Phenotypically Distinguishing Leukemia-Initiating Cells from Inert Leukemia Blasts in a HOXB4-Transgene Dependent Leukemia Model

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Contents

1. Background

1a. Hematopoietic Hierarchy

The hematopoietic or blood-forming system has a hierarchy with cell stages that vary in self-renewing capacity and degree of differentiation. At the beginning of the hierarchy are the most primitive cells: the long-term hematopoietic stem cells (LT-HSCs). These HSCs have three key characteristics that are relevant to this paper: sustained self-renewing capacity, multipotency, and the endogenous expression of the homeobox genes. Multipotency allows HSCs to differentiate into any of the lineage-committed progenitors: myeloid, lymphoid, or erythroid lineages. Self-renewal refers to the capacity of HSCs to replicate into copies of themselves that retain identical self-renewing capacity and multipotency. It is this self-renewing property of HSCs that supports the permanent, sustained function of the hematopoietic system because only HSCs exhibit a permanent reconstituting capacity through successive generations and as a result, maintain persistent immortalized clones (Wiseman et al. 1999, Lim et al. 2017). Differentiation of HSCs into specific hemopoietic lineages is accompanied by a diminished self-renewing capacity, a shortened reconstituting life span in which they can reconstitute clones of themselves over a shorter period before they die (Seita and Weissman 1999), and loss of homeobox expression (Lim et al. 2017).

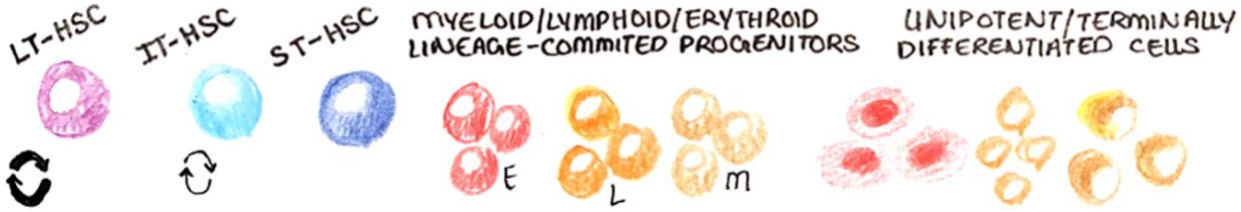


Figure 1. ***Cell stages in the hematopoietic hierarchy.*** *Cells on the left are most primitive and cells on the right are more mature. Self-renewal diminishes and HOXB4-expression is downregulated as cells advance from left to right.*

Down the hierarchy, HSCs eventually differentiate into lineage-committed progenitors (LCPs), which later differentiate into unipotent terminally differentiated cells that belong in one lineage and are functionally particular like red blood cells, megakaryocytes, platelets, B, and T lymphocytes *(Fig 1).* As we move from HSCs to LCPs to terminally differentiated cells, the expression of the homeobox genes is downregulated. The lineage-committed progenitor stage in which the homeobox genes are silenced is also the stage during which self-renewing capacity diminishes. The Iscove lab questioned if there is a relationship between the silencing of the homeobox genes and the diminished self-renewal in LCPs.

1b. HOXB4-Dependent Leukemia Model

Well-known as the genes responsible for segment development, the homeobox genes recently gained attention for their role in hematopoiesis. HOXB4 –a transcription factor in the homeobox family– has been implicated in enhancing self-renewal (Antonchuk et al. 2001, 2002). Considering both principles: the association of HOXB4 in regulating self-renewal **and** the diminishing self-renewal paired with the downregulation of HOXB4 in later stages of the hierarchy, the Iscove Lab examined whether the diminished self-renewing capacity further down the hierarchy is related to the loss of HOXB4 expression. To test this, expression of HOXB4 had been enforced in lineage-committed progenitors where HOXB4 isn't naturally expressed. This was done by means of infecting normal donor HSCs with a HOXB4VENUS retrovirus. VENUS is a fluorescent protein that serves as a visual proxy for the expression of the HOXB4 transgene.

The expression of VENUS allowed us to identify and visualize cells infected with the HOXB4 retrovirus. The IRES –the internal ribosomal site integrated in the virus– will sometimes fail to express the HOXB4 gene following it, so there might be cells that express HOXB4 but not VENUS. The opposite, however, is not a possibility: VENUS+ cells will never not express HOXB4. Because of this, "VENUS+" in this paper always refers to HOXB4-expressing cells.

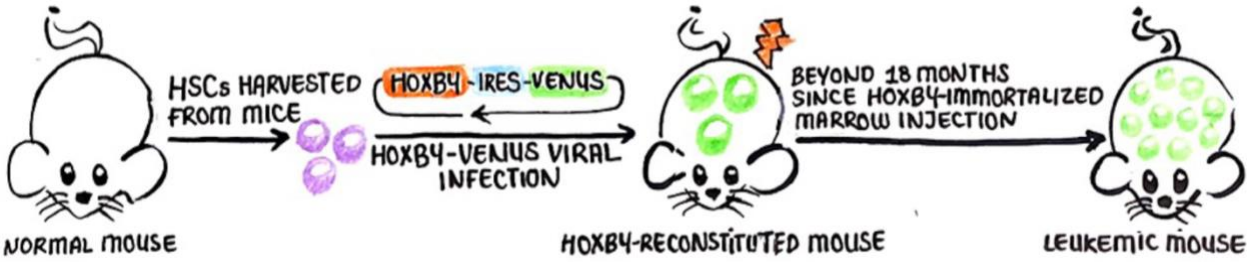


Figure 2. Normal HSCs were harvested from a donor mouse, infected with a HOXB4VENUS retrovirus, and injected into an irradiated recipient mouse. The HOXB4-transgene also exerts its expression in lineage-differentiated progeny of HSCs, which -as a result- are immortalized by means of self-renewal conferred to them by the transduction. Beyond 18 months, this HOXB4-reconstituted BM accumulates enough mutations to become leukemic BM. This is a HOXB4-transgene dependent leukemia model (mAML1).

A recipient mouse had been irradiated as to deplete its own bone marrow (BM), and the donor HOXB4-infected HSCs had been injected to generate HOXB4-reconstituted marrow in the recipient. The differentiated progeny of these HOXB4-infected HSCs will also intergenerationally express the HOXB4 transgene. In the earlier cell stages of the hierarchy, endogenous HOXB4 is expressed, so there was no significant effect when HOXB4 was transduced in HSCs (Lim et al. 2017). However, since LCPs don't express endogenous HOXB4, the lineage-differentiated progeny of HOXB4-infected HSCs responded to the enforced expression of the transgene and acquired a self-renewing capacity characteristic of HSCs (Lim et al. 2017). This means they were able to replicate indefinitely into more LCPs with sustained self-renewal, creating immortalized clones of themselves. These immortalized hemopoietic clones accumulate gene mutations which eventually become leukemogenic (Argiropoulos and Humphries 2007). Leukemic transformation has been observed in the Iscove lab model after in vivo passage of HOXB4-immortalized marrow cells into recipient mice for periods beyond 18 months. The mAML1 line of murine acute myeloid leukemia was derived in this way. One key property of this leukemia is its continuing dependence on the HOXB4 transgene. After excision of HOXB4 using a cre-loxP strategy, the leukemia regresses and disappears in host mice. Another key property is the evidence for proliferative hierarchy typical of clinical AML (Lapidot et al. 1994; Jordan 2007; Wiseman et al. 2014; Jackson et al. 2016). This HOXB4-dependent leukemia had been thawed or maintained in transplantation from one mouse to another long before I joined the lab for this project.

AML has been reported to preserve remnants of normal precursor hierarchy. Typically, only a minority of cells can regenerate a leukemia population after transplant. The mAML1 line was analyzed by limiting dilution analysis in which varying numbers of cells were transplanted into irradiated recipient mice. The analysis estimated that only 1 in every 63 mAML cells engrafted and regenerated the leukemia, suggesting that this line shares the hierarchical structure typical of human AML samples. The analysis also showed that a single mAML1 leukemia initiating cell regenerates a lethal leukemia burden within 30 -33 days in a mouse. The leukemia

blasts, which also make up the leukemia population, do not contribute to the fate of the leukemia and are inert in this respect.

1c. Windows of Expression of Markers of Interest

My project is to characterize the surface antigen phenotype of the rare LICs in the HOXB4-transgene dependent mAML1 model in a way that would allow these cells to be distinguished from most of the inert leukemia blasts, and further allow identification of the LIC's counterpart in the normal hemopoietic hierarchy. The six antigens I looked at to begin composing a distinguishing phenotype for the LICs are cKit, Sca1, CD150, Flt3, CD34, and FcyR. These markers are of interest because they mark primitiveness, maturation, and divergence points of differentiation across the hematopoietic hierarchy. Additionally, some of these markers signify entrance into or exit from quiescence –or dormancy– and are known to track the cycling of cells.

Sca1 or Stem Cell Antigen-1 is used to enrich for HSCs (Spangrude et al. 1988, Mazzanti 2019). It marks LT-HSCs (which reconstitute differentiated clones for 4 -11 months posttransplant), IT-HSCs (whose clones persist for 6–8months and then die thereafter), and ST-HSCs (which reconstitute differentiated hematopoietic clones for 4-6 weeks) (Benveniste et al. 2010). Hematopoietic cells that are Sca1- tend to be more differentiated and further down the hierarchy (Holmes and Stanford 2007, Mazzanti 2019). Similarly, cKit or stem cell factor receptor marks stem cells but continues to mark lineage-committed progenitor cells. The combined expression of cKit and Sca1 continues all the way through ST-HSC. Once cells become lineage-differentiated progenitors, Sca1 expression ceases, but cKit expression continues. This means that the difference between Sca1+cKit+ cells and Sca1-cKit+ cells correspond to a differentiation shift from ST-HSC to lineage-restricted progenitors (Mazzanti 2019).

CD150 is a more primitive marker than cKit and Sca1 that marks LT-HSC and IT-HSC. ST-HSC, LCPs, and terminally differentiated cells are CD150-.

Flt3 marks the portion of LT-HSC that cycle (i.e. that are quiescent). Further across the hierarchy, Flt3 expression ceases in IT-HSCs and ST-HSCs, but persists in lymphoid-committing progenitors. Since CD34 is often expressed in cycling cells, it marks ST-HSC and LCPs because these markers indicate an exit from quiescence (Mazzanti 2019).

FcγR is typically expressed on mature myeloid or B lymphoid lineage-restricted cells (Nimmerjahn and Ravetch 2006, Mazzanti 2019). Increasing FcγR expression also corresponds to further differentiation. LT-HSC don't express FcγR at all. LCPs (which are cKit+Sca1-) express FcyR.

Studying the literature for the windows of expression of these milestone stem-cell and differentiation hematopoietic markers is aligned with the objective of this project because it forms a baseline from which the phenotypic profile of the LIC can be explained. For example, if we find that the LIC is cKit+Sca1-CD150-, then we might ask if the leukemia-initiating cell's counterpart in a normal hierarchy is a progenitor or if the leukemia-initiating cell itself is a progenitor *(Fig 3).* Contextualizing the phenotype of the LIC with what has been covered in the

literature about the markers that make up this distinguishing phenotype allows us to ask more informed and targeted questions.

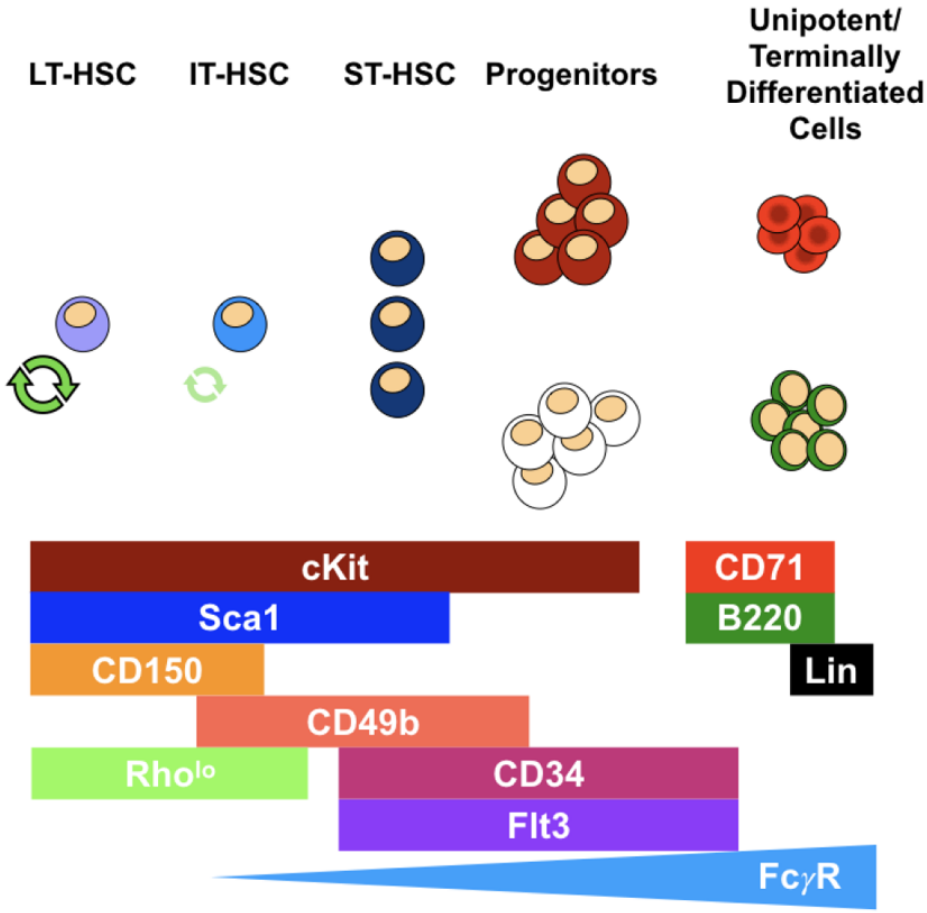


Figure 3. Expression of markers in primitive to mature stages of the hematopoietic hierarchy. (Mazzanti 2019)

2. Methodology

2a. Harvesting Leukemic HOXB4-reconstituted Marrow

The HOXB4-dependent leukemia described in part (1b) had been thawed or maintained in transplantation from one mouse to another long before I joined the lab for this project. For my project, I harvested leukemic cells from one of the leukemic HOXB4-reconstituted CD45.2:*Gpi*^{b} donor mice by flushing bone marrow (BM) from femurs and tibias using sort media then filtered through 40um cell strainer and lysed RBCs with ACK buffer so that only the WBCs remained. At this point, bone marrow is whole because it is not yet sorted into fractions.

2b. Lineage Marker Depletion

Then, whole BM was mixed with cocktail of biotinylated antibodies against lineage markers (CD5, CD45R (or B220), CD11b, Gr-1, 7-4, and Ter119), and then with Lin-biotin magnetic MicroBeads in the Miltenyi Lineage Cell Depletion Kit. Lin^{+} cells were discarded using the Miltenyi autoMACS Pro Separator, and Lin^{-} cells were then resuspended in sort media and stained with fluorophore-conjugated antibodies. This ensured that in the next step, only the lineage negative cells are sorted into fractions.

2c. Sorting and Gating Strategy

This lineage negative leukemic BM had been sorted in a binary or ternary fashion on BD FACSAriaII or III cell sorters into a population that expresses a specific marker and a population that does not –in the case of binary markers– and populations with low, medium, or high expression of a specific marker –in the case of ternary markers *(Fig 4)*. Both sorters were run by staff from the SickKids-UHN Flow and Mass Cytometry Facility. Sorted cells were suspended in a master mix of injection media and a radioprotective dose of 2 x10^6 normal non-leukemic BM. Then, the two binary or three ternary fractions were injected into two or three cohorts of CD45.1/*Gpi*^{a} recipient mice irradiated by 8Gys. For example, CD34+ cells were injected into a cohort of the mice and CD34- cells were injected into another cohort. All gating strategy used during each marker sort is shown in figures 5-8.

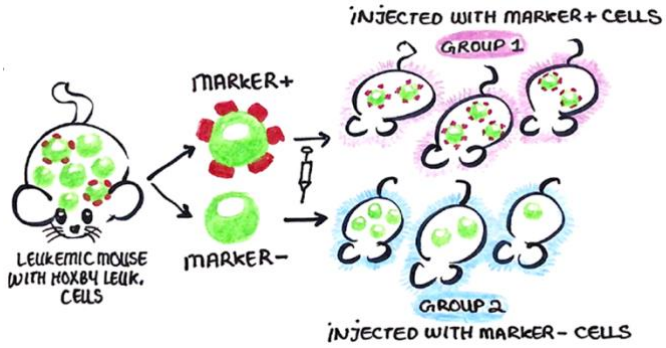
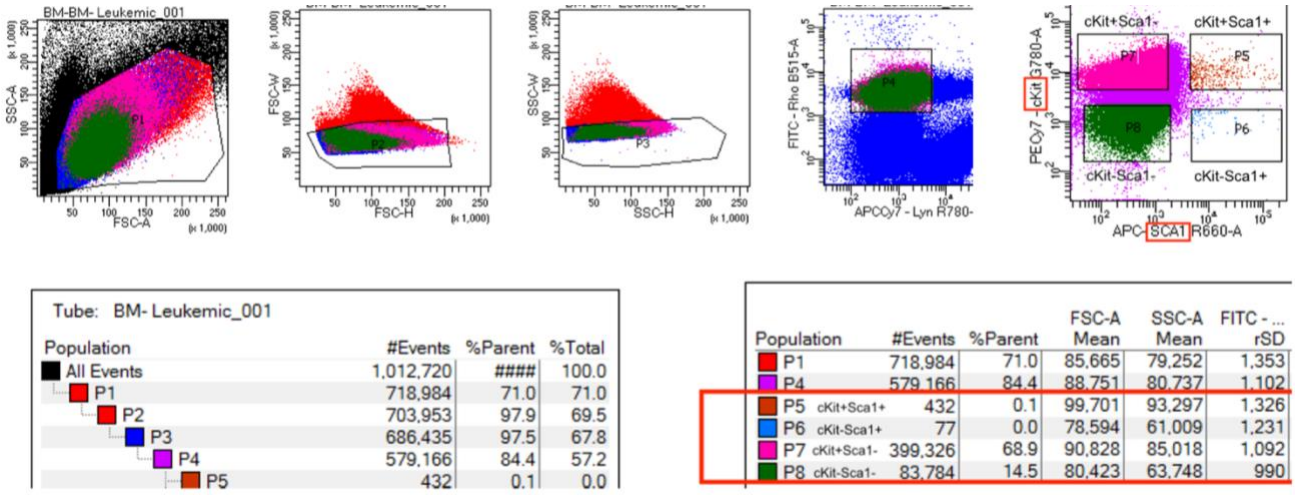


Figure 4. Leukemic HOXB4-reconstituted BM was harvested and sorted into marker fractions which were then transplanted into separate cohorts.

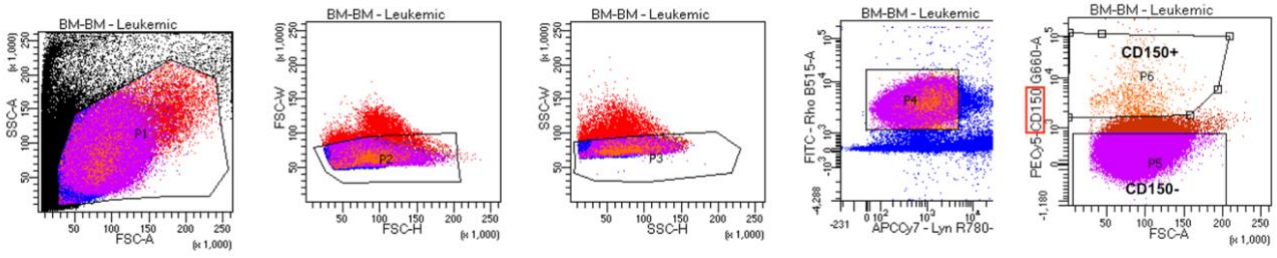


Tube: BM- Leukemic_001

Population	#Events	%Parent	%Total
All Events	1,012,720	####	100.0
P1	718,984	71.0	71.0
P2	703,953	97.9	69.5
P3	686,435	97.5	67.8
P4	579,166	84.4	57.2
P5	432	0.1	0.0

Population	#Events	%Parent	FSC-A Mean	SSC-A Mean	FITC - ... rSD
P1	718,984	71.0	85,665	79,252	1,353
P4	579,166	84.4	88,751	80,737	1,102
P5 cKit+Sca1+	432	0.1	99,701	93,297	1,326
P6 cKit-Sca1+	77	0.0	78,594	61,009	1,231
P7 cKit+Sca1-	399,326	68.9	90,828	85,018	1,092
P8 cKit-Sca1-	83,784	14.5	80,423	63,748	990

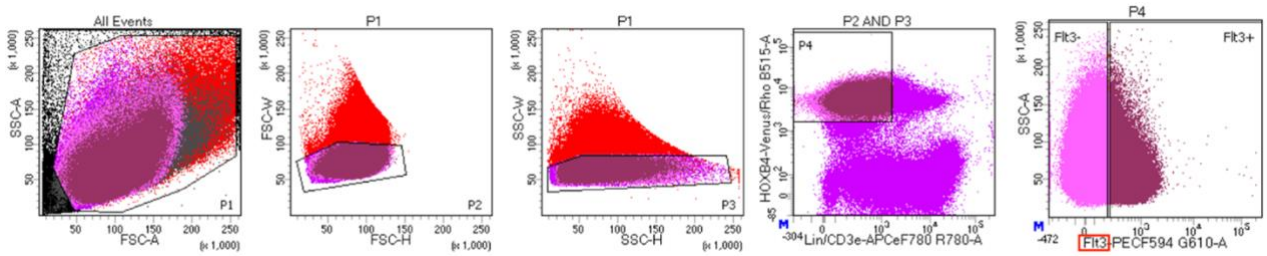
Figure 5. Gating Strategy for cKit and Sca1 Surface Markers*. P1 is to only gate the lymphocytes. P2 and P3 remove doublets and debris. P4 is to select only the lineage negative and VENUS+ cells. P5 gates the cKit+Sca1+ cells. P6 gates the cKit-Sca1+ cells. P7 gates the cKit+Sca1- cells. P8 gates the cKit-Sca1- cells.*



Tube: BM - Leukemic

Population		#Events	%Parent	%Total
All Events		719,257	####	100.0
P1		555,314	77.2	77.2
P2		550,556	99.1	76.5
P3		545,149	99.0	75.8
P4		471,135	86.4	65.5
P5	CD150-	450,947	95.7	62.7
P6	CD150+	993	0.2	0.1

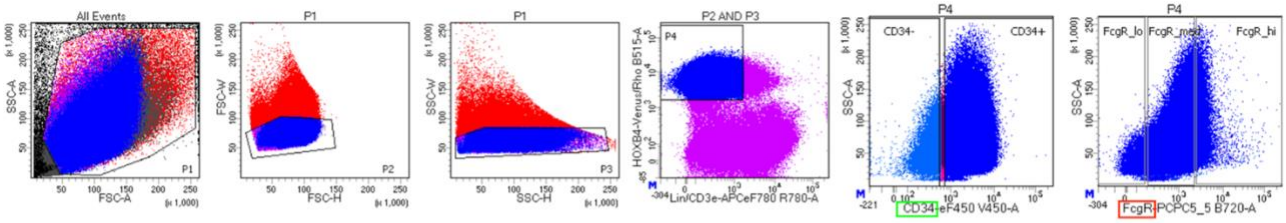
Figure 6. Gating Strategy for CD150 surface marker. *P1 is to only gate the lymphocytes. P2 and P3 remove doublets and debris. P4 is to select only the lineage negative and VENUS+ cells. P5 gates the cells NOT expressing CD150. P6 gates the cells expressing CD150.*



Tube: Leu_Flt3_sort

Population	#Events	%Parent	%Total
All Events	2,500,000	####	100.0
P1	2,355,221	94.2	94.2
P2	2,269,857	96.4	90.8
P3	2,256,100	95.8	90.2
P2 AND P3	2,241,309	95.2	89.7
P4	2,120,565	94.6	84.8
Flt3-	1,902,785	89.7	76.1
Flt3+	185,090	8.7	7.4

Figure 7. Gating Strategy for Flt3 Surface Marker. *P1 is to only gate the lymphocytes. P2 and P3 remove doublets and debris. P4 is to select only the lineage negative and VENUS+ cells. In the P4 gate, the Flt3- and Flt3+ cells are gated.*



Tube: Leu1_sort

Population	#Events	%Parent	%Total
All Events	1,839,936	####	100.0
P1	1,556,033	84.6	84.6
P2	1,521,707	97.8	82.7
P3	1,515,868	97.4	82.4
P2 AND P3	1,505,224	96.7	81.8
P4	1,334,527	88.7	72.5
FcgR_lo	16,773	1.3	0.9
FcgR_med	1,253,055	93.9	68.1
FcgR_hi	48,415	3.6	2.6
CD34-	58,728	4.4	3.2
CD34+	1,238,853	92.8	67.3

Figure 8. Gating Strategy for FcyR and CD34 Surface Marker. *P1 is to only gate the lymphocytes. P2 and P3 remove doublets and debris. P4 is to select only the lineage negative and VENUS+ cells. In the P4, the lineage negative and VENUS+ cells are gated. In P4, the three FcyR fractions are gated in a FcyR vs SSC plot. Also in P4, binary CD34 fractions are gated in a CD34 vs SSC plot.*

2d. Blood Analysis

The mice were bled first after 10-12 days since injections and every 3-6 days since then to collect data points of how leukemic the mice become over time. A full 75-150uL capillary of peripheral blood was collected from saphenous veins of mice and suspended in 3mL of FACS Buffer. The cells were spun, lysed with ACK buffer for 5 minutes, and resuspended in FACS buffer. Cells were then filtered through a falcon 5ml polystyrene round-bottom tube with cell-strainer cap and resuspended in FACS. An example of the gating strategy used in blood analysis for a mouse which had been injected with CD34+ fraction is shown in figure 9.

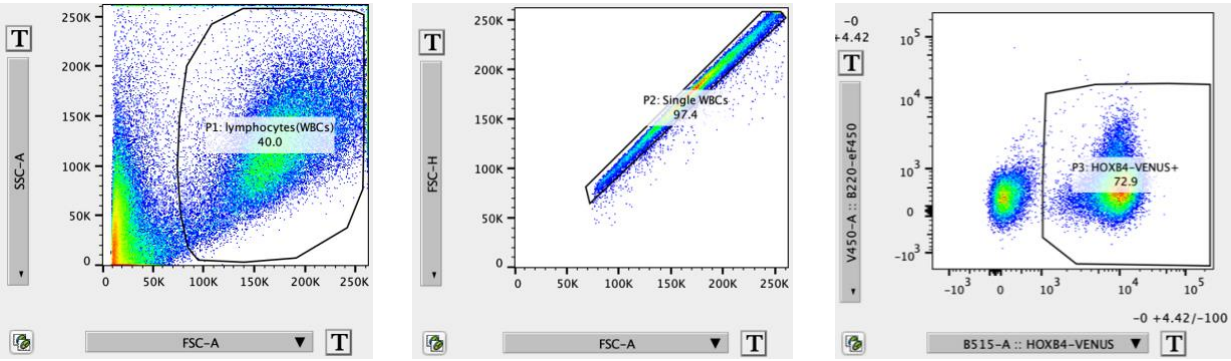


Figure 9. P1 selects only the WBCs, leaving the RBCs that weren't lysed by ACK in the lower left corner. P2 gates only the SINGLE WBCs, excluding out doublets or debris. P3 gates the VENUS+ population out of the single WBCs only. In this case, it doesn't matter what is plotted as the second parameter against the B515-A: HOXB4-VENUS because in blood analysis of the WBCs, we are only looking at one parameter: the presence or absence of the HOXB4-transgene in each cell (visually represented as the presence or absence of VENUS).

The group that becomes leukemic will show a VENUS+ leukemic population like in the right panel of figure 9. This VENUS+ population indicates that the HOXB4-transduced leukemia donor cells had expanded to detectable numbers in the recipient since injection. This detectability of a VENUS+ population reflects the transfer of leukemia-initiating cells. For example, presence of functional LICs in the CD34+ fraction would indicate that LICs express the CD34 antigen. Considering that the recipient mouse itself doesn't inherently circulate VENUS+ cells, any increasing VENUS+% in the recipient indicates leukemic reconstitution from the injected HOXB4-transduced donor cells. Only the LICs can divide into clones like this and sustain them. Therefore, increasing VENUS+% indicate the presence of LICs. This process of harvesting BM, depleting lineage markers, sorting, and analyzing blood had been repeated for each of the six markers: cKit, Sca1, CD150, Flt3, CD34, and FcyR.

Results

The numbers of the LICs sorted into each marker fraction were estimated to determine which fractions contain the majority of the LICs. The mice developed leukemia at different speeds. That is, the rate at which this leukemia developed differed among the separate fractions.

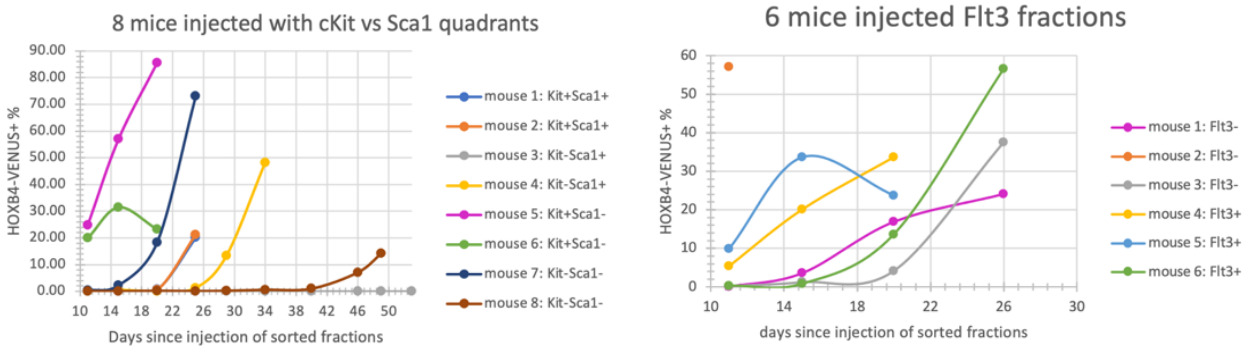


Figure 10. VENUS+ percentages are recorded over the number of days since injections of the sorted fractions to track rate at which the mice become leukemic.

Using these different rates at which the leukemia developed, an estimate of how many LICs were sorted into each fraction was calculated. One of the Iscove lab findings that helps serve as a baseline for this calculation is that injecting only one leukemia-initiating cell into a recipient mouse makes it 50% VENUS+ by day 30-33 since injection. For simplicity, the method of calculation is shown in figure 11 using only four mice which had been injected with four different fractions.

A

	11			15			20			25			26			29			34		
fraction	%	%/(100-%)	ln[%/(100-%)]	%	%/(100-%)	ln[%/(100-%)]	%	%/(100-%)	ln[%/(100-%)]	%	%/(100-%)	ln[%/(100-%)]	%	%/(100-%)	ln[%/(100-%)]	%	%/(100-%)	ln[%/(100-%)]	%	%/(100-%)	ln[%/(100-%)]
Kit-Sca1+	-	-	-	-	-	-	-	-	-	1.26	0.0	-4.4	-	-	-	13.4	0.2	-1.9	48.2	0.9	-0.1
Kit+Sca1-	24.7	0.3	-1.1	57.0	1.3	0.3	85.5	5.9	1.8	-	-	-	-	-	-	-	-	-	-	-	-
Kit-Sca1-	0.4	0.0	-5.5	2.2	0.0	-3.8	18.3	0.2	-1.5	73.1	2.7	1.0	-	-	-	-	-	-	-	-	-
Flt3-	0.2	0.0	-6.2	1.2	0.0	-4.4	4.2	0.0	-3.1	-	-	-	37.5	0.6	-0.5	-	-	-	-	-	-

B

	from plot A slope	td
baseline	0.47	1.47
Kit+Sca1-	0.32	2.17
Kit-Sca1-	0.47	1.49
Flt3-	0.37	1.88
mean	0.41	1.75

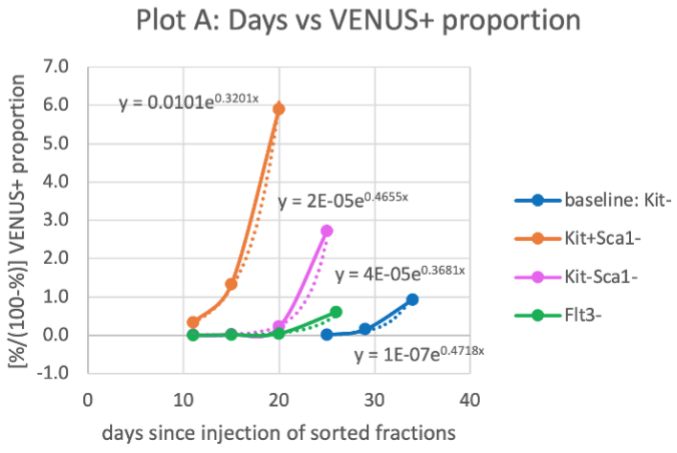
C

td= 0.6931/slope
ln(b)=y(mean ln) - (slopemean*xmean)
x(1)=(ln(1)-lnb)/meanslope
doublings=offset from baseline/meanTd
offset from baseline: baseline x(1) - fraction x(1)
[x(mean), y(exp ln mean)]: 1st point in imposed slopes
[x(1), 1]: 2nd point in imposed slopes

D

x(mean)	y(mean ln)	y(exp ln mean)	ln(b)	x(1)	y=1	offset from baseline	doublings	LSCs injected: 2^doublings
29.33	-2.1	0.12	-14.02	34.50	1	0.00	0	1
15.33	0.3	1.37	-5.92	14.56	1	19.94	11.38	2672.504472
17.75	-2.5	0.09	-9.67	23.78	1	10.72	6.12	69.46936653
18.00	-3.6	0.03	-10.88	26.77	1	7.73	4.41	21.28163703

E



F

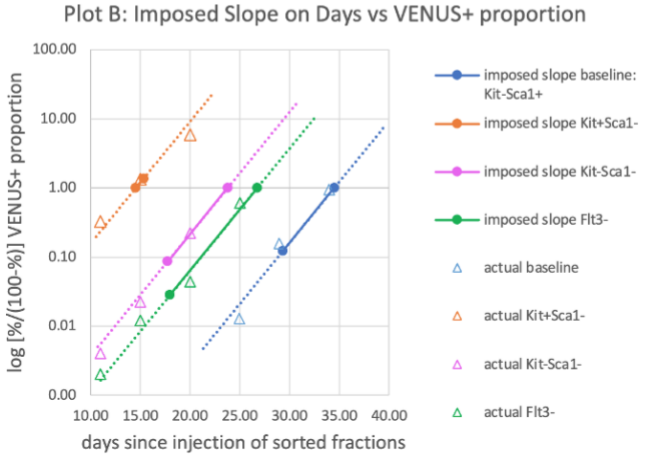


Figure 11. Steps to calculate the LICs per mouse using the baseline that 1 LIC injected into a mouse makes it 50% VENUS-HOXB4+ by day 30. Examples of four mice injected with three different fractions are shown here. In **A&E** *%/(100-%) was plotted instead of just % because %/(100-%) tracks the VENUS+% in a linear, direct fashion. The last column in* **D** *represents the number of LICs injected in each of the mice in the labelled fractions.* **C** *is a list corresponding to the formulas used in* ***A*** *and* ***B***. **E** *is the plot of days vs units of VENUS+. The slopes in the first column of B are derived from this plot.* **F** *is a plot in which the average slope was imposed to easily compare other mice to the baseline mouse. The distance difference between a fraction and the baseline at the x(1) intercept is the "offset from baseline" column in* ***D*** *that allows us to estimate the LICs injected per fraction.*

After calculating the raw LIC numbers per fraction, I converted them into percentages of the total LICs associated with one marker. For example, out of the total LICs in the cKit marker fractions, 98.3% were in the cKit+ fraction and 1.7% were in the cKit- fraction. Continuing with the rest of the binary markers, most of the LICs were sorted into the Sca1- fraction, the CD150- fraction, the Flt3- fraction, and the CD34+ fraction.

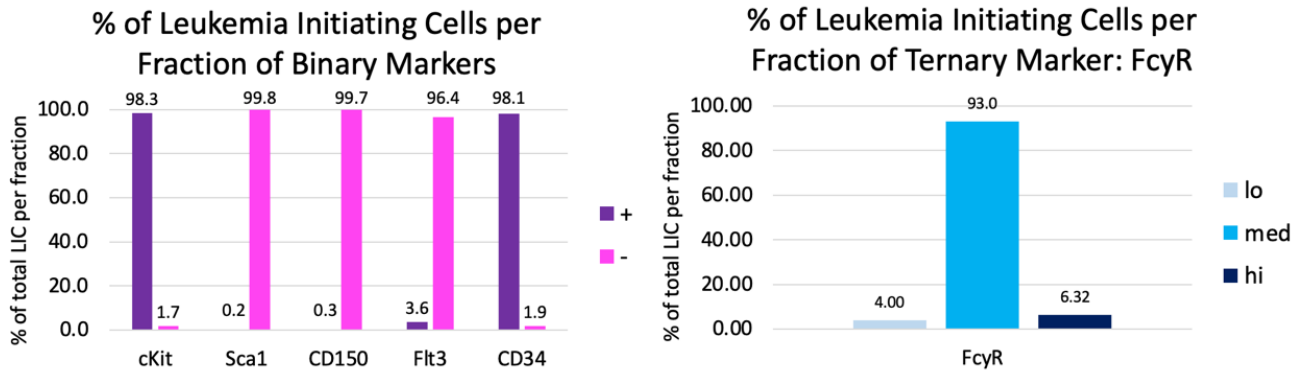
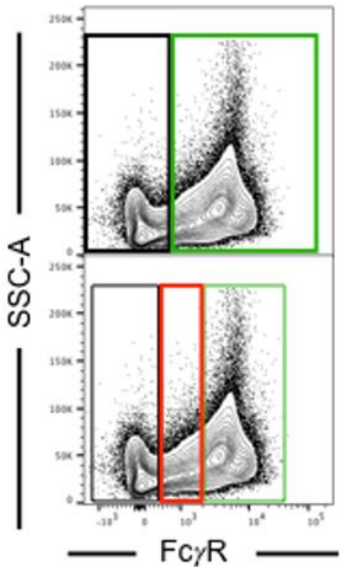


Figure 12. Percentages of how many LICs per fraction in one marker.



For FcyR, instead of a binary sort, a ternary sort was performed. This is because the Iscove lab found that FcyR expression falls over three distinct populations (FcyRhi, FcyRmed, FcyRlo) rather than two (Mazzanti 2019). Out of the three FcyR marker fractions, 93% of the LICs were in the FcyRmed population.

Figure 13. ***Comparison of a binary sort of FcyR and a ternary FcyR sort****. After a ternary sort, reconstitution in some lineages previously attributed to the broad FcyR+ fraction (which grouped FcyRmed and FcyRhi) proved to be coming from specifically the FcyRmed population. (Mazzanti 2019).*

The binary or ternary sort of individual markers and the calculations that indicate which fraction enriched for the majority of the LICs suggest a tentative compound phenotype that distinguishes the leukemia initiating cells from the inert leukemia blasts. The distinguishing phenotype based on the six markers sorted for in this project is cKit+, Sca1-, CD150-, Flt3-, CD34+, FcyRmed.

Discussion

The purpose of this project is to distinguish the leukemia-initiating cells from the inert leukemia blasts. Because the LICs are the minority in the leukemia population, markers that show a minority population with a significantly different level of expression than the majority are good candidates for distinguishing LICs from blasts. In FlowJo, this corresponds to a marker where the VENUS+ leukemia cell population shows heterogeneity. Heterogeneity would not yield a condensed single population of events but a bimodal continuum where some cells are clearly + and others are – for a certain parameter. Ideally, heterogeneity would manifest in two distinct populations, one of which often shows a minor population. An example of a parameter that shows heterogeneity is the VENUS parameter on the y-axis in figure 14C because it shows two distinct populations vertically spread over the plot. If the marker is one that significantly distinguishes LICs from blasts, the marker must fulfill two criteria: first, it would yield two distinct populations and second, the LICs would end up in the minor population

In this experiment, the characteristic markers that appear in hematopoietic stem cell literature were sorted for. The only markers that expressed some heterogeneity are the cKit and Sca1 cells in figure 14E. However, neither of them fulfilled the second criterion of becoming a distinguishing marker: enriching for the LICs in the minority population. From panel 14E, cKit- is the minority population, but as per figure 12, most of the LICs ended up in the cKit+ fraction, so most of the LICs are still not significantly isolated from the majority population. Similarly, the minor population in panel E is Sca1+, but figure 12 shows that most LICs fell into the Sca1- fraction, so Sca1 didn't significantly distinguish the LICs from the majority population. The rest of the markers sorted for –Flt3, CD34, CD150, and FcyR– do not even show heterogeneity but exhibit a condensed continuum of events that don't show distinct or bimodal negative and positive populations.

Given that our compound distinguishing phenotype is cKit+, Sca1-, CD150-, Flt3-, CD34+, FcyRmed, the level of marker expression of the leukemia-initiating cells always tracks that of the majority population. For example, cKit+Sca1- is the majority population in panel 14E, and the LICs also fall in the cKit+Sca1- quadrant. Similarly, the majority population in figure 14D is Flt3-, which is also the fraction that enriches for LICs. Again, the majority populations are CD34+, CD150-, and FcyRmed, all of which also correspond to the phenotype of LICs shown in Figure 12.

In figure 14, the population cKit+, Sca1-, CD150-, Flt3-, CD34+, FcyRmed corresponds to the gate labelled "FcyRmed" in panel H. The percentage of the remaining population from the starting total after all the LIC compound phenotype filters have been added yields 24.7%. This percentage signifies the extent to which this compound phenotype purifies the leukemia initiating cells from the blasts.

Future Directions

Given that the LICs have the majority phenotype for all six markers despite their minor proportion in the leukemia cell population, the markers sorted for didn't significantly distinguish the LICs. Further refinement of the heterogeneous markers by means of conducting further

fractionation of the subset in which the majority of the LICs were transplanted might be useful. For example, resorting for cKit, but gating only the cKit expressing cells and dividing them into three further refined gates as in figure 15 might help identify which level of expression in the cKit+ fraction characterizes the LICs.

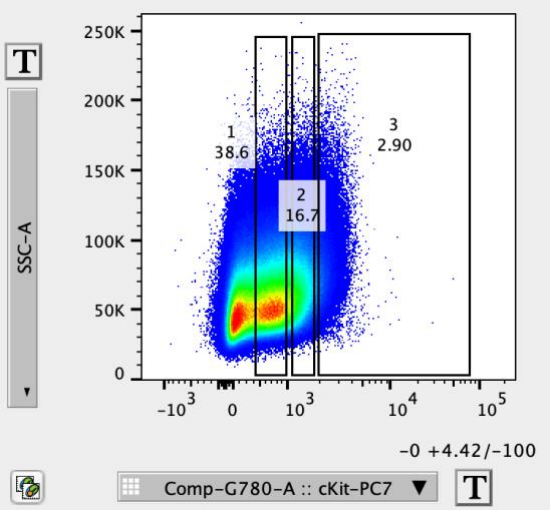
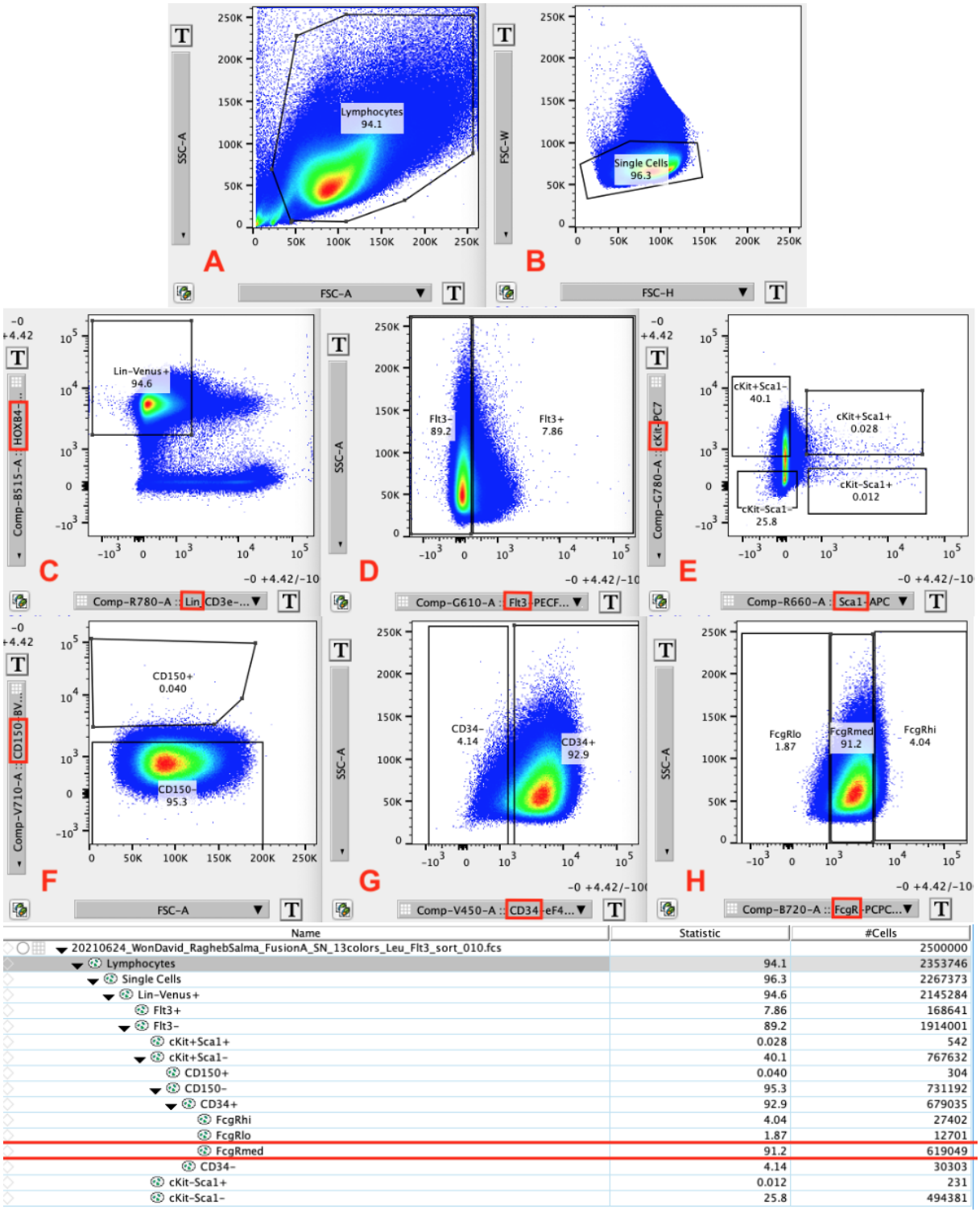


Figure 15. Refining the cKit+ population into three different subsets with progressively increasing cKit expression. Gates 1 to 3 combined had been the cKit+ gate in the binary sort for cKit.

In terms of sorting for other markers, since this HOXB4-dependent model is an acute myeloid leukemia, our target is to look at myeloid-associated markers. Both Gr-1 and CD11b mark myeloid subsets (Bronte et al. 2016). Also, spleen myeloid populations exhibit high CD11b expression (Hey et al. 2016). Because of their association with isolating for myeloid cells, it is worth sorting for them to see how they mark the components of the myeloid leukemia population. Also, CD123 (or IL-3R) is expressed in malignant populations of AML and other cancers and has been studied as a target marker in potential treatment for hematological cancers. The literature covers that CD123 characterizes both leukemic blasts and leukemia-initiating cells (Testa et al. 2019, Naik et al. 2019). It is worth investigating if the levels of expression might differ between blasts and LICs in this m-AML1 HOXB4-dependent model. Finally, it has been demonstrated that the $CD34^{+}$ $CD38^{-}$ phenotype enriches for most of the leukemia-initiating cells in AML samples. Because of the association of CD38 with a potential distinguishing marker of LICs, it is also worth sorting for.

Isolating the LICs further from the inert blasts will allow us to study what differentiates the cells that reconstitute the leukemia from those that are inert even though they both make up the leukemia population. How might the phenotypic profiles of each of them contribute to their different roles in maintaining leukemic clones? Further, the characterization of LICs will allow comparison between the LIC phenotype and the phenotypes of the different stages across the cell hierarchy of the normal hematopoietic system. Identifying a cell stage in the normal hematopoietic hierarchy with which the phenotype of the LIC aligns opens the opportunity to compare the transcriptome and epigenetic profiles of both the LICs and the corresponding normal cell. What's different between the transcriptomes of the immortal LICs and the regular counterpart cell? These questions which imply a deeper comparison at the level of genetic expression require that we first identify a normal cell stage to which the LIC is phenotypically similar enough; this requires the purification and characterization of the LIC.



Name	Statistic	#Cells
20210624_WonDavid_RaghebSalma_FusionA_SN_13colors_Leu_Flt3_sort_010.fcs		2500000
Lymphocytes	94.1	2353746
Single Cells	96.3	2267373
Lin-Venus+	94.6	2145284
Flt3+	7.86	168641
Flt3-	89.2	1914001
cKit+Sca1+	0.028	542
cKit+Sca1-	40.1	767632
CD150+	0.040	304
CD150-	95.3	731192
CD34+	92.9	679035
FcgRhi	4.04	27402
FcgRlo	1.87	12701
FcgRmed	91.2	619049
CD34-	4.14	30303
cKit-Sca1+	0.012	231
cKit-Sca1-	25.8	494381

Figure 14. After the data of the sorts had been recorded, I recreated on FlowJo all the gates of the compound phenotype: cKit+, Sca1-, CD150-, Flt3-, CD34+, and FcyRmed. In panel C, only the selected population proceeded to panel D. In panel D only the Flt3- gate proceeds to panel E because based on figure 12, the phenotype of LICs is Flt3-. All the events in panel E are Flt3-. All the events in F are cKit+Sca1-. Finally, in panel H, only the FcyRmed gate shows the cells that are cKit+, Sca1-, Flt3-, CD34+, CD150-, FcyRmed – the compound phenotype for LICs.

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