

Cord blood hemopoietic cell receptor expression is associated with early life atopic risk and lung function

To the editor,

The lineage commitment of cord blood (CB) hemopoietic progenitor cells (HPCs) and mobilization of differentiated pro-allergic effector cells is orchestrated by genetic and environmental factors. HPCs differentiate into inflammatory effector cells both in the bone marrow and in peripheral tissues and thus can regulate central and peripheral inflammation. Dendritic cells (DCs) arise from HPC in the bone marrow and differentiate into myeloid (conventional) DC (mDC) and plasmacytoid DC (pDC). HPC lineage commitment underlies the “bone marrow” hypothesis of allergic disease, which posits that environmental challenges, either prenatally or shortly after birth, lead to the skewed production and mobilization of HPC and their progeny, contributing to ongoing allergic inflammatory responses.

We previously reported associations between HPC frequency and their expression of innate immune receptors in neonates with atopic mothers and in adult asthmatics following allergen challenge.^{1,2} Here, we report the results of our prospective investigation into whether the cord blood of neonates carries patterns of cell expression separating at-risk infants from healthy. We used 60 children participating in the Canadian Healthy Infant Longitudinal Development (CHILD) Study³ and examined HPC and DC receptor

expression at birth in relation to the development of atopy and allergic disease, and to lung function (as measured by lung clearance index (LCI)) at ages 1 and 3. LCI is a metric which indicates the degree of heterogeneity in lung ventilation: A higher LCI corresponds to worse lung function.⁴

Among the 60 CB samples, 54 were successfully analyzed using flow cytometry (Table 1). Flow cytometry dot plots for expression of IL5R, GM-CSFR, IL17RB, and ST2 on the surface of CB HPC are in Figure S3. We defined atopic at-risk subjects as having a positive skin prick test (SPT) and/or atopic dermatitis (AD) and/or wheeze. There were 29 and 23 subjects with LCI data at ages 1 and 3, respectively.

We observed a greater percentage of CB IL5RA⁺ HPC ($P = .025$) in atopic at-risk infants as compared to healthy controls at age 1 (Figure 1A). This association persists when applying a more stringent case definition (using only SPT). Since IL5RA is a marker of commitment to eosinophil lineage, this finding suggests augmented Eo/B differentiation at birth in at-risk infants. CB of atopic at-risk children at age 1 also showed a lower proportion of GM-CSFR⁺ ($P = .029$) and ST2⁺ ($P = .031$) HPC (Figure 1C,D) compared to controls. Using phenotypes at age 3, these associations were not significant (Table S2) after accounting for maternal history of atopy/asthma, suggesting

TABLE 1 Characteristics of children using atopy phenotype at ages 1 and 3 years

Characteristic	One-year atopy			Three-year atopy		
	Cases (N = 25)	Controls (N = 29)	P value	Cases (N = 25)	Controls (N = 29)	P value
Sex—Males N (%)	17 (68%)	13 (45%)	.087	12 (63%)	18 (51%)	.407
Gestational Age, mean (weeks)	39.4	39.3	.680	39.3	39.4	.792
Gestational Weight, mean (grams)	3568.8	3502.7	.612	3503.7	3549.4	.742
Maternal Atopy N (%)	16 (64%)	20 (69%)	.990	12 (71%)	25 (76%)	.693
Paternal Atopy N (%)	16 (64%)	15 (52%)	.215	11 (69%)	20 (61%)	.579
SPT positive	18 (72%)	0	—	10 (53%)	0	—
Atopic dermatitis	13 (52%)	0	—	8 (42%)	0	—
SPT + AD	8 (32%)	0	—	4 (21%)	0	—
Wheeze	4 (16%)	0	—	8 (42%)	0	—
% HPC of MNC	63.1	68.5	.640	62.8	67.8	.689

Note: P values refer to t test for statistical significance.

Abbreviations: HPC, hemopoietic progenitor cells; MNC, mononuclear cells.

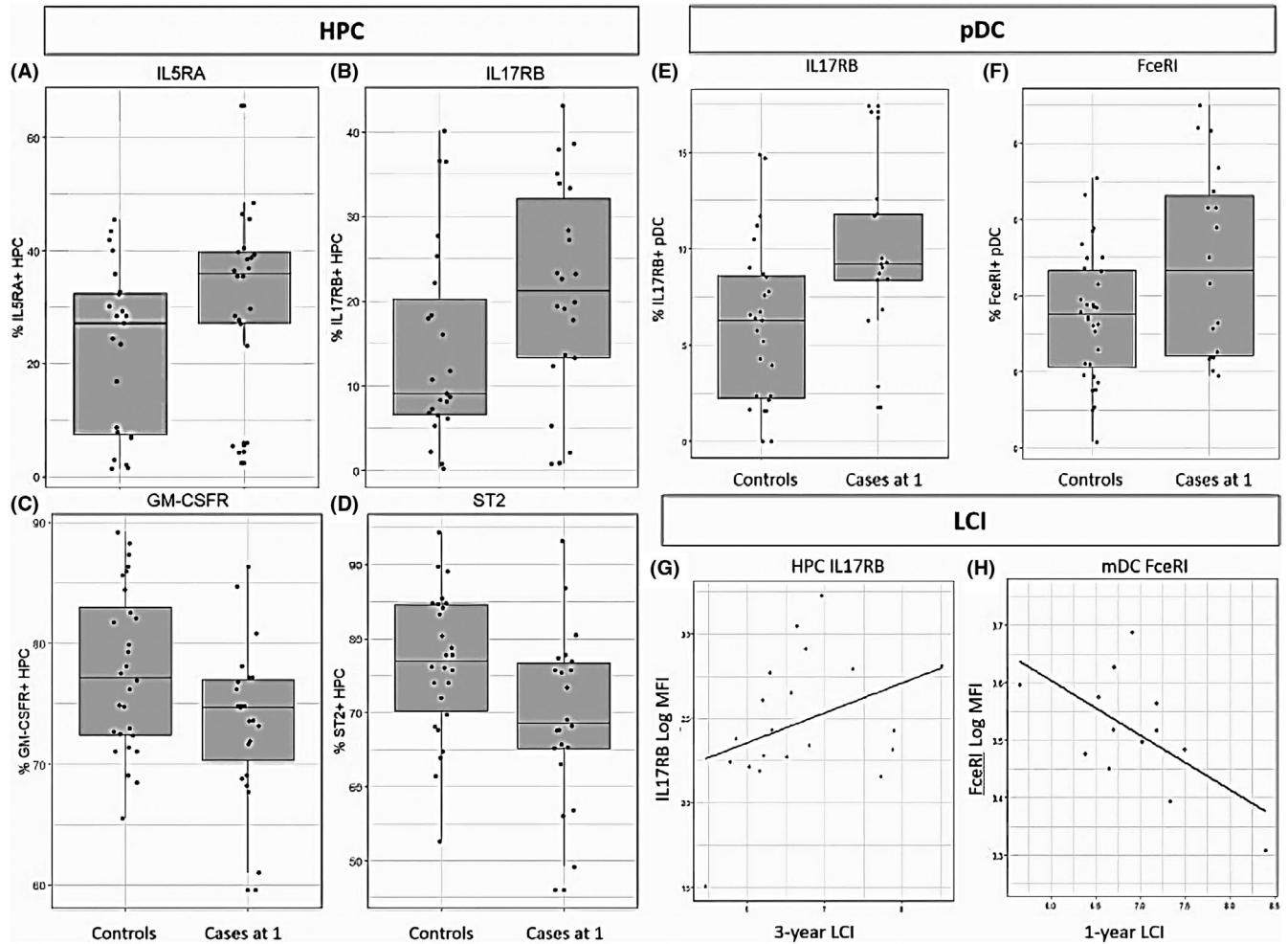


FIGURE 1 Percentage of HPC expressing the receptors IL5RA (A), IL17RB (B), GM-CSFR (C), and ST2 (D), and percentage of DC expressing IL17RB (E) and FcεRIα (F) in the CB of children who developed atopy, AD, or wheeze at 1 year of age, compared with healthy controls. LCI panel: association of LCI with expression levels (log mean fluorescence intensity); (G) IL17RB on CB HPC with LCI at age 3 and (H) FcεRIα on CB mDC with LCI at age 1. Twenty subjects with IL17RB flow cytometry data and 17 subjects with FcεRIα flow cytometry data had available LCI data. HPC: hemopoietic progenitor cells; LCI: lung clearance index; P: p value; Padj: P value adjusted for sex and maternal and paternal history of allergy/asthma; pDC: plasmacytoid dendritic cells

these differences are mainly due to maternal factors. IL5R expression represents the hallmark of commitment of multipotent progenitors to the eosinophil lineage, while ST2 and GM-CSFR expression seem to be gradually decreasing with advancing stages of eosinophilopoiesis.^{5,6} Accordingly, our data suggest a prenatal commitment to enhanced eosinophilopoiesis and hence loss of the CD34 progenitor marker.

IL17RB⁺ HPC and pDC proportions were also greater in the CB of atopic infants at age 1 ($P = .049$ and 0.007 , respectively) (Figure 1B,E). The pDC association was mainly due to maternal history of atopy/asthma (adjusted $P = .066$) and did not persist at age 3. These data may indicate a previous exposure to the IL17RB ligand, IL25⁷; a future avenue of research could be to verify this speculation, for instance, by investigating whether allergic at-risk cord blood is indeed more responsive to ligands such as IL25 and IgE. Subjects with higher levels of IL17RB on HPC at birth also had higher LCI at age 3 ($P = .043$) (Figure 1G), regardless of maternal history. In vivo

studies also show an important role of pDC in antiviral immunity via the IL25/IL17RB axis and propose targeting pDC for the treatment of acute asthma. Interestingly, the IL25 receptor is expressed on the surface of activated or inflammatory Th2 T cells and is a marker of mice "memory" or "inflammatory" ILC2, a select subset of ILC2 exhibiting all the characteristics of memory T cells, with more robust and rapid cytokine production in response to activation.⁸ These memory cells lack expression of ST2, a marker that we found expressed by a lower proportion of HPC in the CB of atopic at-risk subjects. These data, again, are indicative of a prenatal bias toward Th2 inflammatory responses in the neonate rendering the at-risk infants' immunologically primed to respond more strongly to neonatal environmental exposures.

We found a greater proportion of FcεRIα⁺ pDC in the CB of atopic at-risk subjects at age 1 ($P = .025$) (Figure 1F); this association did not persist at age 3. Using only positive SPT at 1, this relationship remains significant. Concerning CB mDCs, subjects with lower

levels of FcεRIα had higher LCI at age 1 ($P = .049$) (Figure 1H). This relationship was attenuated by maternal history (adjusted $P = .057$), in agreement with a previous study assessing the association of maternal allergy with FcεRIα on CB basophils and pDC.⁹

We reported that early allergic sensitization in combination with atopic dermatitis is associated with asthma risk at age 3 in the CHILD study. The current study highlights alterations in CB HPC and DC at birth, some of them due to maternal effects; thus, the *in utero* environment appears to relate to atopic risk of the infant in early life, and this influence is detectable at birth, in alignment with the developmental origin of health and disease framework.

We also found that greater lung ventilation heterogeneity at ages 1 and 3 is associated with allergic risk as evidenced by the relationship with IL17RB and FcεRIα MFI on CB HPC and DC, respectively. MFI indicates relative levels of surface receptors' expression and accordingly their ability to be activated by their ligands or possibly a previous priming encounter with an inflammatory stimulus. This suggests that early allergic risk may be a pathway for ongoing airway inflammation, resulting in lung function differences in early age. Whether LCI alterations signify poorer lung function or predict future lung disease is uncertain. Longitudinal lung imaging data would be beneficial in assessing any correlation of LCI with clinical lung outcomes.

Overall, our findings strongly suggest that in CB there is already evidence of "immune memory" or priming, manifest as increased levels of pro-inflammatory receptors on HPC and DC. The immune system is thus primed to facilitate development of atopy and allergic disease. Continuing allergic stimuli may further perpetuate these processes until clinical phenotypes emerge. Hence, cord blood cell expression patterns could be part of a battery of allergic disease risk stratification for babies at birth.

KEYWORDS

allergic disease, atopy, CHILD Study, cord blood, dendritic cell, hemopoietic progenitor cell

CONFLICT OF INTEREST

The authors have no conflicts of interest to disclose.

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Loubna Akhabir^{1,2} 

Elli Rosenberg¹ 

Damian Tworek³ 

Delia Heroux¹

Jyoti Balhara¹

Kelly M. McNaghy⁴

Malcolm R. Sears¹

Diana L. Lefebvre¹

Allan B. Becker⁵

Stuart E. Turvey⁶ 

Piush J. Mandhane⁷

Theo J. Moraes⁸

Padmaja Subbarao⁸

Guillaume Paré^{2,9,10}

Judah A. Denburg¹

¹Department of Medicine, McMaster University, Hamilton, ON, Canada

Email: denburg@mcmaster.ca

²Population Health Research Institute, McMaster University, Hamilton, ON, Canada

³Department of General and Oncological Pulmonology, Medical University of Lodz, Lodz, Poland

⁴Biomedical Research Centre, The University of British Columbia, Vancouver, BC, Canada

⁵Department of Pediatrics and Child Health, Children's Hospital Research Institute of Manitoba, University of Manitoba, Winnipeg, MB, Canada

⁶Department of Pediatrics, BC Children's Hospital, University of British Columbia, Vancouver, BC, Canada

⁷Department of Pediatrics, University of Alberta, Edmonton, AB, Canada

⁸Department of Pediatrics, Hospital for Sick Children, University of Toronto, Toronto, ON, Canada

⁹Thrombosis and Atherosclerosis Research Institute, Hamilton Health Sciences, McMaster University, Hamilton, ON, Canada

¹⁰Department of Health Research Methods, Evidence, and Impact, McMaster University, Hamilton, ON, Canada

Correspondence

Judah A Denburg, 1280 Main Street W. Hamilton, Ontario L8S 4K1 Canada.

Email: denburg@mcmaster.ca

ORCID

Loubna Akhabir  <https://orcid.org/0000-0002-4378-6758>

Damian Tworek  <https://orcid.org/0000-0001-9862-6703>

Stuart E. Turvey  <https://orcid.org/0000-0003-1599-1065>

Loubna Akhabir and Elli Rosenberg contributed equally to this work.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

Supplementary information for manuscript:

Cord Blood Hemopoietic Cell Receptor Expression is Associated with Early Life Atopic Risk and Lung Function

MATERIAL AND METHODS

Study design

The CHILD study is a national, longitudinal, general population-based cohort that recruited 3,455 pregnant mothers at four sites (Toronto, Manitoba, Vancouver, and Edmonton) and followed their infants from pregnancy onwards ¹. Biological samples, environmental exposure data, nutrition, psychosocial factors, and general health of mother and child were collected through assessments and detailed questionnaires administered at recruitment and at several time points including 5 years after birth ². All participating parents provided signed informed consent. A sub-sample of 60 infants recruited at the Toronto site from 2009 to 2012, selected from subjects with available CB and clinical data, were included in this study. The study was approved by the Research Ethics Boards of the Hospital for Sick Children, Toronto, Ontario, and McMaster University, Hamilton, Ontario.

Subject classification

Clinical phenotypes assessed at the 1- and 3-year study visits were used in this study. Standardized allergen skin prick tests (SPT) were performed on all consenting children for common inhalant and food allergens (ALK Abello Pharmaceuticals, Canada) using Duotip II devices (Lincoln Diagnostics, Canada). At age 1, children were tested for 10 allergens: *Alternaria tenuis*, cat hair, dog epithelium, *Dermatophagoides farina*, *Dermatophagoides pteronyssinus*, German cockroach, peanut, soybean, egg white and cow's milk. At age 3, a further 7 aeroallergens were added: *Cladosporium*, mixed *Penicillium*, *Aspergillus fumigatus*, Midwest trees, mixed grass, weeds and mixed ragweed. A subject developing any wheal ≥ 2 mm greater than the negative control was considered to have a positive SPT. Histamine (1mg/ml) was used as a positive control and glycerin as a negative control. Infants were examined by a healthcare professional for findings consistent with atopic dermatitis and/or wheezing.

In order to study children who are most at risk to develop allergic disease later in life, we defined cases as the subjects who showed a positive SPT result to one or more allergens, and/or were

diagnosed with atopic dermatitis and/or wheeze, and they are referred to as “atopics at-risk”. Those who were negative for allergen sensitization by SPT and showed no symptoms of atopic dermatitis or wheezing episodes were designated as controls. As a sensitivity analysis, we defined cases more strictly, as the subjects who showed at the least a positive SPT result, excluding subjects with AD and/or wheeze who have negative SPT.

Lung clearance index

Lung clearance index (LCI) is a parameter derived from the multiple breath washout (MBW) test³, and is thought to reflect the degree of homogeneity in lung ventilation: a higher LCI corresponds to greater ventilation heterogeneity (worse lung function). MBW testing was performed in CHILD subjects at the 1- year and 3-year visits as previously described in detail⁴. MBW tests were performed using a face mask (Silkomed, Benson Medical Industries, Markham, ON Canada) sealed with therapeutic putty (Air Putty, Sammons Preston Canada, Mississauga, ON, Canada). A mass spectrometer (AMIS 2000; Innovision ApS, Odense, Denmark) was used for the MBW test with a gas mixture of 4% sulfur hexafluoride, 4% helium, 21% oxygen and balance nitrogen. LCI values were computed as the cumulative exhaled volume needed to reduce the end-tidal concentration of the tracer gas to 1/40th of the initial concentration divided by the functional residual capacity.

Cord blood collection and processing

CB was collected directly from the umbilical vein by needle aspiration after clamping of the cord, prior to placenta delivery. CB mononuclear cells were isolated by density centrifugation and cryopreserved in 90% fetal bovine serum and 10% dimethyl sulfoxide. Samples were stored in liquid nitrogen until shipping and analyses.

Flow cytometry

Six-color flow cytometry was performed on thawed CB mononuclear cells using a previously standardized method⁵. For HPC, optimal amounts of antibodies to CD34, CD45, GM-CSFR, IL3R, IL5RA, IL17RB, ST2, TSLPR, TLR2, TLR4, and TLR9 (Table S1) and their isotype controls were used. For DC, optimal amounts of antibodies to CD11c, CD45, CD80, CD86, FcεRIα, IL3R, IL17RB (Table S1) and their isotype controls were used. The gating strategies are shown in Figures S1 and S2 for HPC and DC respectively. We undertook the optional additional step of taking the high forward low scatter again (fourth panel of Fig S1) to further filter out

artifacts from cell debris and focus on the CD34⁺ HPC. We defined mDC as Lin1⁻ HLA-DR⁺ CD11c⁺ and pDC as Lin1⁻ HLA-DR⁺ CD123⁺. All DC expressed CD80 and CD86. Flow cytometry analysis was performed blinded to phenotypic status.

Statistical analysis

Exploratory analyses were performed for data distributions, and pair-wise Pearson correlations between receptor levels. MFI values were log-transformed prior to analysis. Outliers were identified using Tukey's fences⁶. Differences between protein levels were assessed using Student's two-sample t-tests, and Mann-Whitney U tests for normally or not normally distributed variables, respectively. Normality was tested using Shapiro-Wilk tests. We used multivariate logistic regressions to assess the effect of confounding variables, sex and parental history of allergy or asthma, on the binary atopy/allergic disease risk outcome, separately at age 1 and age 3. Alpha level of significance was 0.05. A limitation of our study is the low number of subjects: therefore, we did not correct for multiple testing. The significant p values are nominal and indicate possible mechanisms of disease that should be investigated further. All analyses were conducted using the R statistical programming language version R 3.4.0.

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Table S1 Information for antibodies used in flow cytometry

Target	Clone	Fluorophore	Company
CD11c	S-HCL-3	PerCP/Cy5.5	Biolegend
CD34	581	PerCP	Biolegend
CD45	HI30	eFlour 450	eBioscience
CD80	L307.4 (aka L307)	PE	BD Biosciences
CD86	Clone 2331 (FUN-1)	APC	BD Biosciences
FcεRIα	AER-37(CRA!)	FITC	eBioscience
GM-CFSR	4H1	FITC	eBioscience
HLA-DR	G46-6	APC-H7	BD Biosciences
Human lineage cocktail	UCHT1/TUK4/DJ130c/LT19/2H7/123C3	Alexa Flour 700	Bio-RAD
IL3R (CD123)	6H6	PE/Cy7	Biolegend
IL5RA	26815	APC	R&D Systems
IL17RB	170220	APC	R&D Systems
ST2	2A5	FITC	MBL
TSLPR	eBio1A6	PE	eBioscience
TLR2	TL2.1	APC	eBioscience
TLR4	HTA125	PE	eBioscience
TLR9	eB72-1665	PE	eBioscience

Table S2 Multivariate regression results for receptors in figure 1 findings for the phenotypes at ages 1 and 3.

CI: Confidence interval; p: p value; HPC: Hemopoietic progenitor cells; pDC: Plasmacytoid dendritic cells;

Cell	Analysis	1-Year phenotypes		3-Year phenotypes	
		beta (95% CI)	p	beta (95% CI)	p
HPC	% IL5R ⁺ / Atopy	10.65(1.38, 19.93)	0.025	-6.09 (-16.87, 4.68)	0.260
HPC	% GM-CSFR ⁺ / Atopy	-2.35 (-6.23, 1.54)	0.230	-1.46 (-6.01, 3.09)	0.522
HPC	% ST2 ⁺ / Atopy	-3.93 (-10.34, 2.48)	0.222	-5.05 (-12.80, 2.71)	0.196
HPC	% IL17RB ⁺ / Atopy	2.88 (-0.21, 5.98)	0.066	1.79 (-1.43, 5.02)	0.266
pDC	% FcεRIα ⁺ / Atopy	7.72 (1.00, 14.44)	0.025	-1.58 (-8.92, 5.76)	0.665
pDC	% IL17RB ⁺ / Atopy	2.89 (-0.29, 6.07)	0.073	3.57 (-2.88, 10.01)	0.263
mDC	FcεRIα Log MFI / LCI	-7.08 (-13.02, -1.13)	0.057	-2.05 (-9.17, 5.08)	0.470
HPC	IL17RB Log MFI / LCI	0.21 (-2.08, 1.65)	0.789	1.25 (0.10, 2.41)	0.039

Figure S1 Flow cytometry multiparameter gating strategy for CB HPC.
 CB: Cord blood; HPC: Hemopoietic progenitor cells;

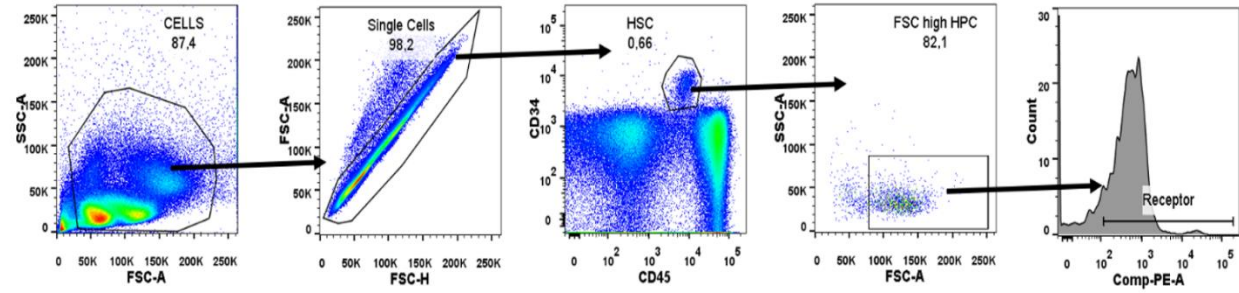


Figure S2 Flow cytometry multiparameter gating strategy for CB DC. 1) Mononuclear cells, 2) Dendritic cells; 3) cDC; 4) pDC
 CB: Cord blood; DC: Dendritic cells; cDC: conventional DC; pDC: Plasmacytoid DC;

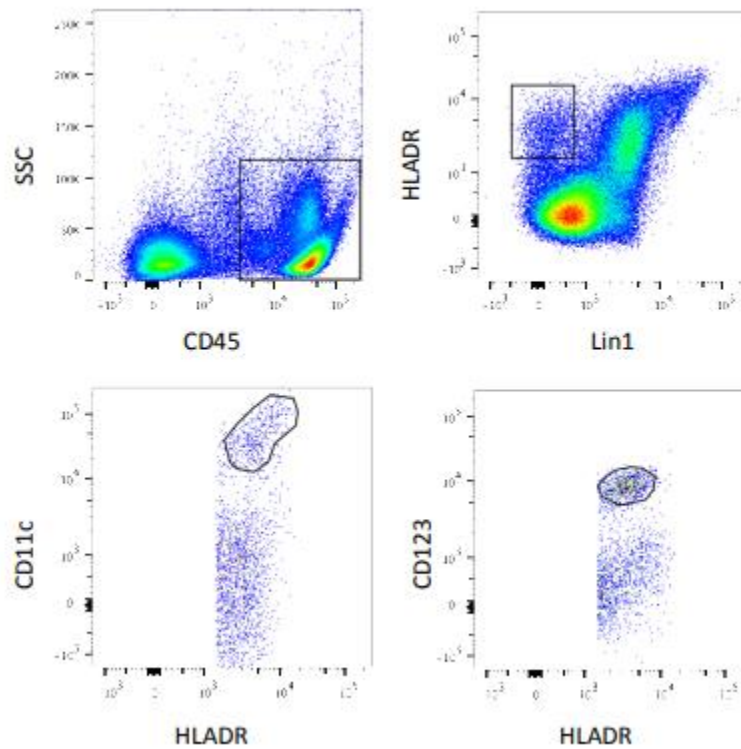


Figure S3 Dot plots for expression of IL5R, GM-CSFR, IL17RB and ST2 on the surface of CB HPC

CB: Cord blood; HPC: Hemopoietic progenitor cells;

