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Lymphocyte subpopulations in premature infants: An observational study

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Abbreviations:
CD Cluster of differentiation
CI Confidence interval
CLD Chronic lung disease
NHS National Health Service
NK Natural killer
PBMC Peripheral blood mononuclear cell

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ABSTRACT

Background and objectives

The infant's immune system evolves over the first months and years of life. Strong positive correlation exists between lymphocyte count, lymphocyte subpopulations and gestational age at birth. Associations with antenatal and postnatal steroid treatment, infection and chronic lung disease have also been described. Few published studies report the effect of increasing postnatal age (PNA) and comorbidities on lymphocyte subpopulations in premature infants beyond the first 4 months of life. This study aimed to describe changes in lymphocyte subpopulations in preterm infants up to 13 months PNA.

Methods

Premature infants (23-34 weeks completed gestation) from 5 centres had lymphocyte subpopulations measured at 2, 5 or 7, 12 and 13 months PNA alongside their vaccine responses in a vaccination trial.

Results

393 blood samples from 151 babies were analysed. There was an increase in absolute numbers of total lymphocytes (median cell count 6.21 $x10^9$/L at 13 months compared with 4.9 $x10^9$/L at 2 months PNA) and $CD3^+$, $CD4^+$, $CD8^+$, natural killer and B cells with increasing age. At 2 months PNA there was a positive correlation between gestation and $CD3^+$ and $CD4^+$ counts (r=0.32 and 0.46 respectively) and proportions (r=0.22 and 0.41 respectively), and $CD4^+$:$CD8^+$ ratios (r=0.57) but a negative correlation with $CD8^+$ proportions (r=-0.32).

Conclusions

This longitudinal study describes the distribution of lymphocyte subpopulations in premature infants and provides reference ranges for the major lymphocyte subsets to help guide clinicians when assessing premature infants for immunodeficiency in the first year of life.

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INTRODUCTION

Infection is a leading cause of morbidity and mortality in young infants and especially in those born prematurely [1–3]. Lymphocytes are key to the development of adaptive immunity and there are major changes in their numbers and function in term infants over the first year of life [4,5]. Reference ranges for lymphocyte counts and subpopulations exist for term infants and describe an increase in absolute counts in most lymphocyte subsets over the first 6 months of life [6,7].

Several studies have explored the effect of gestational age on lymphocyte subpopulations at birth [8–17]. Overall these studies show increasing concentrations of $CD3^+$, $CD4^+$, natural killer (NK) and B cells with increasing gestation.

Factors other than gestational age may affect lymphocyte subset populations. The use of antenatal steroids, for example, has been associated with increased cord blood T cells together with diminished T cell proliferation [11,13,18,19].

Few studies have described the changes in lymphocyte subpopulations after birth in premature infants who may have several months of ex-utero life before reaching term equivalent. Lymphocytes in these babies are likely to be influenced by both the infants' in-utero condition and their subsequent postnatal course. Existing studies have included only a small numbers of babies and have limited the follow-up period to the first 6 months of life [17,20–22], thus the 'normal' subpopulation values in ex-premature infants are unknown [23].

This study aimed to describe the lymphocyte subpopulations in premature infants over the first 13 months of postnatal life and to determine the impact of antenatal and postnatal exposures and comorbidities.

MATERIALS AND METHODS

This was a planned sub-study of a vaccination trial in which premature infants received the UK primary immunisation schedule and were randomised to 3 different schedules of the 13-valent pneumococcal conjugate vaccine (PCV13) (supplementary table 1). It included all infants enrolled at 5 of the 12 vaccination study sites. The clinical teams identified potentially eligible infants and parents were provided with information by the research teams. Infants were eligible for inclusion if they were born before 35^{+0} weeks gestation, were medically fit for vaccination as defined by UK Department of Health guidelines [24] and were between 7 and 12 weeks PNA at the first vaccination dose. The study was approved by the East of England - Essex regional ethics committee (REC reference 07/HO301.11) and written informed consent was obtained from the infants' parents or legal guardians prior to recruitment. The clinical trial was registered (EudraCT number 2007-007535-23).

Blood sampling and laboratory methods

Each infant had up to 4 blood samples obtained: at 2 months of age, at 5 or 7 months of age (1 month after their final primary vaccinations) and prior to and 1 month after their booster vaccinations at 12 months of age (supplementary table 1). Whole blood (1-2 mL) was collected by venepuncture or capillary sampling into EDTA bottles for lymphocyte subpopulation quantification. All participants were well at the time of phlebotomy with no active infection as assessed by the clinical team. Due to the challenges of obtaining sufficient blood volumes from premature infants, not all babies had samples collected at every time point and, due to the design of the study, fewer infants had blood sampling at 7 months of age than at 5 months of age.

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Blood samples for lymphocyte analysis were processed within 12 hours of collection in the local NHS laboratory and analysed using their routine flow cytometry practice (as advised by the manufacturer and local standard operating procedures, table 1).

Cell populations measured are shown in supplementary table 2[22]. Whilst all centres enumerated $CD3^+$, $CD3^+CD4^+$ and $CD3^+CD8^+$ cells, due to local constraints different markers were used for detection of B cells and NK cells. For B cells, 4 centres measured $CD19^+$ and one $CD20^+$ cells, whilst for NK cells 4 centres measured $CD56^+$ and one $CD56^+CD16^+$ cells. For the purpose of overall trends these have been combined into 'B cells' and 'NK cells'.

Statistical analysis

Due to the design of the main vaccination trial there was considerable overlap of ages at the final 2 blood samples. Therefore for the purposes of this sub-study, the 12 and 13 month samples were categorised according to age, rather than their relationship to vaccination. A result was considered a '12 month' sample if it was taken between 353 and 387 days of age, and a "13 month" sample if taken after 387 days of age. If participants had more than one sample within each age category only the first was included.

The data were not all normally distributed and nonparametric tests of statistical significance were used. Correlations were calculated using Spearman's rank correlation coefficient. For regression analysis $\log_e$ transformed values were used. For B cells and NK cell regression analysis, as different cell populations were measured, $CD19^+$ and

$CD56^+$ were analysed due to the smaller number of $CD20^+$ and $CD56^+CD16^+$ results (368 and 366 results compared with 49 and 46 results respectively).

To assess associations between subpopulations, clinical conditions and comorbidities univariable regression analysis was performed on the 2 month and 5 month results for the following variables: sex, small for gestational age (SGA, defined as birth weight less than the 10^{th} centile for gestation), chronic lung disease (CLD, defined as requiring supplemental oxygen or respiratory support at 28 days of age) and receipt of antenatal steroids. All analyses were adjusted for gestation and study centre. Backwards stepwise multivariable regression was performed for variables with significance in univariable analysis ($p \leq 0.2$).

All data were analysed using STATA version 13 (Stata Inc).

RESULTS

A total of 419 analysable samples were collected from 151 infants. The number of infants contributing 1, 2, 3 or 4 samples was 25, 34, 42 and 50 infants respectively. 26 samples were excluded as they were the second sample collected between 353 and 387 days of age. The characteristics of the infants are shown in table 2.

Effect of gestational and postnatal age

The lymphocyte subpopulation results for different gestational and postnatal ages are shown in table 3, table 4 and figure 1. Overall, there was an increase in the total lymphocyte count and all measured cell populations over the first 13 months of life. There was an increase in the percentage of $CD3^+CD8^+$ and NK cells and a decrease in

the percentage of $CD3^+CD4^+$ cells with increasing age. The $CD4^+$:$CD8^+$ ratio correlated negatively with age.

At 2 months of age there was a positive correlation between gestation and $CD3^+$ count and percentage (r=0.32 and 0.22, p=0.001 and 0.028 respectively), $CD3^+CD4^+$ count and percentage (r=0.46 and 0.41 respectively, p<0.001 for both), and a negative correlation with the proportion of $CD3^+CD8^+$ cells (r=-0.32, p=0.001), resulting in positive correlation between CD4:CD8 ratio and birth gestation (r=0.57, p<0.001). The impact of gestation was rarely significant (p<0.05) after 2 months of age. The only significant associations were the proportion of B cells at 7 months (r=-0.41, p=0.022), NK proportions at 12 months (r=0.22, p=0.023) and $CD3^+CD8^+$ and B cell proportions at 13 months (r=-0.29 and 0.28, p=0.011 and 0.014 respectively).

There were no differences between infant lymphocyte profiles according to randomisation group (data not shown).

Associations between co-morbidities, chronic lung disease, antenatal steroids, and lymphocyte subpopulations

There were negative associations between CLD and $CD3^+$ count (fold effect – FE -0.78 i.e. 22% reduction in $CD3^+$ count if CLD present, 95% CI 0.65-0.94; p=0.011), $CD3^+CD4^+$ count (FE 0.78, 95% CI 0.64-0.95; p=0.016) and $CD19^+$ count (FE 0.72, 95% CI 0.53-0.98; p=0.037) and between receipt of antenatal steroids and $CD3^+CD4^+$ percentage (FE 0.86, 95% CI 0.74-1.00, p=0.047) at 2 months PNA only. Postnatal steroids were rarely administered to infants (4 participants). There was no association between SGA and any lymphocyte subpopulation.

In multivariable regression the association between $CD3^+CD4^+$ percentage and antenatal steroids was no longer apparent. Univariable regression revealed no significant associations at 5 months of age.

Effect of study centre

Centres 1 and 5 had significantly different results from the other centres for the following subpopulations: $CD3^+$%, $CD3^+CD4^+$% (5 only), $CD3^+CD8^+$% and $CD3^+CD8^+$ count (1 only) and $CD19^+$% (5 only). In addition, there were significant differences between centre 4 and the other centres for $CD56^+$% and $CD56^+$ count. These differences remained despite adjustment for the different gestational age distributions at each centre.

DISCUSSION

To our knowledge, this is the largest study of lymphocyte subpopulations in premature infants reported to date and provides data up to 13 months of age which may assist clinicians in assessing the immune status of infants who were born prematurely. With increasing age (either gestational or postnatal), an increase in the numbers of lymphocytes in all subpopulations was observed. The degree of increase varied between subpopulations, resulting in an overall increase in cytotoxic T cells and NK cells as a proportion of the total count and a decrease in the proportion of T helper cells over the first year of life.

Several studies have reported changes in lymphocyte subpopulations over the first year of life in term infants. Comparison with these studies is limited by their broad age categories and a focus towards comparison with adult values [4,25,26]. However, the

pattern of changes seen in the preterm infants studied are in keeping with those described in term infants [5–8]. However, whilst our data show an increase in NK cells over the first year of life, the converse is described in term infants [6,27]. The reason for this difference is not clear, but it is worth noting that within our dataset a significant rise in NK cells appears to occur after 12 months of age, influencing the overall trend.

The effect of gestation on subpopulations was most apparent at the earliest samples when many of the infants had not yet reached term-equivalent. This is consistent with the differences reported between premature and term infants at birth [8,9,13,16] as well as in previous preterm infant longitudinal studies [20,22]. Berrington *et al.* reported lower total lymphocyte count, T cells, B cells and T helper cells at 2 months of age in 18 preterm infants compared with term infants; some of these populations (total lymphocytes, T cells and T helper cells) remained significantly lower at their final sample at 6 months of age [22]. Walker *et al.* demonstrated positive correlations between cell count and gestation for all subpopulations up to 12 weeks of age; however, possibly due to the small numbers of participants (25 preterm infants), these results did not reach statistical significance[20].

We found no consistent effect of gestation on the number or proportion of B cells at any time point. Berrington *et al.* did report lower B cell counts in premature infants at 2 months of age, which had resolved by 6 months of age but, similar to our data, Walker *et al.* found similar B cell counts across all gestations [20,22].

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Although the absolute numbers of lymphocytes vary significantly with age, the relative proportions of the lymphocyte subsets remain more constant and will thus provide a simpler reference for clinicians. It is important for a reference range to be applicable to the whole population; it should be noted that the infants in our study were clinically well at the time of sampling but a significant proportion had a number of co-morbidities that are commonly associated with prematurity. In addition, the multicentre approach permits results to be more generalisable.

Chronic lung disease was associated with lower $CD3^+$, $CD3^+CD4^+$ and $CD19^+$ counts. A previous study found decreased $CD2^+$ and $CD3^+CD4^+$ counts in premature infants with CLD at 3 weeks of age and Pelkonen *et al.* described similar findings in the lymphocyte subpopulations of ex-premature school children which were associated with CLD [17,28]. It may be that CLD per se, or an aspect of its treatment, may result in these immunological findings. An intriguing alternative hypothesis is that these abnormalities of immune function are present before the onset of CLD, predisposing to its development and to the recurrent respiratory infections that such infants are prone to [29]. As infants were first sampled after the diagnosis of CLD had been made it is not possible to ascertain if these abnormalities were present earlier.

Unlike other studies, we did not find any association between antenatal steroids and lymphocyte subpopulations in our cohort. Previous studies analysed cord blood samples and it may be that any significant effect of antenatal steroids had resolved by 2 months of age [8,13].

LIMITATIONS

Our study has some potential limitations. It was conducted as a sub-study and, as such, there was no opportunity for pre-selecting different blood sampling time-points. Early neonatal blood samples would have allowed a more complete description of the subpopulations from birth to one year of age. Fortunately there is a pre-existing evidence base for cord blood of preterm infants at various gestational ages [8–13]. The constraints of NHS laboratories also meant that the investigation of more refined subpopulations e.g. regulatory T and B cells, and functional studies were not possible.

Additionally, laboratory analysis was performed within the routine clinical framework using different sample preparation and analysis techniques. Accordingly, differences were found between the centres for nearly all subpopulations despite some centres using similar analytical laboratory protocols. Correcting for other possible centre-dependant factors (e.g. gestation) using multiple regression did not eliminate these differences. It is known that a variety of factors can affect the inter-laboratory quantification of absolute lymphocyte subpopulations [30–32] and other multicentre studies have experienced similar inter-centre variation, with testing centre the most significant factor in analysis of differences between cell populations [6]. Studies have negated this challenge by freezing peripheral blood mononuclear cells (PBMCs) and centralising samples prior to analysis; or performing single centre studies on whole blood [5,22]. Unfortunately the volume of blood required to extract PBMCs is prohibitively large in low weight infants. In order to address the inter-centre variation, the testing centre was included within the regression analysis. These factors should be considered when designing future studies.

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Families were approached to take part in the study before 3 months of age. Babies that were too unstable to be vaccinated at this time were unable to participate. This may have reduced the number of patients with complex clinical courses. To minimise this, recruitment was stratified by age group to ensure extremely premature infants were appropriately represented.

Finally, this study did not include any term infants and as such direct comparison with them is not possible.

CONCLUSION

This is the largest reported cohort to describe lymphocyte subpopulations in premature infants and its longitudinal nature provides reference ranges for the major lymphocyte subsets. The effect of gestation on subpopulations is most pronounced in early life (up to approximately 6 months postnatal age), corresponding with when infants are most vulnerable to infection. This may be compounded in infants with co-morbidities, especially chronic lung disease, and further study is required to determine the nature of this relationship.

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Author contributions

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A Kent coordinated the study, analysed the data and drafted the manuscript. P Heath and S Ladhani developed the initial protocol. All authors helped with the recruitment of and/or blood sampling from participates, contributed to the manuscript review and revision and approved the final version.

Conflict of Interest Statements

Andrew J Pollard: AJP has previously conducted studies on behalf of Oxford University funded by vaccine manufacturers but does not receive any personal payments or travel support. AJP chairs the UK Department of Health's (DH) Joint Committee on Vaccination and Immunisation (JCVI); the views expressed in this manuscript do not necessarily reflect the views of JCVI or DH

Matthew D Snape: MDS acts as chief and principal investigators for clinical studies from both non-commercial funding bodies and commercial sponsors (i.e. Novartis Vaccines, GlaxoSmithKline, Sanofi-Pasteur, Sanofi-Pasteur MSD, and Pfizer Vaccines) conducted on behalf of the University of Oxford. MDS also undertakes consultancy and advisory work for several commercial sponsors; any speaking honoraria, travel and accommodation reimbursements are paid to the University of Oxford Department of Paediatrics. MDS does not receive any financial support from vaccine manufacturers.

Shamez N Ladhani: SLN has conducted studies on behalf of St Georges, University of London funded by vaccine manufacturers but does not receive any personal payments or travel support.

Paul T Heath: PTH has conducted studies on behalf of St Georges, University of London funded by vaccine manufacturers but does not receive any personal payments or travel support.

Alison Kent, Tim Scorrer, Paul Clarke, Karen Few, Stephen Hughes, Anu Varghese and Esse Menson have no conflicts of interests to declare.

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What is already known on this topic:

- At birth, younger gestation is associated with lower concentrations of $CD3^+$, $CD4^+$, natural killer (NK) and B cells and increased $CD4^+$:$CD8^+$ ratios
- Existing reference ranges describe major changes in lymphocyte subpopulation numbers in term infants over the first year of life
- Limited information is available on the normal range of lymphocyte subpopulations in preterm infants after the first few months of life

What this study adds:

- Provides reference ranges for lymphocyte subpopulations in premature infants up to 1 year of age
- Total lymphocyte, $CD3^+$, $CD4^+$, $CD8^+$, B and NK cell counts increase up to 1 year of age but subpopulation proportions are more stable
- The effect of gestation is largest at 2 months of age and chronic lung disease is associated with a reduction in $CD3^+$, $CD4^+$ and $CD19^+$ cells

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Table 1: Flowcytometer methodology by centre.
BC: Beckman Coulter Inc, Miami, USA; BD: Becton Dickinson Inc, San Jose, USA; Siemens AG Erlangen, Germany. FITC: Fluorescein isothiocyanate, PerCP-Cy5.5: peridinin chlorophyll protein cyanin 5.5, APC: allophycocyanin, PE: phycoerythrin.

Table 2: Participant characteristics by gestational age at birth.
Median (range) or n (%).
[a]Significant difference between groups ($p<0.05$).

Table 3: Lymphocyte subpopulations by postnatal and gestational age at birth.
Absolute count $x10^9$/L. Median (5-95th centile). Lymphocytes: $CD45^+$. B cells: $CD19^+$, $CD20^+$. NK cells: $CD56^+$, $CD56^+CD16^+$. p value calculated using Spearman rank correlation coefficient for postnatal age.

Table 4: Lymphocyte subpopulations by postnatal and gestational age at birth. Percentage of total lymphocyte count. Median % (5-95th centile). Lymphocytes: $CD45^+$. B cells: $CD19^+$, $CD20^+$. NK cells: $CD56^+$, $CD56^+CD16^+$. p value calculated using Spearman rank correlation coefficient for postnatal age.

Figure 1: Lymphocyte subpopulations at different gestational and postnatal ages (median percentage). A: Birth gestation <28 weeks, B: Birth gestation 28^{+0}-31^{+6} weeks, C: Birth gestation: 32^{+0} – 34^{+6} weeks

Table 1

Centre	Sample preparation	Analyser	Analysis Software	Lymphocyte count
1	BD FACS lyse BD individual monoclonal antibody reagent	BD FACScalibur	MultiSET	Siemens Advia 2120
2	BD multitest 6-colour TBNK reagent ($CD3^+$ FITC / $CD16^+$ & $CD56^+$ PE / $CD4^+$5 PerCP-C^{TM}5.5 / $CD4^+$ PE-Cy^{TM}7 / CD19 / $CD8^+$ APC-Cy7) With BD Trucount tubes	BD FACScanto II	BD FACScanto v2.4	Sysmex XE2100
3	BC CYTO-STAT tetraCHROME reagent ($CD4^+$5-FITC/$CD4^+$-RDI/$CD8^+$-ECD/$CD3^+$-PC5)	BC FC500	TetraCXP	BC LH750
4	BC CYTO-STAT tetraCHROME reagent ($CD4^+$5-FITC/$CD4^+$-RDI/$CD8^+$-ECD/$CD3^+$-PC5)	BC FC500	TetraCXP	BC DxH
5	BD multitest $CD3^+$/$CD8^+$/$CD4^+$5/$CD4^+$ BD multitest $CD3^+$/$CD16^+CD56^+$/$CD4^+$5/$CD19^+$ With BD Trucount tubes	BD FACScanto II	BD FACScanto v2.4	NA (single platform)

Table 2

	<28 weeks	28-31+6 weeks	32+0-34+6 weeks
N	32	64	55
Gestation (weeks)	26.5 (23.3-27.9)	29.7 (28-31.6)	33.4 (32-34.9)
Birth weight (g)[a]	840 (490-1398)	1298 (521-2070)	1895 (1335-2680)
Sex – male	19 (59)	27 (42)	29 (53)
SGA	10 (31)	13 (20)	10 (18)
CLD*	31 (97)	21 (33)	1 (2)
Antenatal steroids	30 (94)	53 (83)	44 (80)
Weight at first blood[a]	1900 (1070-2764)	2280 (845-3670)	3130 (2000-4620)
Blood A samples (n)	24	48	33
Blood A age (days)[a]	64 (54-83)	61 (48-84)	60 (49-86)
Blood B samples – 5m (n)	15	30	23
Blood B age – 5m (days)	159 (135-175)	158 (135-180)	149 (132-199)
Blood B samples – 7m (n)	11	11	16
Blood B age – 7m (days)[a]	207 (196-241)	206 (187-215)	195 (179-224)
Blood C samples – 12m (n)	20	48	37
Blood C age – 12m (days)	365 (353-387)	364 (354-385)	368 (353-387)
Blood D samples – 13m (n)	17	36	24
Blood D age – 13m (days)	399 (389-439)	401 (388-443)	407 (389-492)

Table 3

Population	Gestation (weeks)	2 months	5 months	7 months	12 months	13 months	p
Total Lymphocytes	<28	4.55 (3.30-6.70)	5.85 (2.70-10.94)	6.20 (4.60-7.80)	6.45 (2.49-8.96)	6.22 (2.12-11.90)	0.003
	28-31+6	4.85 (2.95-8.50)	5.58 (2.20-9.60)	5.70 (4.36-8.36)	5.73 (3.70-9.93)	6.37 (3.01-11.65)	0.001
	32+0-34+6	5.29 (3.25-9.10)	6.00 (3.00-7.40)	5.68 (3.40-11.10)	5.88 (2.60-10.00)	5.91 (2.80-10.90)	0.19
	All	4.90 (3.10-8.30)	5.75 (2.80-9.52)	5.77 (3.40-10.60)	5.88 (2.62-9.55)	6.21 (2.80-11.65)	<0.001
$CD3^+$	<28	2.76 (1.78-3.89)	3.34 (1.76-6.92)	3.55 (2.74-5.46)	4.50 (1.50-6.92)	3.87 (1.14-7.45)	<0.001
	28-31+6	3.13 (1.90-4.81)	3.45 (1.22-6.90)	3.66 (2.81-5.92)	3.75 (1.88-7.27)	4.30 (1.89-6.58)	<0.001
	32+0-34+6	3.40 (1.18-7.40)	3.40 (2.05-5.18)	3.82 (2.01-7.88)	3.36 (1.55-8.08)	3.57 (1.64-6.77)	0.58
	All	3.13 (1.78-5.48)	3.40 (1.63-6.70)	3.66 (2.45-6.51)	3.82 (1.88-7.07)	3.81 (1.76-7.40)	<0.001
$CD3^+CD4^+$	<28	1.65 (1.20-2.65)	2.29 (1.16-4.42)	2.50 (1.81-3.78)	2.85 (0.95-5.03)	2.58 (0.65-5.12)	0.001
	28-31+6	2.17 (1.30-3.70)	2.59 (0.99-4.99)	2.65 (1.98-3.85)	2.47 (1.23-5.12)	2.81 (1.16-4.14)	0.011
	32+0-34+6	2.46 (0.90-6.28)	2.45 (1.42-4.22)	2.44 (1.29-5.88)	2.07 (1.17-5.50)	2.26 (0.70-4.75)	0.23
	All	2.16 (1.22-4.14)	2.45 (1.17-4.74)	2.51 (1.61-4.93)	2.47 (1.23-5.12)	2.54 (1.04-4.81)	0.013
$CD3^+CD8^+$	<28	0.94 (0.40-1.34)	1.10 (0.54-3.54)	1.09 (0.79-1.59)	1.42 (0.50-1.98)	1.31 (0.47-3.25)	<0.001

	28-31+6	0.76 (0.47-1.19)	0.85 (0.21-2.03)	1.06 (0.51-2.05)	1.02 (0.52-2.05)	1.21 (0.48-2.39)	<0.001
	32+0-34+6	0.88 (0.28-1.71)	0.76 (0.36-1.38)	1.01 (0.36-2.05)	1.09 (0.28-2.41)	0.83 (0.53-2.24)	0.021
	All	0.84 (0.44-1.33)	0.86 (0.34-1.85)	1.04 (0.51-2.05)	1.09 (0.45-2.07)	1.19 (0.48-2.50)	<0.001
B cells	<28	1.31 (0.65-3.03)	1.45 (0.67-3.34)	1.99 (0.98-3.26)	1.63 (0.62-3.02)	1.57 (0.61-3.65)	0.33
	28-31+6	1.29 (0.52-3.66)	1.66 (0.48-3.31)	1.60 (0.80-2.52)	1.55 (0.59-2.80)	1.61 (0.38-4.52)	0.14
	32+0-34+6	1.58 (0.32-2.32)	1.33 (0.50-2.65)	1.64 (0.54-3.50)	1.58 (0.52-2.73)	1.81 (0.74-3.17)	0.014
	All	1.32 (0.52-3.00)	1.45 (0.50-3.02)	1.71 (0.65-3.26)	1.57 (0.59-2.80)	1.66 (0.46-3.65)	0.006
NK cells	<28	0.26 (0.04-0.59)	0.27 (0.02-0.66)	0.30 (0.05-1.47)	0.22 (0.10-1.42)	0.46 (0.09-1.31)	0.008
	28-31+6	0.23 (0.06-0.60)	0.23 (0.04-1.42)	0.23 (0.05-0.66)	0.28 (0.08-0.75)	0.38 (0.12-1.57)	0.001
	32+0-34+6	0.25 (0.04-0.82)	0.22 (0.06-0.50)	0.26 (0.03-1.65)	0.35 (0.08-1.16)	0.41 (0.13-1.51)	<0.001
	All	0.25 (0.05-0.68)	0.23 (0.04-0.66)	0.28 (0.04-1.47)	0.29 (0.08-0.90)	0.39 (0.12-1.51)	<0.001

Table 4

Population	Gestation (weeks)	2 months	5 months	7 months	12 months	13 months	p
$CD3^+$	<28	59.0 (38.0-79.0)	65.0 (52.0-77.0)	60.4 (47.0-73.0)	67.4 (45.0-79.5)	63.7 (50.0-80.0)	0.04
	28-31+6	64.1 (47.0-82.0)	65.0 (42.0-86.3)	62.0 (59.0-70.8)	67.0 (51.0-80.6)	65.2 (46.1-89.0)	0.42
	32+0-34+6	65.5 (53.0-86.0)	66.4 (46.0-81.0)	67.5 (42.0-79.0)	63.0 (52.0-78.0)	60.0 (46.0-71.0)	0.026
	All	64.0 (45.0-82.0)	65.0 (46.0-83.0)	62.1 (47.0-74.0)	65.0 (52.0-80.0)	63.1 (46.1-82.0)	0.86
$CD3^+CD4^+$	<28	39.1 (24.0-54.0)	44.0 (29.0-64.0)	40.6 (31.8-52.0)	43.6 (27.0-57.0)	42.0 (25.0-54.0)	0.30
	28-31+6	46.5 (31.2-63.0)	49.0 (29.0-64.8)	43.0 (37.0-56.4)	47.4 (36.0-57.3)	45.0 (27.6-62.0)	0.13
	32+0-34+6	50.5 (36.0-69.0)	51.0 (35.0-68.0)	45.0 (32.0-56.0)	42.0 (26.0-55.0)	41.5 (16.0-56.0)	<0.001
	All	46.0 (26.0-63.0)	49.0 (33.0-67.0)	43.0 (32.0-56.0)	44.0 (32.0-57.0)	43.0 (21.0-56.2)	0.002
$CD3^+CD8^+$	<28	19.1 (12.0-32.0)	19.0 (15.0-31.0)	17.8 (14.0-23.0)	20.1 (13.8-28.6)	22.5 (15.0-31.0)	0.11
	28-31+6	15.2 (10.0-26.0)	16.0 (9.0-27.0)	17.0 (11.0-27.0)	17.7 (11.7-26.0)	19.0 (12.0-30.0)	<0.001
	32+0-34+6	15.8 (10.0-22.0)	15.5 (9.0-25.0)	17.5 (7.5-31.0)	18.0 (11.0-28.0)	17.0 (12.0-28.0)	0.032
	All	16.0 (10.0-26.0)	16.0 (9.0-26.0)	17.4 (11.0-30.0)	18.0 (12.0-28.0)	19.0 (12.0-31.0)	<0.001
B cells	<28	27.5 (17.0-48.0)	26.6 (19.0-38.0)	31.4 (17.2-42.0)	24.4 (12.5-38.0)	24.1 (10.0-42.8)	0.24
	28-31+6	27.8 (15.3-43.0)	29.1 (11.0-48.0)	28.0 (18.3-35.0)	26.4 (14.6-42.0)	26.4 (7.0-38.6)	0.26
	32+0-34+6	27.6 (10.0-35.0)	24.0 (8.1-45.0)	23.6 (16.0-37.7)	28.5 (17.0-35.0)	30.1 (18.1-43.0)	0.07
	All	27.8	27.0	27.8	26.9	27.2	0.84

		(15.0-43.0)	(11.0-45.0)	(17.2-37.7)	(15.0-39.5)	(10.1-42.8)	
NK cells	<28	5.2 (1.0-12.2)	5.0 (0.7-7.0)	5.0 (1.0-21.3)	4.4 (1.5-20.6)	7.7 (3.0-25.9)	0.12
	28-31+6	5.0 (1.0-10.8)	4.0 (1.0-15.3)	4.0 (1.0-7.9)	5.0 (2.0-11.2)	6.9 (2.3-16.6)	0.07
	32+0-34+6	4.0 (1.0-14.5)	4.0 (1.0-10.0)	4.9 (0.3-34.6)	5.7 (2.0-25.1)	8.1 (2.0-24.1)	<0.001
	All	0.25 (0.05-0.68)	0.23 (0.04-0.66)	0.28 (0.04-1.47)	0.3 (0.1-0.9)	0.4 (0.1-1.5)	0.001
$CD4^+$:$CD8^+$	<28	1.93 (1.27-2.85)	2.23 (0.96-4.02)	2.36 (1.69-3.00)	2.07 (1.37-3.49)	2.1 (0.8-3.0)	0.68
	28-31+6	3.00 (1.54-5.07)	3.38 (1.76-5.22)	2.61 (1.52-4.84)	2.60 (1.51-4.14)	2.5 (1.3-3.9)	<0.001
	32+0-34+6	3.11 (2.33-5.00)	3.34 (1.79-5.58)	2.78 (1.09-4.55)	2.49 (0.93-4.23)	2.4 (0.8-4.7)	<0.001
	All	2.77 (1.54-4.90)	3.05 (1.76-5.57)	2.53 (1.27-4.57)	2.46 (1.40-4.14)	2.35 (0.79-4.05)	<0.001

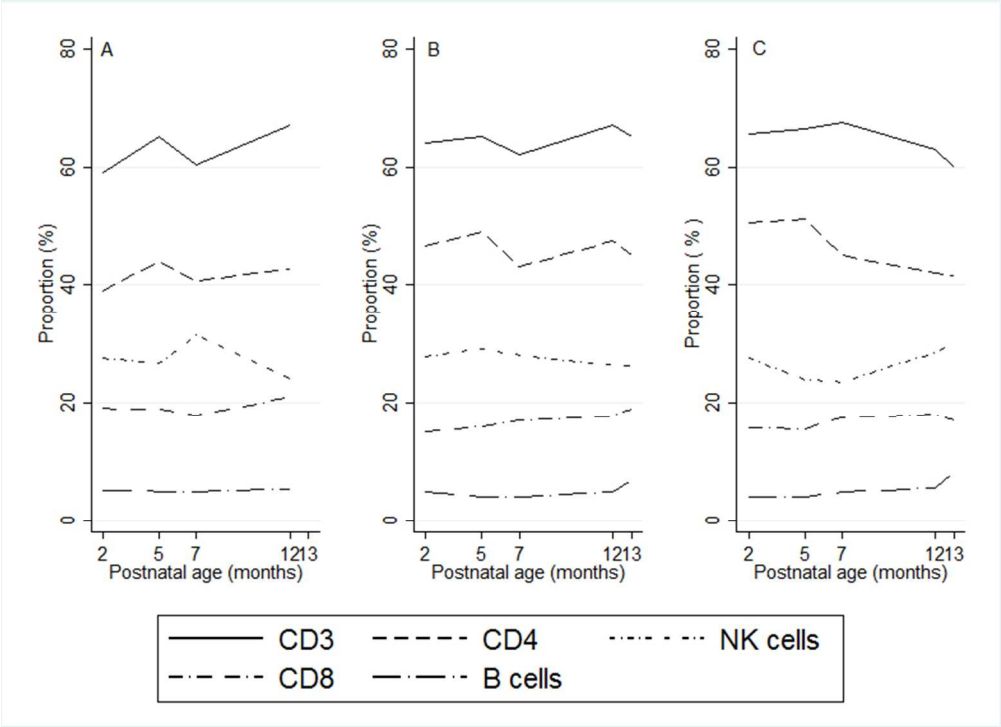

Figure 1: Lymphocyte subpopulations at different gestational and postnatal ages (median percentage). A: Birth gestation <28 weeks, B: Birth gestation 28+0-31+6 weeks, C: Birth gestation: 32+0 – 34+6 weeks
277x201mm (72 x 72 DPI)

Supplementary figures

Supplementary table 1: Study design. Pediacel (Sanofi Pasteur MSD), Prevenar13 (Pfizer Inc), Menjugate (Novartis Diagnostics and Vaccine, Siena, Italy), Priorix (GlaxoSmithKline Ltd) and Menitorix (GlaxoSmithKline Ltd)

Supp Table 1

Approx. age (months)	2	3	4	5	6	7	12	13
Group 1	**Blood sample** Pediacel™ Prevenar13™	Pediacel™ Menjugate™	Pediacel™ Menjugate™ Prevenar13™	**Blood sample**			**Blood sample** Priorix™ Menitorix™ Prevenar13™	**Blood sample**
Group 2	**Blood sample** Pediacel™ Prevenar13™	Pediacel™ Menjugate™ Prevenar13™	Pediacel™ Menjugate™ Prevenar13™	**Blood sample**			**Blood sample** Priorix™ Menitorix™ Prevenar13™	**Blood sample**
Group 3	**Blood sample** Pediacel™ Prevenar13™	Pediacel™ Menjugate™	Pediacel™ Menjugate™ Prevenar13™		Prevenar13™	**Blood sample**	**Blood sample** Priorix™ Menitorix™ Prevenar13™	**Blood sample**

Supplementary table 2: Lymphocyte subpopulations measured

Supp Table 2

Cell population	CD Marker
Total lymphocyte count	Side scatter and CD45
T cell	CD3
T helper cell	CD3/CD4
Cytotoxic T cell	CD3/CD8
Natural killer cell	CD56 and/or CD16
B cell	CD19 or CD20