Title

Reference intervals for lymphocyte subsets in preterm and term neonates without immune defects

Journal

Journal of Allergy and Clinical Immunology, 144(6)

ISSN

0091-6749

Authors

Amatuni, George S
Sciortino, Stanley
Currier, Robert J
et al.

Publication Date

2019-12-01

DOI

10.1016/j.jaci.2019.05.038

Peer reviewed

HHS Public Access

Author manuscript

J Allergy Clin Immunol. Author manuscript; available in PMC 2020 December 01.

Published in final edited form as:

J Allergy Clin Immunol. 2019 December ; 144(6): 1674–1683. doi:10.1016/j.jaci.2019.05.038.

Reference Intervals for Lymphocyte Subsets in Preterm and Term Neonates Without Immune Defects

George S. Amatuni, BS[a,b], **Stanley Sciortino, PhD, MPH**[c], **Robert J. Currier, PhD**[a], **Stanley J. Naides, MD**[d], **Joseph A. Church, MD**[e], **Jennifer M. Puck, MD**[a,*]

[a]Department of Pediatrics, Institute for Human Genetics, and Cardiovascular Research Institute, University of California San Francisco, and UCSF Benioff Children's Hospital, San Francisco, CA

[b]Stem Cell Institute, Department of Cell Biology, Einstein College of Medicine, Bronx, NY

[c]Genetic Disease Screening Program, California Department of Public Health, Richmond, CA

[d]Immunology Department, Quest Diagnostics Nichols Institute, San Juan Capistrano, CA

[e]Department of Pediatrics, University of Southern California Keck School of Medicine, and Children's Hospital Los Angeles, Los Angeles, CA.

Abstract

Background—In 6.5 years of newborn screening for severe combined immunodeficiency (SCID) in California, 3,252,156 infants had DNA from dried blood spots (DBS) assayed for T-cell receptor excision circles (TRECs). Infants with TRECs below a designated cutoff on a single DBS, or 2 DBS samples with insufficient PCR amplification, or known genetic risk of immunodeficiency had peripheral blood complete blood counts and lymphocyte subsets assayed in a single flow cytometry laboratory. Cases in which immune defects were ruled out were available for analysis.

Objective—We wished to determine reference intervals for lymphocyte subsets in racially/ethnically-diverse preterm and term newborns who proved to be unaffected with any T-lymphopenic immune disorder.

Methods—Effective gestational age was defined as gestational age at birth plus postnatal age at time of sample collection. After determining exclusion criteria we analyzed demographic and clinical information, complete and differential white blood counts, and lymphocyte subsets for 301 infants, with serial measurements for 33 infants. Lymphocyte subset measurements included total

[*]**Address correspondence to:** Jennifer M. Puck, MD, Division of Allergy, Immunology and Blood and Marrow Transplantation, UCSF Department of Pediatrics, Box 3118, 555 Mission Bay Blvd South, Rm SC-252K, San Francisco, CA 94143, Phone: 415 502-2090; Jennifer.Puck@ucsf.edu.

Disclosures:

Dr. Naides has been employed by Quest Diagnostics. Dr. Puck discloses spousal employment at InVitae, a clinical DNA sequencing company. The other authors have no conflicts of interest to disclose.

T cells, helper and cytotoxic T-cell subsets, naïve and memory phenotype of each T-cell subset, B cells and NK cells.

Results—Reference intervals were generated for absolute numbers and percentages of lymphocyte subsets from infants with effective gestational ages 22–52 weeks. Sex and ethnicity were not significant determinants of lymphocyte subset counts in this population. Lymphocyte counts rose postnatally.

Conclusion—This study provides a baseline for interpreting comprehensive lymphocyte data in preterm and term infants, aiding clinicians to determine which newborns require further evaluations for immunodeficiency.

Clinical Implication—Reference intervals in preterm and term neonates for T-cell counts, including proportions of cells with naïve and memory markers, facilitate evaluations of newborns suspected to have serious T-lymphopenic conditions.

Capsule Summary

A new compilation of reference intervals for neonatal lymphocyte subsets, including naïve and memory helper and cytotoxic T cells, has been derived from California newborn screening and follow-up of infants who proved to be unaffected.

Keywords

Flow cytometry; Memory T cell; Naïve T cell; Neonatal immunity; Newborn screening; Preterm birth; Reference range/reference interval; Severe combined immunodeficiency (SCID); T-cell receptor excision circle (TREC); T-cell subsets

Introduction

A number of studies have reported lymphocyte subset values for healthy infants and children.[1–3] However, the available reference intervals have limitations, including small numbers of individuals tested, insufficient numbers to permit separation of age groups of infants under 3 months, and omission of preterm or low birthweight (BW) infants. Some, but not all, reports have found that lymphocyte subset intervals varied between infants of different ethnic makeup, sex, environmental exposures and geographical area, although predominant factors driving the differences could not be defined.[4] An important source of variation was inconsistency between multiple contributing laboratories.[1]

Population-based newborn screening (NBS) for severe combined immunodeficiency (SCID) and T-cell lymphopenia (TCL) is now performed throughout the USA[5] and is being increasingly adopted in many countries.[6] NBS for SCID is based on detection of T-cell receptor excision circles (TRECs), a biomarker for T-cell lymphopoiesis, in DNA extracted from infant dried blood spots.[7,8] Insufficient or absent TRECs in the screening test are correlated with subsequent measurements showing low circulating T-cell numbers and few recent thymic emigrant T cells bearing naïve markers such as CD45RA.[9–14] However, direct measurement of lymphocyte subsets with quantitation of naïve and memory helper and cytotoxic T-cell subsets is critical for definitive diagnosis. Therefore, there is a need to

establish standardized reference intervals for all newborns, including preterm infants and infants of low BW.

NBS for SCID has been conducted in the state of California since August, 2010.[9,14] Flow cytometry is incorporated within the screening program as a follow-on test for all infants with TRECs below a designated cutoff on a single DBS with adequate PCR control, 2 DBS samples with insufficient PCR amplification, or a clinical suspicion or genetic risk factor for primary T-cell immunodeficiency. Cutoffs have been set to avoid missing cases with true SCID or significant TCL. Thus, while highly sensitive, the TREC screen flags some immunologically normal newborns for follow-up, as well as infants with transient TCL that can be associated with preterm birth alone. As previously published, lymphocyte subsets for infants born in California have been measured at a single contract laboratory.[9,14] Substantial numbers of infants receiving this testing have proven to have no diagnosed immune system defects or medical conditions, while others had only diagnoses related to prematurity and low BW. We have now analyzed the lymphocyte subset data of these infants to provide an improved set of newborn lymphocyte reference intervals, taking into account the newborns' effective gestational age (EGA), BW, sex and race/ethnicity.

Methods

Study population

A total of 3,252,156 dried blood spot (DBS) specimens were collected as part of newborn screening from essentially all infants born in California between August 15, 2010 and March 31, 2017, except those whose parents opted out for religious reasons and completed a form accepting responsibility for any harm coming to the child as a result of refusal to test. DBS were analyzed for TREC counts in the Genetic Disease Laboratory (GDL) of the Genetic Disease Screening Program (GDSP) within the California Department of Public Health (CDPH) in Richmond, CA, prior to June 2015, after which the EnLite kit (PerkinElmer) was adopted, allowing for TREC testing to be performed at regional laboratories, with oversight from the GDSP. TREC thresholds were adjusted during this period to optimize the assay's sensitivity and specificity.

Information collected from all infants by the GDSP under IRB exemption (California Committee for the Protection of Human Subjects) included birthweight, sex, gestational age at birth, whether the infant was in a regular nursery or neonatal intensive care unit (NICU), parent-designated race or ethnic background, and a clinical summary from maternity providers. Race/ethnicity was a multiple-choice check box on the GDSP State Test Request Form. We used a hierarchical approach to categorize race when more than one box was checked as follows: Hispanic, black, Asian, followed by white. Native Americans were always included in our "Other" category, if checked, as were those with missing and unknown race/ethnicity.[18]

Flow Cytometry

Per NBS protocol, a peripheral blood sample was obtained from infants (n=562) identified by GDSP to have TREC results below the threshold for follow-up testing within the NBS

program or to be at risk for a genetic immune deficiency based on family history. The blood was shipped by courier to the Quest Diagnostics Nichols Institute (San Juan Capistrano, CA). For each specimen an automated complete and differential white blood count (CBC/diff, Coulter STKS/LH750 hematology analyzer, Coulter Technology Center, Miami, FL) was performed. A blood smear was reviewed for blasts or other abnormal cell morphology. Lymphocyte subset analysis was performed by flow cytometry, with a sample from a healthy control included with each batch of patient samples. See Supplementary Methods and Supplementary Table S1 for details.

Statistical methods

Newborn results in this study were not randomly sampled, but rather were obtained from healthy babies with transient non-normal TREC NBS or recognized genetic risk, such as having a sibling affected with a T-lymphopenic disorder. Univariate and multi-variate analyses were conducted using ANOVA in SAS version 9.4 (SAS Institute Inc., Cary, NC) to examine whether race/ethnicity, sex, or EGA or BW had independent associations with the results of flow cytometry. Paired sign tests for sequential flow results were based on first and last test per child and were calculated by hand using a binomial distribution. Graphics were created using SAS and MS Excel.

Results

Study cohort

Inclusion and exclusion criteria were established prior to examining the data. Infants born at any gestational age (GA) and weight were included, regardless of their likelihood to be under greater physical stress compared to term newborns. Conditions expected to occur in prematurely born subjects, such as respiratory insufficiency requiring ventilatory support or feeding intolerance requiring intravenous or enteral nutritional support did not render them ineligible. However, any infant for whom a primary or secondary clinical concern was recorded that could be associated with SCID or TCL was excluded from this study (Table 1).[14] Additionally, Table 1 shows excluded infants who did not fit the general profile of a reasonably healthy newborn from the provided clinical information. Reasons for omission from study cohort included an identified clinical syndrome that has been reported to cause immunodeficiency[15–17] (n=21), any of various severe congenital conditions (n=33), conditions accompanied by fluid accumulation or vascular leak that might affect blood counts (n=8), infant death prior to discharge from the hospital (n=10), immune abnormalities without overall T-cell lymphopenia (n=7), and miscellaneous reasons (n=6).

If a baby was indicated to be “term” by a physician with no GA given, the baby’s gestational age at birth was imputed to be 40 weeks for the purpose of our calculations. Likewise, if a baby was indicated to be in the regular nursery with a BW greater than 2500 grams, the gestational age was imputed to be 40 weeks. If a baby was indicated to be “term” and greater than 2500 grams in BW, we imputed the nursery to be a regular nursery if not otherwise recorded. If a baby was born at GA of 32 weeks or less, or had BW less than or equal to 1500 grams, we imputed the nursery to be NICU if not otherwise recorded. GA imputation

was required for 2 infants, and nursery type for an additional 2, both imputed to be in regular nurseries.

GA and BW cohorts

The analysis cohorts were established after missing data imputation for eligible infants with case data available for GA, BW, NICU status, race/ethnicity, sex and liquid blood lymphocyte subset determinations. Study subjects were divided into groups according to effective GA (EGA), defined as GA at birth plus postnatal age at time of flow test, as follows: 22–28 weeks (n=31), 29–31 weeks (n=53), 32–36 weeks (n=58), 37–39 weeks (n=51), 40–41 weeks (n=75), 42–43 weeks (n=44), and 44–52 weeks (n=26). There were 268 (89%) infants with one test only, 29 (10%) with 2 tests, and 4 (1%) with 3 tests, totaling 338 tests of lymphocyte subsets. We analyzed tests as individual observations for the EGA cohort (n=338). A second, BW cohort (n=301) was created as a subset of the EGA cohort, retaining only the flow cytometry determination conducted closest to the time of birth.

Comparison of race/ethnicity and sex of study subjects

Approximately equal numbers of newborns were identified in NICUs (n=151) and regular nurseries (n=150) (Table 2). This observation is indicative of the higher likelihood of false TREC screen-positive results among infants in NICU, including those born prematurely.[14] Twice as many male (n=201) as female (n=100) newborns were included in the eligible cohort. Males were more likely to be treated in a NICU and to be of earlier GA and lower BW than females in this cohort. As shown in Table 2, univariate analysis of mean EGA and BW by NICU showed statistically significant differences, with earlier mean EGA (NICU 33 weeks; regular nursery 44 weeks; $p < 0.001$) and lower mean BW (NICU 1,127 g; regular nursery 3,160 g; $p < 0.001$). There were no significant differences for mean EGA and BW by race/ethnicity or sex.

Lymphocyte subset distribution vs. effective gestational age and birthweight

Table 3 shows lymphocyte subset counts (A) and percentages (B) by EGA, and Table 4 shows lymphocyte subset counts (A) and percentages (B) by BW. Whereas the absolute numbers of lymphocytes tended to increase with EGA and BW, the percentages of a given subset were essentially constant across the tracked EGA and BW groups, as shown in Figure 1 for total T cells and helper and cytotoxic T-cell subsets, and in Supplemental Figures S1–S4, where data are also shown for B and NK cells.

Scatter plots and distributions of flow cytometry results

Scatter plots indicated a positive correlation, shown by diagonal clustering of dots, among absolute counts of total lymphocytes and each of the subsets: total T cells (CD3), helper T cells (CD4), cytotoxic T cells (CD8), naïve helper T cells (CD4-CD45RA), and naïve cytotoxic T cells (CD8-CD45RA) (Figure 2). The numbers of cells of each subset, also represented by the bar graphs in Figure 2, demonstrated essentially normal distributions. In contrast, there was little to no correlation among other subgroups, including B cells, NK cells, and CD4 and CD8 cells expressing the memory marker CD45RO (Supplementary Figure S5, in which diagonal clustering is not apparent). Thus, while numbers of T cells and

naïve T-cell subsets were correlated with each other, B-cell, NK-cell, and memory T-cell numbers were independent (statistics not shown).

Regression analysis

Multivariate ANOVA was used to explore the relationship between sex, race/ethnicity and EGA at time of testing for each of the lymphocyte subsets CD3, CD4, CD8, CD19 and CD16-CD56 (Table 5A, Supplementary Table S2A). Similarly, these subsets were analyzed for relationships between sex, race/ethnicity and BW (Table 5B, Supplementary Table 2B). NICU status was omitted from the final models due to co-linearity with both BW and EGA. In each analysis, sex and race/ethnicity class variables were non-significant. However, effective GA and BW were statistically significant for T cells, as shown in Table 5, and also significant in all other subsets in Supplementary Table S2, except for CD19, which was not significant in the BW cohort (Supplementary Table S2B).

Serial determinations of peripheral blood lymphocyte profiles

While the main analyses were cross-sectional and lacked a time element to imply causality, the sub-sample of 33 babies who did have multiple flow determinations over time showed trends towards improvement of several flow cytometry measures. In eligible cohort, 29 infants had two and 4 had three tests. Most measurements showed increase in lymphocyte subset counts. All subsets in Figure 3 and Supplemental Figure S6 had significant p-values in a match, before and after Sign test (CD3: n=33, $p<.0001$; CD4-CD45RA: n=33, $p<.05$; CD8-CD45RA: n=33, $p<.001$; CD19: n=33, $p<.01$; CD16-CD56: n=33, $p<.05$), although the rise in numbers of B cells was modest and NK cells only barely significant.

Discussion

This study contributes detailed lymphocyte profiles permitting establishment of reference intervals for T, B and NK lymphocytes, as well as naïve and total CD4 helper and CD8 cytotoxic T lymphocytes for very young infants, including those of preterm birth and with low birthweight. We had available a large, racially/ethnically diverse cohort in California. Our immunocompetent cohort differs from the general population by having been selected due to initial TREC newborn screening results that were abnormal or incomplete, or by having a relative with an immune disorder. Nonetheless, infants with an established or suspected condition that could affect lymphocyte determinations were eliminated, leaving only the immunocompetent ones for inclusion. The infants studied differed from the general newborn population due to the preponderance of babies identified in NICUs whose TREC results were below the threshold for SCID NBS. However, it is well-known that NICU babies have higher rates of false-positive results than do infants from regular nurseries. Screen-positive TREC results initiated subsequent follow-up testing according to criteria established by immunologists associated with the California GDSP. The babies included in the study were subsequently determined to be healthy and immunocompetent, and our sample thus provides flow cytometry results and intervals for healthy subjects who were initially classified as at-risk to have a T-lymphopenic disorder. While results from our cohort may not reflect the entire newborn population, this study provides useful guidance for the

interpretation of flow cytometry results for newborns most likely to be identified by programs conducting newborn screening for SCID.

Importantly, and in contrast to most prior publications of infant lymphocyte reference intervals, all of the liquid blood samples in this study were obtained and transported in standardized fashion and analyzed in a single flow cytometry laboratory. The lack of distinction in lymphocyte counts between infants of different racial/ethnic background in this study is in contrast to other published studies, but may be explained by the fact that all of our data were analyzed in a single setting. It is possible that racial/ethnic variation seen in prior studies could be an artifact of sample handling delays or systematic analytical differences that predominantly affected one group over another or by chance due to small sample sizes.

Additionally, in this study we have shown that EGA and BW are significant predictors of flow cytometry results. Some of the differences seen in previous studies may be consequences of differential rates of prematurity and low birthweight, which were not analyzed previously. Multivariate analysis showed that flow cytometry results were not influenced by demographic factors such as race/ethnicity and sex but were strongly related to both EGA and to BW. This is in contrast to prior reports.[19] However, EGA and BW are strongly correlated with each other and were analyzed separately, each with its own contribution to flow cytometry results. Having both of these analyses available may prove useful in clinical contexts where either EGA or BW is not available.

Flow cytometry results for the main subsets increased as the newborns achieved an EGA approaching term and a birthweight normally found among term infants. Flow results for the main subsets increased together alongside increasing EGA and BW, as suggested by increasing absolute counts with little concomitant change in relative subset proportions.

While the majority of EGA and all BW data were cross-sectional and lacked a time element to imply causality, the small sub-sample of babies who did have multiple flow results over time showed a trend towards improvement of several flow cytometry measures, and the paired Sign Test confirmed that these increasing values among the small number of babies were not likely to have occurred by chance. B-cell absolute numbers rose slightly but significantly with increasing EGA, while NK cells remained nearly constant (Supplemental Figure S6). To our knowledge, these trends have not previously been recognized.

Our data emphasize the importance of looking at cell numbers rather than percentages, which did not show significant changes in any subsets as EGA or BW increased. Moreover, it is also important to stain lymphocytes with surface markers such as CD45RA, which differentiate naïve T cells, newly emerged from the thymus, from memory phenotype cells, which express the isotype CD45RO and have undergone activation and expansion in the peripheral circulation.

It is anticipated that Tables 3(A, B) and 4(A,B) in this publication will constitute a useful reference. With the widespread adoption of newborn screening for SCID and disorders with clinically significant T-cell lymphopenia, the reference intervals provided here should help neonatologists and immunologists provide optimal care, both by recognizing and protecting

infants with immunodeficiency while avoiding excessive anxiety and immune testing when not necessary.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

The authors thank the dedicated and careful personnel of the California Newborn Screening Program and the flow cytometry team at Quest Diagnostics Nichols Institute for outstanding technical assistance.

Declaration of Funding Sources:

JMP received support from R01 AI105776; U54 AI082973 for the Primary Immune Deficiency Treatment Consortium, a member of the Rare Diseases Clinical Research Network (RDCRN) funded by NIAID and ORDR, NCATS, NIH; the Jeffrey Modell Foundation; the Lisa and Douglas Goldman Fund; and the Michelle Platt-Ross Foundation. JAC received support from the Jeffrey Modell Foundation.

Abbreviations

ALC	(absolute lymphocyte count)
ANOVA	(analysis of variance)
APC	(allophycocyanin)
BW	(birthweight)
CBC/diff	(complete and differential blood count)
CDPH	(California Department of Public Health)
CD	(cluster of differentiation, defining a numbered sequence of cell surface protein markers)
CHD	(congenital heart defect)
DBS	(dried blood spot)
EDTA	(ethylenediaminetetraacetic acid)
EGA	(effective gestational age)
FACS	(fluorescence-activates cell sorting)
FITC	(fluorescein isothiocyanate)
GA	(gestational age)
GDL	(Genetic Disease Laboratory)
GDSP	(Genetic Disease Screening Program)
NBS	(newborn screening)

NICU	(neonatal intensive care unit)
NK	(natural killer)
PE	(phycoerythrin)
PerCP	(peridinin-chlorophyll-protein complex)
SCID	(severe combined immunodeficiency)
TCL	(T-cell lymphopenia)
TREC	(T-cell receptor excision circle)
TTTS	(twin-to-twin transfusion syndrome)
VATER	(vertebral, anorectal, tracheal, esophageal, and renal abnormalities)
WBC	(white blood cell)

References

1. Shearer WT, Rosenblatt HM, Gelman RS, Oyomopito R, Plaeger S, Stiehm ER, et al. Pediatric AIDS Clinical Trials Group. Lymphocyte subsets in healthy children from birth through 18 years of age: the Pediatric AIDS Clinical Trials Group P1009 study. J Allergy Clin Immunol. 2003 11;112(5):973–80. [PubMed: 14610491]
2. Huenecke S, Fryns E, Wittekindt B, Buxmann H, Königs C, Quaiser A, et al. Percentiles of lymphocyte subsets in preterm infants according to gestational age compared to children and adolescents. Scand J Immunol. 2016 11;84(5):291–298. doi: 10.1111/sji.12474. [PubMed: 27548364]
3. Huo Y, Patel K, Scott GB, Van Dyke RB, Siberry GK, Burchett SK, et al. Lymphocyte subsets in HIV-exposed uninfected infants and HIV-unexposed uninfected infants. J Allergy Clin Immunol. 2017 8;140(2):605–608.e3. doi: 10.1016/j.jaci.2017.01.033. Epub 2017 Mar 6. [PubMed: 28279682]
4. Mandala WL, Ananworanich J, Apornpong T, Kerr SJ, MacLennan JM, Hanson C, et al. Control lymphocyte subsets: can one country's values serve for another's? J Allergy Clin Immunol. 2014 9;134(3):759–761.e8. doi: 10.1016/j.jaci.2014.06.030. [PubMed: 25171870]
5. IDF SCID Newborn Screening Campaign. Towson, MD: Immune Deficiency Foundation; 2018 [Cited 2019 Jan 27]. Available from: https://primaryimmune.org/idf-advocacy-center/idf-scid-newborn-screening-campaign
6. Rechavi E, Lev A, Simon AJ, Stauber T, Daas S, Saraf-Levy T, et al. First year of Israeli newborn screening for severe combined immunodeficiency-clinical achievements and insights. Front Immunol. 2017 11 6;8:1448. doi: 10.3389/fimmu.2017.01448. [PubMed: 29167666]
7. Chan K, Puck JM. Development of population-based newborn screening for severe combined immunodeficiency. J Allergy Clin Immunol. 2005 2;115(2):391–8. [PubMed: 15696101]
8. Puck JM. Laboratory technology for population-based screening for severe combined immunodeficiency in neonates: The winner is T-cell receptor excision circles. J Allergy Clin Immunol. 2012 3;129(3):607–616. doi: 10.1016/j.jaci.2012.01.032. Epub 2012 Jan 29. [PubMed: 22285280]
9. Kwan A, Church JA, Cowan MJ, Agarwal R, Kapoor N, Kohn DB, et al. Newborn screening for severe combined immunodeficiency and T-cell lymphopenia in California: results of the first 2 years. J Allergy Clin Immunol. 2013 7;132(1):140–50. doi: 10.1016/j.jaci.2013.04.024. [PubMed: 23810098]

10. Kwan A, Abraham RS, Currier R, Brower A, Andruszewski K, Abbott JK, et al. Newborn screening for severe combined immunodeficiency in 11 screening programs in the United States. JAMA. 2014 8 20;312(7):729–38. doi: 10.1001/jama.2014.9132. [PubMed: 25138334]

11. Verbsky J, Thakar M, Routes J. The Wisconsin approach to newborn screening for severe combined immunodeficiency. J Allergy Clin Immunol. 2012 3;129(3):622–7. doi: 10.1016/j.jaci.2011.12.004. Epub 2012 Jan 11. [PubMed: 22244594]

12. Vogel BH, Bonagura V, Weinberg GA, Ballow M, Isabelle J, DiAntonio L, et al. Newborn screening for SCID in New York State: experience from the first two years. J Clin Immunol. 2014 4;34(3):289–303. doi: 10.1007/s10875-014-0006-7. Epub 2014 Mar 1. [PubMed: 24578017]

13. Hannon WH, Abraham RS, Kobrynski L, Vogt RF Jr, Adair O, Aznar C, et al. Newborn blood spot screening for severe combined immunodeficiency by measurement of T-cell receptor excision circles; Approved guideline. CLSI document NBS06-A. Wayne, PA: Clinical and Laboratory Standards Institute, 2013.

14. Amatuni GS, Currier RJ, Church JA, Bishop T, Grimbacher E, Nguyen AA, et al. Newborn screening for severe combined immunodeficiency and T-cell lymphopenia in California, 2010–2017. Pediatrics. 2019;143(2):e20182300. doi: 10.1542/peds.2018-2300. [PubMed: 30683812]

15. Jyonouchi S, Orange J, Sullivan KE, Krantz I, Deardorff M. Immunologic features of Cornelia de Lange syndrome. Pediatrics. 2013 8;132(2):e484–9. doi: 10.1542/peds.2012-3815. [PubMed: 23821697]

16. Chrzanowska KH, Krajewska-Walasek M, Kuś J, Michałkiewicz J, Maziarka D, Wolski JK, et al. Kabuki (Niikawa-Kuroki) syndrome associated with immunodeficiency. Clin Genet. 1998 4;53(4):308–12. [PubMed: 9650771]

17. Bitton N, Alexander S, Ruggiero S, Parameswaran A, Russo A, Ferguson F. Case report: Noonan-like multiple central giant cell granuloma syndrome. Pediatr Dent. 2012 Sep-Oct;34(5):144–7. [PubMed: 23211900]

18. Race and Ethnic Standards for Federal Statistics and Administrative Reporting; Office of Management and Budget (OMB), Directive No. 15. Atlanta, CA: CDC, 2016 [Cited 2018 Oct 8]. Available from: https://wonder.cdc.gov/wonder/help/populations/bridged-race/Directive15.html

19. Preterm Birth. Child Health USA 2013. Rockville, MD: U.S. Department of Health and Human Services, 2013 [Cited 2019 Feb 9]. Available from: https://mchb.hrsa.gov/chusa13/perinatal-health-status-indicators/p/preterm-birth.html

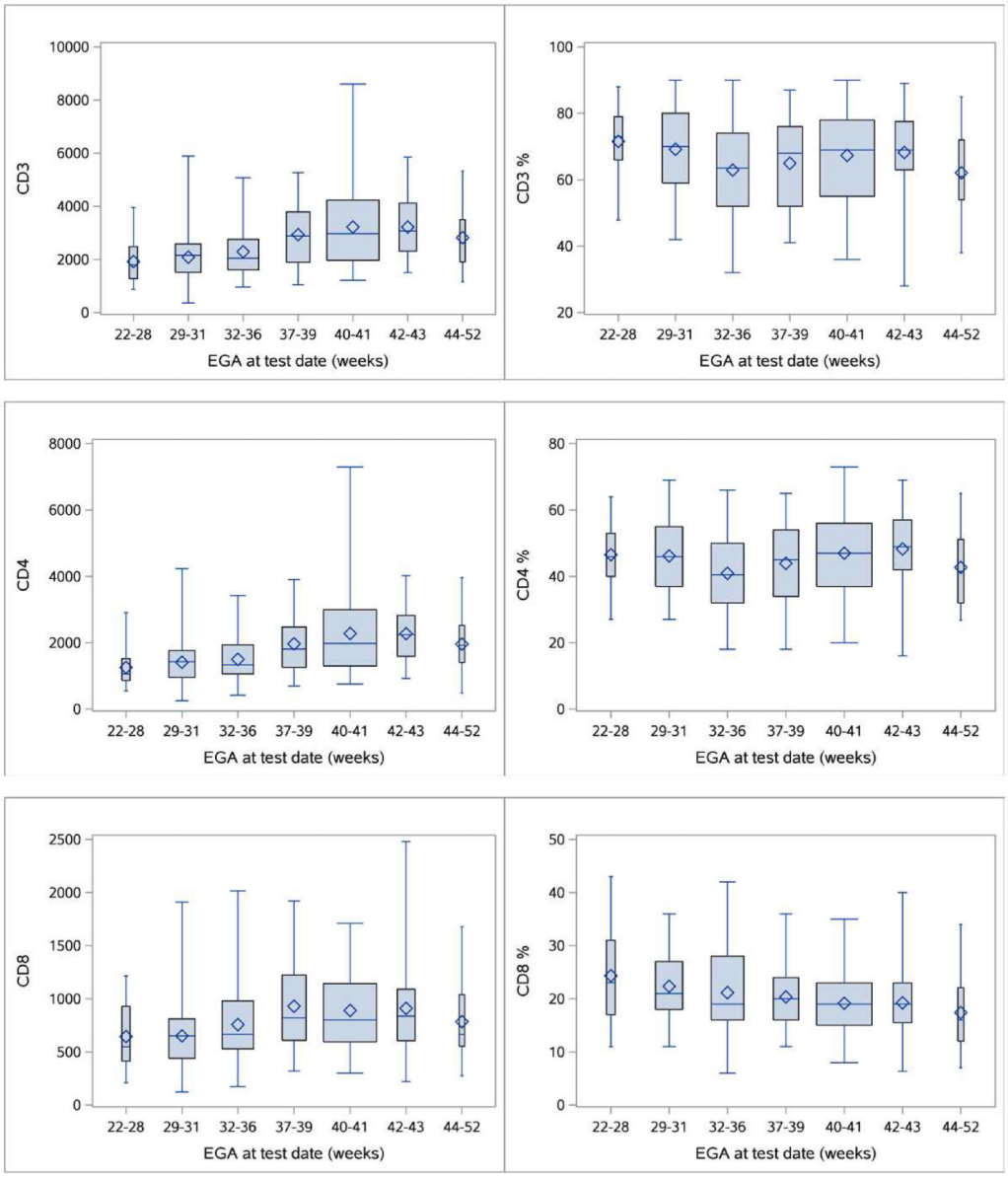

Figure 1. Absolute counts, left panels, and percentages, right panels, of each T cell subset at increasing effective gestational age groupings, EGA.

CD3, CD3 T cells/μl of peripheral blood; CD3 %, CD3/absolute lymphocyte count x100% (ALC); CD4, CD4 T cells/μl; CD4 %, CD4/ALC x100%; CD8, CD8 T cells/μl; CD8 %, CD8/ALC x100%. Shaded boxes encompass the 25th to 75th percentiles, the width of each proportional to number of measurements included.

Horizontal line within each box, median; diamond within each box, mean. Whisker extensions show lowest and highest values.

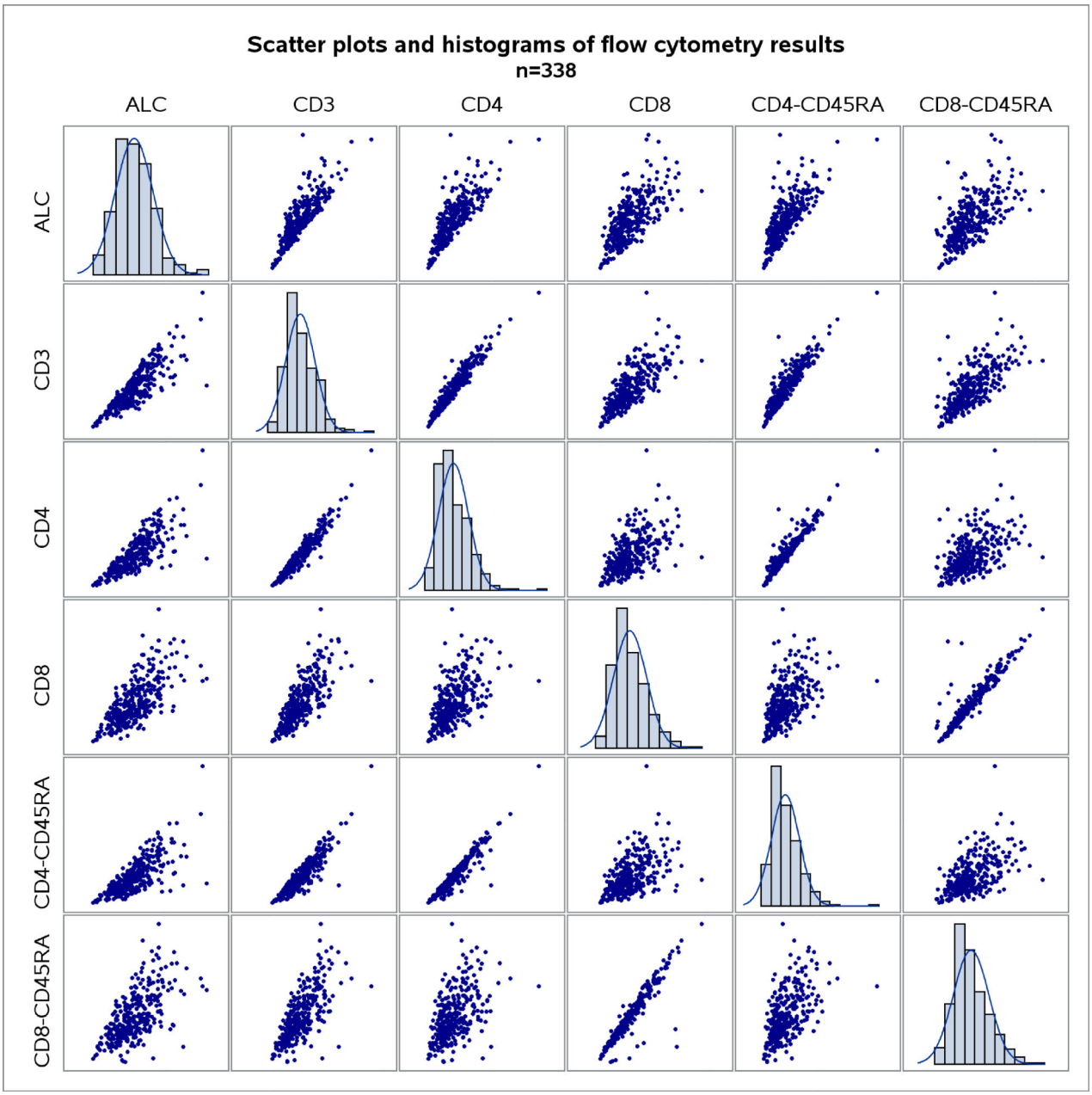

Figure 2. Scatter plots and distributions in deciles of flow cytometry results.

ALC, absolute lymphocyte count, cells/μl; CD3, CD3 T cells/μl; CD4, CD4 T cells/μl; CD8, CD8 T cells/μl; CD4-CD45RA, CD4 T cells/μl that also express CD45RA; CD8-CD45RA, CD8 T cells/μl that also express CD45RA.

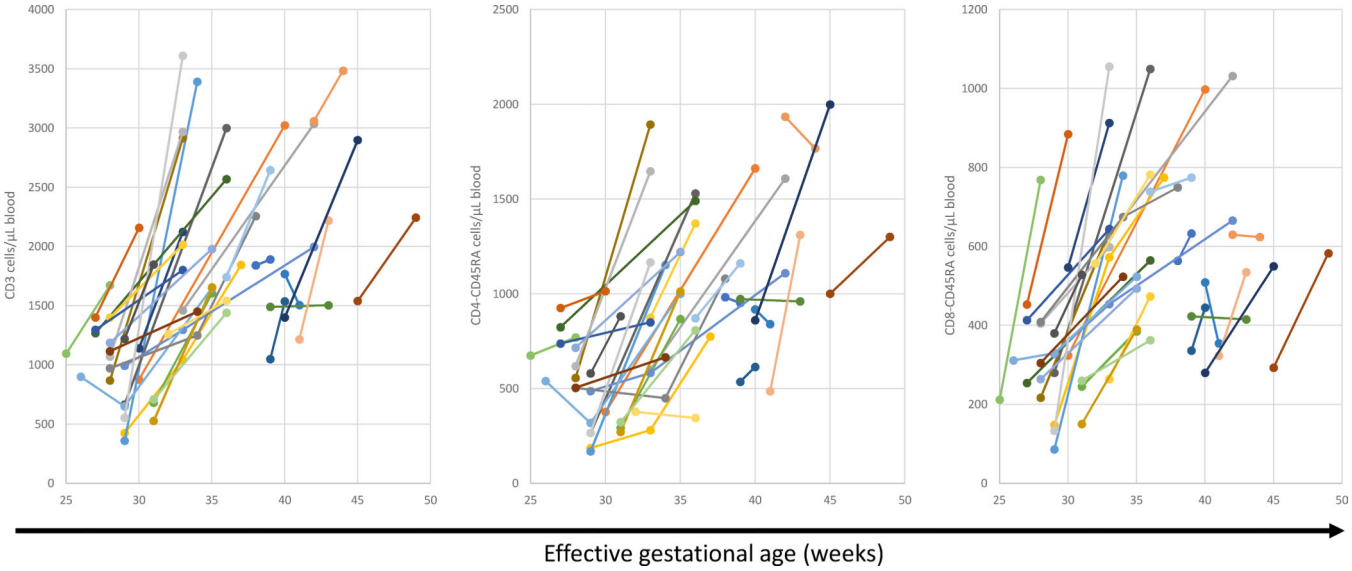

Figure 3.

Serial counts of CD3 (left panel), CD4-CD45RA (middle panel), and CD8-CD45RA (right panel) T cells at increasing effective gestational ages, in 33 infants with multiple measurements. Each set of connected points represents measurements from a single infant, with each infant's data given a consistent color in all panels.

J Allergy Clin Immunol. Author manuscript; available in PMC 2020 December 01.

Table 1:

Eighty-five infants excluded from reference interval cohort.

Category (number of infants)	Infants with each diagnosis
Recognized syndromes and associations (21)	12 DiGeorge syndrome
	5 Trisomy 21
	1 Each of Cornelia de Lange syndrome; Kabuki syndrome; Noonan syndrome; VATER (vertebral, anorectal, tracheal, esophageal, renal) association
Severe congenital abnormalities* **(33)**	11 Congenital heart defect, isolated
	4 Congenital heart defect with additional anomalies§
	14 Other major birth defects (ambiguous genitalia, brain malformation, diaphragmatic hernia, gastroschisis, Hirschprung's disease, intestinal atresia/ obstruction, liver malformation, pyriform aperture stenosis, skeletal deformity)
	2 Endocrinopathy (thyroid, parathyroid)
	2 Infection (chorioamnionitis, cytomegalovirus)
Fetal conditions with fluid accumulation* **(8)**	6 Hydrops, anasarca, ascites
	2 Twin-to-twin transfusion syndrome
Other circumstances (23)	10 Expired in hospital
	7 Abnormal leukocyte profile+ (2 severe congenital neutropenia, 2 with low or no B cells, and 1 each of absent NK cells, absent CD8 cells, fluctuating B and T cell numbers)
	2 Missing data (1 no flow cytometry; 1 no date of birth)
	2 Out-of-state birth
	1 Non-newborn (7-year-old immigrant)
	1 Methamphetamine withdrawal

*Infants with these conditions, which can be associated with T-cell lymphopenia, had T-cell counts above 1,500/μl, and thus were not referred by the California Genetic Disease Screening Program to immunology centers for further SCID evaluation.

§Non-syndromic cases.

+Although total CD3 T-cell counts were above 1,500/μl, leukocyte values that were extreme outliers caused these infants to be excluded.

Table 2.

Demographics of the cohorts of infants analyzed.

	BW Cohort		Mean BW	EGA Cohort		Mean EGA
Categories	**Number of infants**	**% within group**	**Grams**	**Number of infants**	**% within group**	**Weeks**
Totals	301	100.0%	2,140	338	100.0%	37
Race/ethnicity *§						
Asian	58	19.3%	2,187	69	20.4%	37
Black	23	7.6%	2,065	24	7.1%	36
Hispanic	135	44.9%	1,946	157	46.4%	36
Other	24	8.0%	2,217	25	7.4%	37
White	61	20.3%	2,522	63	18.6%	38
Nursery type*						
NICU	151	50.2%	1,127	180	53.3%	33
Regular	150	49.8%	3,160	158	46.7%	41
Sex						
Female	100	32.1%	2,212	112	33.1%	37
Male	201	67.9%	2,104	226	66.9%	37
EGA (weeks)§						
22–28	28	9.3%	650	31	9.2%	
29–31	44	14.6%	710	53	15.7%	
32–36	44	14.6%	966	58	17.2%	
37–41	47	15.6%	2,533	51	15.1%	
40–41	74	24.6%	3,046	75	22.2%	
42–43	40	13.3%	3,218	44	13.0%	
44–52	24	8.0%	3,291	26	7.7%	
Birthweight (g)						
<550	40	13.3%		52	15.4%	32
551–800	49	16.3%		62	18.3%	31
801–1250	27	9.0%		29	8.6%	32
1251–2500	40	13.3%		43	12.7%	38
2501–3000	36	12.0%		36	10.7%	40
3001–3500	61	20.3%		68	20.1%	42
>3500	48	15.9%		48	14.2%	42

*p <0.001 based on an F-test for each category (differences within other categories not significant).

§Multiple race/ethnicity categorized as single race in a hierarchy as follows: Hispanic, black, Asian, followed by white. Native Americans were included in the “Other” category, as were those with missing and unknown race/ethnicity.

EGA, Effective gestational age at time of test; BW, Birthweight; for the BW Cohort (sampled once closest to birth date) GA and EGA were the same.

Table 3.

Lymphocyte counts in cells/pl at increasing effective gestational ages (EGA) in children without immune disorders.

A. Absolute counts, median (5%-95% confidence interval), for peripheral blood lymphocytes and lymphocyte subsets.								
EGA (weeks)	**22–28**	**29–31**	**32–36**	**37–39**	**40–41**	**42–43**	**44–55**	**Total**
White cell count x 10^3 *	15 (7–30)	10 (5–21)	9(6–23)	10 (6–17)	11 (6–16)	8(6–14)	9(4–12)	10 (6–20)
Lymphocyte count	2,500 (1,200–4,200)	3,219 (800–5,800)	3,450 (1,800–6,486)	4,400 (2,700–6,400)	4,500 (2,600–7,770)	4,500 (2,800–7,500)	4,750 (2,300–7,600)	3,900 (1,700–7,000)
CD3 T cells	1,798 (900–3,608)	2,158 (528–3,828)	2,048 (1,250–4,080)	2,886 (1,512–4,872)	2,968 (1,512–6,092)	3,071 (1,595–5,104)	2,620 (1,540–5,063)	2,450 (1,071–4,781)
CD4 T cells	1,060 (564–2,542)	1,430 (340–2,436)	1,335 (612–2,701)	1,806 (1,025–3,498)	1,980 (912–4,725)	2,240 (1,056–3,900)	1,923 (943–3,650)	1,620 (638–3,650)
CD8 T cells	548 (255–1,131)	650 (152–1,110)	667 (312–1,389)	820 (400–1,792)	800 (352–1,632)	836 (416–1,736)	667 (336–1,482)	735 (290–1,590)
CD19 B cells	322(82–930)	426 (44–2,000)	729 (114–3,178)	677 (132–1,530)	656 (120–1,512)	755 (232–2,240)	893 (224–2,594)	624 (96–2,008)
CD16-CD56 NK cells	195 (44–470)	192(40–900)	254 (84–624)	408 (131–1,271)	315 (98–1,330)	222(76–750)	334 (138–1,204)	278 (66–1,066)
CD4-CD45RA T cells	770 (504–2,100)	1041 (266–2,068)	1,006 (378–2,279)	1,386 (536–2,989)	1,677 (571–4,169)	1,756 (836–3,225)	1,534 (792–3,411)	1,253 (396–3,111)
CD8-CD45RA T cells	453 (217–990)	570 (148–954)	620 (264–1,297)	750 (345–1,679)	729 (313–1,476)	767 (368–1,629)	593 (282 –1,289)	663 (264–1,421)
CD4-CD45RO T cells	183 (68–830)	199 (35–520)	194 (76–460)	252 (110–546)	277 (99–945)	260 (120–664)	214 (81–420)	227 (78–638)
CD8-CD45RO T cells	28(0–119)	23 (0–119)	40 (0–108)	42 (0–261)	44 (0–140)	48 (0–140)	40 (0–98)	38 (0–127)
Infants per group	31	53	58	51	75	44	26	338
B. Percentages, median (5%-95% interval), for subsets of peripheral blood lymphocytes.								
EGA (weeks)	**22–28**	**29–31**	**32–36**	**37–39**	**40–41**	**42–43**	**44–55**	**Total**
CD3 as % of ALC *	73% (51–85%)	70% (45–87%)	64% (36–87%)	68% (42–86%)	69% (45–88%)	69% (46–84%)	61% (40–83%)	69% (43–87%)
CD4 as % of ALC	46% (33–62%)	46% (28–65%)	40% (24–60%)	45% (25–61%)	47% (28–70%)	49% (28–66%)	42% (27–64%)	46% (27–65%)
CD8 as % of ALC	23% (12–39%)	21% (13–33%)	19% (10–36%)	20% (12–32%)	19% (11–31%)	19% (9–28%)	16% (10–26%)	20% (11–34%)
CD19 as % of ALC	13% (6–34%)	14% (4–40%)	22% (4–50%)	16% (3–34%)	16% (2–31%)	18% (5–39%)	20% (6%-40%)	17% (4–39%)
CD16-CD56 as% of ALC	6% (2–19%)	7% (2–20%)	7% (3–21%)	10% (3–33%)	7% (3–26%)	5% (2–13%)	9% (3–22%)	7% (2–24%)
CD4-CD45RA as % of total CD4	79% (50–95%)	75% (61–93%)	82% (52–91%)	80% (42–94%)	84% (51–97%)	83% (61–92%)	84% (67–93%)	81% (53–94%)
CD4-CD45RA as % of ALC	35% (21–83%)	36% (19–59%)	31% (16–56%)	35% (16–53%)	40% (18–62%)	39% (21–58%)	35% (21–54%)	36% (18–59%)

CD8-CD45RA as % of total CD8 **	93% (76–100%)	93% (80–100%)	94% (70–100%)	91% (72–100%)	92% (72–100%)	92% (83–100%)	92% (78–100%)	93% (75–100%)
CD8-CD45RA as % of ALC	23% (10–37%)	19% (12–31%)	18% (8–34%)	17% (11–31%)	17% (10–30%)	17% (8%-25%)	14% (7–26%)	18% (10–32%)
CD4-CD45RO as % of CD4	18% (7–36%)	17% (4–35%)	14% (5–30%)	14% (6–31%)	14% (5–31%)	12% (6–25%)	12% (4–43%)	14% (5–32%)
CD4-CD45RO as % of ALC	8% (3–23%)	8% (2–17%)	5% (2–13%)	7% (3–21%)	6% (3–21%)	6% (2–15%)	5% (2–13%)	6% (2–16%)
CD8-CD45RO as % of CD8	4% (0–13%)	3% (0–12%)	4% (0–17%)	4% (0–33%)	5% (0–17%)	5% (0–20%)	5% (0–21%)	4% (0–20%)
CD8-CD45RO as % of ALC	1% (0–4%)	1% (0–3%)	1% (0–3%)	1% (0–9%)	1% (0–3%)	1% (0–3%)	1% (0–2%)	1%(0–3%)
Infants per group	31	53	58	51	75	44	26	338

* Cell counts are shown as median (5–95% confidence interval) cells/pl in peripheral blood for each EGA group.

* Cell percentages are median (5% - 95% confidence interval) cells/ml in peripheral blood for each EGA group. ALC, absolute lymphocyte count.

** Intervals have been truncated at 100%, although percentages occasionally exceeded 100% due to flow cytometry technology.

Table 4.

Lymphocyte counts in cells/ml at increasing birthweights (BW) in children without immune disorders.

A. Absolute counts, median (5%-95% interval), for subsets of peripheral blood lymphocytes.								
Birtdweight (grams)	**0 – 550**	**551 – 800**	**801–1250**	**1251–2500**	**2501–3000**	**3001–3500**	**>3500**	**Total**
White cell count x 10^3 *	11 (6–19)	10 (5–17)	10 (6–16)	9 (6–16)	12 (6–23)	9 (5–23)	10 (7–23)	10 (6–20)
Lymphocyte count	4,069 (2,550–7,400)	4,650 (2,800–8,600)	4,600 (2,700–7,300)	4,365 (2,600–6,600)	3,100 (1,500–6,400)	3,700 (2,000–6,496)	2,950 (1,150–5,128)	4,000 (2,000–7,100)
CD3 T cells	2,718 (1,577–5,143)	3,358 (1,612–6,527)	3,213 (1,512–4,752)	2,988 (1,650–4,712)	2,250 (957–3,611)	2,226 (1,484–3,384)	1,914 (885–3,322)	2,541 (1,220–4,872)
CD4 T cells	2,003 (1,040–4,064)	2,283 (992–4,817)	1,932 (928–3,658)	2,072 (1,116–3,705)	1,406 (513–2,430)	1,689 (1,036–2,478)	1,176 (450–2,209)	1,699 (761–3,658)
CD8 T cells	813 (414–1,633)	956 (440–1,792)	774 (336–1,736)	791 (414–1,429)	702 (255–1,375)	669 (420–1,360)	661 (261–1,323)	759 (319–1,596)
CD19 B cells	516 (138–1,908)	644 (192–3,010)	684 (126–1,748)	664 (140–1,512)	608 (96–2,376)	774 (210–2,200)	378 (77–1,807)	615 (108–2,016)
CD16-CD56 NK cells	312(83–903)	306 (92–1,292)	350 (102–1,272)	286 (117–1,066)	217 (45–581)	200 (52–646)	227 (47–928)	279 (72–1,015)
CD4-CD45RA T cells	1583 (687–3,421)	1761 (836–4,196)	1594 (608–3,225)	1752 (571–3,150)	1029 (353–2,062)	1220 (778–2,100)	919 (309–1,578)	1321 (525–3,150)
CD8-CD45RA T cells	787 (365–1,538)	834 (410–1,738)	707 (300–1,629)	700 (275–1,326)	585 (212–1,037)	611 (291–1,178)	600 (247–1,250)	685 (276–1,424)
CD4-CD45RO T cells	255 (131–528)	248 (100–1,806)	252 (97–657)	303 (121–945)	187 (80–578)	219 (68–460)	212 (58–558)	241 (83–638)
CD8-CD45RO T cells	49 (0–201)	45(0–162)	41 (0–140)	48 (0–98)	30 (0–136)	33 (0–178)	26 (0–75)	39 (0–127)
Infants per group	40	36	61	48	49	27	40	301
B. Percentages, median (5%-95% interval), for subsets of peripheral blood lymphocytes.								
Birthweight (grams)	**0 – 550**	**551 – 800**	**801 –1250**	**1251–2500**	**2501–3000**	**3001–3500**	**3500+**	**Total**
CD3 as % of ALC *	71% (48–88%)	70% (46–87%)	67% (41–88%)	72% (46–84%)	69% (43–84%)	69% (44–84%)	69% (45–87%)	70% (45–87%)
CD4 as % of ALC	52% (31–64%)	47% (30–72%)	45% (25 –69%)	51% (29–65%)	40% (27–64%)	46% (2–59%)	43% (25–63%)	46% (27–65%)
CD8 as % of ALC	20% (12–34%)	20% (11–31%)	18% (11–30%)	19% (11–30%)	20% (10–36%)	18% (12–32%)	25% (15–35%)	20% (11–34%)
CD19 as % of ALC	12% (3–33%)	14% (2–35%)	17% (4–37%)	16% (4–29%)	19% (4–49%)	20% (10–42%)	14% (3–40%)	16% (4–38%)
CD16-CD56 as% of ALC	7% (2–25%)	6% (2–30%)	8% (2–33%)	7% (3–22%)	6% (2–16%)	5% (2–19%)	8% 3–22%)	7% (2–24%)
CD4-CD45RA as % of total CD4	83% (60–93%)	87% (55–97%)	81% (61–93%)	84% (37–95%)	77% (54–92%)	82% (60–92%)	76% (51–91%)	82% (53–94%)
CD4-CD45RA as % of ALC	40% (21–54%)	39% (28–63%)	36% (16–58%)	41% (18–59%)	31% (19–62%)	35% (17–58%)	33% (16–56%)	37% (18–59%)

CD8-CD45RA as % of total CD8 **	91% (75–100%)	92% (75–100%)	92% (75–100%)	93% (75–100%)	92% (76–100%)	93% (29–100%)	94% (72–100%)	92% (75–100%)
CD8-CD45RA as % of ALC	17% (11–32%)	19% (10–30%)	16% (10–27%)	17% (9–26%)	19% (9–34%)	17% (10–31%)	21% (14–35%)	18% (10–31%)
CD4-CD45RO as % of CD4	14% (6–27%)	13% (5–32%)	13% (5–27%)	14% (4–31%)	16% (7–35%)	17%(4–26%)	18% (4–38%)	14% (5–32%)
CD4-CD45RO as % of ALC	6% (3–13%)	6% (2–23%)	6% (2–12%)	7% (2–24%)	6% (3–14%)	6% (2–14%)	8% (2–16%)	6% (2–15%)
CD8-CD45RO as % of CD8	5% (0–32%)	4% (0–24%)	5% (0–21%)	5%(0–15%)	4% (0–22%)	4%(0–40%)	4% (0–12%)	5% (0–20%)
CD8-CD45RO as % of ALC	1% (0–5%)	1% (0–3%)	1% (0–5%)	1% (0–2%)	1% (0–4%)	1% (0–6%)	1% (0–3%)	1% (0–4%)
Infants per group	40	36	61	48	49	27	40	301

* Cell counts are median (5% - 95% confidence interval) cells/pl in peripheral blood for each BW group.

* Median (5% - 95% confidence interval) percentages as designated of lymphocytes in peripheral blood for each BW group.

** Intervals have been truncated at 100%, although percentages occasionally exceeded 100% due to flow cytometry technology.

Table 5.

ANOVA models for effective gestational age (EGA) and birthweight (BW) cohorts.

A. EGA Cohort, n=338			
CD3 ANOVA parameters			
Category	**Parameter**	**Estimate**	**Standard Error**
Intercept	**Intercept**	2876.72	265.35
Sex	**Female**	−67.56	131.17
	Male	0	
EGA (weeks)	**22–28**	−857.40	300.38
(p <0.001)	**29–31**	−729.53	270.20
	32–36	−501.06	266.18
	37–41	82.00	271.60
	40–41	358.71	257.20
	42–43	410.35	280.96
	44–55	0	
Race/ethnicity§	**Asian**	209.33	199.86
	Black	−75.06	271.64
	Hispanic	−185.89	171.80
	Other	204.27	268.15
	White	0	
B. BW cohort, n-301			
CD3 ANOVA parameters			
Category	**Parameter**	**Estimate**	**Standard Error**
Intercept	**Intercept**	3125.47	204.29
Sex	**Female**	−66.45	139.11
	Male	0	
Birthweight *	**<550**	−1124.92	240.38
(grams) (p	**551–800**	−893.54	228.31
<0.001)*	**801–1250**	−728.51	271.97
	1251–2500	−70.63	241.96
	2501–3000	322.77	248.82
	3001–3500	−80.04	216.69
	>3500	0	
Race/ethnicity§	**Asian**	347.53	207.84
	Black	−127.53	275.37
	Hispanic	−60.55	176.11
	Other	269.96	270.52
	White	0	

* Category-level significant p-value shown if p <0.05, based on type-III sum of squares in the multivariate model.

[§]Multiple race/ethnicity categorized as single race in a hierarchy as follows: Hispanic, black, Asian, followed by white; Native Americans were included in the “Other” category, as were those with missing or unknown race or ethnicity.

EGA, effective gestational age at time of test; BW, birthweight.