

Association of race/ethnicity with innate immune tumor microenvironment of children with B-acute lymphoblastic leukemia

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ABSTRACT

Background Black and Hispanic children with B-acute lymphoblastic leukemia (B-ALL) experience worse outcomes compared with their non-Hispanic white (NHW) counterparts. Immune-based approaches have begun to transform the therapeutic landscape in children with B-ALL. Recent studies identified several alterations in both innate and adaptive immune cells in children with B-ALL that may impact disease risk and outcome. However, the impact of racial/ethnic background on immune microenvironment is less studied, as children of minorities background have to date been severely under-represented in such studies.

Methods We performed high-dimensional analysis of bone marrow from 85 children with newly diagnosed B-ALL (Hispanic=29, black=18, NHW=38) using mass cytometry with 40 and 38-marker panels.

Results Race/ethnicity-associated differences were most prominent in the innate immune compartment. Hispanic patients had significantly increased proportion of distinct mature CD57 +T-bet+DR+ NK cells compared with other cohorts. These differences were most apparent within standard risk (SR) patients with Hispanic SR patients having greater numbers of CD57 +NK cells compared with other cohorts (43% vs 26% p=0.0049). Hispanic and Black children also had distinct alterations in myeloid cells, with a significant increase in a population of non-classical activated HLA-DR +CD16+myeloid cells, previously implicated in disease progression, compared with NHW counterparts. Racial background also correlated with altered expression of inhibitory checkpoint PD-L1 on myeloid cells.

Conclusion There are surprisingly substantial race/ethnicity-based differences in innate immune cells of children with newly diagnosed B-ALL. These differences urge the need to enhance accrual of children from minorities background in immunotherapy trials and may impact their outcome following such therapy.

INTRODUCTION

B-acute lymphoblastic leukemia (B-ALL) is a leading cause of cancer in children.¹ Prior studies have shown that racial and ethnic background has strong association with survival of children with B-ALL, with worse

outcomes in Hispanic (H) and black (B) children compared with their non-Hispanic white (NHW) counterparts.²⁻³ Several factors such as socioeconomic status, access to healthcare, treatment compliance, differences in tumor genetics, response to chemotherapy, genetic polymorphisms, and tumor genetics have been studied to understand these differences.⁴⁻¹² However, there remains an unmet need to better understand the potential biological associations of racial/ethnic background and address these disparities.

Immune-based approaches have begun to transform the therapeutic landscape in childhood cancer including B-ALL.¹³ Recent studies have identified several alterations in the immune microenvironment of children with B-ALL.¹⁴⁻¹⁵ These differences include changes in both T cells (such as loss of naïve T cells and increase in terminal effector T cells), and innate immune cells, such as NK and myeloid cells.¹⁴⁻¹⁵ Some of these differences have been correlated with underlying disease risk and outcome in children with B-ALL.¹⁴⁻¹⁶⁻¹⁷ Properties of the immune microenvironment are also expected to impact outcomes with the emerging application of immune-based therapies in these patients. However, children from non-white background have been under-represented in prior analyses of immune microenvironment and clinical trials testing novel immune therapies in B-ALL.¹⁸ Whether race/ethnicity impacts the properties of immune microenvironment in these children may benefit from application of high-dimensional single-cell approaches.

METHODS

Patients and samples

Diagnostic bone marrow specimens from children with B-ALL were provided by the Children's Healthcare of Atlanta Pediatric Biorepository (patient characteristics in online supplemental table 1) under an institutional IRB-approved protocol. Written informed consent or patient assent and consent from the parent/legal guardian were obtained before collection of samples, in accordance with the Declaration of Helsinki. Clinical patient characteristics including race, ethnicity and NCI risk status were obtained from institutional database. An additional cohort of biospecimens (n=17) was obtained from Children's Oncology Group (COG) biobank. Selection of samples analyzed in this study was based on availability of adequate diagnosis marrow in the institutional biorepository, collected between 2016 and 2019, focusing initially on all patients with racial/ethnic minority with available biospecimens and then inclusion of specimens from NHW children randomly selected and matched for patient characteristics including NCI risk status. The racial/ethnic diversity of the overall study population (20% black children) is representative of that seen in our clinics. The information available on specimens from COG biobank was limited to age, gender, race, NCI risk status and ETV-RUNX1 status.

Mass cytometry

Thawed mononuclear cells were stained with 40-marker and 38-marker panels using metal-conjugated antibodies at manufacturer-suggested concentrations (Fluidigm). For all newly conjugated antibodies, titrations were performed to determine the optimal concentration of antibody to be used for the studies. Details on the panels and specific antibodies/clones are provided in online supplemental table 2. Briefly, after staining for surface markers, the cells were fixed, permeabilized, and washed. After staining for intracellular markers, the cells were incubated with intercalation solution, mixed with EQ Four Element Calibration Beads, and acquired with a Helios mass cytometer. Cisplatin was used to stain non-viable cells and DNA content identified using iridium-labeled intercalator. To minimize batch effects, the same lot of antibody was used for all samples and samples from different groups (B, H, NHW) were thawed together and stained with the same antibody cocktail.

Data analysis

Data from mass cytometry were analyzed with CYTOBANK software using both manual gating as well as algorithms such as viSNE, based on Hispanic, NHW, and black categorization as well as NCI risk status (standard risk—SR, high risk—HR). After gating of singlet viable events with DNA intercalator uptake, individual clusters of cell types within individual patients were identified. Clusters were contrasted between patients to identify subsets of patients with concordant profiles of immune cells. Data were also pooled to create concatenated

files from the marrow cells of patients in each of the racial/ethnic categories, using equal numbers of cells from each patient. Statistical analysis of mass cytometry data was performed using two-dimensional graphing and statistics software GraphPad Prism.

RESULTS AND DISCUSSION

Bone marrow mononuclear cells from 85 patients (H=29, NHW=38, B=18) with newly diagnosed B-ALL prior to initiation of chemotherapy were stained and characterized by mass cytometry (patient characteristics in online supplemental table 1). There were no significant differences in patient characteristics between the racial/ethnic groups. Tumor cells were gated out as CD45^{dim}CD19⁺CD10⁺ to evaluate the non-tumor compartment in the marrow. There were no significant differences in the overall proportion of T cells, NK cells or myeloid cells between ethnic/racial groups (online supplemental figure 1).

Differences in NK cells

ViSNE clustering of NK cells (defined within the non-tumor compartment by CD45^{hi}CD3⁺CD14⁺CD19⁺CD56⁺ events) identified three distinct NK populations (figure 1A). These populations were defined by their surface and cytosolic immune markers as (1) immature NK cells (CD56^{hi}CD16⁺CD57⁺), (2) early cytotoxic NK cells (CD56^{dim}CD16⁺CD57⁺Granzyme⁺), and (3) mature CD57⁺NK cells (CD56^{dim}CD16⁺CD57⁺granzyme⁺) (figure 1B). The immature NK cells expressed higher levels of NKG2D and CD27 as previously described¹⁹ while CD57⁺NK cells expressed higher amounts of Tbet and HLADR in addition to granzymeB and lacked expression of CD27.¹⁹ Differences in NK cell subsets were most evident in the Hispanic group. Hispanic patients had higher proportions of CD57⁺ mature NK cells when compared with other groups, (42%±3% vs 33±2%; p=0.006) (figure 1C). These differences were most apparent within SR patients with Hispanic SR patients having greater numbers of CD57⁺NK cells compared with B and NHW SR patients (mean H SR 44±5% vs 25±2% p=0.001) (figure 1D–E).

Differences in myeloid cells

The myeloid cell compartment was defined within the non-tumor compartment by CD45^{hi}CD3⁺CD56⁺ cells that were positive for either CD11c or CD33. ViSNE clustering of myeloid cells identified five distinct clusters (termed M1–M5) based on patterns of cell surface markers (figure 2A). This included a CD14⁺CD16⁺classical monocyte population (M3), CD11c⁺DR^{low} population (M4), CD33⁺C-kit⁺population (M2), CD11b⁺/11c⁺CD15⁺population (M5) and a CD11c⁺CD16⁺DR^{hi} inflammatory/non classical myeloid population (M1) (figure 2B). M1 myeloid population also expressed higher levels of PD-L1 as well as HLA-E (figure 2B). Further analysis showed that NHW

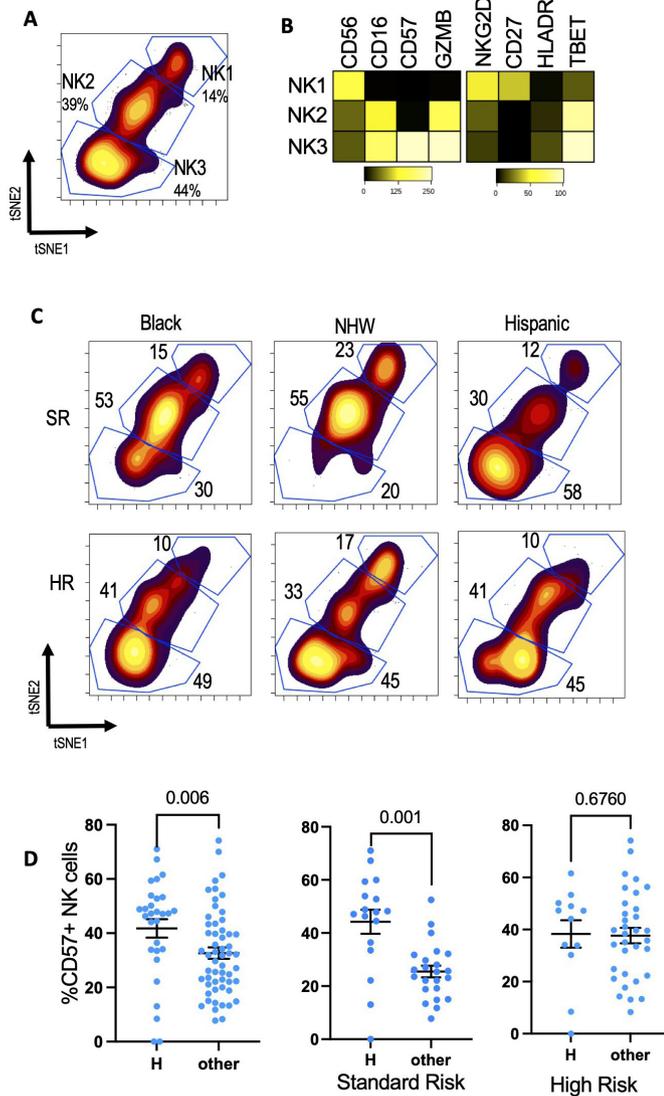


Figure 1 Changes in NK cells bone marrow mononuclear cells obtained at diagnosis (Hispanic=29, black=18, NHW=38) were stained with metal conjugated antibodies and data acquired using Helios mass cytometry machine. The data were normalized using the calibration beads and analysis performed using Cytobank software and VISNE algorithm. Concatenated files with equal numbers of cells from individual samples were used to visualize the data. **(A)** Density plot of NK cells from H, B and NHW patients showing three distinct subsets of NK cells (NK1; CD56^{hi}CD16⁻CD57⁻GZMB⁻), NK2; CD56^{Dim}CD16⁺CD57⁻GZMB⁺ and NK3; CD56^{Dim}CD16⁺CD57⁺GZMB⁺). Numbers denote percentages of NK cells in the specific cluster. **(B)** Heatmap showing expression of various markers in the NK subsets. **(C)** Density plot showing distribution of NK cells within the 3 distinct clusters by race/ethnicity and disease risk (standard risk; SR high risk; HR). Numbers in the plot denote percentages of NK cells in the specific cluster. **(D)** Dot plot showing CD57+NK cells as percent of total NK cells in all Hispanic patients compared to black and NHW(others) as well as in SR and HR Hispanic as compared to SR and HR blacks and NHW(others). Figure shows mean and standard error of the mean. Figure shows mean and SE of the mean, B, black; H, Hispanic; NHW, non-Hispanic white.

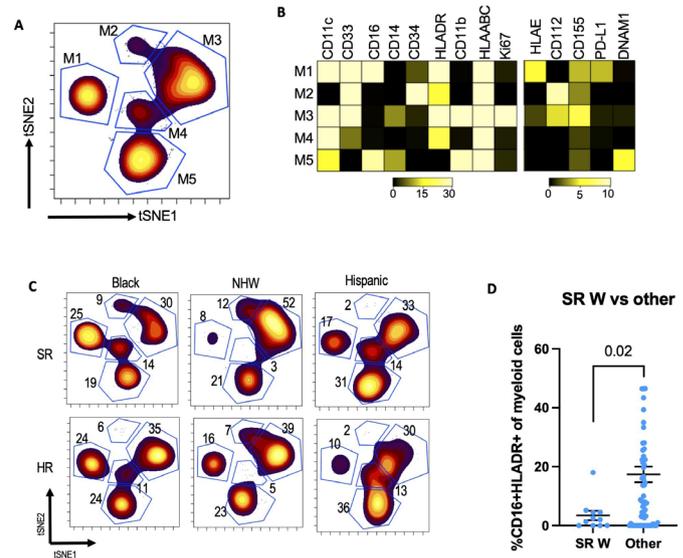


Figure 2 Changes in myeloid cells bone marrow mononuclear cells (Hispanic=29, black=18, NHW=38) were analyzed using single cell mass cytometry or CyTOF as described above. Figure shows VISNE visualization of the myeloid cells. **(A)** Density plot of myeloid cells from H, B and NHW patients showing five distinct subsets of myeloid cells (M1–M5). **(B)** Heat map shows expression of different markers that help distinguish five distinct myeloid clusters (M1; 16+DR^{hi} non classical/inflammatory myeloid cells; M2 34+immature/progenitor myeloid cells, M3. CD14+classical monocytes, M4 CD11C+16- myeloid cells and M5 CD11C+CD11B+16+DR- myeloid cells). **(C)** Density plot showing proportion of myeloid cells by race/ethnicity and risk. Numbers denote percentages of myeloid cells in the specific cluster. **(D)** Dot plot showing proportions of CD16+DR+ myeloid cells as percent of total myeloid cells in individual SR white patients compared with all others. Figure shows mean and SE of the mean. B, black; H, Hispanic; HR, high risk; NHW, non-Hispanic white; SR, standard risk.

SR patients have significantly lower proportions of CD16+DR+ myeloid cells (M1) compared with other groups (mean NHW SR 3±2% vs Others 17%±3% p=0.02) (figure 2C–D).

Differences in T cells

ViSNE analysis of T cells revealed four CD8 and two CD4 clusters progressing from CCR7/CD45RA+naïve cells to CD57+CD45RO-RA+terminally differentiated cells (online supplemental figure 2A, B). These subsets revealed expected changes in transcription factors and surface/cytosolic markers (online supplemental figure 2B), but in contrast to findings with innate immune cells, these clusters were comparable between racial/ethnic groups (online supplemental figure 2C).

Differences in immune checkpoints, transcription factors and co-stimulatory genes

Both CD4+ and CD8+ T cells expressed PD-1, TIGIT as well as LAG-3 as major inhibitory immune checkpoints, while TIGIT was the dominant inhibitory checkpoint on NK cells and molecules in the TIGIT axis (CD112, CD155)



were also prominent on myeloid cells (online supplemental figure 3). The expression of inhibitory checkpoints on CD4/CD8+T cells was comparable between racial/ethnic (R/E) cohorts (online supplemental figure 4). CD4/CD8+T cells also did not differ in terms of costimulatory molecules (CD27, CD28, 4-1BB) and key transcription factors (TCF1, T-bet, Eomes) (online supplemental figures 5 and 6). While checkpoints (LAG3, PD-1, TIGIT and DNAM-1) were comparably expressed on NK cells from different R/E cohorts (online supplemental figure 7A), NK cells from Hispanic patients expressed lower proportion of NKG2D, consistent with lower proportion of immature NK cells in this cohort¹⁹ (online supplemental figure 7B). Myeloid cells showed comparable expression of molecules on TIGIT axis and HLA-E in different R/E cohorts (online supplemental figure 8). Myeloid cells from SR-NHW cohort however had lower expression of PD-L1, consistent with prior data showing lower proportion of inflammatory myeloid cells in this cohort (online supplemental figure 9). Together, these data suggest that the prominent differences based on race/ethnicity are in the innate immune system.

These studies use a high-dimensional single cell proteomic analysis to identify several race/ethnicity-based differences in the immune microenvironment in children with B-ALL. Surprisingly, major race/ethnicity-based differences were found in the innate immune marrow non-tumor compartment. These differences are of interest as both NK and myeloid cells have been implicated in the immune-pathogenesis of B-ALL and therapies specifically targeting innate immunity are now entering the clinic.^{16 17} These differences may therefore not only contribute to poor disease-free survival in Hispanic and Black children, but also impact outcomes following treatment with emerging immune therapies.

In recent studies, alterations in NK cells have been linked to worse outcome in pediatric B-ALL.^{16 20} Single cell sequencing studies have also characterized altered transcriptional profiles in NK cells in B-ALL consistent with NK dysfunction.¹⁴ However, these initial studies were based on B-ALL samples from the Children's Oncology Group tissue bank and samples from non-white racial/ethnic groups were underrepresented. The finding that Hispanic children had increased proportion of CD57 +mature NK cells is therefore of interest. This is a distinct subset of human NK cells with lower proliferative potential and decreased ability to secrete interferon gamma, but higher cytolytic function, that may expand in the setting of chronic viral infections.¹⁹ The mechanism underlying differences in NK differentiation in Hispanic children needs further study but may be related in part to differences in killer inhibitory receptor genotypes or environmental exposures in these children.²¹ In prior studies, changes in NK cells were more prominent in children with AML than in B-ALL.¹⁴ Therefore, it would also be of interest to evaluate if the race/ethnicity-associated differences in NK cells that we observed in B-ALL are also present in children with AML. Race/ethnicity related

differences in NK biology may also have clear implications for the emerging application of NK-based therapies in leukemia.

These data have several implications for testing novel immune approaches to treat B-ALL in children. The finding that TIGIT and LAG-3 are prominently expressed by immune cells in children with B-ALL suggest that further studies to better understand the role that these clinically targetable immune checkpoint molecules play in the leukemic microenvironment should be explored. Combination studies involving TIGIT and LAG-3 blockade have recently shown promise in adult cancer and should be formally tested in pediatric leukemia.²² Our finding that black and Hispanic children have an increase in distinct populations of non-classical activated myeloid cells is also of interest, as this specific population was recently linked to leukemic progression in both patient specimens and preclinical models.¹⁷ In preclinical models, macrophage-targeted approaches led to improved outcome.¹⁷ Therefore, strategies targeting the myeloid compartment may be particularly useful in Hispanic or black children. NK-based immune therapies have already shown promise in adult cancers and are rapidly moving to the clinic.²³ Our data suggest that racial/ethnic differences in NK biology may impact the translation of these strategies in the clinic, including in children.

Strengths of the current study are evaluation of specimens from patients with racially diverse representation obtained from a well-characterized biorepository and use of high-dimensional approaches validated in prior studies.^{14 24 25} Weaknesses include a relatively small sample size, although to our knowledge, this dataset represents one of the largest cohorts of non-white children analyzed at diagnosis of B-ALL with high-dimensional approaches. In prior studies, we have shown that risk status has a major impact on immune microenvironment.¹⁴ While the clinical features of different racial/ethnic cohorts were comparable, there could be yet unknown differences in cancer genetics in these cohorts. Nonetheless, these data need to be confirmed in another independent dataset, and as such should be viewed as hypothesis generating. Together, our novel finding is that there are surprisingly substantial differences in the innate immune compartment in Hispanic and black children with B-ALL. These findings implore the urgent and unmet need to include higher proportions of these children in current studies of immune microenvironment and emerging immune therapies, particularly those that specifically target innate immunity.

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Contributors JRG designed and performed experiments, analyzed data and wrote the manuscript, RR and DBD performed experiments and analyzed data, HSS and

SMC helped design study, collected patient material and edited the manuscript, DBD collected patient material and edited manuscript, KD designed and supervised the study, analyzed data and wrote the manuscript.

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Competing interests None declared.

Patient consent for publication Not applicable.

Ethics approval Diagnostic bone marrow specimens from children with B-ALL were provided by the Children's Healthcare of Atlanta Pediatric Biorepository under an institutional IRB-approved protocol. Written informed consent or patient assent and consent from the parent/legal guardian were obtained before collection of samples, in accordance with the Declaration of Helsinki.

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Supplementary Tables and Figures

Supplementary Table 1. Patient Characteristics

Supplementary Table 2. Antibodies used for single cell mass cytometry

Supplementary Figure 1: Proportion of T, Myeloid, and NK cells in CD45+ non-tumor compartment

Supplementary Figure 2: Changes in T cell subsets in CD45+ non-tumor compartment

Supplementary Figure 3: Expression of inhibitory immune checkpoints on CD4+ and CD8+ T cells, NK and myeloid cells.

Supplementary Figure 4: Expression of inhibitory immune checkpoints on CD4+ and CD8+ T cells by race/ethnicity.

Supplementary Figure 5: Expression of co-stimulatory molecules on CD4+ and CD8+ T cells by race/ethnicity.

Supplementary Figure 6: Expression of transcription factors on CD4+ and CD8+ T cells by race/ethnicity.

Supplementary Figure 7a: Expression of immune checkpoints on NK cells by race/ethnicity.

Supplementary Figure 7b: Expression of NKG2D on NK cells by race/ethnicity.

Supplementary Figure 8: Expression of CD112, CD155 and HLA-E on myeloid cells by race/ethnicity.

Supplementary Figure 9a: Expression of PD-L1 on myeloid cells in standard-risk NHW patients versus others.

Supplementary Figure 9b: Expression of CD112, CD155 and HLA-E on myeloid cells in standard-risk NHW patients versus others.

Table 1. Patient Characteristics

Characteristic	All patients (n=85)	Black (n=18)	Non-Hispanic White (n=38)	Hispanic (n=29)
Baseline demographics				
Age: years; median (range)	7(1.2-20.5)	5(1.4-17.5)	8(1.2-20.5)	7 (1.9-23)
Gender: number(%)				
Male	47(55%)	11(61%)	19 (50%)	17(59%)
Female	38(45%)	7(39%)	19(50%)	12(41%)
NCI risk status: number(%)				
Standard risk	40(47%)	9(50%)	14(37%)	16(55%)
High risk	45(53%)	9(50%)	24(63%)	13(45%)
Genetics: number(%)				
ETV-RUNX1	19(24%)	4(24%)	8(23%)	7(28%)
BCR-Abl	1(2%)	0	1(3%)	0
KMT2A rearranged	1(2%)	1(6%)	0	0
Trisomy 4/10	15(25%)	2(12%)	9(24%)	4(11%)

Information regarding age, race, gender and NCI risk status was available on all patients. ETV6-RUNX1 status was available on 81 patients and BCR-Abl, KMT2A and Trisomy 4/10 was available on 61 patients.

There is no statistical difference between the cohorts (Black/Non Hispanic white/Hispanic) with respect to baseline demographics, Risk status or defined tumor genetics.

Table 2: Antibodies used for CyTOF

	Antibody	Clone		Antibody	Clone
1	CD45	HI30	32	CD22	HIB22
2	HLAABC	W6/32	33	CCR7	G043H7
3	CCR6	G034E3	34	CD14	M5E2
4	CD155*	SKII.4	35	CD28	CD28.2
5	CD19	HIB19	36	CLEC9A	8F9
6	cKIT	104D2	37	TBET	4B10
7	CD45RA	HI100	38	CD112*	TX31
8	CD38	HIT2	39	FOXP3	PCH101
9	CD69	FN50	40	CD33	WM53
10	CD4	RPAT4	41	EOMES**	WD1928
11	CD8	RPAT8	42	TdT	E171519
12	CD11C	Bu15	43	CD95	DX2
13	TCF1#	7F11A10	44	CD45RO	UCHL1
14	CD34	581	45	CD141	M80
15	CD200	OX104	46	NKG2D	ON72
16	CCR4	L291H4	47	CD11b	ICRF44
17	SOX2	O30678	48	Ki67	B56
18	LAG3#	11C3C65	49	CD127	A019D5
19	CD123	6H6	50	DNAM1*	11A8
20	CD14	M5E2	51	CD25	2A3
21	CD95	DX2	52	iNKT	6B11
22	TCRgD	11F2	53	CD20	2H7
23	BDCA2	201A	54	CXCR5	RF8B2
24	TIGIT	MBSA43	55	PD-L2	24F.10C12
25	CD3#	UCHT1	56	CD57	HCD57
26	HLAE*	3D12	57	Granzyme	GB11
27	CD27	L128	58	HLADR	L243
28	PD-L1	29E.2A3	59	PD-1	EH12.2H7
29	CXCR3	G025H7	60	CD56	HCD56
30	CD10	HI10a	61	CD16	3G8
31	4-1BB	4B41			

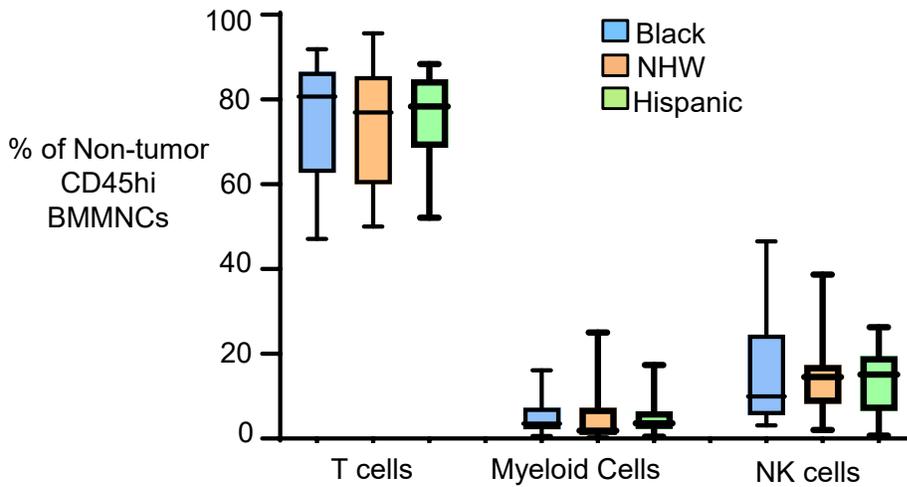
Antibodies were purchased from Fluidigm unless otherwise noted.

*Antibodies purchased from Biolegend.

**Antibodies purchased from Ebioscience.

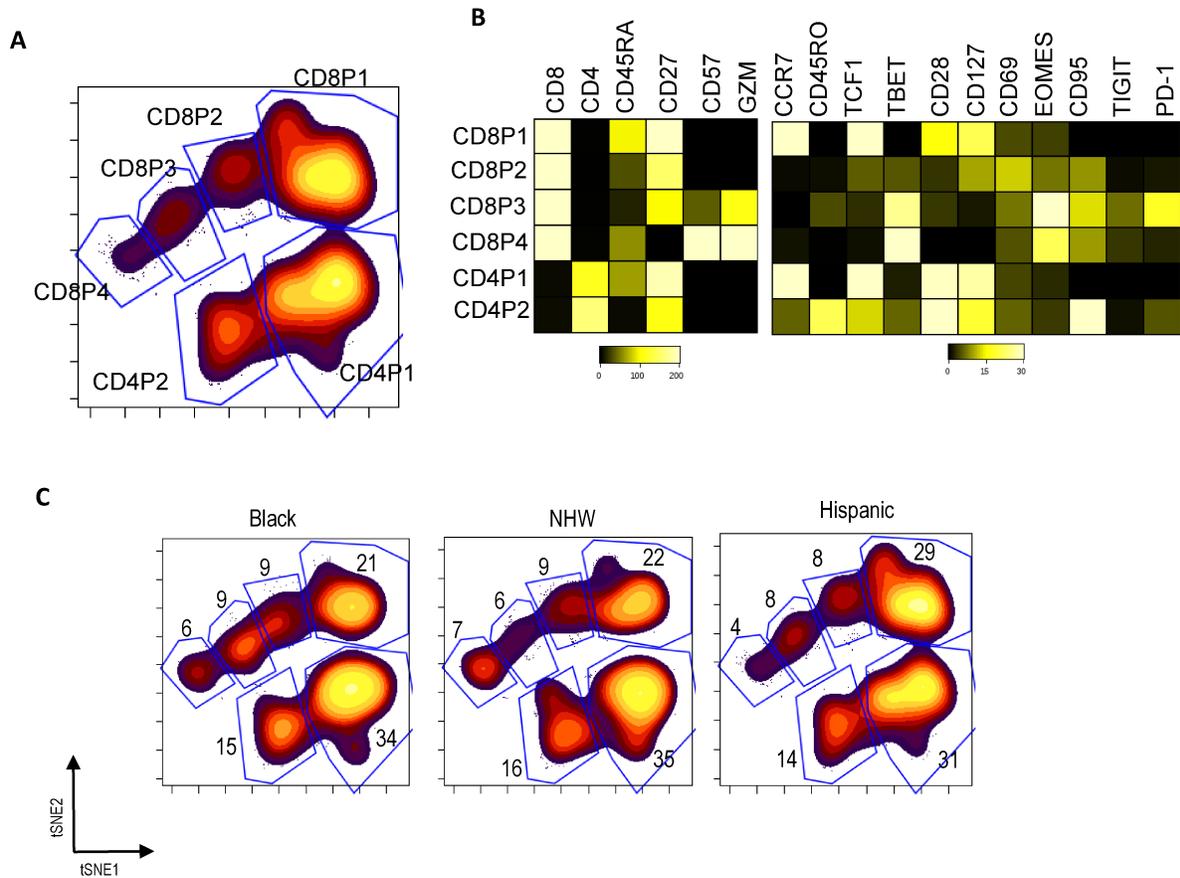
#Antibodies conjugated in the lab.

Supplementary Figure 1



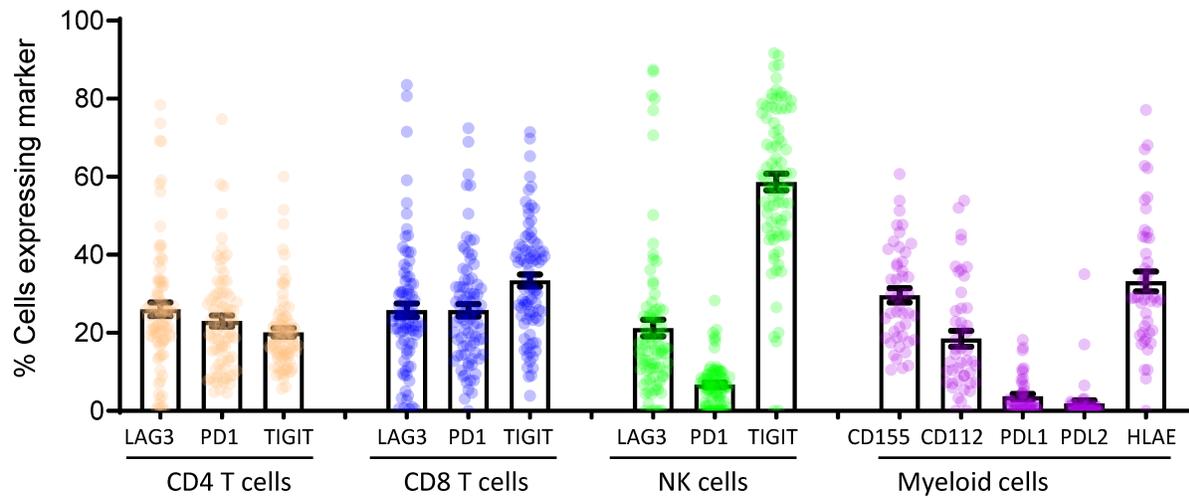
Supplementary Figure 1. Proportion of T cells, myeloid cells and NK cells in CD45^{hi} non-tumor compartment of the bone marrow in Black, Non-Hispanic White (NHW) and Hispanic children with B-ALL.

Supplementary Figure 2



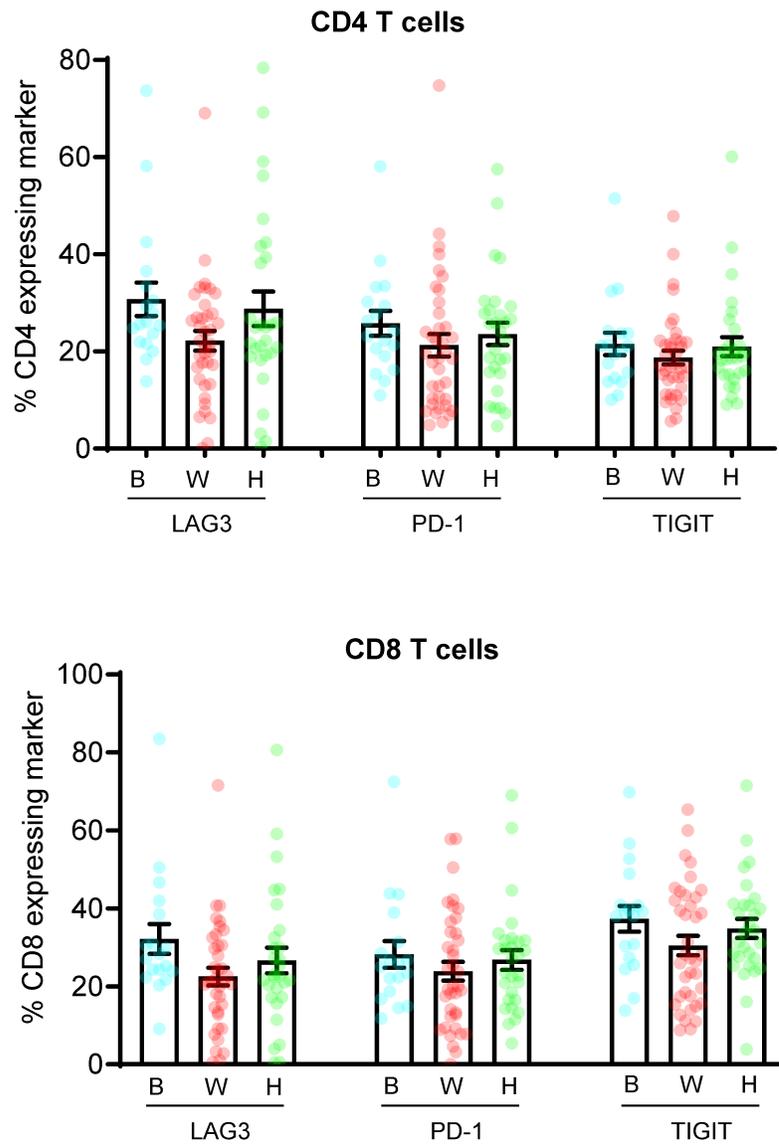
Supplementary Figure 2. Changes in T cell subsets in CD45^{hi} non tumor bone marrow compartment. **A.** VISNE analysis of T cells showing 4 distinct CD8 T and 2 distinct CD4 T cell populations. **B.** Heat map showing phenotypes of the CD8 and CD4 populations/clusters shown in the VISNE plot. **C.** Proportions of CD8 and CD4 T cells in the distinct clusters in Black, Non Hispanic White (NHW) and Hispanic patients.

Supplementary Figure 3



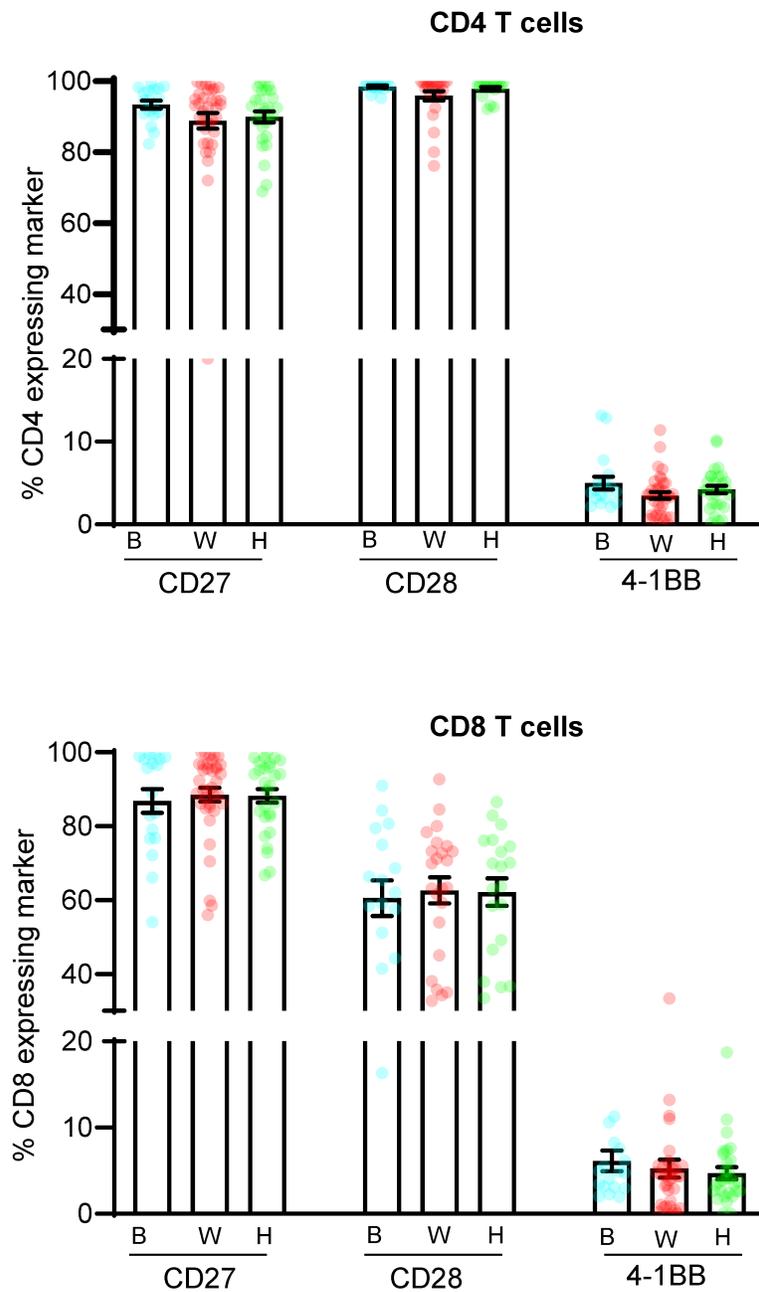
Supplementary Figure 3. Expression of inhibitory immune checkpoint molecules on bone marrow CD4, CD8, NK and myeloid cells in patients with B-ALL. Each dot represents individual patient sample.

Supplementary Figure 4



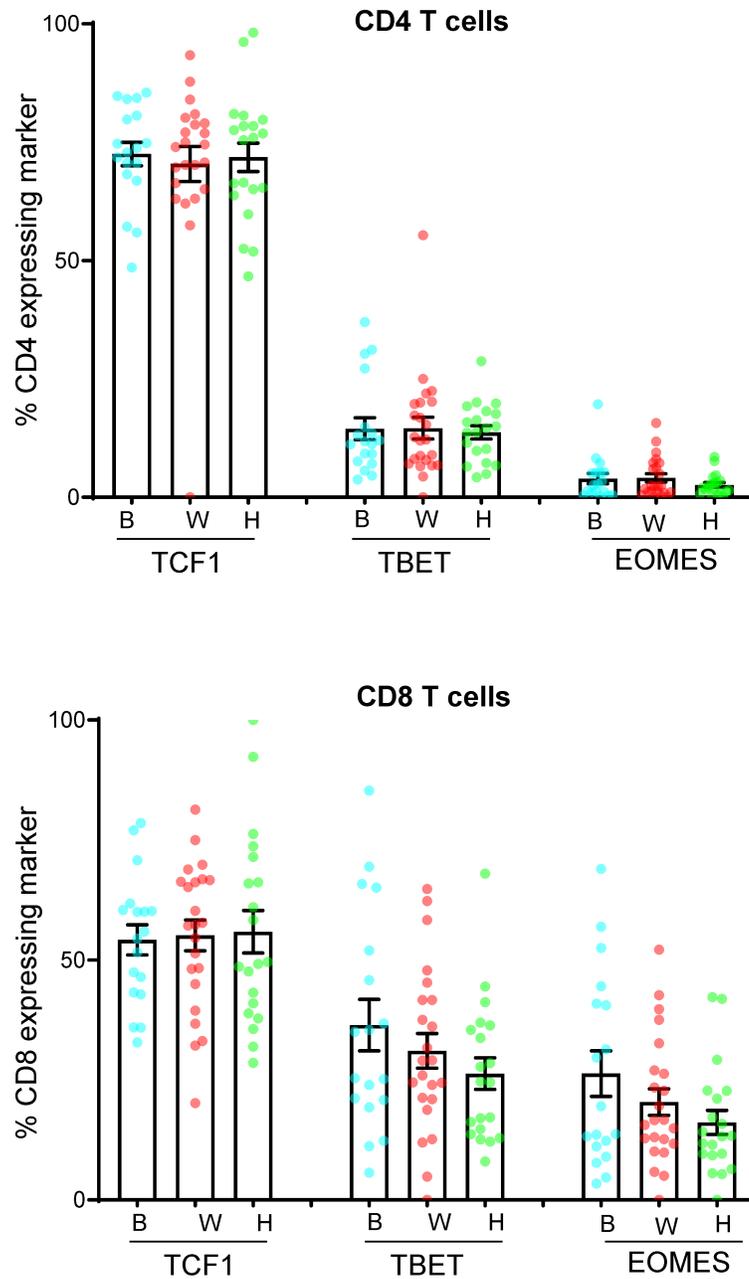
Supplementary Figure 4. Expression of inhibitory immune checkpoint molecules on bone marrow CD4 and CD8 T cells in Black(B), Non Hispanic White(W) and Hispanic (H) patients with B-ALL. Each dot represents individual patient sample.

Supplementary Figure 5



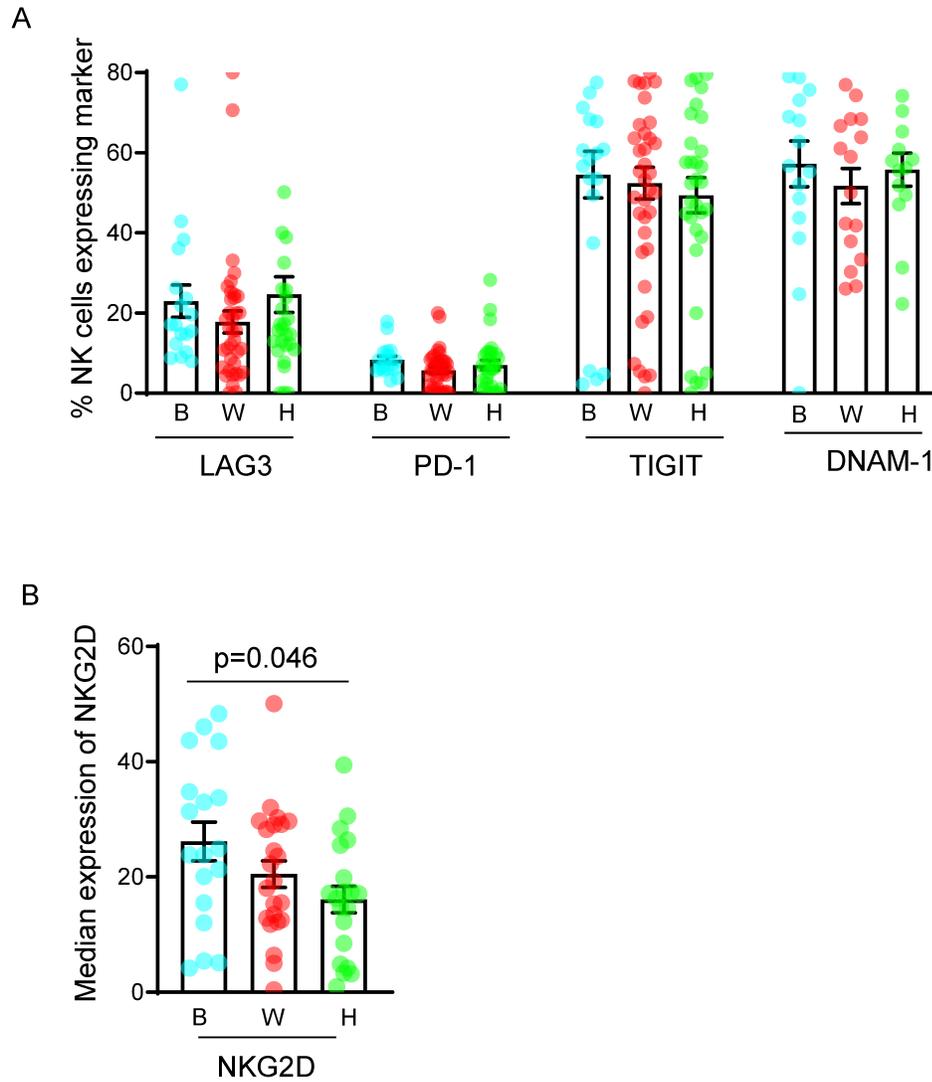
Supplementary Figure 5. Expression of co-stimulatory molecules on bone marrow CD4 and CD8 T cells in Black(B), Non Hispanic White(W) and Hispanic (H) patients with B-ALL. Each dot represents individual patient sample.

Supplementary Figure 6



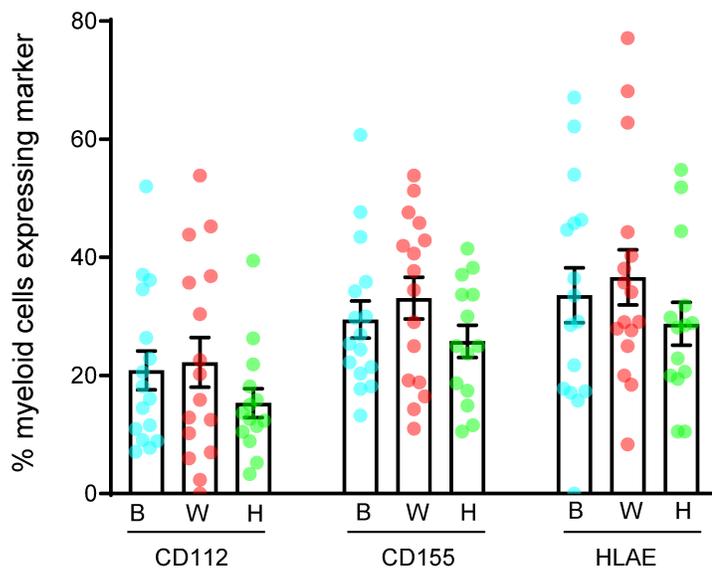
Supplementary Figure 6. Expression of transcription factors in bone marrow CD4 and CD8 T cells in Black(B), Non Hispanic White(W) and Hispanic (H) patients with B-ALL. Each dot represents individual patient sample.

Supplementary Figure 7



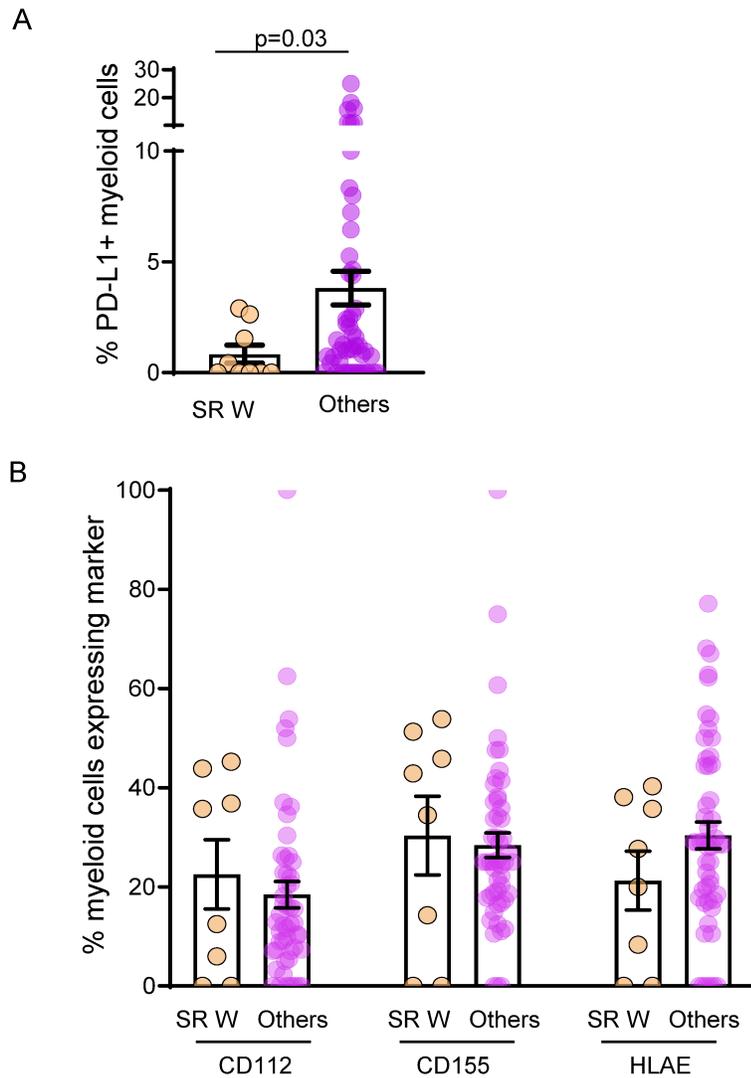
Supplementary Figure 7. **A.** Expression of checkpoint molecules on bone marrow NK cells in Black(B), Non Hispanic White(W) and Hispanic (H) patients with B-ALL. Each dot represents individual patient sample. **B.** Median expression of NKG2D on NK cells.

Supplementary Figure 8



Supplementary Figure 8. Expression of CD112, CD155 and HLAE on bone marrow myeloid cells in Black(B), Non Hispanic White(W) and Hispanic (H) patients with B-ALL. Each dot represents individual patient sample.

Supplementary Figure 9



Supplementary Figure 9. **A.** Expression of PD-L1 on bone marrow myeloid cells from standard risk white (SR W) patients and others (Black, Hispanic and High risk White patients). **B.** Expression of CD112, CD155 and HLA-E on bone marrow myeloid cells. Each dot represents individual patient sample.