10]. However, this combination of markers is not absolute as long-term repopulating activity has also been reported in the CD90− HSC/MPP fraction, albeit at a lesser frequency [11]. Moreover, CD38 expression is variable between donors and gating can be arbitrary as there is no consensus on a cut-off for CD38− primitive cells [12, 13]. Sialomucin or CD164, has been suggested as an alternative to CD38-based gating strategies in HSC transplantation settings [13] and we have included this marker in our panel. CD69, while typically referred to as a lymphocyte activation marker [14], recently has been described as marking a transient population of mid-gestation HSC/MPPs [15] and thus might provide some insight into the developmental staging.

In addition to these relatively broad positive and negative selection markers, we also propose the use of more specific HSC enrichment markers to expand our phenotyping panel. CD49f has been reported as a highly specific marker for HSCs, with the capacity to enrich HSCs to a purity of ∼10% (1 in 10.5 cells) when added to the aforementioned selection scheme [11]. Originally described in CB, this marker is also expressed on BM HSCs [16] and FL HSCs [4] and is a common addition to HSC enrichment panels. Another powerful HSC enrichment scheme that has recently been described is driven by CD133 and GPI-80 expression [17]. CD133 surface expression is known to mark uncommitted HSCs from FL to BM [18–20] and GPI-80 expression specifically marks self-renewing HSCs in the FL [21]. The combination of these (18lin-CD34+CD38-CD133+GPI-80+) resulted in the purification of CB HSCs to the level of ∼1/5 cells [17]. CD201, also known as endothelial protein C receptor (EPCR), presents another marker with potent HSC enrichment potential. First described to mark mouse HSCs [22], CD201 was later reported to also mark human HSCs in both CB [23] and FL [24]. Assessment of CD201 expression at different developmental time points revealed that unlike in mice where its expression remains stable throughout development, the percentage of cells that are CD201+ declines over time and few CD201− expressing cells can be found in adult BM [24]. Integration of transcriptomic and cell surface level data obtained from the multimodal profiling of FL HSCs revealed that CD201 marks a subset of cells with the highest engraftment potential in the FL and transplantation experiments in the context of this work suggested that the

engraftable HSC enrichment capacity of CD201 parallels or even exceeds that of GPI-80 [4].

In our panel design, we have assigned the HSC/MPP markers to fluorochromes with as little spillover among each other as possible since we expected unpredictable patterns of co-expression of these molecules as well as a variety of expression levels (Table 1). The panel design has been optimized in several iterations to eliminate artifacts that were observed due to spillover. Clone selection was informed by the consensus in the field as well as data generated previously [4].

We have included an extensive list of lineage-specific markers in our phenotyping panel. HSC phenotyping usually assumes the use of "lineage exclusion," and a routine approach is to combine multiple antibody specificities within one "dump" fluorescence channel by using same fluorochrome conjugates or conjugates with similar emission spectra. This technique accommodates larger marker sets when the parameter capacity is limited. However, many of those markers are not exclusively expressed on committed lineages and this solution carries a risk of excluding HSC/MPPs with ectopic expression of traditional 'lineage' markers. For instance, CD45 is used as a marker for immune populations in general, but its expression can also be found, to a lower extent, on progenitors. Also, the varying ranges of expression, when combined in one channel of detection, often presents a difficulty in "gating"/subsetting the lineage negative fraction. Since we employed a five laser spectral cytometry system, we were not limited by parameter capacity and assigned all lineage markers to individual fluorochromes. To map the lineages of committed hematopoietic cells, we used CD45 (pan-hematopoetic marker), CD3 (T cells), CD56 (NK cells), CD19 (B cells), CD33 (monocytes), CD14 (monocytes), CD66c (granulocytes), CD10 (ProB progenitors), CD42b (platelets), CD41 (platelets), and CD235a (erythroid cells), summarized in Table 2 [25–27].

To illustrate the performance of our proposed phenotyping setup, we have stained CD34+ enriched human fetal liver cells as well as human adult PBMCs with our 22-color spectral panel and acquired the data on a 5 L Cytek Aurora analyzer (Cytek Biosciences). Cell isolation, staining and data acquisition were performed as reported previously [4, 25] (see Supplemental Material for details) and data were analyzed in OMIQ cloud platform. Both samples were concatenated, all immunophenotyping fluorescence parameters were dimensionally reduced into a UMAP space and clustered using Phenograph algorithm. The PBMC datapoints served as landmarks that helped to visualize commitment trajectories of FL cells in the same UMAP space (Figure 1A). Phenograph clustering identified 39 clusters in the concatenated samples (Figure 2A), most of which were exclusive to

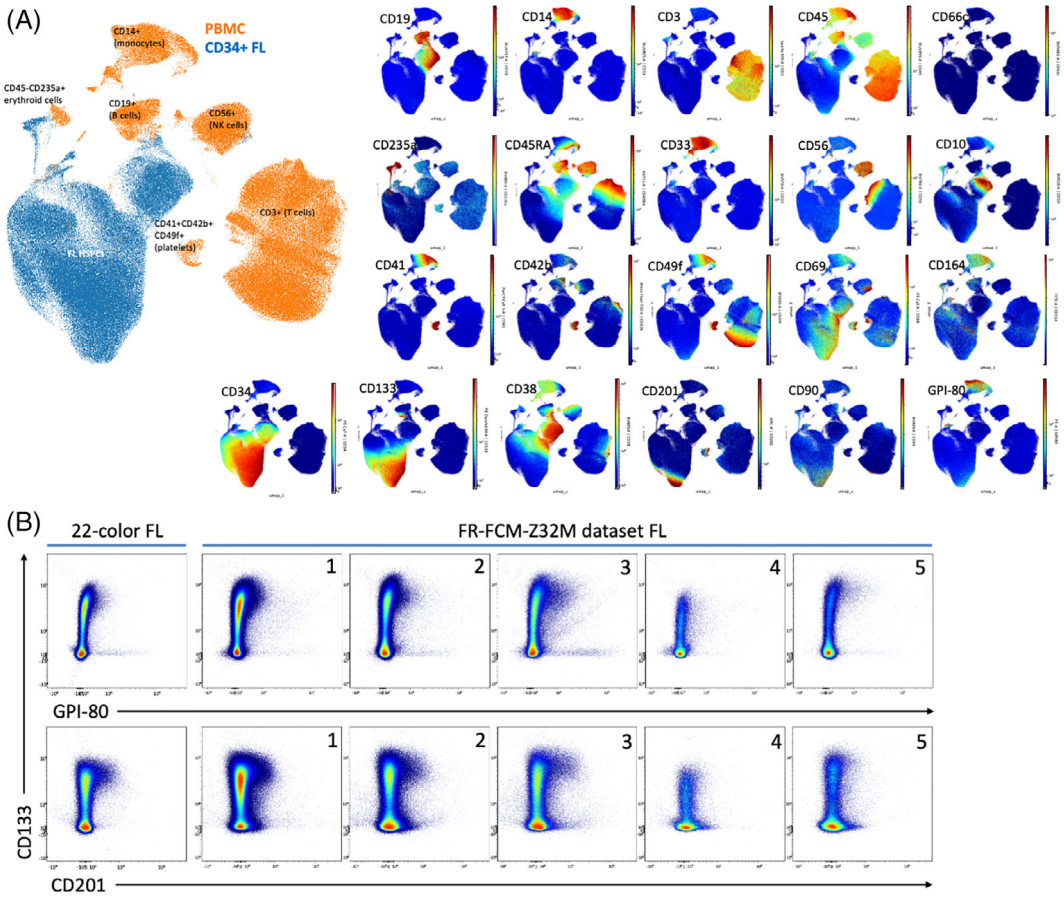

FIGURE 1 Twenty-two-color spectral cytometry immunophenotyping of human fetal liver HSC/MPP cells. (A) UMAP projections of PBMC and FL samples labeled with 22 color spectral cytometry panel. Expression levels of tested surface markers is shown as heatmaps overlaid on the UMAP plot depicting live, single cells or platelets. (B) Bivariate plots showing expression of CD133, GPI-80 and CD201 in FL sample stained with 22-color panel (left panel) or previously reported [4] FL phenotypes (same reagents used for the depicted markers)

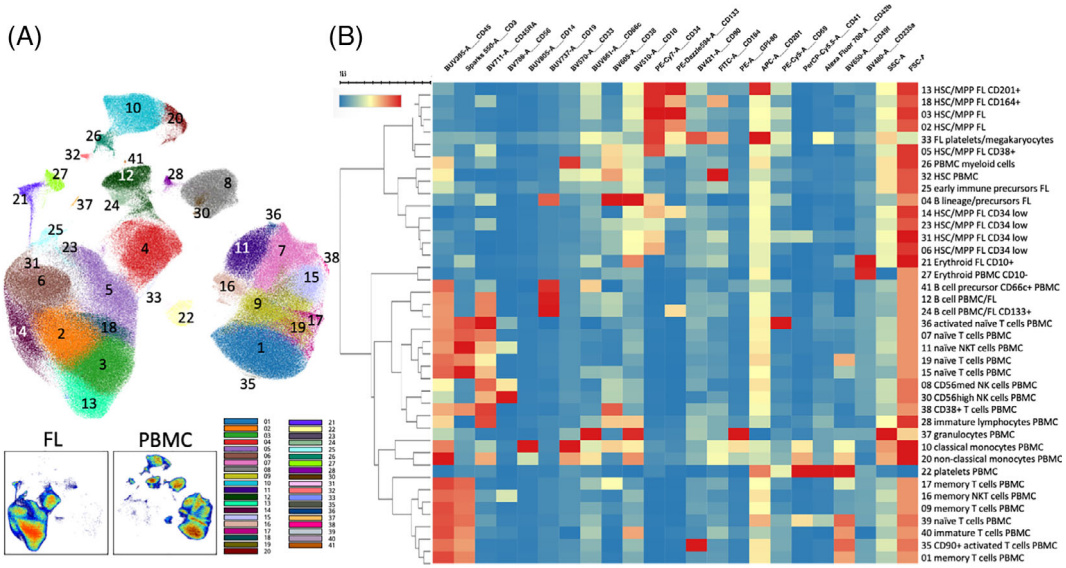

FIGURE 2 Human fetal liver HSC/MPP population landscape. (A) Marker expression Phenograph clustering overlaid on UMAP plot depicting live, single cells or platelets. Insets at the bottom illustrate the distinction between FL cells and PBMCs. The latter were included to visualize commitment trajectories of FL cells in the same UMAP space. (B) Heatmap visualization of normalized expression levels of tested markers on Phenograph clusters defined from live, single cells or platelets. Unsupervised hierarchical clustering of the cell clusters in the heatmap is depicted by the dendrogram on the *left*. All expression level intensities are normalized per column

one of the sample types, but others contained cells from both sources. In Figure 2B, representing a clustered heatmap of the median marker expression across clusters, we have marked the predominant, but not exclusive, source of cells for each cluster. It is important to note that there may exist sample-to-sample variability of marker expression across FL specimens, and our 22-color dataset only represents a single FL sample. When compared with the dataset collected with the early version of our panel (available as FR-FCM-Z32M Flow Repository public dataset) that contains 5 FL specimens, the ratios of certain populations may vary across samples; for instance, the GPI-80+ CD133+ and CD201+ CD133+ cell subsets proportions differ across the specimens (Figure 1B and [4]). However, qualitatively the identified combinatorial phenotypes of HSC/MPP subsets exist in most samples.

Overall, we observed the expected patterns of expression of previously described markers in the HSC/MPP compartment as well as the combinatorial signatures of multiple HSC markers that have not been yet assessed in a single panel.

3 | CONCLUSIONS

In this report, we present a summary of current trends in phenotyping human HSC/MPPs. We provide a detailed list of surface markers to assist the study design, as well as share our 22-color spectral cytometry panel that has been designed to encompass expression of all proposed markers in a single tube assay. The HSC/MPP marker selection spans historically used HSC markers as well as more recently described alternatives for HSC enrichment. All HSC markers included in our panel are well-defined markers that have been extensively characterized in terms of enrichment capacity for cells with self-renewal and multi-lineage reconstitution potential, obviating the need to repeat functional characterization in the context of this work.

We illustrate the performance of our multi-parameter phenotyping panel on CD34+ enriched human fetal liver cells, creating a dataset that serves as an example of marker distribution across the human HSC/MPP continuum at this developmental stage.

Importantly, the spectral cytometry panel described here allows for the characterization of HSCs in different tissues, ranging in location and developmental time, and can easily be expanded to accommodate alternative/newly discovered HSC/MPP markers, or incorporate additional markers to profile non-conventional HSC sources.

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AUTHOR CONTRIBUTIONS

Kim Vanuytsel: Conceptualization (equal); investigation (lead); methodology (equal); resources (equal); writing – original draft (lead); writing – review and editing (equal). **Anthony K Yeung:** Investigation (supporting); writing – review and editing (supporting). **Todd W Dowrey:** Investigation (supporting); writing – review and editing (supporting). **George J Murphy:** Conceptualization (supporting); resources (lead); writing – review and editing (supporting). **Anna C**

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CONFLICT OF INTEREST

The authors declare no competing interests.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

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