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Immunophenotyping of very and extremely preterm infants at risk of developing sepsis and necrotising enterocolitis – a prospective, single-centre cohort study

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Summary

OBJECTIVE: To characterise adaptive immunity in very and extremely preterm neonates and assess potential immunological markers that predispose them to infection.

STUDY DESIGN: Peripheral blood lymphocytes in 30 very to extremely preterm neonates (mean birth weight 815 g) were analysed using 9-colour flow cytometry at days of life 0–7, 8–14, and 15–28. Patients were prospectively recruited at the University Hospital of Zurich from 2021 to 2023. Statistical analyses included group comparisons and longitudinal assessments.

RESULTS: Six of the thirty preterm neonates (20%) developed early- or late-onset sepsis, of whom one (3%) also exhibited signs of necrotising enterocolitis. T and B lymphocytes were predominantly composed of naïve T cells and unswitched CD27- B cells, respectively. The CD4/CD8 ratio was initially elevated, especially in extremely preterm neonates, and declined significantly after week 1. EPT neonates also had significantly fewer natural killer cells compared with very premature infants. Although no distinct immunological features were identified as predisposing to infection, B-cell proportions were significantly higher in infected neonates during the second week of life, possibly reflecting an immune response to infection.

CONCLUSION: Adaptive immunity in preterm neonates is characterised by profound immaturity, including an almost complete absence of immunological memory and low CD8 T-cell frequency, which may limit effective pathogen elimination and increase susceptibility to infection. Immunophenotyping provides valuable insights into the immune processes in this vulnerable population and, when combined with clinical observations and routine laboratory findings, could aid in the earlier identification of high-risk neonates and guide targeted interventions.

Introduction

Preterm infants, particularly those with very low birth weight and/or gestational age, are highly susceptible to serious infections such as sepsis and necrotising enterocolitis [1, 2]. This vulnerability is multifactorial, involving maternal and microbiological factors, but is also linked to the immaturity of the neonatal immune system [3–7]. Despite a partial understanding of these factors, detailed insights into the early immune development of very PT infants, particularly during episodes of suspected or confirmed infection, remain limited.

Prematurity and low birth weight are significant risk factors for infection-related mortality and morbidity in neonates [1, 3, 5], with mortality rates for necrotising enterocolitis reaching 15–30%, even in high-resource settings [2, 8]. In Switzerland for example, approximately 0.4% of children are born with extremely low (<1000 g) birth weight [9], and even higher rates are reported

in other regions of the world [10]. The immaturity of both innate and adaptive immunity in these infants contributes significantly to their vulnerability to infection. At birth, all newborn infants display deficiencies in T-cell activation, B-cell antibody production, and T/B-cell interactions [1]. However, PT infants have significantly lower absolute B- and T-cell counts compared with term infants [3, 4, 11]. Notably, cytotoxic T cells (CD8+) are more severely affected than helper T cells (CD4+), leading to an elevated CD4/CD8 ratio [12–15]. Additionally, T-cells in these infants are predominantly naïve CD45RA+ T cells, lacking immunological memory [3, 16]. A study by Scheible et al. showed how alterations in T-cell maturation in preterm infants might influence respiratory morbidity in later infancy [17]. B-cell class switching and antibody production are also impaired in PT infants, partly due to reduced CD40 ligand expression, which hinders effective T/B-cell interactions [18, 19]. Combined with lower levels of maternal immunoglobulin G (IgG) transfer, these immunological features lead to inefficient phagocytosis and delayed pathogen elimination, elevating the risk of neonatal infections such as sepsis and necrotising enterocolitis [1, 3, 5].

Necrotising enterocolitis is characterised by extensive necrosis of intestinal tissue, which can lead to rupture, bleeding, and subsequent sepsis, and affects 5–10% of neonates with a birth weight of less than 1500 g [8]. Although the pathogenesis of necrotising enterocolitis remains incompletely understood, it likely involves a complex interplay among microbial colonisation, maternal antibodies, and neonatal immunity [20]. Recent findings indicate that impaired regulation of intestinal microbiota – primarily *Enterobacteriaceae* – by maternal immunoglobulin A (IgA) leads to gut dysbiosis and is associated with necrotising enterocolitis [21]. Dysregulation in mucosal and peripheral lymphocytes may also play a role, although evidence for this remains limited [20]. Analysis of resected ileal tissue from necrotising enterocolitis patients has revealed altered T-cell differentiation and a reduction in mucosal regulatory T cells (Tregs), which are crucial for maintaining intestinal homeostasis, compared with controls [22, 23]. A study by Bochennek et al. further demonstrated that patients with necrotising enterocolitis showed reduced peripheral natural killer (NK) cell and naïve cytotoxic T-cell frequencies [6]. In addition, sepsis in premature infants has been shown to influence NK and γδ T-cell frequencies and functional characteristics [24].

A deeper understanding of peripheral lymphocyte subsets in PT infants, particularly those with very low birth weight or born extremely preterm, as well as of how infections such as necrotising enterocolitis and LOS impact these populations, is needed to enable the early identification of at-risk infants and guide potential preventive interventions. In this study, multicolour flow cytometry was used to characterise peripheral lymphocyte populations and their subpopulations in very preterm infants, both with and without suspected or confirmed sepsis and/or necrotising enterocolitis.

Methods

Study design and patient cohort

This is a substudy of a larger prospective, explorative cohort study conducted at the neonatal intensive care unit (NICU) department of the University Hospital of Zurich, one of the major perinatal centres in Switzerland, which provides care for (extremely) preterm and other high-risk infants. The study was conducted in collaboration with ETH Zurich from 2021 to 2023. The project involved collecting biological materials (stool, stomach fluid, urine, and blood) from very-low-birth-weight infants. The study received approval from the local ethics committee of Zurich (BASEC 2020-01764) and was performed in accordance with the Declaration of Helsinki. Informed consent was obtained from the legal guardians of all participating infants. All neonates with a birth weight <1500 g who were born during the study period and from whom legal consent for blood sampling was obtained were eligible. Due to the limited size of this population, a formal sample size calculation was not feasible. Peripheral blood samples (100–200µl, EDTA) were collected serially from 30 very-low-birth-weight infants at different time points during their stay in the neonatal intensive care unit (NICU): days of life 1 to 7, DOL 8 to 14, and DOL 15 to 28 (see figure S1 in the appendix). For some patients, additional samples were also collected from DOL 29 until discharge. Blood sampling was conducted alongside the collection of other biological materials as part of the overarching project. For the comparison of immune cell profiles across different developmental stages, patients were stratified into three gestational age categories: <26 weeks, 26–28 weeks, and 29–31 weeks.

Sample preparation and acquisition

Fresh blood samples were immediately mixed with Cytodelics Whole Blood Stabiliser at the collection site in a 1:1 ratio, incubated at room temperature for approximately 10 minutes, and subsequently stored at –80°C for later analysis, following the manufacturer's protocol (Cytodelics AB; https://www.cytodelics.com/sites/cytodelics.com/files/2023-10/Protocol%20WBCS%20P01%20-%20Sample%20Stabilization_v1.11.pdf) [25, 26]. For staining and analysis, frozen samples were thawed at 37 °C, then centrifuged in PBS for 4 minutes. Cells were resuspended in 100 µl of a staining mix (adapted Euroflow panel for main lymphocyte subpopulations) [27–29] and incubated for 30 minutes. Stained samples were fixed, lysed, and washed according to the Cytodelics Whole Blood Processing protocol [29]. Data acquisition was performed using a BD FACSDiva cytometer. Data analysis was conducted with FlowJo software (version 10.10.0) using a gating strategy, as suggested by the Human Immunology Project [30]. Further details on the gating strategy are provided in figure S2 in the appendix.

Immunophenotyping

The primary immunodeficiency (PID) orientation tube (Cytognos), a Euroflow primary immunodeficiency antibody panel, was used with the following fluorochrome-antibody specificity: CD3 APC; CD4-IgM PerCP-Cy5,5; CD8-IgD FITC; CD16 PE; CD19-TCRγδ PE-Cy; CD27 BV421; CD45 ACP-C750; CD45RA BV510; CD56 PE. Additionally, a CD38 BV711 (BD) single antibody was included to enhance B-cell differentiation. Nine-colour flow cytometry was performed to identify the following lymphocyte subsets (see also table S1 in the appendix): CD45+ total lymphocytes; CD45+/CD3+ total T lymphocytes; CD45+/CD3+/TCRγδ-/CD4+ helper T lymphocytes; CD45+/CD3+/TCRγδ-/CD8+ cytotoxic T lymphocytes; CD45+/CD3+/TCRγδ-/CD4-/CD8- double-negative T lymphocytes; CD45+/CD19+ total B lymphocytes, and CD45+/CD16+/CD56+ NK cells. Subpopulations of helper and cytotoxic T cells included CD27+/CD45RA+ naïve, CD27+/CD45RA- central memory (CM), CD27-/CD45RA- effector memory (EM), and CD27-/CD45RA+ terminally differentiated (TD) helper T and cytotoxic T cells, respectively. Within the CD45+/CD19+ B lymphocytes, CD27- B cells, CD27+/CD38- memory B cells (MBCs), and CD27+/CD38++ plasma cells (PC) were determined. For CD27- B cells, CD27-/IgM+/IgD+ pre-germinal B cells and CD27-/IgM-/IgD- switched CD27- B cells were identified**.** MBCs were further divided into IgM-/IgD- switched MBCs, IgM++/IgD+ unswitched MBCs, and IgM-/IgD++ MBCs.

Statistical analysis

All statistical analyses were performed using R (version 2024.09.0+375) with standard packages for data cleaning, manipulation, and visualisation. Because of the incomplete availability of absolute lymphocyte counts across all records (see figure S3 in the appendix for available data and trends in absolute lymphocyte counts), relative cell frequencies were used for analysis. Data variability was represented using medians and interquartile ranges (IQRs). For comparisons between independent groups, the Mann–Whitney U test was applied. Paired comparisons, such as changes in cell populations between visits, were analysed using the Wilcoxon signed-rank test. Associations between categorical variables, such as gestational age and infection status, were assessed using Fisher's exact test. All p-values were two-sided, with statistical significance defined as $p < 0.05$. Analyses were restricted to participants with complete data at all three sampling time points. No imputation was performed for missing values.

Ethics approval

This study received approval from the local ethics committee of Zurich (BASEC 2020-01764) and was performed in accordance with the Declaration of Helsinki. Informed consent was obtained from the legal guardians of all participating infants.

Results

Patient characteristics

Thirty preterm neonates were included in this study, with three blood samples collected at three different time points to represent immunological development in the first month of life. The cohort consisted of 23 extremely preterm neonates (<26 weeks, n = 10, 33%; 26–28 weeks, n = 13, 43%) and 7 very preterm neonates (29–31 weeks, n = 7, 23%). Comprehensive patient data are presented in table 1.

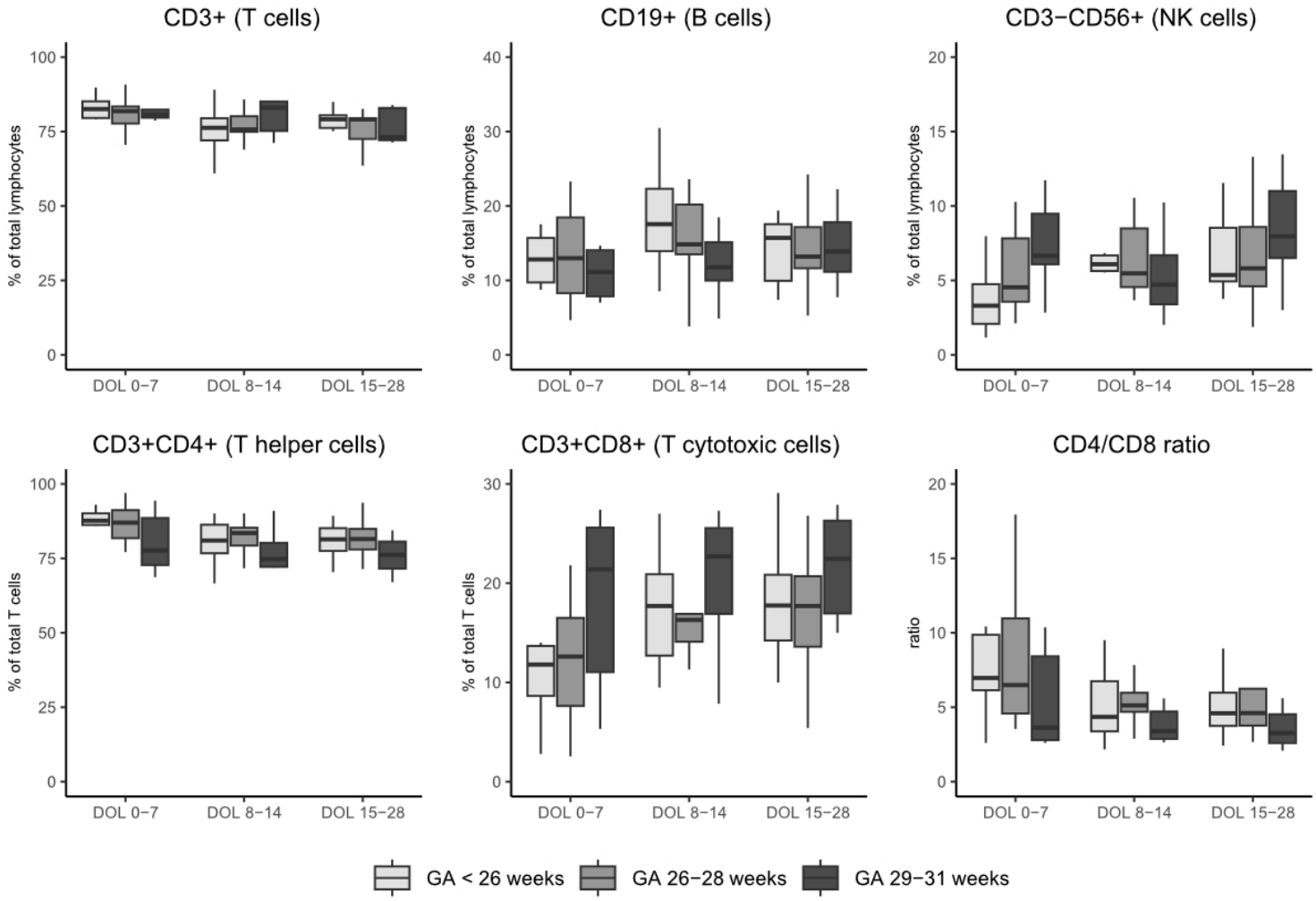
Table 1: Patient characteristics. One patient had both sepsis and suspected necrotising enterocolitis (Bell stage I). Sepsis cases included four patients with culture-proven sepsis: *Staphylococcus epidermidis* (n = 2), co-infection with *Staphylococcus epidermidis* and *Staphylococcus caprae* (n = 1), and *Streptococcus anginosus* (n = 1). Additionally, three patients had negative blood cultures but demonstrated strong clinical signs of sepsis and received antimicrobial treatment for ≥5 consecutive days. Chorioamnionitis was defined clinically when signs of intra-amniotic infection were present (e.g. perinatal fever or high infection parameters of the mother) and/or histologically. Complete antenatal steroids were defined as ≥2 doses with a 24-hour interval and last dose >24 hours before birth.

Features		**All study participants (n = 30), n (%)**
Male, n (%)		12 (40)
Gestational age (range), n (%)	23 weeks (23+3 to 23+5)	2 (7)
	24 weeks (24+3 to 24+5)	3 (10)
	25 weeks (25+0 to 25+6)	5 (17)
	26 weeks (26+2 to 26+4)	6 (20)
	27 weeks (27+0 to 27+6)	5 (17)
	28 weeks (28+4 to 28+5)	2 (7)
	≥29 weeks (29+0 to 31+5)	7 (23)
Birth weight, g	Median (range)	815 (370–1550)
Perinatal factors, n (%)	Caesarean section	26 (87)
	Chorioamnionitis	15 (50)
	APGAR at 1′, median (range)	6 (1–9)
	APGAR at 10′, median (range)	9 (4–10)
	Complete antenatal steroids	20 (66)
	Endotracheal intubation	13 (43)
	Cardiac compression	1 (3)
	Adrenaline administration	0 (0)
Infection, n (%)	Sepsis	6 (20)
	Necrotising enterocolitis	1 (3)

Main lymphocyte subpopulations at different gestational ages

For the analysis of immunological trends during the first month of life (figure 1), neonates (n = 30) were divided into three gestational age groups: <26 weeks (n = 10), 26–28 weeks (n = 13), and 29–31 weeks (n = 7). Across all groups, CD3+ T-cell and CD19+ B-cell frequencies remained relatively stable during the first month. CD56+ NK cells showed significantly lower frequencies at DOL 0–7 in more preterm infants (< 26 weeks and 26–28 weeks) compared with infants born at ≥29 weeks gestational age (p = 0.049). However, NK cell counts approached similar frequencies across all age groups at subsequent time points, with no significant differences noted at later visits. Within the T-cell population, lower frequencies of CD8+ cytotoxic and higher frequencies of CD4+ helper T cells were observed at DOL 0–7 in both extremely preterm groups compared with older neonates. Notably, interindividual variability within the CD8+ cell group was high. This resulted in an elevated CD4/CD8 ratio in extremely preterm neonates at DOL ≤7 (median values: <26 weeks = 7.5; 26–28 weeks = 6.9) compared with neonates born ≥29 weeks (median = 3.63). By the second week of life (DOL 8–14), a significant reduction in the CD4/CD8 ratio was observed in both extremely preterm groups (<26 weeks: p = 0.027, median = 4.4; 26–28 weeks: p = 0.017, median = 5.1). No significant changes of the CD4/CD8 ratio were detected between DOL 8–14 and the final visit (DOL 15–28). In the B-cell compartment, the majority were CD27- B cells (median = 98% of total B cells, TBCs), with a small proportion of CD27+/CD38- memory B cells (median = 1% of TBCs), which predominantly co-expressed IgD/IgM, indicating an unswitched phenotype. As expected, CD27+/CD38++ plasma cells were observed at very low frequencies (median = 0.2% of TBCs).

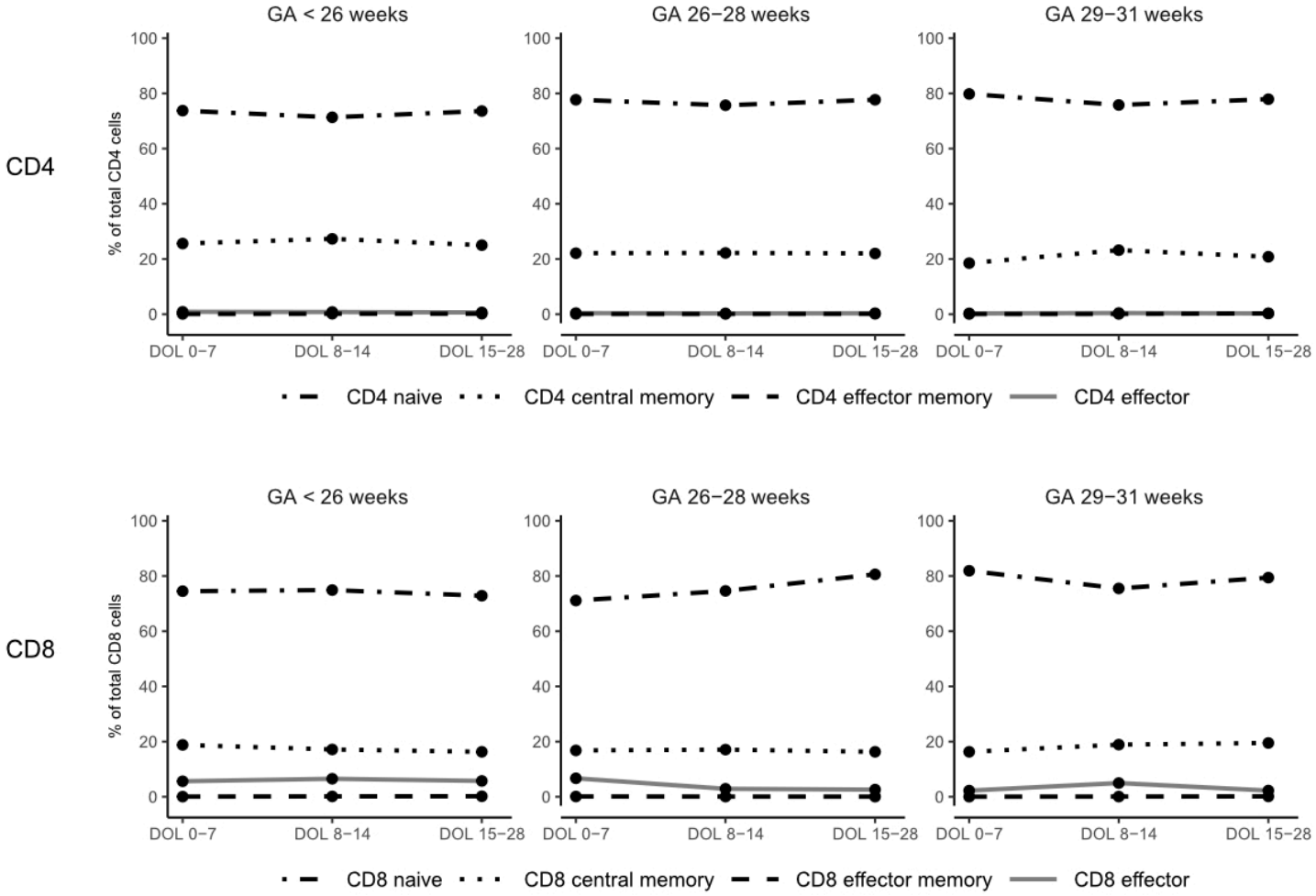
Figure 1: Main lymphocyte populations according to gestational age. Boxes show the interquartile range (IQR) with the median line; whiskers extend to 1.5×IQR, and outliers beyond 1.5xIQR were excluded for clarity. Abbreviations: DOL, day of life; NK, natural killer; GA, gestational.



CD4 helper and CD8 cytotoxic cell differentiation in preterm infants

Throughout the first month of life, the CD4+ and CD8+ T-cell populations in preterm infants were predominantly composed of naïve T cells (figure 2). This pattern was consistent across all preterm age groups and time points. The proportion of central memory T cells in both CD4+ and CD8+ populations ranged between 15% and 20% across all groups, with no significant temporal changes. Effector memory T cells and terminally differentiated (TD) T cells within the CD4+ population remained very low throughout the study period (<1% of CD4+ T cells). By contrast, CD8+ TD T cells exhibited slightly higher frequencies, with an overall median of 4.7% of CD8+ T cells. One patient, born at 24+5 weeks of gestational age and without signs of infection, demonstrated unexpectedly high frequencies of CD8+ TD cells throughout the first month (DOL 3, 44.9%; DOL 10, 59.5%; DOL 25, 52.0% of CD8+ T cells).

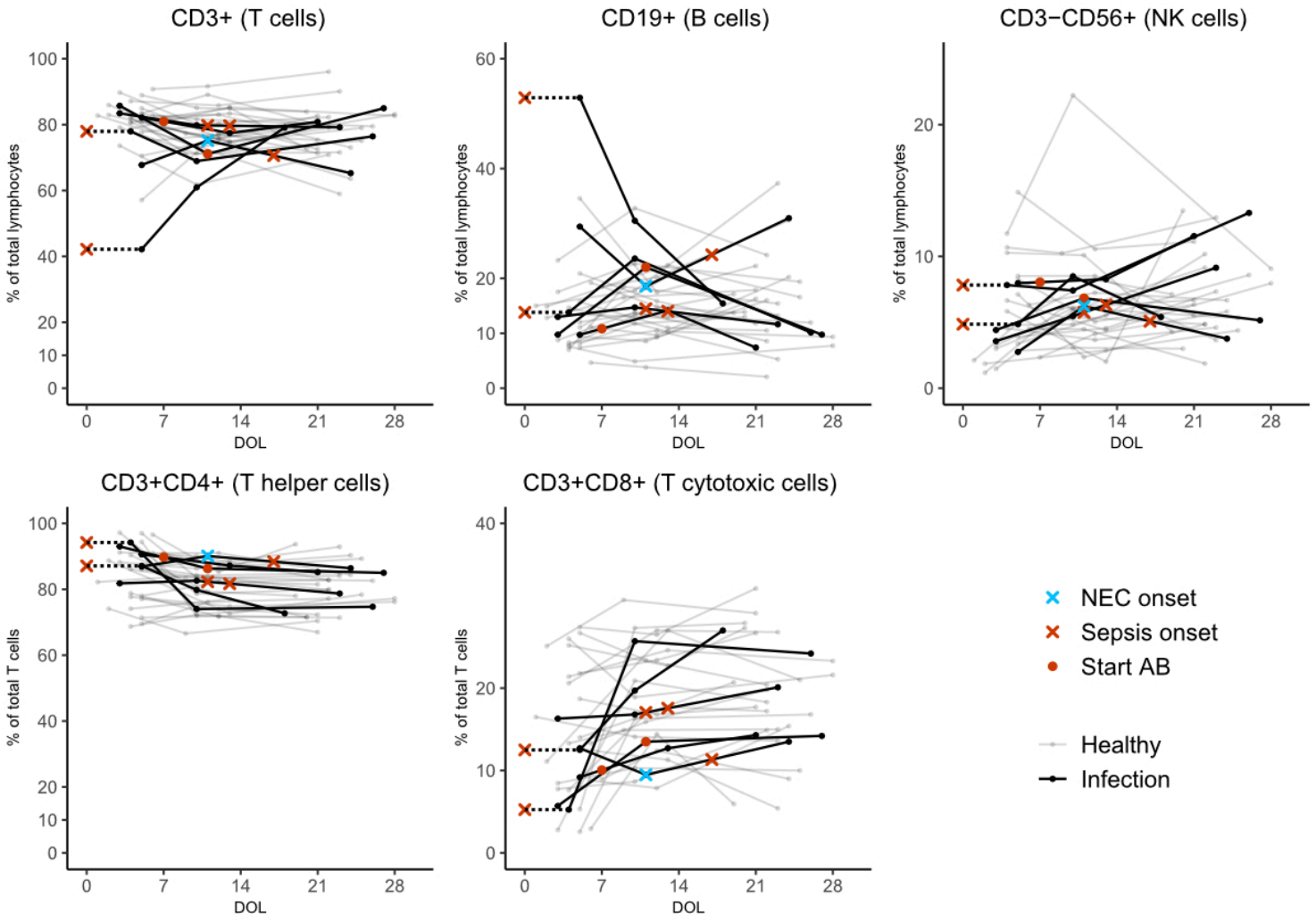
Figure 2: Differentiation of CD4+ and CD8+ T cells across different gestational age groups during the first month of life. Subpopulations include naïve, central memory (CM), effector memory (EM), and terminally differentiated (TD) T cells. Abbreviations: GA, gestational age. Medians were used for data visualisation. For clarity, variance was not displayed in this figure.



Infections

Six patients had confirmed or suspected sepsis and/or necrotising enterocolitis (infection group, see table 1). Among these, four patients had culture-proven sepsis: *Staphylococcus epidermidis* (n = 2; DOL 0 and DOL 11), a co-infection with *Staphylococcus epidermidis* and *Staphylococcus caprae* (n = 1; DOL 17), and *Streptococcus anginosus* (n = 1; DOL 0). The patient with *Staphylococcus caprae* sepsis also exhibited signs of necrotising enterocolitis (Bell stage 1) at DOL 11. Two patients were treated with antibiotics for ≥5 consecutive days without pathogen identification and were therefore classified as having culture-negative sepsis. One patient with clinical suspicion of sepsis at DOL 69, who was treated with antibiotics for <5 days without pathogen identification, was excluded from the infection group. No significant correlations were found between gestational age or birth weight groups and infection risk. Longitudinal data on lymphocyte subpopulations are shown in figure 3. Notably, one patient with sepsis onset at birth (DOL 0) displayed the highest B-cell frequency (53% of all lymphocytes) at DOL 5, possibly in response to infection, with a gradual normalisation over the first 3 weeks of life (DOL 10, 30%; DOL 18, 15%). Although no definitive trends or correlations were observed with infection onset, the infection group had significantly higher B-cell frequencies at DOL 8–14 compared with neonates without infection (p = 0.04, medians: infection group 20%; healthy group 14%). Both groups exhibited a gradual increase in CD8+ T cells and a corresponding decrease in the CD4/CD8 ratio during the first month of life. However, this trend reached statistical significance only in the healthy group between DOL 0–7 and DOL 8–14.

Figure 3: Main lymphocyte subsets in preterm neonates with and without infection. Main lymphocyte subsets are shown for preterm neonates with sepsis and/or necrotising enterocolitis (infection group) compared with those without infection. Infection onset is indicated to provide context for observed immunological changes. For the two patients with culture-negative sepsis, the exact onset of infection was unclear; therefore, the initiation of antibiotic therapy (lasting ≥5 consecutive days) is marked instead. Abbreviations: DOL, day of life; NK, natural killer; NEC, necrotising enterocolitis; AB, antibiotics



Discussion

In our explorative cohort of thirty very preterm neonates with birthweight ≤1550 g, flow cytometric analysis of peripheral lymphocyte populations in the first month of life revealed a considerable immaturity of adaptive immunity, with naïve T cells (both CD4+ and CD8+) and unswitched CD27- B cells dominating the T- and B-cell compartment, respectively. No distinct immunological features predisposing infants to infection were observed; however, infants with infections exhibited significantly higher B-cell proportions in the second week of life, possibly indicating an immunological response to previous or ongoing infection.

Consistent with findings from previous studies [3, 4], the CD4+ and CD8+ T-cell populations in preterm neonates were almost completely devoid of immunological memory, with extremely low proportions of EM and TD T cells. This deficiency may contribute to their vulnerability to infection, as efficient pathogen recognition and elimination remain limited. However, term neonates have also been shown to express similarly high naïve T-cell proportions (albeit in higher absolute numbers) [31–33], suggesting that the immaturity of CD4+ and CD8+ T cells may be due to limited postnatal antigenic stimulation rather than a feature unique to preterm birth. Notably, lower gestational age was associated with reduced CD8+ T-cell frequencies during the first week of life, resulting in an increased CD4/CD8 ratio, particularly in extremely preterm neonates [11–15]. In this

context, Bochennek et al. demonstrated a significant correlation between low naïve CD8+ T-cell frequencies and necrotising enterocolitis occurrence [6], but this effect was not observed in our study, and its clinical significance remains unclear.

Within the B-cell compartment, we observed extremely low frequencies of memory and plasma B cells, in line with limited immunoglobulin (Ig) production in preterm neonates, making these infants highly dependent on passively transferred maternal Ig. However, IgG transfer is reduced in preterm neonates, as it is interrupted by preterm delivery [34], thereby impairing IgG-mediated pathogen recognition and elimination. Another study showed that IgA-mediated control of intestinal microbiota often fails in preterm and low-birth-weight infants, contributing to the pathogenesis of necrotising enterocolitis [17]. Interestingly, preterm neonates with sepsis and/or necrotising enterocolitis had significantly higher B-cell proportions in their second week of life, likely as a humoral immune response to a previous or ongoing infection. However, further longitudinal studies are needed to clarify this finding in a larger sepsis cohort.

Additionally, we assessed NK cells and found extremely preterm neonates expressed significantly fewer NK cells in their first week of life compared to older neonates. Although extremely preterm infants were generally at a higher risk for infection, no statistically significant correlation was observed between low NK cell frequencies and infection risk in our study. In contrast, Bochennek et al. found that low NK cell frequencies significantly increased the risk of late-onset sepsis [6], suggesting that NK cell-mediated mechanisms of innate immunity may play a role in neonatal susceptibility to infection.

Several limitations should be considered in this study. First, the sample sizes were relatively small, limiting statistical power. Second, clinical features such as chorioamnionitis, severe respiratory distress/intubation, or mode of delivery may have influenced immunological processes but could not be statistically addressed due to low sample sizes. Third, for logistical reasons, samples were stabilised with a whole blood stabiliser and then frozen for weeks to months before data acquisition, which may have reduced sample quality, although the stabiliser kit has been validated in previous studies [26, 27]. However, obtaining blood samples from preterm neonates, particularly those with extremely low birth weights, is challenging, and few longitudinal data on lymphocyte phenotyping in this population exist. Last, as whole-blood samples were not routinely collected alongside study samples, few data on absolute lymphocyte counts exist, and the analysis was performed using frequencies rather than absolute numbers. Even though the use of relative frequencies has drawbacks (e.g. limited information on small populations and difficult-to-interpret changes in populations separately), it provides important insights into immune cell composition and dynamics and how these influence individual susceptibility to infection. Despite these limitations, this study is among the few to assess peripheral blood samples rather than cord blood or resected tissue in this difficult-to-study population.

In conclusion, the immunophenotyping of very and extremely preterm neonates during their first month of life highlighted how immaturity within both T- and B-cell subsets contributes to their heightened susceptibility to infection. Although no distinct immunological profiles predictive of infection were identified, certain features associated with extreme preterm birth, such as a high CD4/CD8 ratio and low NK cell frequency, may contribute to the multifactorial pathogenesis of neonatal infections. These findings warrant further studies to identify high-risk immunological constellations in this extremely vulnerable population.

Data availability statement

Due to the sensitive nature of patient data and ethical considerations, the underlying dataset cannot be publicly shared. Deidentified aggregated data, along with data dictionaries, where applicable, and the analytical code are available from the corresponding author upon reasonable request for scientific purposes consistent with the approved ethical framework.

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Author contributions: Study concept and design: VvN, JT, ES, NM, SS; acquisition and analysis of data: JS, CK, NM, SS, VvN, JT; interpretation of data: SS, JT, ES, GN; drafting of manuscript: SS, JT; revision of manuscript: SS, VvN, ES, GN, JT

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Potential competing interests

All authors have completed and submitted the International Committee of Medical Journal Editors form for disclosure of potential conflicts of interest. No potential conflict of interest related to the content of this manuscript was disclosed.

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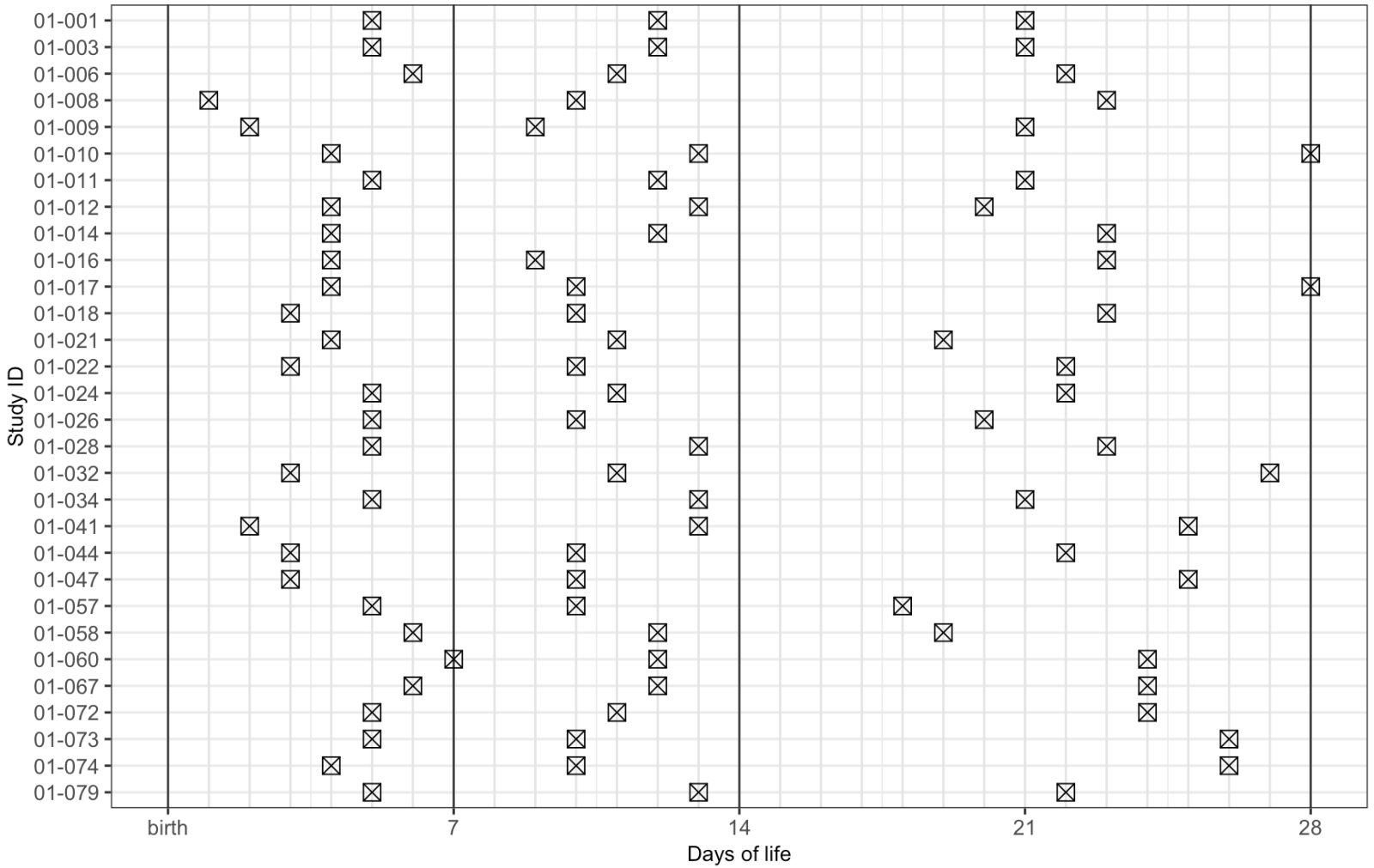
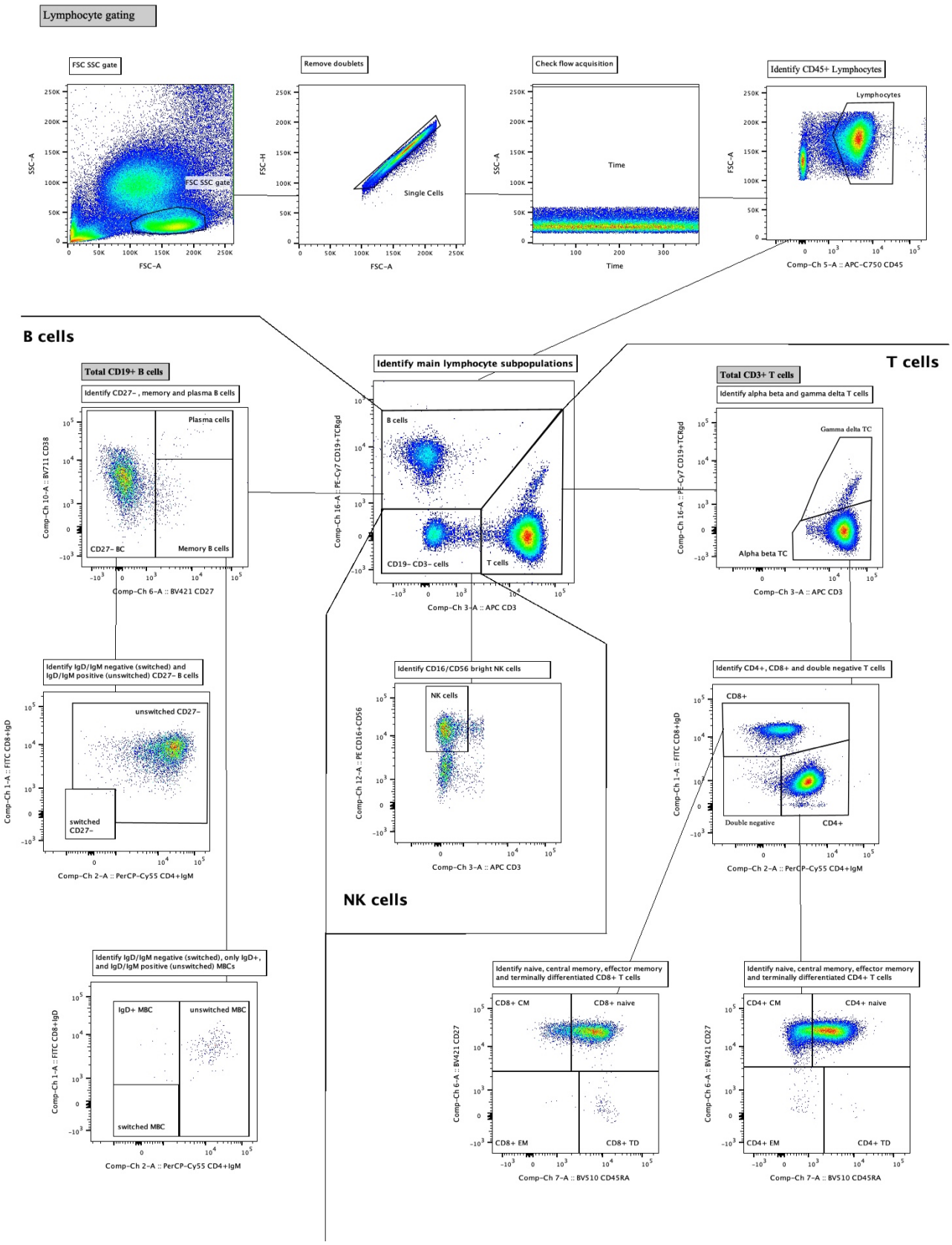
Appendix

Table S1. Immunophenotyping of lymphocyte subsets. Abbreviations: NK, natural killer; IgM, immunoglobulin M; IgD, immunoglobulin D; MBC, memory B cells; CM, central memory; EM, effector memory; TD, terminally differentiated; TCR, T cell receptor.

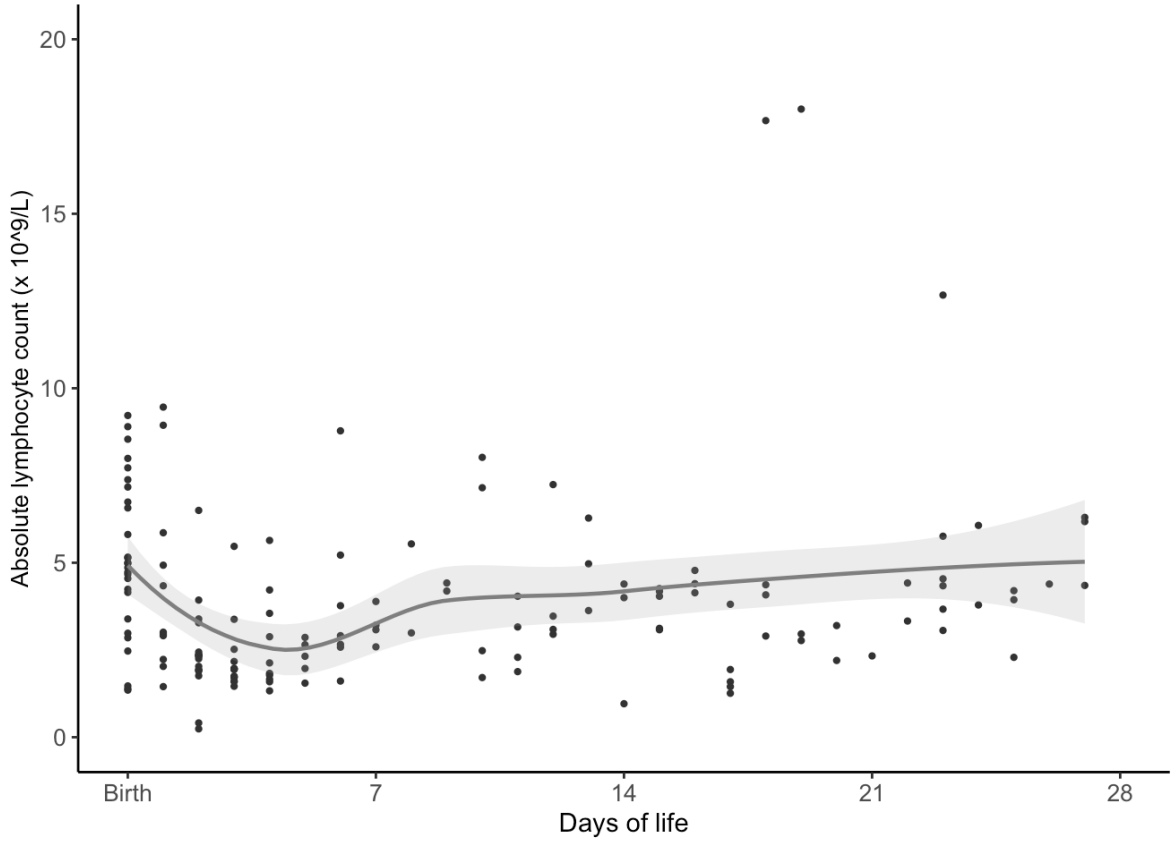
Subset			Phenotype
NK cells			CD45+/CD3-/CD19-/CD16+CD56+
B cells			CD45+/CD3-/CD19+
	Pre-germinal B cells		CD27-/IgM+/IgD++
	Switched CD27- B cells		CD27-/IgM-/IgD-
	Memory B cells		CD27+/CD38-
		Switched MBC	IgM-/IgD-
		Unswitched MBC	IgM+/IgD+
		IgD MBC	IgM-/IgD+
	Plasma cells		CD27+/CD38++
T cells			CD45+/CD3+/CD19-
	CD4+		CD4+/CD8-/TCRgd-
		Naive CD4	CD27+/CD45RA+
		CM CD4	CD27+/CD45RA-
		EM CD4	CD27-/CD45RA-
		TD CD4	CD27-/CD45RA+
	CD8+		CD4-/CD8+/TCRgd-
		Naive CD8	CD27+/CD45RA+
		CM CD8	CD27+/CD45RA-
		EM CD8	CD27-/CD45RA-
		TD CD8	CD27-/CD45RA+
	Double negative T cells		CD4-/CD8-/TCRgd-
	TCRgd		CD3+/TCRgd+



Supplementary figure 1. Samples.



Supplementary figure 2. Lymphocyte gating. Abbreviations: FSC, forward scatter; SSC, side scatter; NK, natural killer.



Supplementary figure 3. Available data and trends in absolute lymphocyte counts.