DNA Methylation Signatures Underpinning Blood Neutrophil to Lymphocyte Ratio During First Week of Human Life

Supplementary Figures

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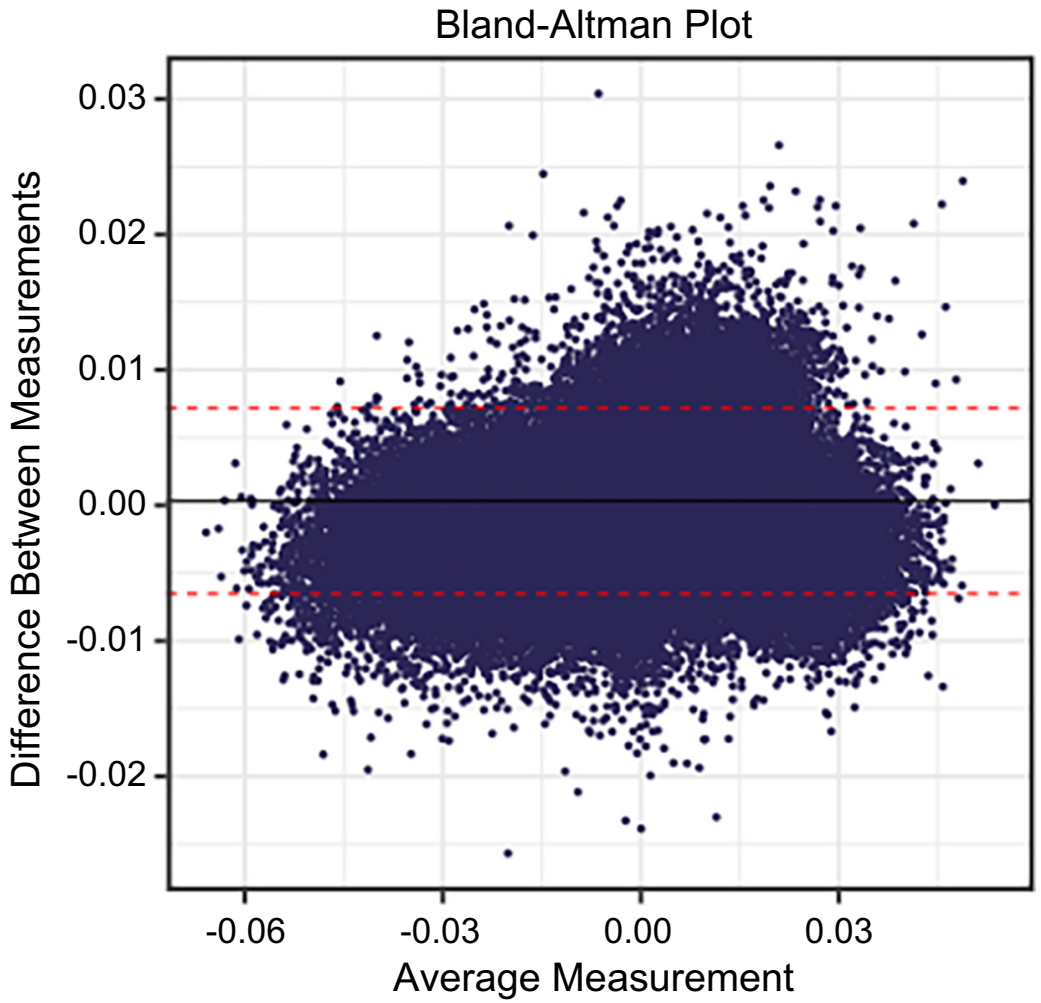
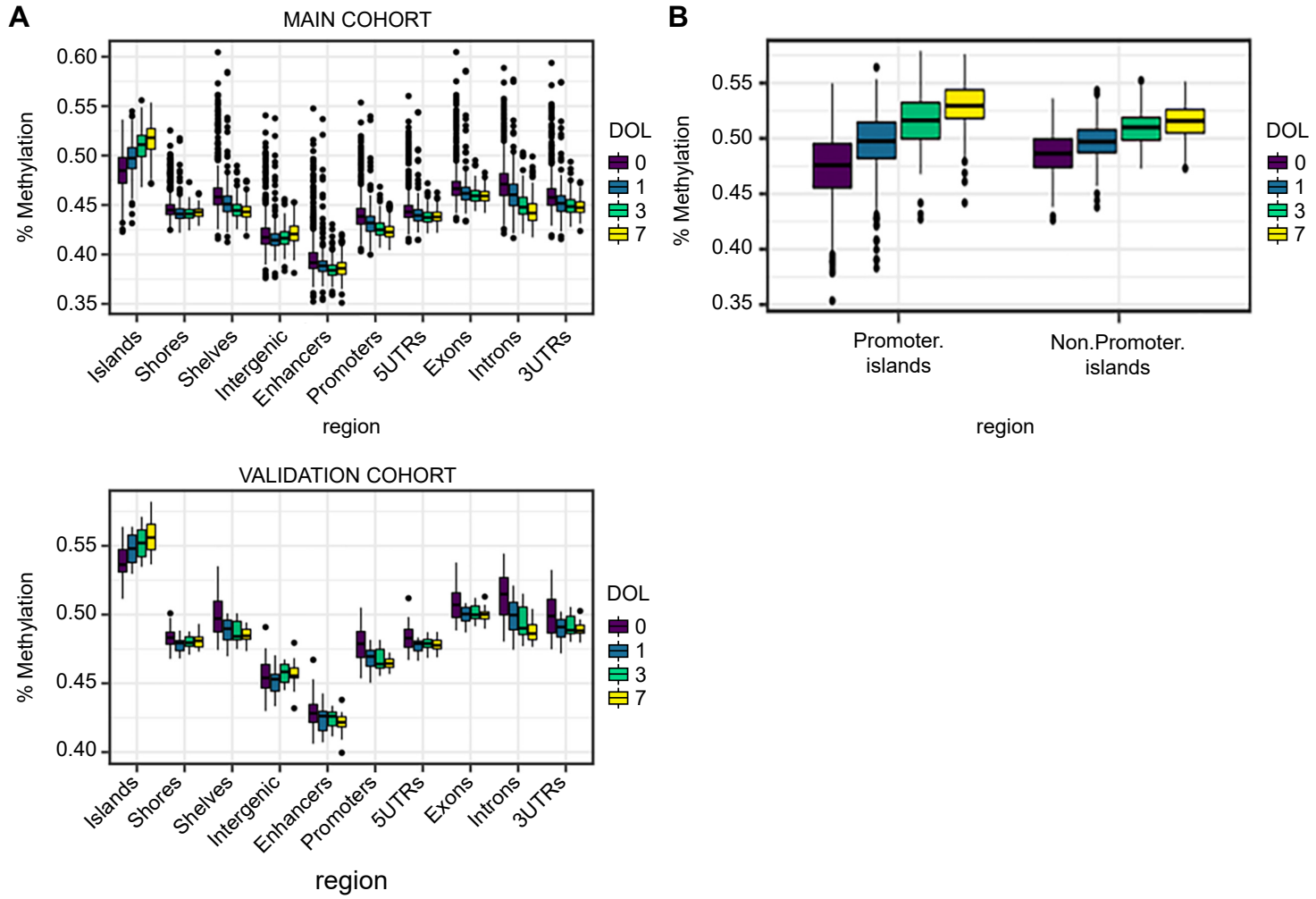
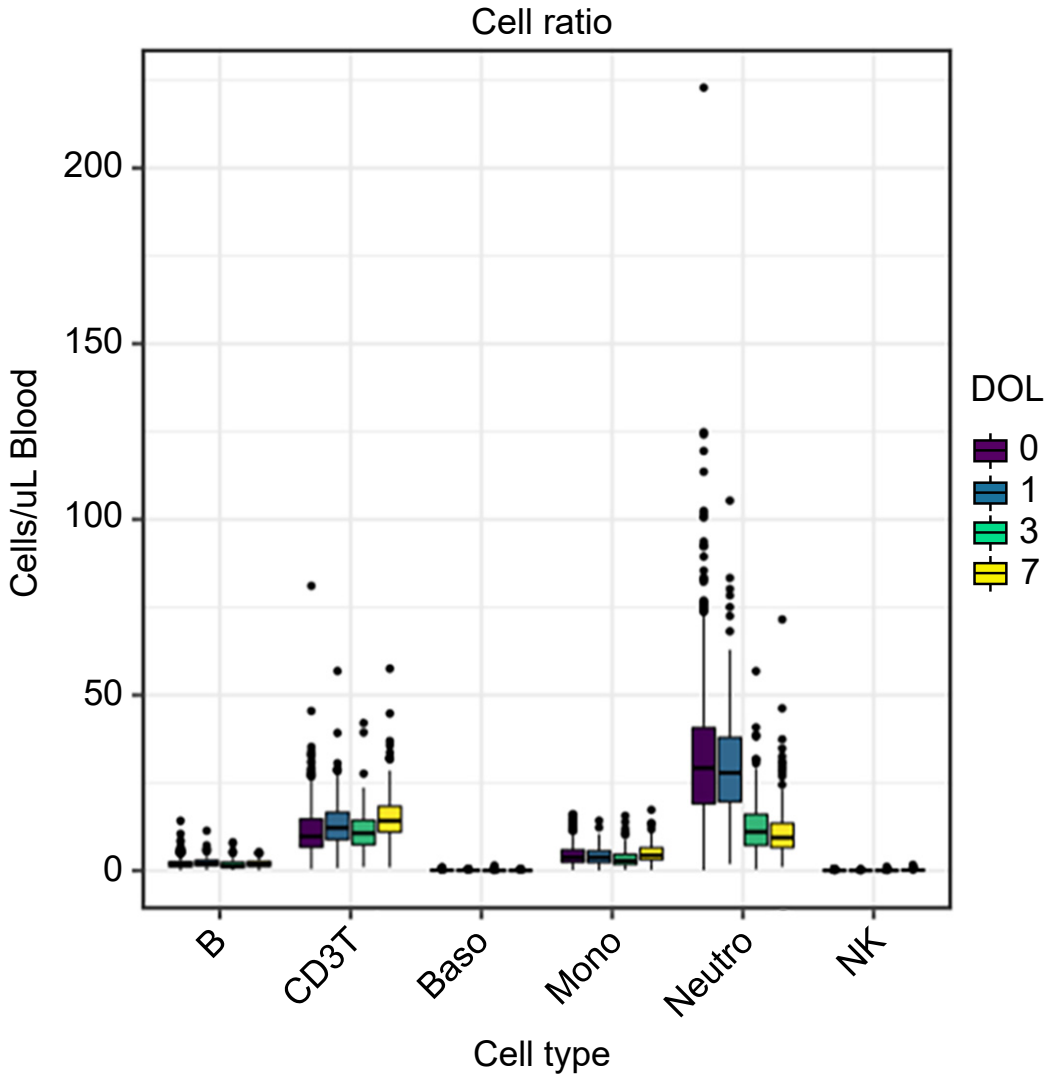


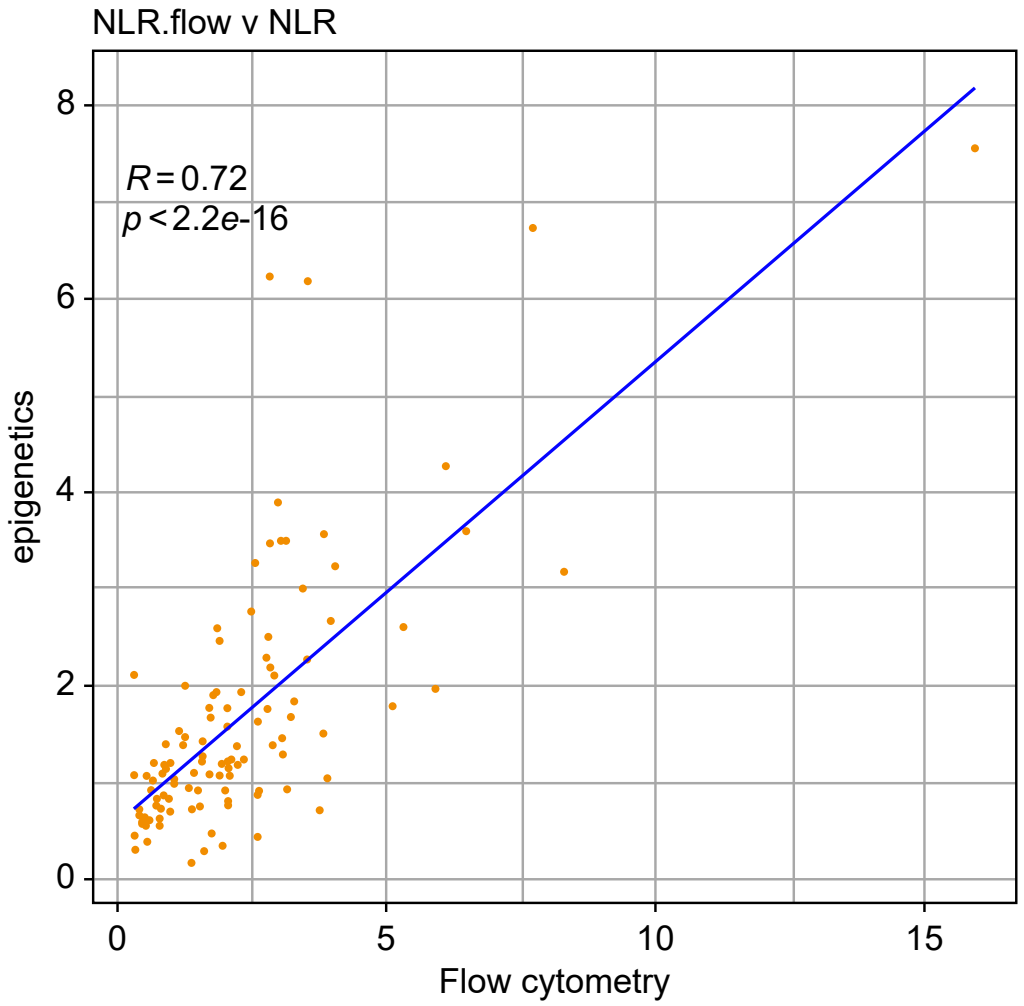
Supplementary Figure 1 – Bland-Altman analysis of agreement between main cohort and replication cohort. Beta values for DOL-associated CpG were modelled using DOL as the predictor, adjusted for sex and sample plate. The same model was fitted to both the main cohort and the replication cohort. Difference in model logFC estimates are plotted on the y-axis and the average between the logFC estimates on the x-axis. The mean difference is shown as the solid line and 95% confidence intervals are shown as dashed red lines.



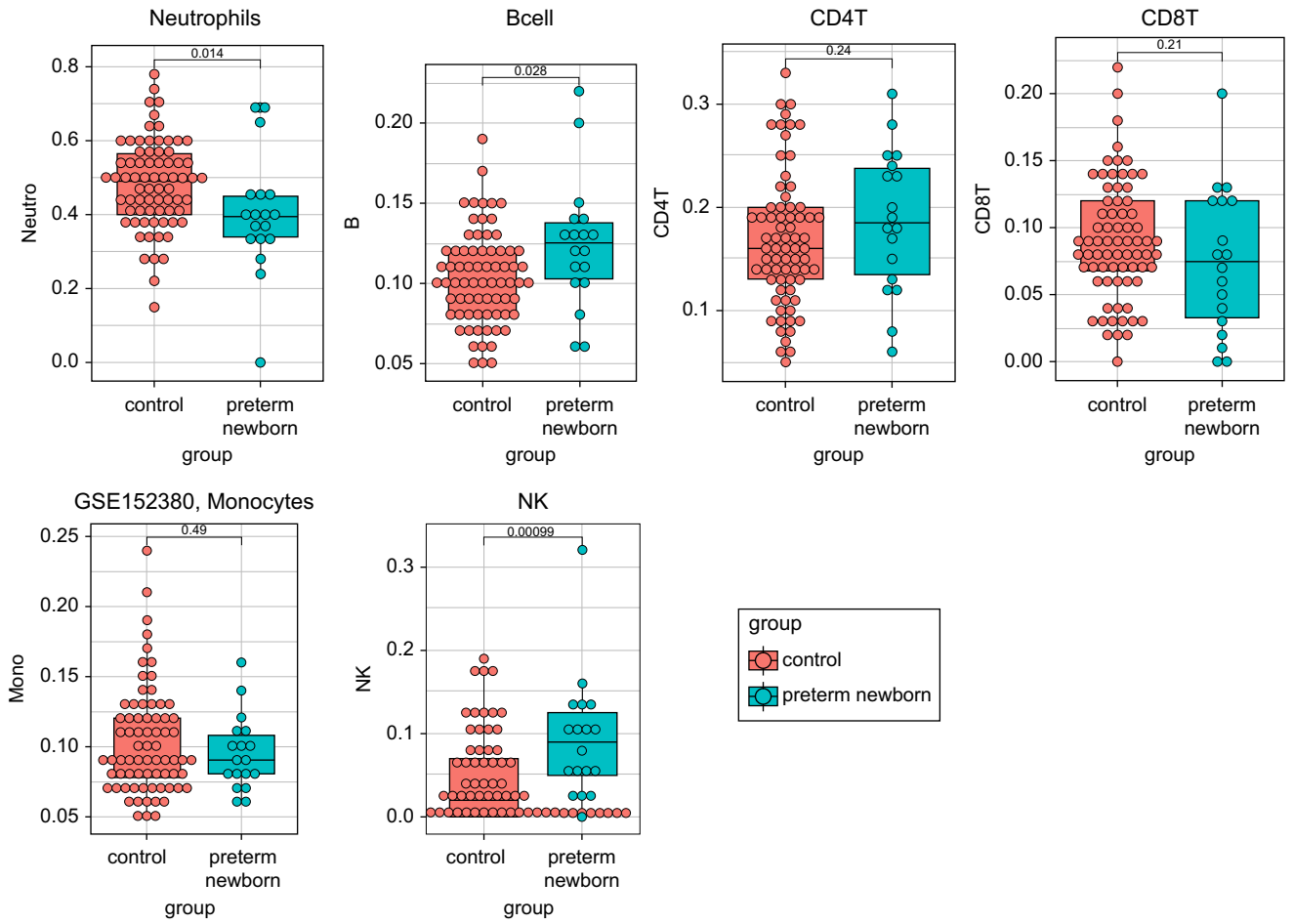
Supplementary Figure 2 – Median methylation change per DOL in different genomic regions. (A) Methylation ratios expressed as percentage (y-axis) for DOL associated CpGs stratified by genomic feature (x-axis). UTR = untranslated region. **(B)** DOL associated changes in CpG islands stratified by promoter associated and non-promoter associated CpG islands. Boxplots show the median and interquartile range (IQR, 25th-75th percentile). Whiskers extend to the most extreme data points within 1.5 times the IQR from the box hinges (the 25th and 75th percentiles). Points outside this range are considered outliers and are plotted individually). Total n=1267 (main cohort), n=86 (validation cohort).



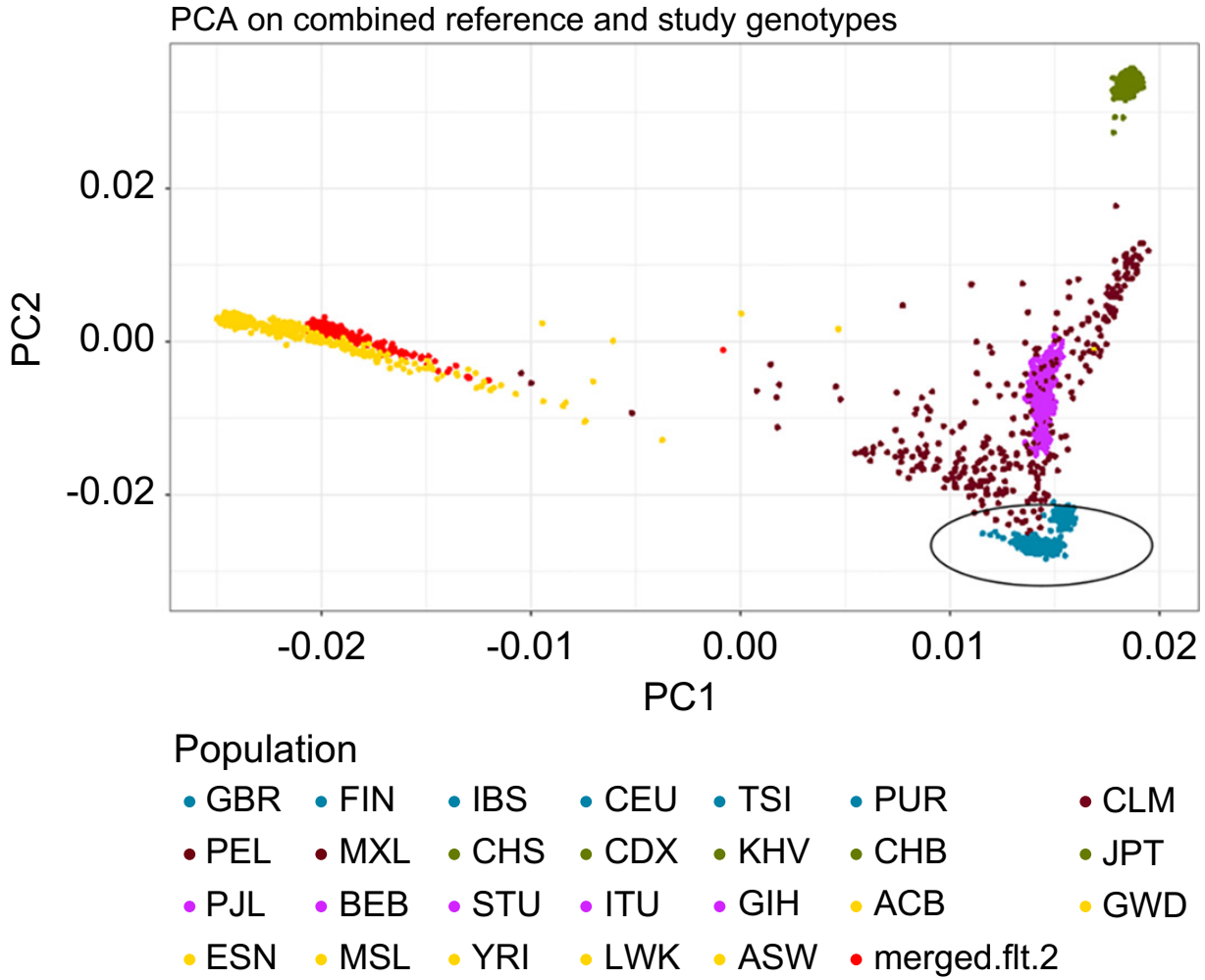
Supplementary Figure 3 – Flow cytometric analysis of cell ratios stratified by Day of Life. Boxplots show the median and interquartile range (IQR, 25th-75th percentile). Whiskers extend to the most extreme data points within 1.5 times the IQR from the box hinges (the 25th and 75th percentiles). Points outside this range are considered outliers and are plotted individually). Total n=1256 samples.



Supplementary Figure 4 – Pearson's' Correlation between Flow cytometry derived NLR and epigenetically inferred NLR. Each point represents a subjects NLR score from the sepsis sub-cohort. Each point is an individual observation. The solid line indicates the linear regression fit for DOL. Two-sided *P* value for the Pearson's correlation coefficient (*R*) is shown. Total n=54.



Supplementary Figure 5 – Inferred cell counts from GSE152380. Umbilical cord whole blood methylation array data were downloaded and cell counts estimated using the EpiDish package. Boxplots show the median and interquartile range (IQR, 25th-75th percentile). Whiskers extend to the most extreme data points within 1.5 times the IQR from the box hinges (the 25th and 75th percentiles). Points outside this range are considered outliers and are plotted individually*). P*-values are one-Wilcoxon Ranke-Sum Test. Total n=90.



Supplementary Figure 6 – Principal component analysis of genetically inferred ancestry. Study population samples were merged with 1000 Genomes reference populations and PCA analysis performed. Study samples are shown in red. The African super-cluster is shown in yellow. Ellipse denotes the European population.

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Supplementary Methods: Flow Cytometry Gating Strategy

Flow cytometry sample acquisition and data analysis were performed by the EPIC-HIPC flow cytometry core and detailed methods are provided in [1]. An overview of flow cytometry methods are provided below.

Sample Processing and Acquisition

Newborns were sampled twice within the first week of life, collecting 2 ml of peripheral blood at birth (DOL0) and at either DOL1, 3, or 7. Briefly, 225 µl of whole blood cells were mixed with culture medium, stained with a fixable viability dye, and fixed prior to storage. Thawed samples were stained with two flow cytometry panels designed to identify adaptive and innate cell populations. Stained cells were washed, resuspended, and acquired on an LSRII flow cytometer.

Quality Control and Automated Gating

Initial quality control involved visual inspection of compensated data using FlowJo software to ensure optimal experimental conditions and identify major populations. Five QC steps were performed:

- **Sample quality:** Samples were visually inspected at thawing and after centrifugation. Samples with incomplete red blood cell lysis or poorly resuspended cell pellets were flagged or excluded.
- **Cytometer performance:** CS&T beads were used to assess instrument performance.
- **Laser stability:** Rainbow beads were used to monitor and adjust for laser fluctuations.
- **Treatment group randomization:** Samples were randomized across plates using a block randomization scheme.
- **Batch effects:** A biological internal control was run in parallel with study samples to assess stability across batches.

An automated pipeline was used for gating and further quality control to reduce subjectivity and time. Data was compensated, transformed, and poor-quality events were removed using flowCut. flowDensity performed automated cell population identification based on predefined gating strategies for each panel (33 populations for Innate, 23 for Adaptive). Samples were excluded based on:

- Insufficient cell numbers for complete gating
- Unusual cell distributions affecting population identification

- Low cell counts in the "Size" gate (threshold: mean - 3*SD)
- Low live cell numbers (threshold: mean - 3*SD)
- Sex mismatch in metadata

Data Analysis

flowTypeFilter generated all possible marker combinations (immunophenotypes) based on flowDensity thresholds. To reduce false positives, populations with low cell counts were excluded (<10 cells/ul for Adaptive, <20 cells/ul for Innate). Cell counts were normalized using counting beads and the remaining data was analyzed using RchyOptimyx.

Note: The detailed descriptions of flowCut[2], flowDensity [3], flowTypeFilter[4], and RchyOptimyx[5] algorithms and their implementation can be found in the respective publications.

References

1 Montante, S. *et al.* Breastfeeding and Neonatal Age Influence Neutrophil-Driven Ontogeny of Blood Cell Populations in the First Week of Human Life. *J Immunol Res* **2024**, 1117796, doi:10.1155/2024/1117796 (2024).

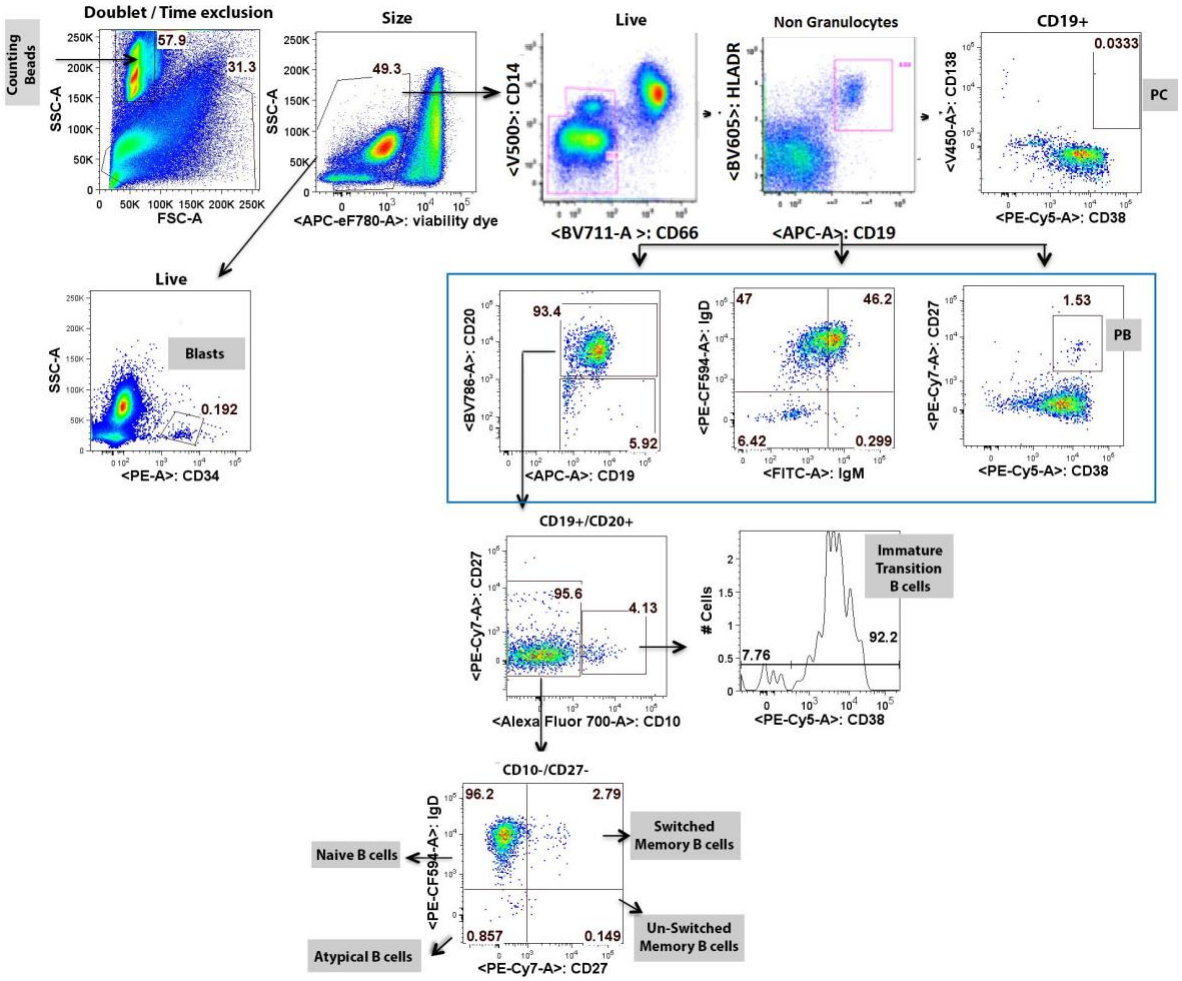
2 Meskas, J., Yokosawa, D., Wang, S., Segat, G. C. & Brinkman, R. R. flowCut: An R package for automated removal of outlier events and flagging of files based on time versus fluorescence analysis. *Cytometry A* **103**, 71-81, doi:10.1002/cyto.a.24670 (2023).

3 Eskandari, G. *et al.* Implementing flowDensity for Automated Analysis of Bone Marrow Lymphocyte Population. *J Pathol Inform* **12**, 49, doi:10.4103/JOPI.JOPI_12_21 (2021).

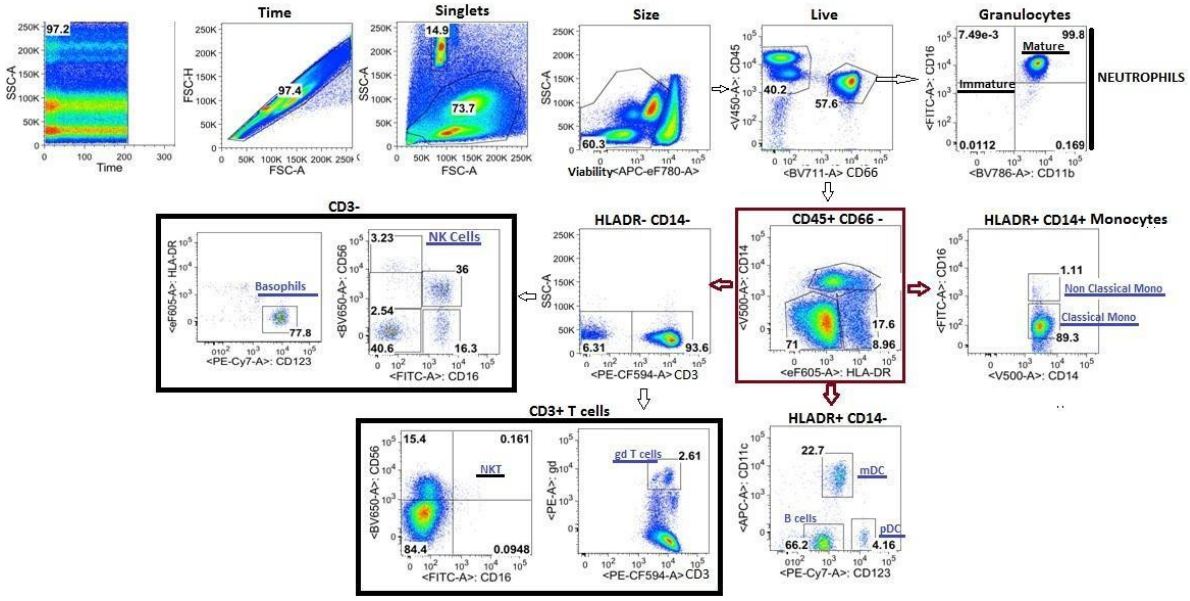
4 flowTypeFilter v. 1 (2023). eve-chen97/flowTypeFilter: Phenotyping Flow Cytometry Assays (github.com)

5 O'Neill, K., Jalali, A., Aghaeepour, N., Hoos, H. & Brinkman, R. R. Enhanced flowType/RchyOptimyx: a BioConductor pipeline for discovery in high-dimensional cytometry data. *Bioinformatics* **30**, 1329-1330, doi:10.1093/bioinformatics/btt770 (2014).

Flow cytometry gating strategy, adaptive immune cell panel



Flow cytometry gating strategy, innate panel



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Amrit Singh

Supplementary Table 1 – Flow cytometry panel markers

INNATE FLOW CYTOMETRY PANEL	
TARGET	**FLUOROCHROME**
CD64	Alexa 700
CD11c	APC
CD123	PE-Cy7
CD3	PE-CF594
gd TCR	PE
CD56	BV650
CD11b	BV786
CD16	FITC
CD45	V450
ADAPTIVE FLOW CYTOMETRY PANEL	
TARGET	**FLUOROCHROME**
CD10	Alexa 700
CD19	APC
CD27	PE-Cy7
CD38	PE-Cy5
IgD	PE-CF594
CD34	PE
IgM	FITC
CD20	BV786
CD138	V450
COMMON MARKERS IN INNATE AND ADAPTIVE PANELS	
TARGET	**FLUOROCHROME**
FVD	APC-eFluor780
CD66	Biotin/ BV711 Streptavidine
CD14	V500
HLADR	eFluor605