SUPPLEMENTAL MATERIAL

SUPPLEMENTAL METHODS

Immune Profiling Cohort

The *Immune Trajectory Cohort* consisted of preterm infants enrolled in the IRoN (Immunoregulation of the Newborn) study conducted at the Department of Pediatrics, University Hospital of Lübeck. Data were collected from infants born between October 1, 2014, and December 31, 2024. Inclusion criteria were preterm birth (<37+0 weeks of gestation), in-hospital treatment, and availability of lymphocyte subset analysis in peripheral blood within the first 49 days of life (n = 511). Exclusion criteria included chromosomal abnormalities, syndromal diseases, or death prior to hospital discharge (n = 12).

Extended Phenotyping Cohort

The *Extended Phenotyping Cohort* included preterm infants born at <32+0 weeks of gestation and birth weight <1,500 g, enrolled in the IRoN study at the Department of Pediatrics, University Hospital of Würzburg. Data were collected from infants born between January 1, 2022, and September 30, 2024 (n = 84). Exclusion criteria were the same as for the *Immune Trajectory Cohort* (n = 6 infants excluded from analysis). Sampling of peripheral blood for flow cytometric analysis occurred as early as possible after birth (median day: 3, range: 1-10) and on day 28-30.

Baseline characteristics of both cohorts and major in-hospital complications related to prematurity are detailed in Table 1.

Definitions

Gestational age (GA) was calculated from the best obstetric estimate according to early prenatal ultrasound and obstetric examination. Small for gestational age (SGA) was defined as a birth weight less than the 10th percentile according to sex-specific standards for birth weight by postmenstrual age in Germany(1). Amniotic infection syndrome (or Triple I) was

suspected by the attending obstetrician as cause of preterm birth based on maternal fever plus fetal tachycardia, a white blood cell count greater than 15 000/µL, or a foul-smelling discharge. When chorionamnionitis was histologically confirmed it was considered as proven.

Neonatal sepsis was defined as blood culture-proven sepsis identified by clinical sepsis criteria and detection of a causative pathogen in one or more blood cultures according to criteria of the German National Nosocomial Infection Surveillance System in Preterm Infants (NEO-KISS). Early-onset sepsis (EOS) was defined as sepsis occurring within the first 72 hours of life, while late-onset sepsis (LOS) was defined as sepsis episode after 72 hours of life(2).

Bronchopulmonary dysplasia (BPD) was defined as requirement of supplemental oxygen and/or ventilation support at 36 weeks' postmenstrual age(3). Intraventricular hemorrhage (IVH) was diagnosed according to Papile classification based on cranial ultrasound findings(4). Necrotizing enterocolitis (NEC) requiring surgery corresponded to clinical NEC classified as Bell stage II or III with the need for laparotomy and macroscopic diagnosis of NEC(5). Focal intestinal perforation (FIP) was defined as spontaneous intestinal perforation requiring laparatomy or peritoneal drainage.

Ethics

Written informed consent was obtained from parents or legal representatives. The study protocols were approved by the local ethics committees at the University of Lübeck (IRON AZ 15-304) and the University Hospital of Würzburg (IRON Würzburg 11/21 me). Blood samples were obtained as part of routine clinical procedures, with additional volumes for research purposes remaining below 1% of total blood volume per sampling, in compliance with European Medicines Agency (EMA) guidelines on medicinal product investigation in preterm and term neonates (PDCO, 2006).

Flow cytometry

Peripheral blood samples within both cohorts were collected in EDTA tubes and processed for flow cytometry within 24 hours of collection.

Immune Profiling Cohort: Absolute and relative lymphocyte counts, including T helper cells and cytotoxic T cells, B cells, and natural killer (NK) cells, were measured during routine centralized laboratory diagnostics when blood samples were collected for clinical purposes. A BD FACSCanto II system (BD Biosciences) equipped with the BD FACS Canto Clinical software was used for analysis of lymphocyte subsets in whole blood utilizing Multitest 6-Color TBNK (T cells and B cells) kits according to the manufacturer's protocols. Representative plots and detailed information on the gating strategy used by the central laboratory are available in the BD Biosciences user manual. Weekly checks of all cytometer performances were performed using BD FACS 7-Color Setup Beads, with alternating use of BD Multi-Check Control and BD Multi-Check CD4 Low Control quality controls twice daily.

Extended Phenotyping Cohort: Lymphocyte counts were determined as part of routine diagnostics (ADVIA 2120i, Siemens Healthineers). Whole blood was stained with three different antibody panels (Supplemental Table 2, Biolegend) for 30 minutes at room temperature. Erythrocytes were lysed with FACS Lysing Solution (BD Biosciences), and cells were washed, centrifuged (1,500 rpm, 5 min), and resuspended in PBS containing 0.5% BSA. Data acquisition was performed on a BD FACSCanto II cytometer (BD Biosciences), and analysis was conducted using FlowJo software (version 10; Tree Star). Regular checks of cytometer performances were performed using 8 peak Rainbow Calibration Beads (Biolegend). Gating strategies are presented in Supplemental Figure 1. Following recommendations of the EuroFlow consortium, Panel 1 gives an overview of main adaptive immune cell populations and memory formation in T and B cell subsets, using a double-staining approach for markers specific to B and T cells(6). Panel 2 assesses the frequencies of thymic output (recent thymic emigrants) and the proportion of T cells expressing the activation marker HLA-DR. Furthermore, it enables comparison of two complementary gating strategies for T cell subsets (based on CD27 and CD45RO or based on CCR7 and CD45RO). Panel 3 measures the polarization status of memory T helper cells using expression patterns of surface chemokine receptors that have been linked to expression of marker cytokines upon *ex vivo* stimulation(7-9). Although low in frequencies, we excluded circulating T follicular helper (cTFH)

cells and the equivalent subset among Tregs (circulating T follicular regulatory (cTFR) cells) by gating on CD45RO+CXCR5- T helper cells and Tregs for a more stringent analysis. The presented analysis strategy enables comparable assessments of adaptive immunity cell subsets in the small volumes of whole blood samples (e.g. 50-100 µl for the 3 panels) available for preterm infants without the need for cell isolation or freezing.

Statistical Analysis

Statistical analyses and graphical representations were performed using R software (version 4.4.3). In the *Immune Profiling Cohort*, outliers within lymphocyte subsets, defined as z-scores < -3.29 or > 3.29, were excluded prior to analysis. Group differences were assessed using one-way ANOVA. In the *Extended Phenotype Cohort* due to smaller sample sizes and analysis in subgroups group differences were assessed using Kruskal-Wallis test. Pairwise subgroup comparisons were conducted using the Wilcoxon rank sum test with Benjamini-Hochberg correction for multiple testing. Developmental trajectories of lymphocyte subset counts were modeled using the Loess method implemented in the geom_smooth function. Missing data were not imputed.

Principal component analysis (PCA) was conducted with the prcomp function (stats package) with scaling to unit variance. Results were visualized using the factoextra (version 1.0.7) package and ggplot2 (version 3.5.1). Complete datasets from the *Immune Profiling Cohort*, including lymphocyte counts and T-, B-, and NK-cell frequencies, were used for PCA.

MetadeconfoundR (version 1.0.2, https://github.com/TillBirkner/metadeconfoundR) was employed to evaluate potential confounding effects of clinical metadata on lymphocyte subset frequencies. The tool employs univariate statistics to identify associations between lymphocyte subsets and sample metadata, subsequently applying nested linear model comparisons to account for confounding effects(10). To minimize potential bias resulting from the fact that the number of measurements obtained at later postnatal ages was higher in more premature infants, we restricted the multivariate analyses to samples collected within the first 35 days of life. AIS, sex, SGA, delivery mode, multiple birth, EOS, LOS, NEC, FIP, BPD, and IVH were

used as binary variables. Where appropriate, categorical variables were adapted to binary classifications (e.g., clinical suspicion and histologically confirmed AIS were combined as AIS). Prematurity (calculated in weeks to 40 weeks of gestation, where higher values indicate greater prematurity) and birth weight (grams) were included as continuous variables. Associations and confounding effects were analyzed separately for each postnatal age group.

SUPPLEMENTAL FIGURES

Supplemental Figure 1 – Flow cytometric gating strategies in *Extended Phenotyping Cohort*

Exemplary depiction of flow cytometric gating and analysis strategies used in FACS panel 1-3. Resulting populations are named. Th: T helper cell; Tcon: conventional T cell; Treg: regulatory T cell; cTFH: circulating T follicular helper cell; cTFR: circulating T follicular regulatory cell, TEMRA: Effector memory T cells re-expressing CD45RA; RTE: Recent thymic emigrants.

Supplemental Figure 2 – Immunophenotypes do not converge due to postmenstrual age

A Boxplots of PC1 values from the PCA (Figure 1 and this figure) grouped by postnatal time of sampling and gestational age at birth. Each point represents an individual sample. Significance was determined using one-way ANOVA to compare subset frequencies between gestational age groups for a given range of time; ns: not significant, ***: $p<0.001$, ****: $p<0.0001$. **B** Relative contributions of immune cell subsets used for PCA to principal components 1 and 2. Positively contributing variables are shown in violet, negatively contributing in petrol. **C** PCA representations of cellular immunophenotyping in preterm infants in the first 49 days of life as in Figure 1 colored for gestational age at birth (weeks). Plots are splitted for different postmenstrual ages (days). Each small circle represents an individual sample, each large circle the geometric mean of all samples in the indicated gestational age group within each plot (calculated when more than 10 measurements were available). PCA: principal component analysis; PC: principal component; GA: gestational age.

Supplemental Figure 3 – Postnatal trajectories of lymphocyte and subset counts

A-F Lymphocyte and lymphocyte subset counts in peripheral blood of preterm infants grouped for different gestational ages at birth in the first 49 days of life. Line indicates smoothed conditional mean computed by Loess method, grey area indicates 95 % confidence interval.

Supplemental Figure 4 – Time intervals for analysis of postnatal trajectories of lymphocyte and subset counts

A-F Lymphocyte counts and lymphocyte subset frequencies in peripheral blood of preterm infants grouped for different gestational ages at birth in the first 49 days of life. Line show smoothed conditional mean computed by Loess method, grey area indicates 95 % confidence interval. Vertical lines define chosen time intervals for further analysis.

Supplemental Figure 5 – Postnatal development of T cell frequencies and CD4/CD8 ratios

Boxplots of **A** T cell frequencies and **B** CD4/CD8 ratios in preterm infants grouped by gestational age at birth and postnatal time of sampling. Each point represents an individual sample. Significance was determined using one-way ANOVA to compare subset frequencies between gestational age groups for a given range of time; ns: not significant, ****: $p<0.0001$.

Supplemental Figure 6 – Prematurity, amniotic infection syndrome and sex shape postnatal cell trajectories of the adaptive immune system

Heatmaps illustrating the results of integrated analysis of immune cell trajectories and clinical metadata using MetadeconfoundR. Stars indicate 'deconfounded' effects, circles indicate effects 'confounded' by other variables. Positive effect sizes are shown in red, negative in blue. **A** shows correlations between cell subsets and clinical metadata at defined postnatal ages. **B** shows the same data rearranged according to cell subsets. FIP: Focal intestinal perforation; BPD: Bronchopulmonary dysplasia; AIS: Amniotic infection syndrome; EOS: Early-onset sepsis; LOS: Late-onset sepsis; SGA: Small for gestational age; IVH: Intraventricular hemorrhage.

Supplemental Figure 7 – Postnatal development of lymphocyte subsets due to AIS

Boxplots of indicated lymphocyte subset frequencies (**A-E**) and CD4/CD8 ratios (**F**) in preterm infants with and without the diagnosis of AIS (red and blue respectively) grouped by gestational age at birth and postnatal time of sampling. Each point represents an individual sample. Significance was determined between infants with and without AIS within subgroups using Wilcoxon test with Benjamini-Hochberg correction for multiple testing strategy; only adjusted p values < 0.05 are shown; *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$.

Supplemental Figure 8 – Postnatal development of lymphocyte subsets due to sex

Boxplots of indicated lymphocyte subset frequencies (**A-E**) and CD4/CD8 ratios (**F**) in male (red) and female (blue) preterm infants grouped by gestational age at birth and postnatal time of sampling. Each point represents an individual sample. Significance was determined between male and female infants within subgroups using Wilcoxon test with Benjamini-Hochberg correction for multiple testing strategy; only adjusted p values < 0.05 are shown; *: p<0.05, **: p<0.01, ***: p<0.001.

Supplemental Figure 9 – Comparison of lymphocyte and subset counts in the *Immune Trajectory Cohort* and the *Extended Phenotyping Cohort*

A-F Lymphocyte and lymphocyte subset counts in peripheral blood of preterm infants grouped for different gestational ages at birth in the first 49 days of life as in Supplemental Figure 2. Grey points represent individual measurements from the *Immune Trajectory Cohort*, red dots individual measurements from the *Extended Phenotyping Cohort*

Supplemental Figure 10 – Predominant naïve B cell phenotype in preterm infants

A-F Lymphocyte subset frequencies and counts in peripheral blood of preterm infants grouped for different gestational ages at birth in the first week of life and at day 28. P values indicate significances determined by Kruskal-Wallis test to compare subset frequencies between gestational age groups within sampling groups. Asterisks mark significant differences to the gestational age group ’23-25’ at the given time point calculated using Wilcoxon rank sum test adjusted with Benjamini-Hochberg correction for multiple testing; only significant adjusted p values are shown; *: p<0.05.

Supplemental Figure 11 – Predominant effector T cell phenotype in preterm infants

A-F Lymphocyte subset frequencies and counts in peripheral blood of preterm infants grouped for different gestational ages at birth in the first week of life and at day 28. **A-B** and **C-D** respectively represent different flow cytometric gating strategies for analysis of corresponding

naïve, effector and memory T cell populations using either CD45RO and CD27 or CD45RO and CCR7 surface expression (Supplemental Figure 1). P values indicate significances determined by Kruskal-Wallis test to compare subset frequencies between gestational age groups within sampling groups. Asterisks mark significant differences to the gestational age group '23-25' at the given time point calculated using Wilcoxon rank sum test adjusted with Benjamini-Hochberg correction for multiple testing; only significant adjusted p values are shown; *: $p<0.05$, **: $p<0.01$.

Supplemental Figure 12 – Memory cell frequencies among $CD4^+$ T helper cells and conventional (non Treg) $CD4^+$ T helper cells

A-B $CD45RO^+CD4^+$ T helper and $CD45RO^+$conventional (non Treg) $CD4^+$ T helper cell frequencies of $CD4^+$ T cells in peripheral blood of preterm infants grouped for different gestational ages at birth in the first week of life and at day 28. Significances were determined as in Supplemental Figure 8 and 9.

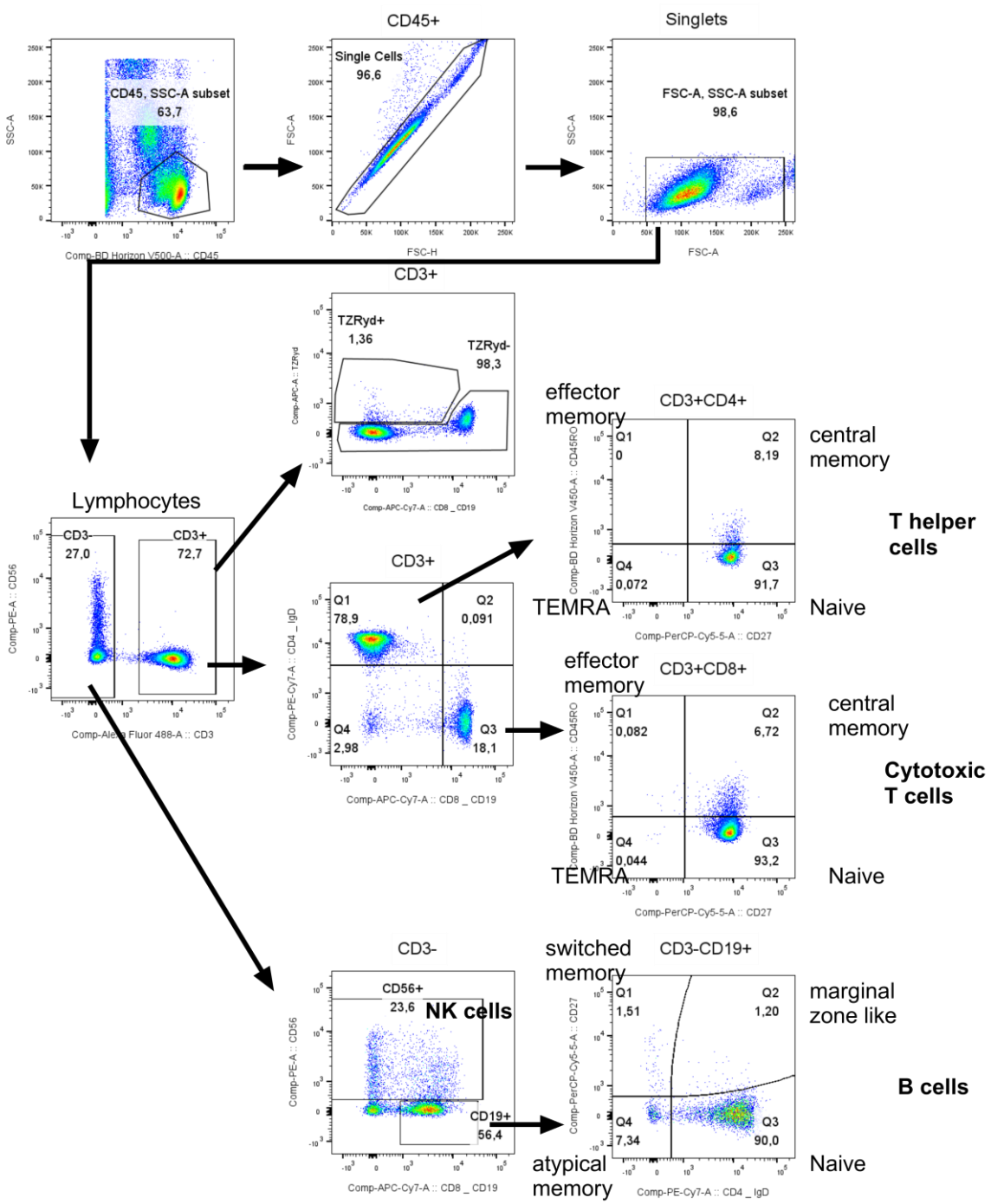
Supplemental Figure 13 – Comparison of lymphocyte subset counts to published reference values for SCID-screening

A-F Cell counts of lymphocytes and indicated lymphocyte subsets according to postmenstrual age at time of blood draw. Every dot represents an individual measurement from the *Immune Trajectory Cohort* in this study. Pink rectangles represent the ranges between the 5th and 95th percentile of the published reference ranges by Amatuni et al., 2019(11).

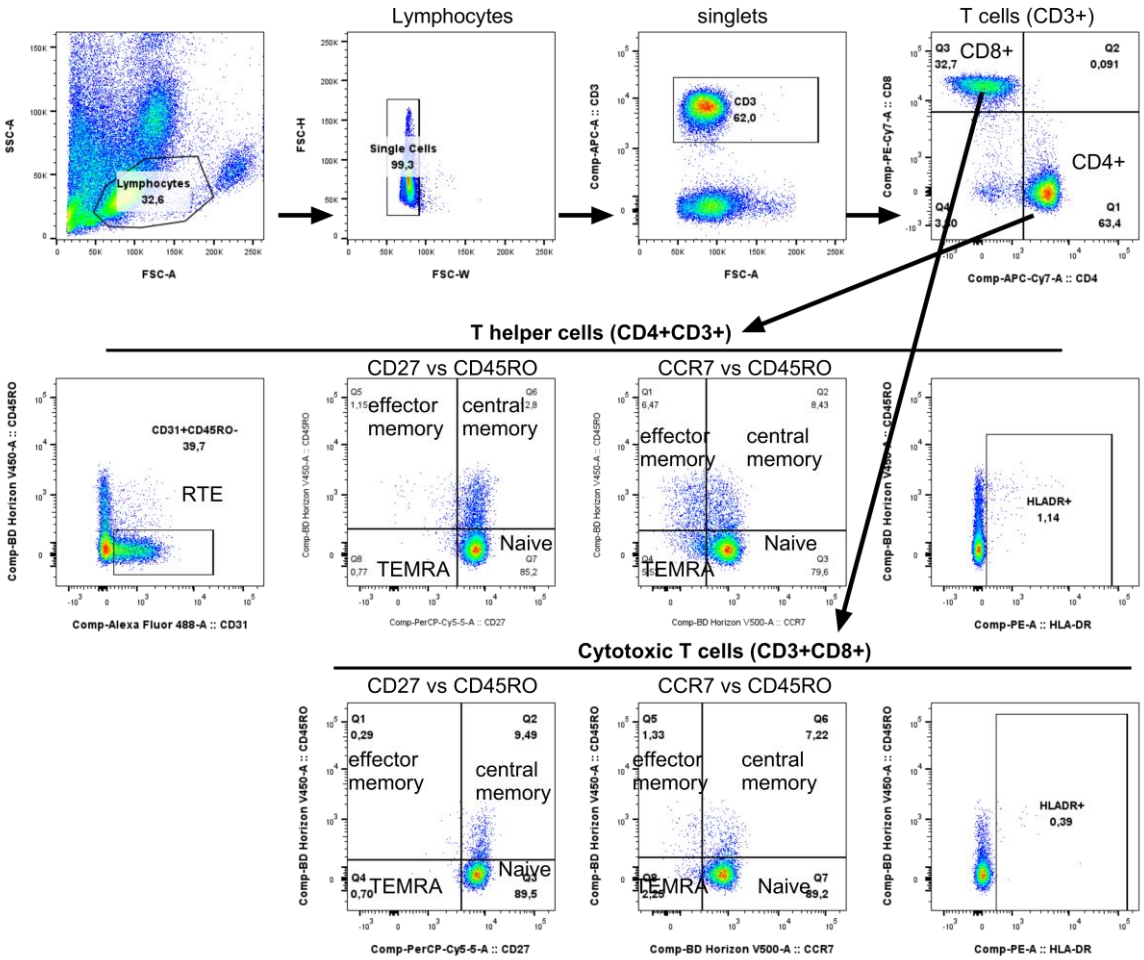
Supplemental Figure 14 – Comparison of lymphocyte subset frequencies to published reference values for SCID-screening

A-D Frequencies of indicated lymphocytes subsets according to postmenstrual age at time of blood draw. Every dot represents an individual measurement from the *Immune Trajectory Cohort* in this study. Pink rectangles represent the ranges between the 5th and 95th percentile of the published reference ranges by Amatuni et al., 2019(11).

Supplemental Figure 1 – Panel 1



Supplemental Figure 1 – Panel 2



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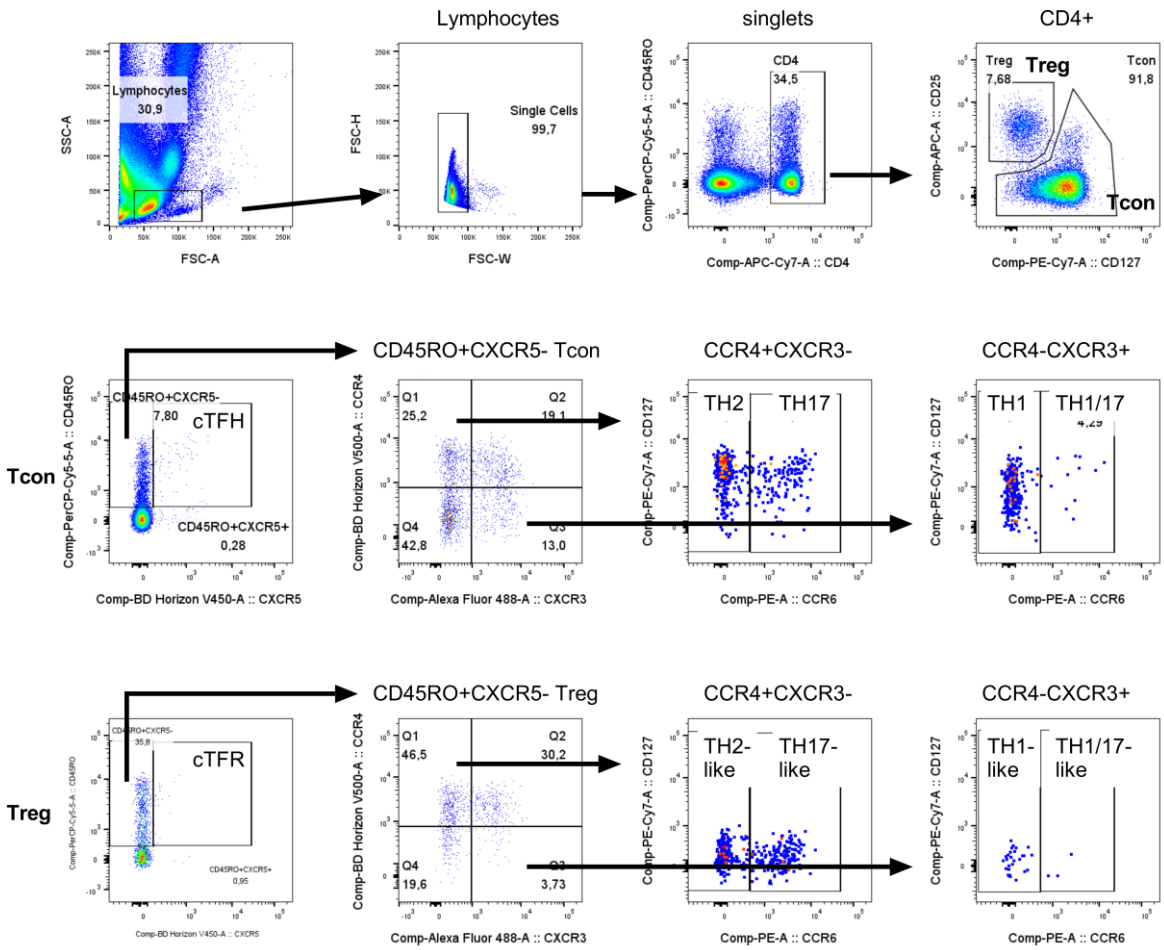
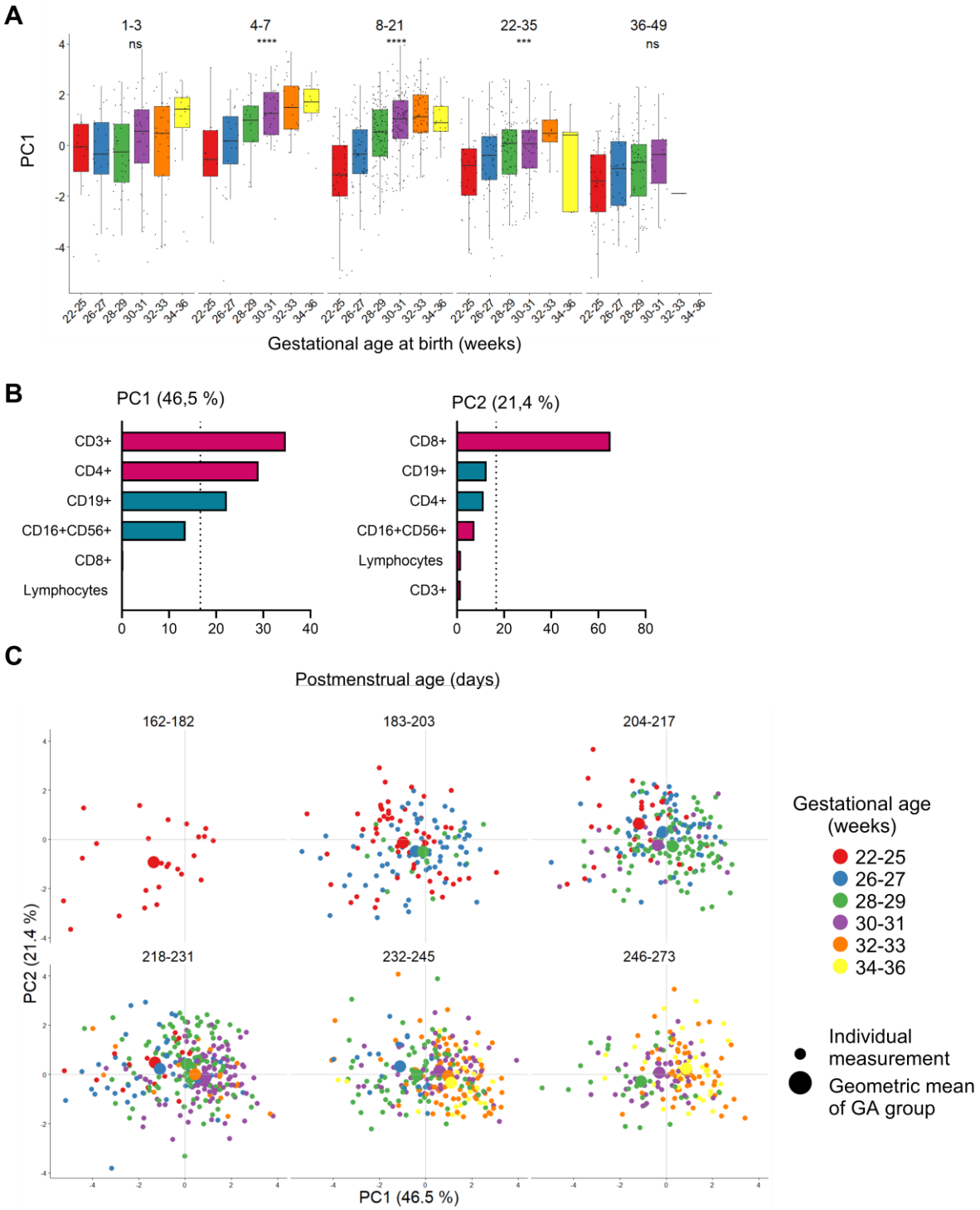


228 Supplemental Figure 2



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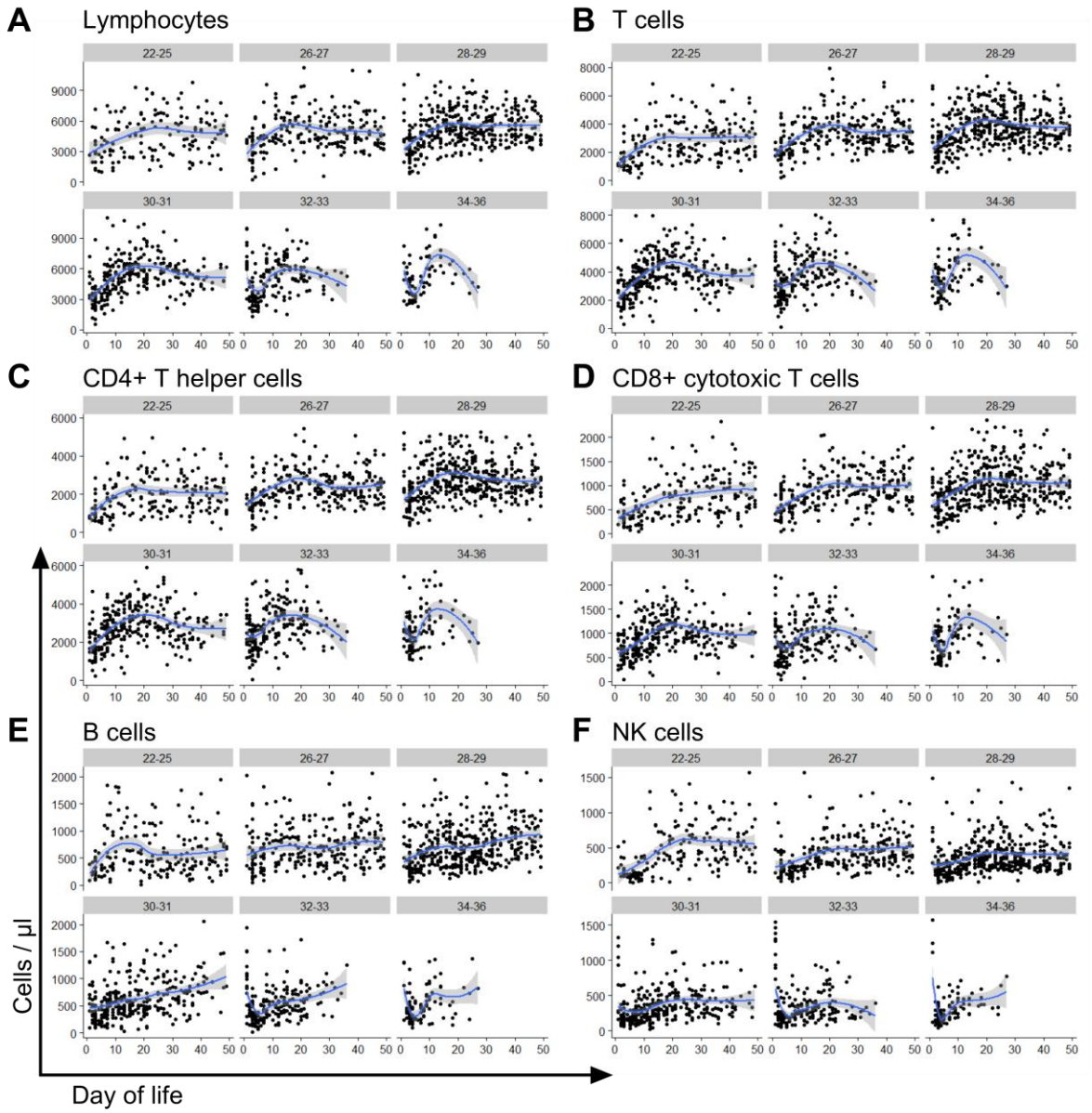

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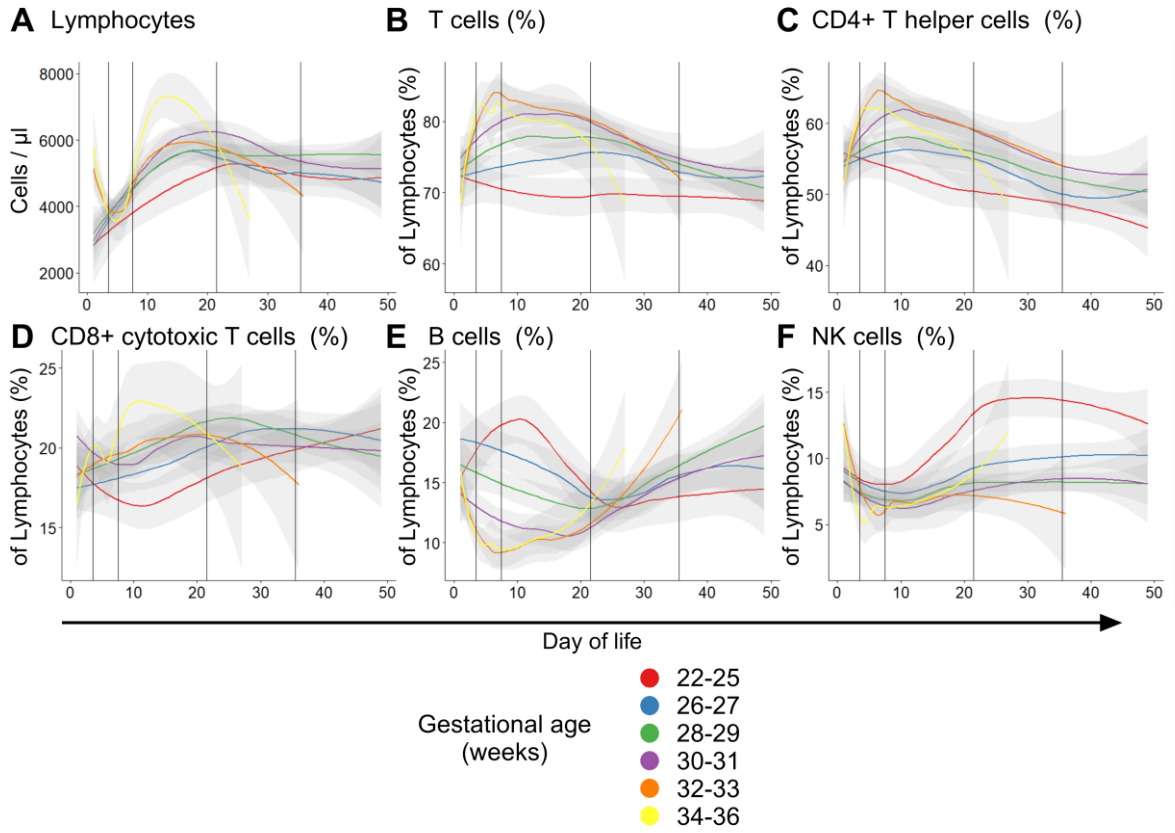

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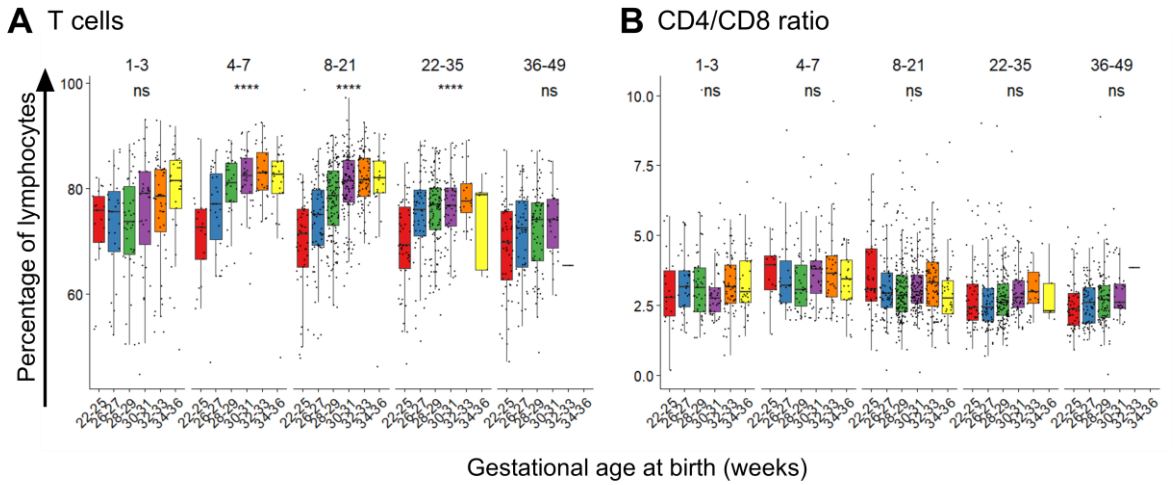

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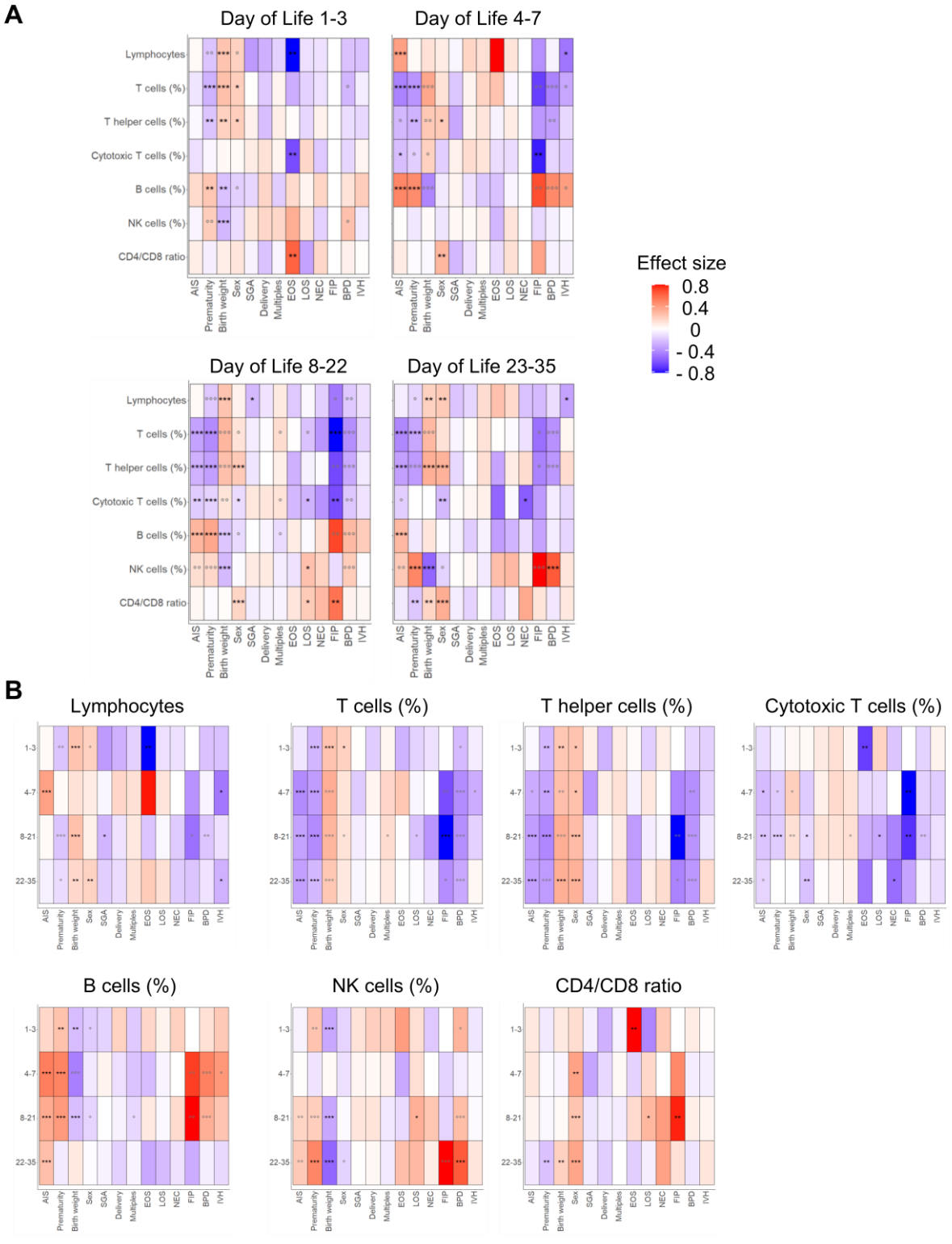
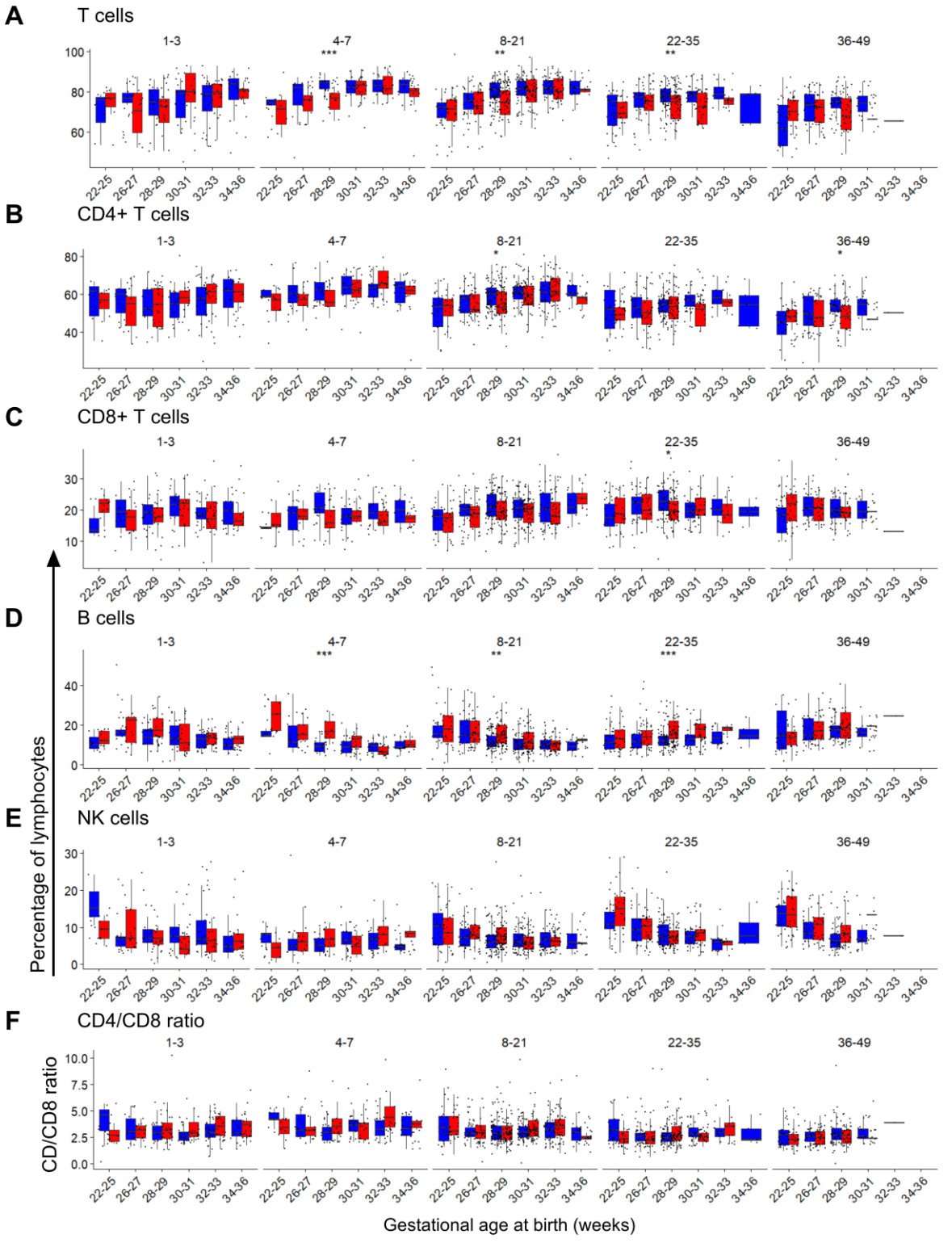


247 Supplemental Figure 7



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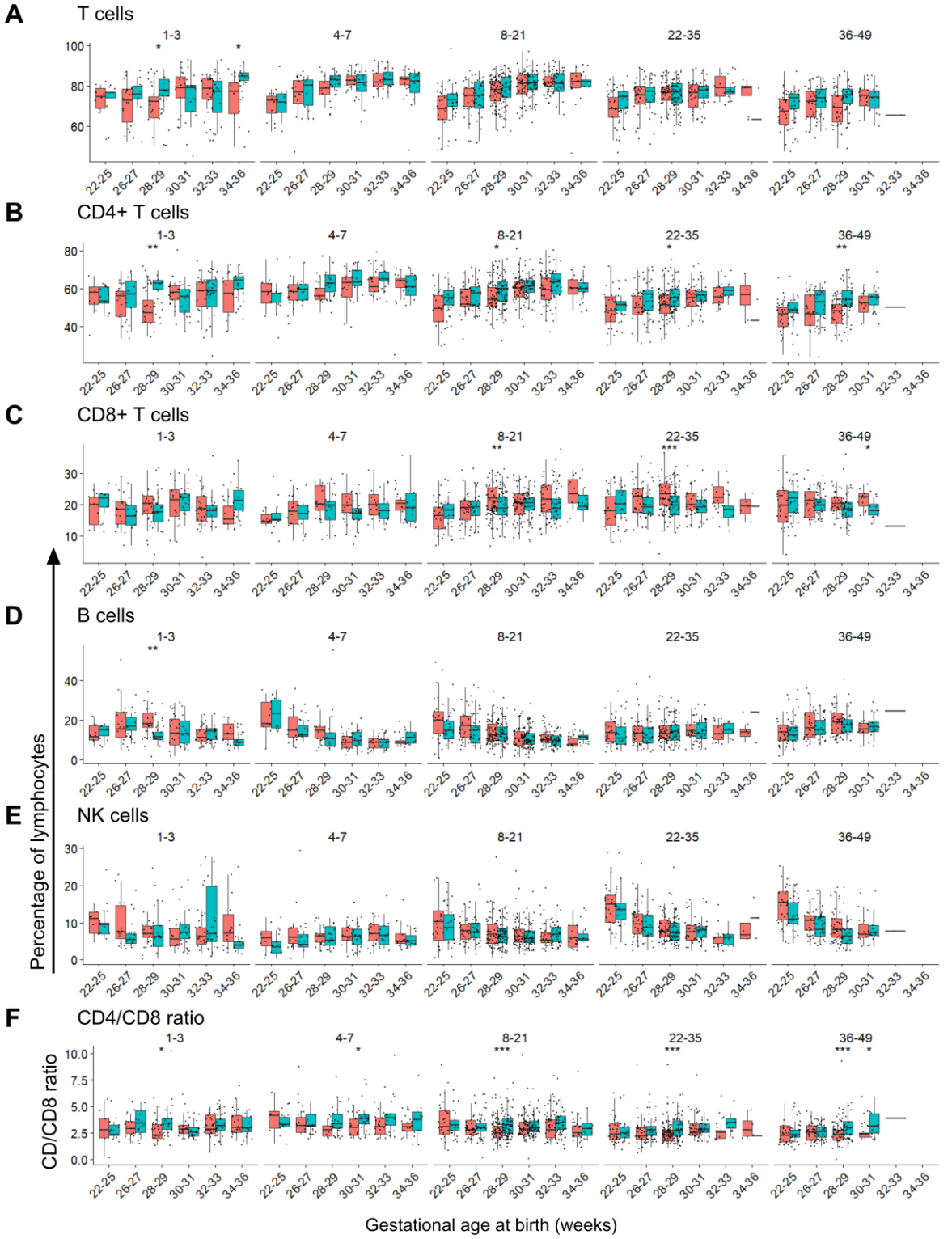
Supplemental Figure 8



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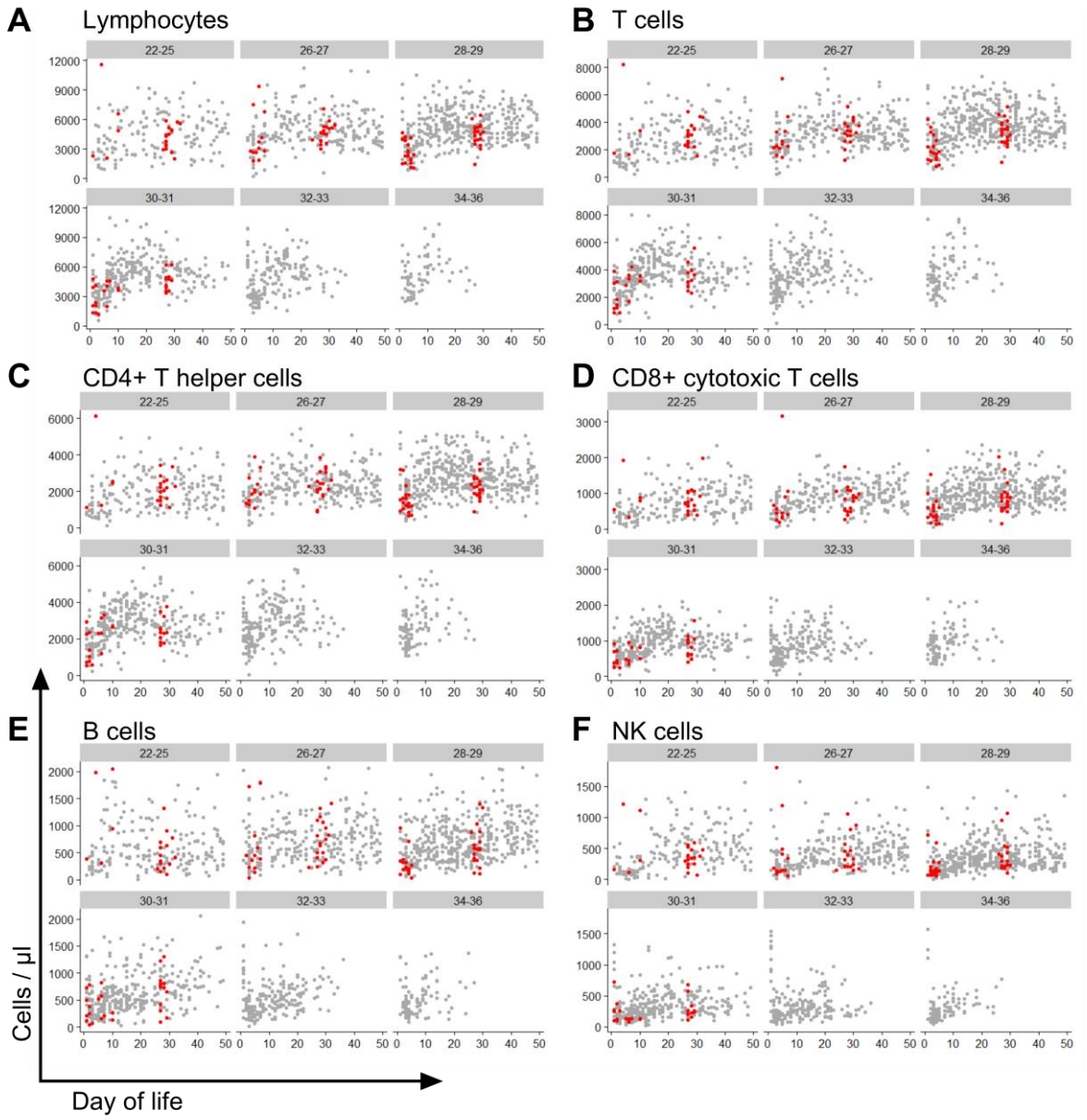

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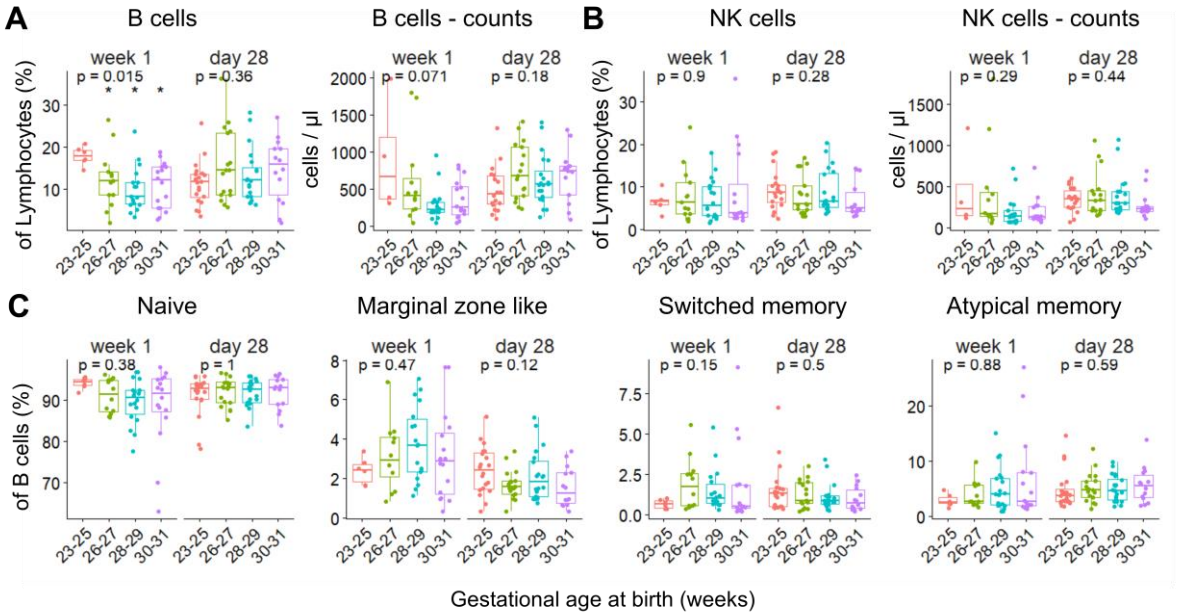
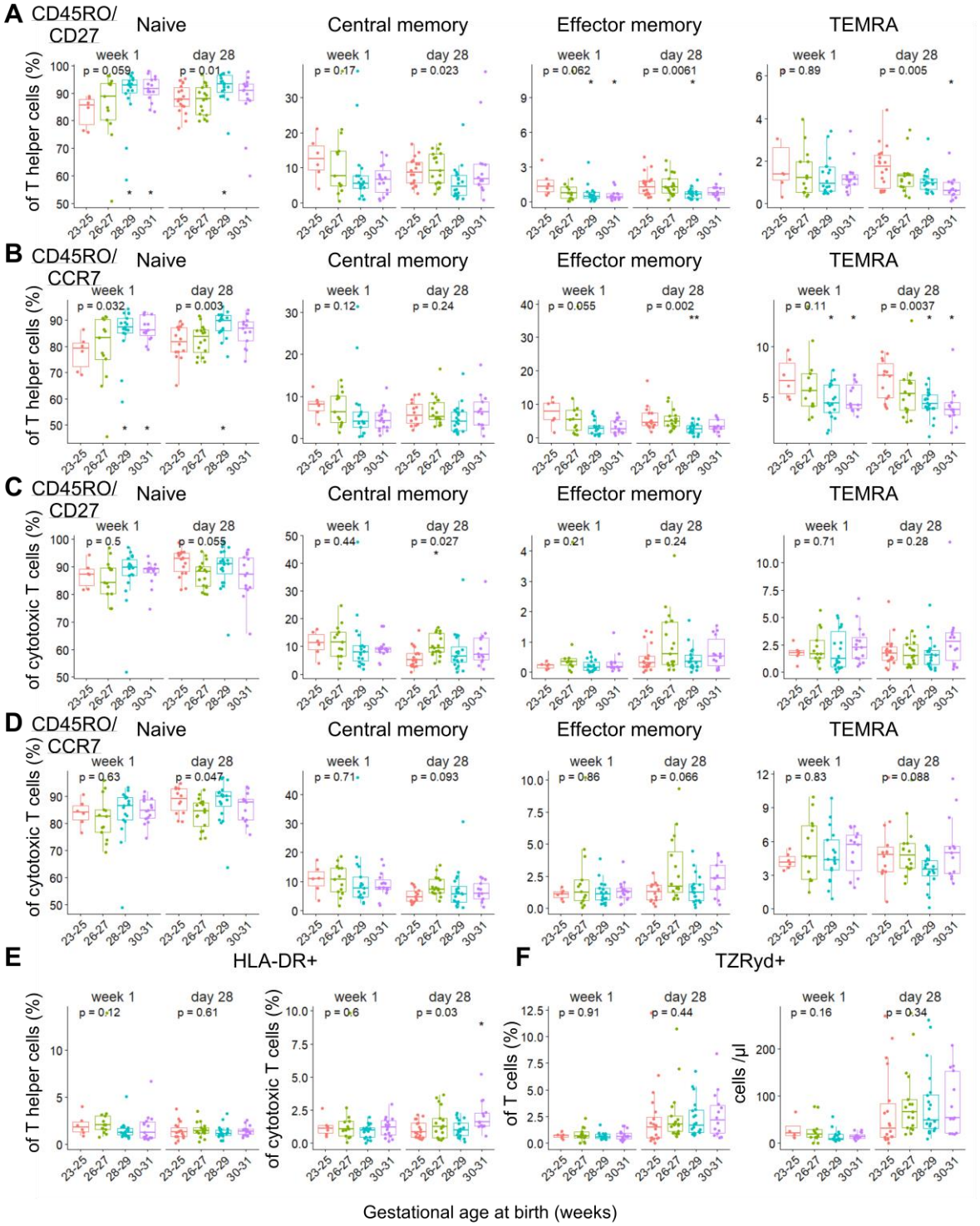


263 Supplemental Figure 11



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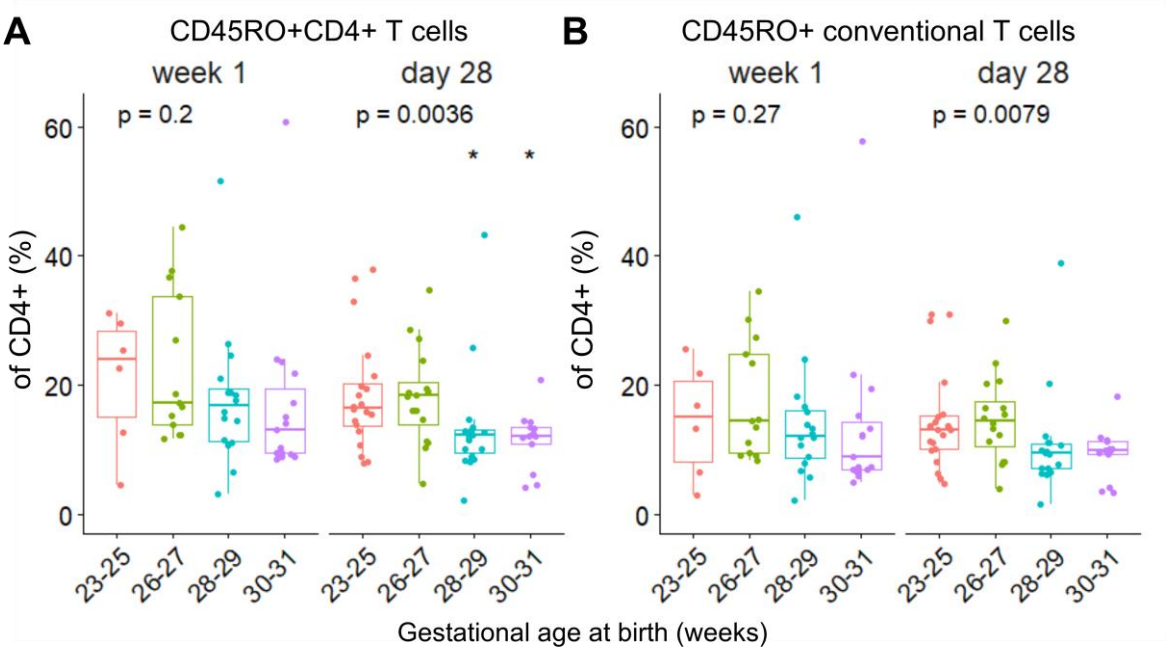

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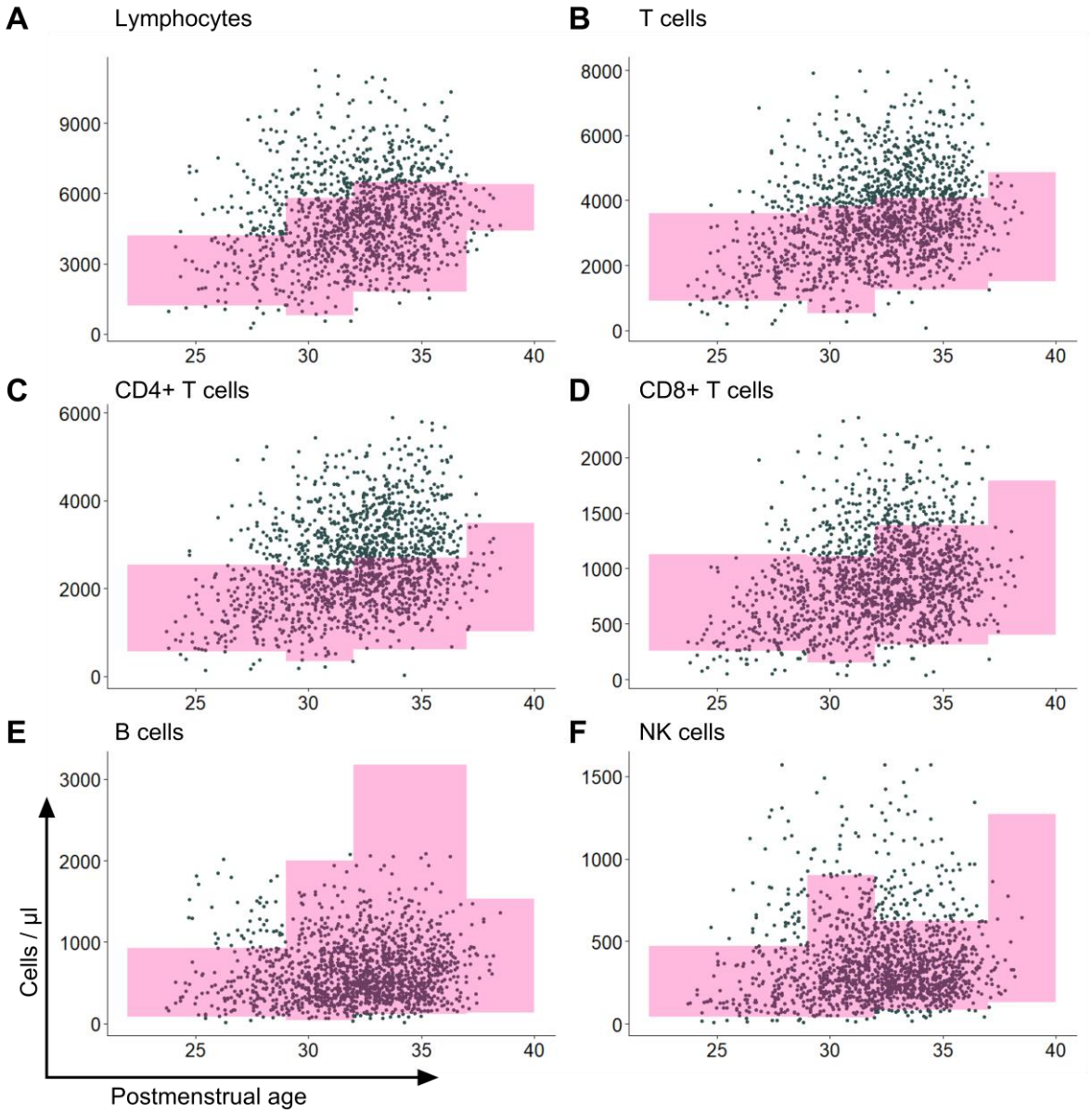

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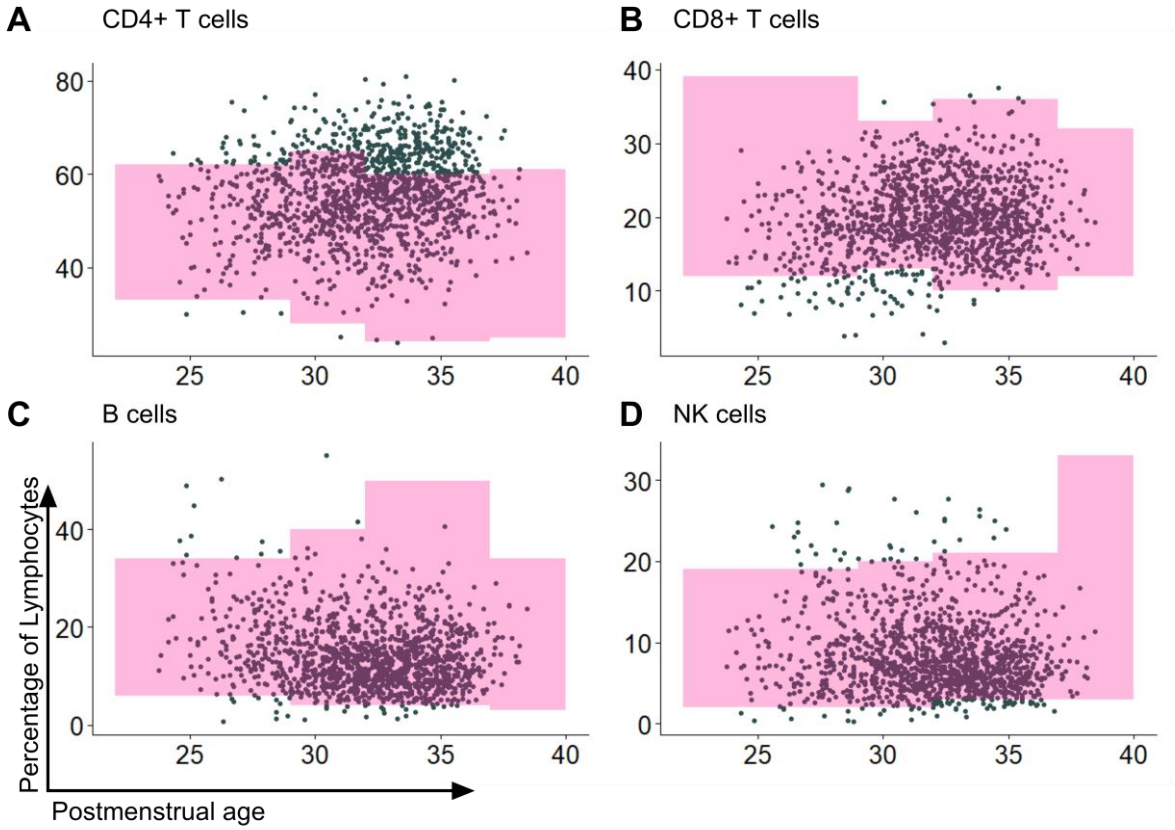

279 SUPPLEMENTAL TABLES

280 Supplemental Table 1

281 Clinical characteristics at birth of gestational age subgroups in the two cohorts. GA: gestational
282 age.

283 Supplemental Table 2

284 Monoclonal antibodies and fluorophores used for whole blood staining for flow cytometric
285 analysis of lymphocyte subsets. All antibodies used were from Biolegend.

Supplemental Tables

Supplemental Table 1

	Subgroup	22-25	26-27	28-29	30-31	32-33	34-36
Cohort 1	n	49	56	106	103	114	71
	GA at birth (median (range))	25 (22.9 - 25.9)	27.1 (26 - 27.9)	28.9 (28 - 29.9)	31 (30 - 31.9)	33 (32 - 33.9)	34.4 (34 - 36.4)
	Weight at birth (median (range))	680 (305 - 1100)	965 (385 - 1205)	1235 (600 - 1630)	1480 (665 - 2400)	1820 (1130 - 2645)	2190 (1270 - 2820)
	sex (% female)	34.7	46.4	52.8	59.2	54.4	42.3
Cohort 2	n	20	17	23	18	-	-
	GA at birth (median (range))	25.1 (23.9 - 25.9)	26.9 (26.1 - 27.9)	29.1 (28 - 29.9)	30.7 (30 - 31.9)	-	-
	Weight at birth (median (range))	640 (315 - 1055)	950 (590 - 1330)	1075 (395 - 1490)	1267.5 (720 - 1470)	-	-
	sex (% female)	40	55.8	56.5	44.4	-	-

Supplemental Table 2

Fluorochrome	Panel 1		Panel 2		Panel 3	
	Target	**Clone**	**Target**	**Clone**	**Target**	**Clone**
FITC	CD3	SK7	CD31	WM59	CXCR3	G025H7
PE	CD56	HCD56	HLA-DR	L243	CCR6	G034E3
APC	TZRγδ	B1	CD3	SK7	CD25	BC96
PerCP-Cy5.5	CD27	M-T271	CD27	M-T271	CD45RO	UCHL1
PE-Cy7	CD4 IgD	OKT4 IA6-2	CD4	OKT4	CD127	A019D5
APC-Cy7	CD8 CD19	SK1 HIB19	CD8	SK1	CD4	OKT4
BV421	CD45RO	UCHL1	CD45RO	UCHL1	CXCR5	J252D4
BV510	CD45	HI30	CD38	HIT2	CCR4	L291H4

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