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

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Background: In 6.5 years of newborn screening for severe combined immunodeficiency in California, 3,252,156 infants had DNA from dried blood spots (DBSs) assayed for T-cell receptor excision circles. Infants with T-cell receptor excision circle values of less than a designated cutoff on a single DBS, 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 sought to determine reference intervals for lymphocyte subsets in racially/ethnically diverse preterm and term newborns who proved to be unaffected by any T-lymphopenic immune disorder.

Methods: Effective gestational age (GA) was defined as GA at birth plus postnatal age at the time of sample collection. After determining exclusion criteria, we analyzed demographic and clinical information, complete and differential white blood cell counts, and lymphocyte subsets for 301 infants, with serial measurements for 33 infants. Lymphocyte subset measurements included total T cells, helper and cytotoxic T-cell subsets, naive and memory phenotype of each T-cell subset, B cells, and natural killer cells.

Results: Reference intervals were generated for absolute numbers and lymphocyte subsets from infants with effective GAs of 22 to 52 weeks. Sex and ethnicity were not significant determinants of lymphocyte subset counts in this population. Lymphocyte counts increased 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. (J Allergy Clin Immunol 2019;144:1674-83.)

Key words: *Flow cytometry, memory T cell, naive T cell, neonatal immunity, newborn screening, preterm birth, reference range/reference interval, severe combined immunodeficiency, T-cell receptor excision circle, T-cell subsets*

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 subjects tested, insufficient numbers to permit separation of age groups of infants less than 3 months of age, and omission of preterm or low birth weight (BW) infants. Some, but not all, reports have found that lymphocyte subset intervals varied between infants of different ethnic makeups, sexes, environmental exposures, and geographic areas, 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 United States[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 (DBSs).[7,8] Insufficient or absent TRECs on screening are correlated with subsequent measurements showing low circulating T-cell numbers and few recent thymic emigrant T cells bearing naive markers, such as CD45RA.[9-14] However, direct measurement of lymphocyte subsets with quantitation of naive 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 TREC values of less than a designated cutoff on a single DBS with adequate PCR control, 2 DBS samples with insufficient PCR

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Abbreviations used
BW: Birth weight
DBS: Dried blood spot
EGA: Effective gestational age
GA: Gestational age
GDSP: Genetic Disease Screening Program
NBS: Newborn screening
NICU: Neonatal intensive care unit
NK: Natural killer
SCID: Severe combined immunodeficiency
TCL: T-cell lymphopenia
TREC: T-cell receptor excision circle

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, although 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 proved to have no diagnosed immune system defects or medical conditions, whereas 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 DBS specimens were collected as part of NBS 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. DBSs were analyzed for TREC counts in the Genetic Disease Laboratory of the Genetic Disease Screening Program (GDSP) within the California Department of Public Health in Richmond, California, before June 2015, after which the EnLite kit (PerkinElmer, Waltham, Mass) was adopted, allowing for TREC tests 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 institutional review board exemption (California Committee for the Protection of Human Subjects) included BW, sex, gestational age (GA) 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 1 box was checked as follows: Hispanic, black, and Asian, followed by white. Native Americans were always included in our "other" category, if checked, as were those with missing and unknown race/ethnicity.[15]

Flow cytometry

Per NBS protocol, a peripheral blood sample was obtained from infants (n = 562) identified by the GDSP to have TREC results of less than the threshold for follow-up testing within the NBS program or to be at risk for a genetic immune deficiency based on family history. Blood was shipped by courier to the Quest Diagnostics Nichols Institute (San Juan Capistrano, California). For each specimen, an automated complete and differential WBC count (Coulter STKS/LH750 hematology analyzer; Coulter Technology Center, Miami, Fla) was performed. A blood smear was reviewed for blasts or other abnormal cell morphology. Lymphocyte subset analysis was performed by using flow cytometry, with a sample from a healthy control subject included with each batch of patient samples. See the Methods section and Table E1 in this article's Online Repository at www.jacionline.org for details.

TABLE I. Eighty-five infants excluded from the reference interval cohort

Category (no. of infants)	Infants with each diagnosis
Recognized syndromes and associations* (n = 21)	DiGeorge syndrome: n = 12
	Trisomy 21: n = 5
	Cornelia de Lange syndrome, Kabuki syndrome, Noonan syndrome, and vertebral, anorectal, tracheal, esophageal, renal association: n = 1 each
Severe congenital abnormalities* (n = 33)	Congenital heart defect, isolated: n = 11
	Congenital heart defect with additional anomalies†: n = 4
	Other major birth defects (ambiguous genitalia, brain malformation, diaphragmatic hernia, gastroschisis, Hirschprung disease, intestinal atresia/obstruction, liver malformation, pyriform aperture stenosis, skeletal deformity): n = 14
	Endocrinopathy (thyroid, parathyroid): n = 2
	Infection (chorioamnionitis, cytomegalovirus): n = 2
Fetal conditions with fluid accumulation* (n = 8)	Hydrops, anasarca, ascites: n = 6
	Twin-to-twin transfusion syndrome: n = 2
Other circumstances (n = 23)	Died in hospital: n = 10
	Abnormal leukocyte profile‡: n = 7 (2 severe congenital neutropenia, 2 with low or no B cells, and 1 each with absent NK cells, absent CD8 cells, fluctuating B- and T-cell numbers)
	Missing data: n = 2 (1 no flow cytometry; 1 no date of birth)
	Out-of-state birth: n = 2
	Nonnewborn (7-year-old immigrant): n = 1
	Methamphetamine withdrawal: n = 1

*Infants with these conditions, which can be associated with TCL, had T-cell counts of greater than 1500 cells/μL and thus were not referred by the California GDSP to immunology centers for further SCID evaluation.
†Nonsyndromic cases.
‡Although total CD3 T-cell counts were greater than 1500 cells/μL, leukocyte values that were extreme outliers caused these infants to be excluded.

Statistical methods

Newborn results in this study were not randomly sampled but rather were obtained from healthy babies with transient nonnormal TREC NBS results or recognized genetic risk, such as having a sibling affected with a T-lymphopenic disorder. Univariate and multivariate analyses were conducted

TABLE II. Demographics of the cohorts of infants analyzed

	BW cohort		Mean BW (g)	EGA cohort		Mean EGA (wk)
Categories	No. of infants	Percentage within group		No. of infants	Percentage within group	
Totals	301	100.0%	2140	338	100.0%	37
Race/ethnicity*†						
Asian	58	19.3%	2187	69	20.4%	37
Black	23	7.6%	2065	24	7.1%	36
Hispanic	135	44.9%	1946	157	46.4%	36
Other	24	8.0%	2217	25	7.4%	37
White	61	20.3%	2522	63	18.6%	38
Nursery type*						
NICU	151	50.2%	1127	180	53.3%	33
Regular	150	49.8%	3160	158	46.7%	41
Sex						
Female	100	32.1%	2212	112	33.1%	37
Male	201	67.9%	2104	226	66.9%	37
EGA (wk)†						
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%	2533	51	15.1%	
40-41	74	24.6%	3046	75	22.2%	
42-43	40	13.3%	3218	44	13.0%	
44-52	24	8.0%	3291	26	7.7%	
BW (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

For the BW cohort (sampled once closest to birth date), GA and EGA were the same.

*$P \leq .001$ based on an F test for each category (differences within other categories were not significant).

†Multiple race/ethnicity was categorized as single race in a hierarchy as follows: Hispanic, black, and Asian, followed by white. Native Americans were included in the "other" category, as were those with missing and unknown race/ethnicity.

with ANOVA in SAS software (version 9.4; SAS Institute, 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 by using a binomial distribution. Graphics were created with SAS and MS Excel (Microsoft, Redmond, Wash).

RESULTS

Study cohort

Inclusion and exclusion criteria were established before examining the data. Infants born at any GA and weight were included, regardless of their likelihood to be under greater physical stress compared with 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 I).[14]

Additionally, Table I shows excluded infants who did not fit the general profile of a reasonably healthy newborn from the provided clinical information. Reasons for omission from the study cohort included an identified clinical syndrome that has been reported to cause immunodeficiency (n = 21),[16-18] 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 before discharge from the hospital (n = 10), immune abnormalities without overall TCL (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 GA 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 of greater than 2500 g, the GA was imputed to be 40 weeks. If a baby was indicated to be "term" and greater than 2500 g in BW, we imputed the nursery to be a regular nursery if not otherwise recorded. If a baby was born at a GA of 32 weeks or less or had a BW of less than or equal to 1500 g, we imputed the nursery to be a NICU if not otherwise recorded. GA imputation was required for 2 infants and nursery type for an additional 2 infants, both imputed to be in regular nurseries.

GA and BW cohorts

Analytic 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 EGA, which was defined as GA at birth plus

TABLE III. Lymphocyte counts in cells per microliter at increasing EGA in children without immune disorders

EGA (wk)	22-28	29-31	32-36	37-39	40-41	42-43	44-55	Total
Absolute counts and medians (5% to 95% CI) for peripheral blood lymphocytes and lymphocyte subsets								
White cell count ($\times 10^3$)*	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	2500 (1200-4200)	3219 (800-5800)	3450 (1800-6486)	4400 (2700-6400)	4500 (2600-7770)	4500 (2800-7500)	4750 (2300-7600)	3900 (1700-7000)
CD3 T cells	1798 (900-3608)	2158 (528-3828)	2048 (1250-4080)	2886 (1512-4872)	2968 (1512-6092)	3071 (1595-5104)	2620 (1540-5063)	2450 (1071-4781)
CD4 T cells	1060 (564-2542)	1430 (340-2436)	1335 (612-2701)	1806 (1025-3498)	1980 (912-4725)	2240 (1056-3900)	1923 (943-3650)	1620 (638-3650)
CD8 T cells	548 (255-1131)	650 (152-1110)	667 (312-1389)	820 (400-1792)	800 (352-1632)	836 (416-1736)	667 (336-1482)	735 (290-1590)
CD19 B cells	322 (82-930)	426 (44-2000)	729 (114-3178)	677 (132-1530)	656 (120-1512)	755 (232-2240)	893 (224-2594)	624 (96-2008)
CD16-CD56 NK cells	195 (44-470)	192 (40-900)	254 (84-624)	408 (131-1271)	315 (98-1330)	222 (76-750)	334 (138-1204)	278 (66-1066)
CD4-CD45RA T cells	770 (504-2100)	1041 (266-2068)	1006 (378-2279)	1386 (536-2989)	1677 (571-4169)	1756 (836-3225)	1534 (792-3411)	1253 (396-3111)
CD8-CD45RA T cells	453 (217-990)	570 (148-954)	620 (264-1297)	750 (345-1679)	729 (313-1476)	767 (368-1629)	593 (282-1289)	663 (264-1421)
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
Percentages, median (5%-95% interval), for subsets of peripheral blood lymphocytes								
CD3 as % of ALC†	73% (51% to 85%)	70% (45% to 87%)	64% (36% to 87%)	68% (42% to 86%)	69% (45% to 88%)	69% (46% to 84%)	61% (40% to 83%)	69% (43% to 87%)
CD4 as % of ALC	46% (33% to 62%)	46% (28% to 65%)	40% (24% to 60%)	45% (25% to 61%)	47% (28% to 70%)	49% (28% to 66%)	42% (27% to 64%)	46% (27% to 65%)
CD8 as % of ALC	23% (12% to 39%)	21% (13% to 33%)	19% (10% to 36%)	20% (12% to 32%)	19% (11% to 31%)	19% (9% to 28%)	16% (10% to 26%)	20% (11% to 34%)
CD19 as % of ALC	13% (6% to 34%)	14% (4% to 40%)	22% (4% to 50%)	16% (3% to 34%)	16% (2% to 31%)	18% (5% to 39%)	20% (6% to 40%)	17% (4% to 39%)
CD16-CD56 as % of ALC	6% (2% to 19%)	7% (2% to 20%)	7% (3% to 21%)	10% (3% to 33%)	7% (3% to 26%)	5% (2% to 13%)	9% (3% to 22%)	7% (2% to 24%)
CD4-CD45RA as % of total CD4	79% (50% to 95%)	75% (61% to 93%)	82% (52% to 91%)	80% (42% to 94%)	84% (51% to 97%)	83% (61% to 92%)	84% (67% to 93%)	81% (53% to 94%)
CD4-CD45RA as % of ALC	35% (21% to 83%)	36% (19% to 59%)	31% (16% to 56%)	35% (16% to 53%)	40% (18% to 62%)	39% (21% to 58%)	35% (21% to 54%)	36% (18% to 59%)
CD8-CD45RA as % of total CD8‡	93% (76% to 100%)	93% (80% to 100%)	94% (70% to 100%)	91% (72% to 100%)	92% (72% to 100%)	92% (83% to 100%)	92% (78% to 100%)	93% (75% to 100%)
CD8-CD45RA as % of ALC	23% (10% to 37%)	19% (12% to 31%)	18% (8% to 34%)	17% (11% to 31%)	17% (10% to 30%)	17% (8% to 25%)	14% (7% to 26%)	18% (10% to 32%)
CD4-CD45RO as % of CD4	18% (7% to 36%)	17% (4% to 35%)	14% (5% to 30%)	14% (6% to 31%)	14% (5% to 31%)	12% (6% to 25%)	12% (4% to 43%)	14% (5% to 32%)
CD4-CD45RO as % of ALC	8% (3% to 23%)	8% (2% to 17%)	5% (2%to 13%)	7% (3% to 21%)	6% (3% to 21%)	6% (2% to 15%)	5% (2% to 13%)	6% (2% to 16%)
CD8-CD45RO as % of CD8	4% (0% to 13%)	3% (0% to 12%)	4% (0% to 17%)	4% (0% to 33%)	5% (0% to 17%)	5% (0% to 20%)	5% (0% to 21%)	4% (0% to 20%)
CD8-CD45RO as % of ALC	1% (0% to 4%)	1% (0% to 3%)	1% (0% to 3%)	1% (0% to 9%)	1% (0%to 3%)	1% (0% to 3%)	1% (0% to 2%)	1% (0% to 3%)
Infants per group	31	53	58	51	75	44	26	338

ALC, Absolute lymphocyte count.

*Cell counts are shown as medians (5% to 95% CIs) in cells per microliter in peripheral blood for each EGA group.

†Cell percentages are medians (5% to 95% CIs) in cells per microliter in peripheral blood for each EGA group.

‡Intervals have been truncated at 100%, although percentages occasionally exceeded 100% because of flow cytometric technology.

TABLE IV. Lymphocyte counts in cells per microliter at increasing BW in children without immune disorders

BW (g)	0-550	551-800	801-1250	1251-2500	2501-3000	3001-3500	≥3500	Total
Absolute counts, medians (5% to 95% CIs), for subsets of peripheral blood lymphocytes								
White cell count (× 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	4069 (2550-7400)	4650 (2800-8600)	4600 (2700-7300)	4365 (2600-6600)	3100 (1500-6400)	3700 (2000-6496)	2950 (1150-5128)	4000 (2000-7100)
CD3 T cells	2718 (1577-5143)	3358 (1612-6527)	3213 (1512-4752)	2988 (1650-4712)	2250 (957-3611)	2226 (1484-3384)	1914 (885-3322)	2541 (1220-4872)
CD4 T cells	2003 (1040-4064)	2283 (992-4817)	1932 (928-3658)	2072 (1116-3705)	1406 (513-2430)	1689 (1036-2478)	1176 (450-2209)	1699 (761-3658)
CD8 T cells	813 (414-1633)	956 (440-1792)	774 (336-1736)	791 (414-1429)	702 (255-1375)	669 (420-1360)	661 (261-1323)	759 (319-1596)
CD19 B cells	516 (138-1908)	644 (192-3010)	684 (126-1748)	664 (140-1512)	608 (96-2376)	774 (210-2200)	378 (77-1807)	615 (108-2016)
CD16-CD56 NK cells	312 (83-903)	306 (92-1292)	350 (102-1272)	286 (117-1066)	217 (45-581)	200 (52-646)	227 (47-928)	279 (72-1015)
CD4-CD45RA T cells	1583 (687-3421)	1761 (836-4196)	1594 (608-3225)	1752 (571-3150)	1029 (353-2062)	1220 (778-2100)	919 (309-1578)	1321 (525-3150)
CD8-CD45RA T cells	787 (365-1538)	834 (410-1738)	707 (300-1629)	700 (275-1326)	585 (212-1037)	611 (291-1178)	600 (247-1250)	685 (276-1424)
CD4-CD45RO T cells	255 (131-528)	248 (100-1806)	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
Percentages, medians (5% to 95% CIs), for subsets of peripheral blood lymphocytes								
CD3 as % of ALC†	71% (48% to 88%)	70% (46% to 87%)	67% (41% to 88%)	72% (46% to 84%)	69% (43% to 84%)	69% (44% to 84%)	69% (45% to 87%)	70% (45% to 87%)
CD4 as % of ALC	52% (31% to 64%)	47% (30% to 72%)	45% (25% to 69%)	51% (29% to 65%)	40% (27% to 64%)	46% (2% to 59%)	43% (25% to 63%)	46% (27% to 65%)
CD8 as % of ALC	20% (12% to 34%)	20% (11% to 31%)	18% (11% to 30%)	19% (11% to 30%)	20% (10% to 36%)	18% (12% to 32%)	25% (15% to 35%)	20% (11% to 34%)
CD19 as % of ALC	12% (3% to 33%)	14% (2% to 35%)	17% (4% to 37%)	16% (4% to 29%)	19% (4% to 49%)	20% (10% to 42%)	14% (3% to 40%)	16% (4% to 38%)
CD16-CD56 as % of ALC	7% (2% to 25%)	6% (2% to 30%)	8% (2% to 33%)	7% (3% to 22%)	6% (2% to 16%)	5% (2% to 19%)	8% (3% to 22%)	7% (2% to 24%)
CD4-CD45RA as % of total CD4	83% (60% to 93%)	87% (55% to 97%)	81% (61% to 93%)	84% (37% to 95%)	77% (54% to 92%)	82% (60% to 92%)	76% (51% to 91%)	82% (53% to 94%)
CD4-CD45RA as % of ALC	40% (21% to 54%)	39% (28% to 63%)	36% (16% to 58%)	41% (18% to 59%)	31% (19% to 62%)	35% (17% to 58%)	33% (16% to 56%)	37% (18% to 59%)
CD8-CD45RA as % of total CD8‡	91% (75% to 100%)	92% (75% to 100%)	92% (75% to 100%)	93% (75% to 100%)	92% (76% to 100%)	93% (29% to 100%)	94% (72% to 100%)	92% (75% to 100%)
CD8-CD45RA as % of ALC	17% (11% to 32%)	19% (10% to 30%)	16% (10% to 27%)	17% (9% to 26%)	19% (9% to 34%)	17% (10% to 31%)	21% (14% to 35%)	18% (10% to 31%)
CD4-CD45RO as % of CD4	14% (6% to 27%)	13% (5% to 32%)	13% (5% to 27%)	14% (4% to 31%)	16% (7% to 35%)	17% (4% to 26%)	18% (4% to 38%)	14% (5% to 32%)
CD4-CD45RO as % of ALC	6% (3% to 13%)	6% (2% to 23%)	6% (2% to 12%)	7% (2% to 24%)	6% (3% to 14%)	6% (2% to 14%)	8% (2% to 16%)	6% (2% to 15%)
CD8-CD45RO as % of CD8	5% (0% to 32%)	4% (0% to 24%)	5% (0% to 21%)	5% (0% to 15%)	4% (0% to 22%)	4% (0% to 40%)	4% (0% to 12%)	5% (0% to 20%)
CD8-CD45RO as % of ALC	1% (0% to 5%)	1% (0% to 3%)	1% (0% to 5%)	1% (0% to 2%)	1% (0% to 4%)	1% (0% to 6%)	1% (0% to 3%)	1% (0% to 4%)
Infants per group	40	36	61	48	49	27	40	301

ALC, Absolute lymphocyte count.
*Cell counts are medians (5% to 95% CIs) in cells per microliter in peripheral blood for each BW group.
†Medians (5% to 95% CIs) as designated of lymphocytes in peripheral blood for each BW group.
‡Intervals have been truncated at 100%, although percentages occasionally exceeded 100% because of flow cytometric technology.

postnatal age at time of the flow test, as follows: 22 to 28 weeks (n = 31), 29 to 31 weeks (n = 53), 32 to 36 weeks (n = 58), 37 to 39 weeks (n = 51), 40 to 41 weeks (n = 75), 42 to 43 weeks (n = 44), and 44 to 52 weeks (n = 26). There were 268 (89%) infants with 1 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 cohort, the BW cohort (n = 301), was created as a subset of the EGA cohort, retaining only the flow cytometric determination conducted closest to the time of birth.

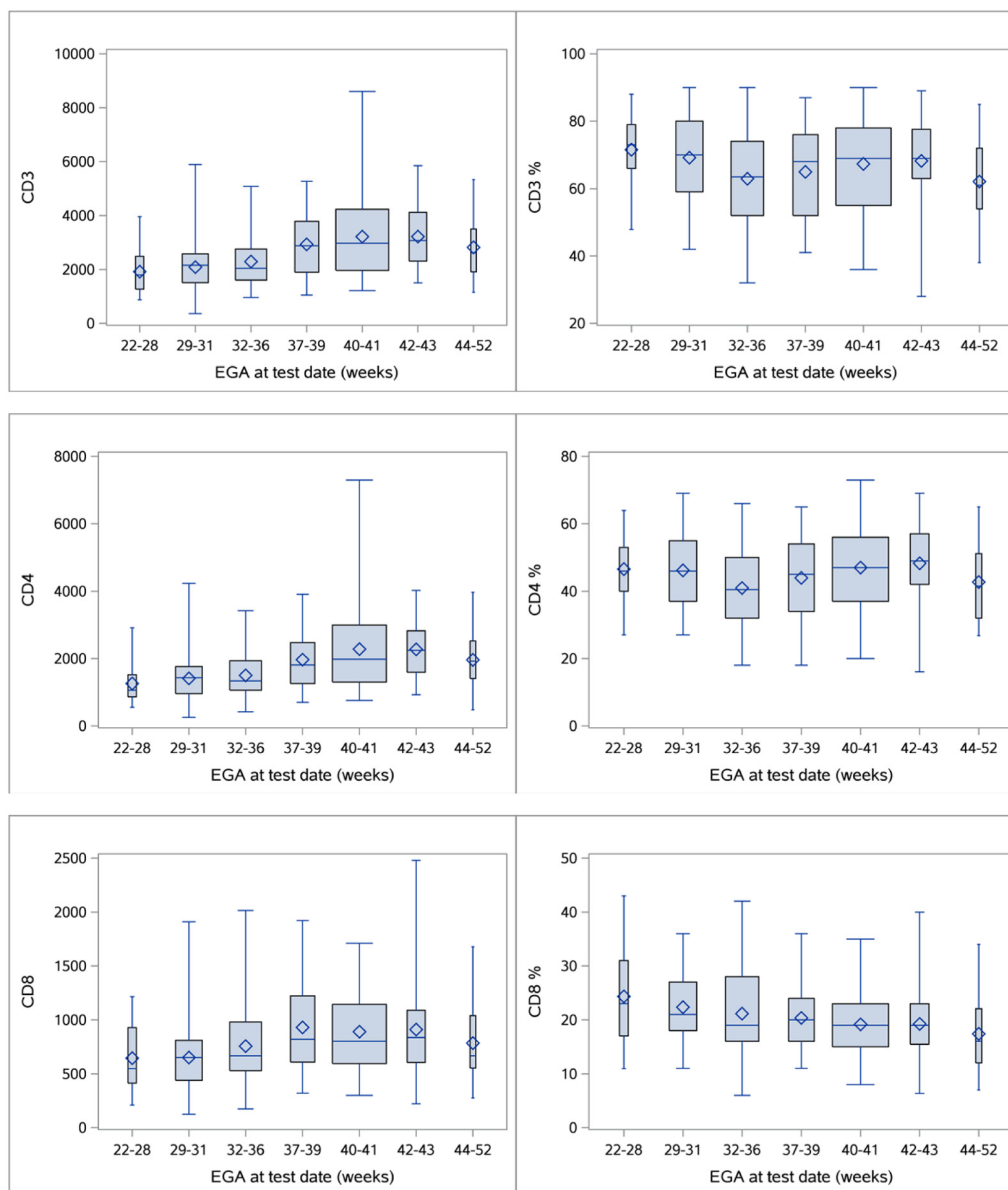

FIG 1. Absolute counts *(left panels)* and percentages *(right panels)* of each T-cell subset at increasing EGA groupings. *CD3*, CD3 T cells per microliter of peripheral blood; *CD3 %*, CD3/absolute lymphocyte count *(ALC)* × 100%; *CD4*, CD4 T cells per microliter; *CD4 %*, CD4/ALC × 100%; *CD8*, CD8 T cells per microliter; and *CD8 %*, CD8/ALC × 100%. *Shaded boxes* encompass the 25th to 75th percentiles, with the width of each proportional to the number of measurements included. *Horizontal line within each box*, Median; *diamond within each box*, mean. *Whisker extensions* show lowest and highest values.

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 II). This observation is indicative of the higher likelihood of false-positive TREC screen results among infants in the NICU, including those born prematurely.[14] Twice as many male (n = 201) as female (n = 100) newborns were included in the eligible cohort. Male subjects were more likely to be treated in a NICU and to be of earlier GA and lower BW than female subjects in this cohort. As shown in Table II, univariate analysis of mean EGA and BW by NICU showed statistically significant differences, with earlier mean EGA (NICU, 33 weeks; regular nursery, 44 weeks; $P < .001$) and lower mean BW (NICU, 1127 g; regular nursery, 3160 g; $P < .001$). There were no significant differences for mean EGA and BW by race/ethnicity or sex.

Lymphocyte subset distribution versus EGA and BW

Table III shows lymphocyte subset counts and percentages by EGA, and Table IV shows lymphocyte subset counts and percentages by BW. Although the absolute numbers of lymphocytes tended to increase with EGA and BW, the percentages of a given

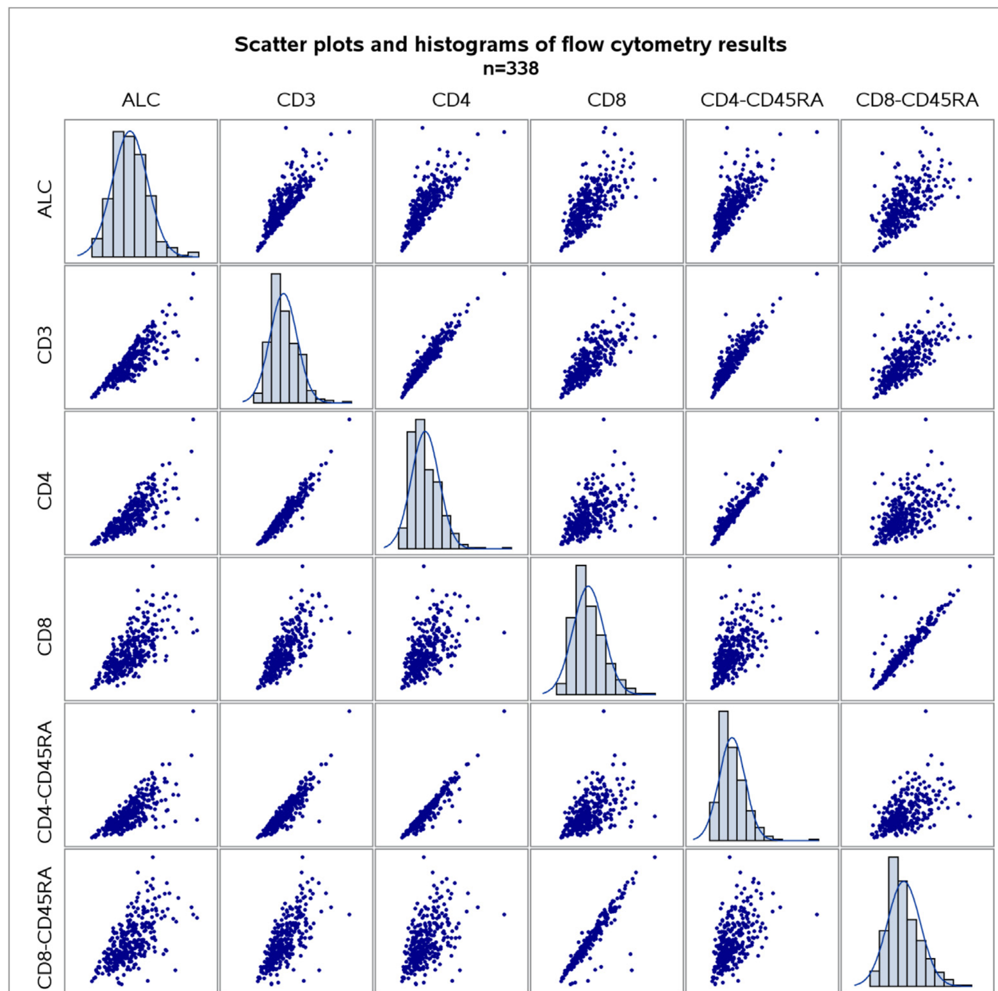

FIG 2. Scatter plots and distributions in deciles of flow cytometric results. *ALC*, Absolute lymphocyte count (cells per microliter); *CD3*, CD3 T cells per microliter; *CD4*, CD4 T cells per microliter; *CD4-CD45RA*, CD4 T cells per microliter that also express CD45RA; *CD8*, CD8 T cells per microliter; and *CD8-CD45RA*, CD8 T cells per microliter that also express CD45RA.

subset were essentially constant across the tracked EGA and BW groups, as shown in Fig 1 for total T cells and helper and cytotoxic T-cell subsets and in Figs E1 to E4 in this article's Online Repository at www.jacionline.org, where data are also shown for B and natural killer (NK) cells.

Scatter plots and distributions of flow cytometric 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), naive helper T cells (CD4-CD45RA), and naive cytotoxic T cells (CD8-CD45RA, Fig 2). Numbers of cells of each subset, also represented by bar graphs in Fig 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 (see Fig E5 in this article's Online Repository at www.jacionline.org, in which diagonal clustering is not apparent). Thus although numbers of T cells and naive 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 the time of testing for each of the lymphocyte subsets (CD3, CD4, CD8, CD19, and CD16-CD56; Table V and see Table E2, *A*, in this article's Online Repository at www.jacionline.org). Similarly, these subsets were analyzed for relationships between sex, race/ethnicity, and BW (Table V and see Table E2, *B*). NICU status was omitted from the final models because of collinearity with both BW and EGA. In each analysis sex and race/ethnicity class variables were nonsignificant. However, EGA and BW were statistically significant for T cells, as shown in Table V, and also significant in all other subsets in Table E2, except for CD19, which was not significant in the BW cohort (see Table E2, *B*).

TABLE V. ANOVA models for the EGA and BW cohorts

CD3 ANOVA parameters			
Category	**Parameter**	**Estimate**	**SE**
EGA cohort (n = 338)			
Intercept	Intercept	2876.72	265.35
Sex	Female	−67.56	131.17
	Male	0	
EGA (wk [$P \le .001$])*	22-28	−857.40	300.38
	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	
BW cohort (n = 301)			
Intercept	Intercept	3125.47	204.29
Sex	Female	−66.45	139.11
	Male	0	
BW (g [$P \le .001$])*	≤550	−1124.92	240.38
	551-800	−893.54	228.31
	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	

*A category-level significant *P* value is shown as a *P* value of .05 or less based on type III sum of squares in the multivariate model.

†Multiple race/ethnicity was categorized as single race in a hierarchy as follows: Hispanic, black, and Asian, followed by white. Native Americans were included in the "other" category, as were those with missing or unknown race or ethnicity.

Serial determinations of peripheral blood lymphocyte profiles

Although the main analyses were cross-sectional and lacked a time element to imply causality, the subsample of 33 babies who had multiple flow determinations over time showed trends toward improvement in several flow cytometric measures. In the eligible cohort 29 infants had 2, and 4 infants had 3 tests. Most measurements showed an increase in lymphocyte subset counts. All subsets in Fig 3 and Fig E6 in this article's Online Repository at www.jacionline.org had significant *P* values in a match before and after the 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 increase in numbers of B cells was modest, and NK cell numbers were only barely significant.

DISCUSSION

This study contributes detailed lymphocyte profiles, permitting establishment of reference intervals for T, B, and NK lymphocytes, as well as naive and total CD4 helper and CD8 cytotoxic T lymphocytes for very young infants, including those of preterm birth and with low BW. We had available a large and racially/ethnically diverse cohort in California. Our immunocompetent cohort differs from the general population by having been selected because of initial TREC NBS 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 because of the preponderance of babies identified in NICUs whose TREC values were less than 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 values 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 thus our sample provides flow cytometric results and intervals for healthy subjects who were initially classified as at risk for a T-lymphopenic disorder. Although results from our cohort might not reflect the entire newborn population, this study provides useful guidance for the interpretation of flow cytometric results for newborns most likely to be identified by programs conducting NBS 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 backgrounds in this study is in contrast to other published studies but might 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 analytic differences that predominantly affected one group over another or by chance because of small sample sizes.

Additionally, in this study we have shown that EGA and BW are significant predictors of flow cytometric results. Some of the differences seen in previous studies might be consequences of differential rates of prematurity and low BW, which were not analyzed previously. Multivariate analysis showed that flow cytometric results were not influenced by demographic factors, such as race/ethnicity and sex, but were strongly related to both EGA and 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 cytometric results. Having both of these analyses available might prove useful in clinical contexts in which either EGA or BW is not available.

Flow cytometric results for the main subsets increased as the newborns achieved an EGA approaching term and a BW 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.

Although the majority of EGA and all BW data were cross-sectional and lacked a time element to imply causality, the small subsample of babies who had multiple flow results over time

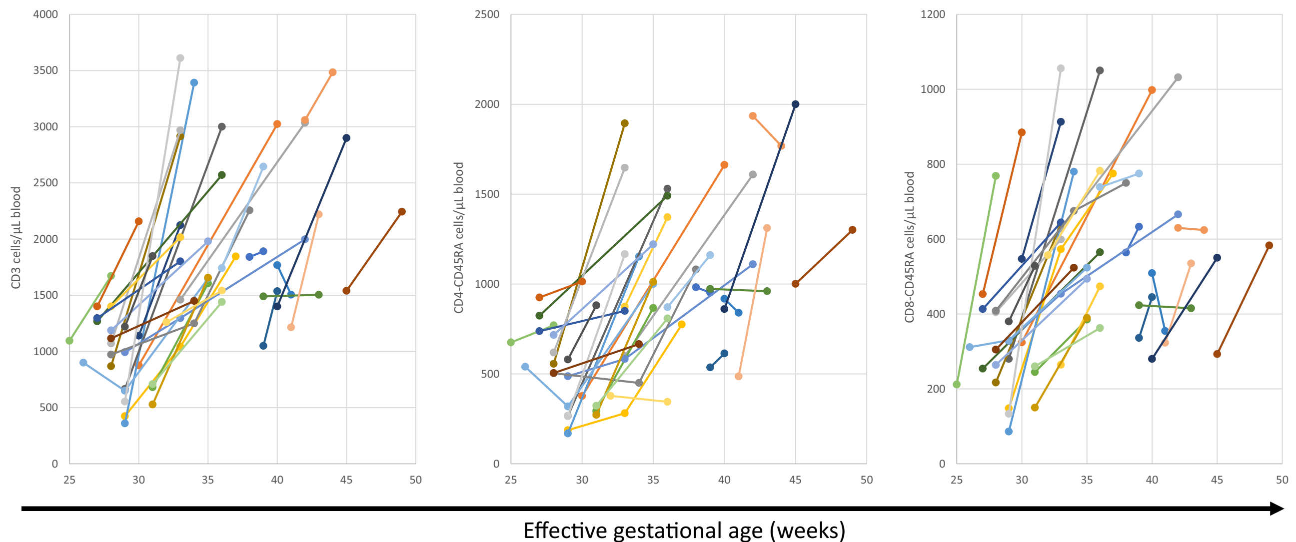

FIG 3. Serial counts of CD3 *(left panel)*, CD4-CD45RA *(middle panel)*, and CD8-CD45RA *(right panel)* lymphocytes at increasing EGAs in 33 infants with multiple measurements. Each set of connected points represents measurements from a single infant, with each infant's data given a consistent color in all panels.

showed a trend toward improvement of several flow cytometric 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 increased slightly but significantly with increasing EGA, whereas NK cells remained nearly constant (see Fig E6). 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 naive 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 III and IV will constitute a useful reference. With widespread adoption of NBS for SCID and disorders with clinically significant TCL, the reference intervals provided here should help neonatologists and immunologists provide optimal care, both by recognizing and protecting infants with immunodeficiency and by avoiding excessive anxiety and immune testing when not necessary.

We 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.

Clinical implications: Reference intervals in preterm and term neonates for T-cell counts, including proportions of cells with naive and memory markers, facilitate evaluation of newborns suspected to have serious T-lymphopenic conditions.

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