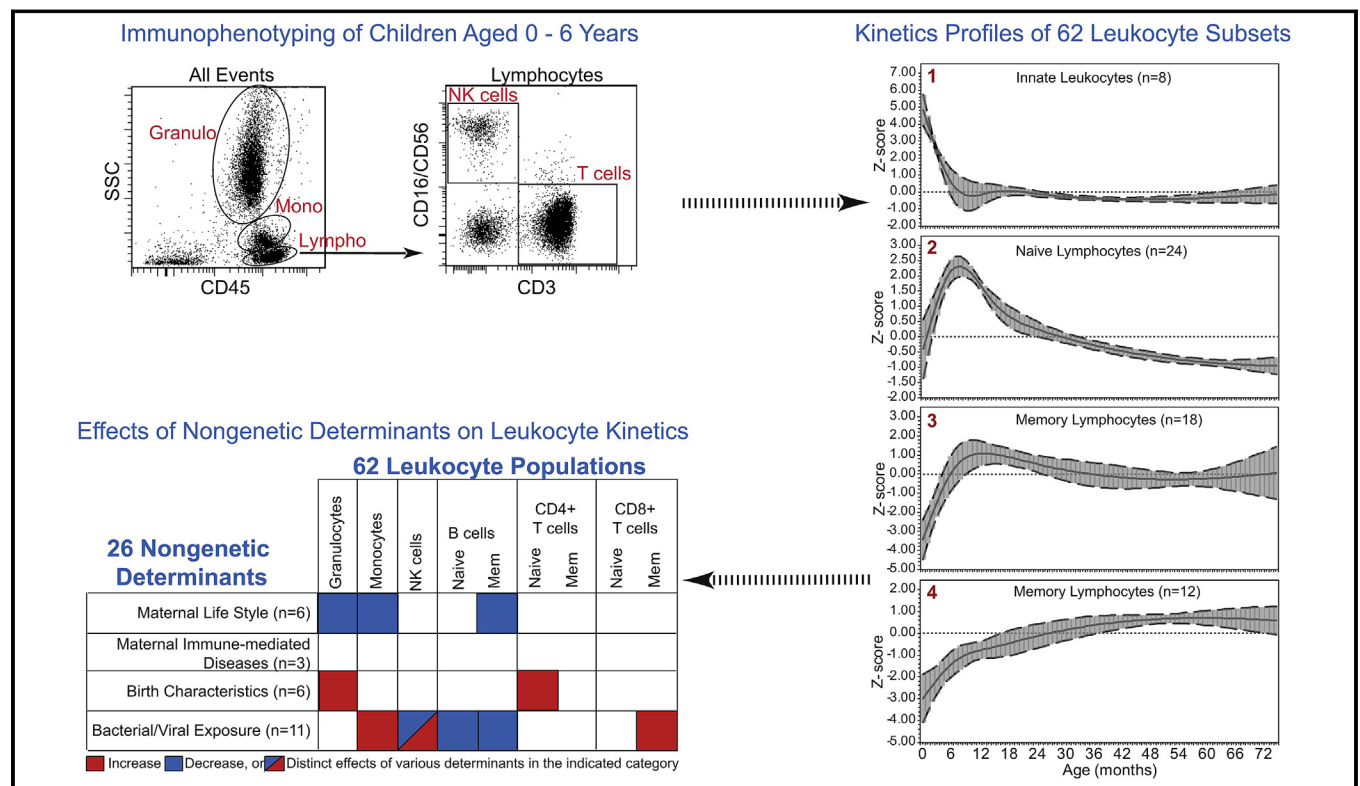


Effects of nongenetic factors on immune cell dynamics in early childhood: The Generation R Study

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GRAPHICAL ABSTRACT



Background: Numbers of blood leukocyte subsets are highly dynamic in childhood and differ greatly between subjects. Interindividual variation is only partly accounted for by genetic factors.

Objective: We sought to determine which nongenetic factors affect the dynamics of innate leukocytes and naive and memory lymphocyte subsets.

Methods: We performed 6-color flow cytometry and linear mixed-effects modeling to define the dynamics of 62 leukocyte subsets from birth to 6 years of age in 1182 children, with 1 to 5 measurements per subject. Subsequently, we defined the effect of prenatal maternal lifestyle-related or immune-mediated determinants, birth characteristics, and bacterial/

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viral exposure–related determinants on leukocyte subset dynamics.

Results: Functionally similar leukocyte populations were grouped by using unbiased hierarchical clustering of patterns of age-related leukocyte dynamics. Innate leukocyte numbers were high at birth and predominantly affected by maternal low education level. Naive lymphocyte counts peaked around 1 year, whereas most memory lymphocyte subsets more gradually increased during the first 4 years of life. Dynamics of CD4⁺ T cells were predominantly associated with sex, birth characteristics, and persistent infections with cytomegalovirus (CMV) or EBV. CD8⁺ T cells were predominantly associated with CMV and EBV infections, and T-cell receptor $\gamma\delta$ ⁺ T cells were predominantly associated with premature rupture of membranes and CMV infection. B-cell subsets were predominantly associated with sex, breast-feeding, and *Helicobacter pylori* carriership.

Conclusions: Our study identifies specific dynamic patterns of leukocyte subset numbers, as well as nongenetic determinants that affect these patterns, thereby providing new insights into the shaping of the childhood immune system. (J Allergy Clin Immunol 2016;■■■:■■■-■■■.)

Key words: Longitudinal leukocyte dynamics, interindividual immunologic variance, innate leukocytes, CD4⁺ T cells, CD8⁺ T cells, B cells, T-cell receptor $\gamma\delta$ ⁺ T cells, nongenetic determinants

The human immune system shows high diversity in cellular composition and functional responses between subjects. Blood leukocytes in young children are highly dynamic in number and composition.¹⁻⁶ Numbers of innate cells, such as neutrophils and natural killer (NK) cells, are higher in neonates than in children or adults^{1,5,7} and already display dynamic changes within the first few days after birth.^{1,5,6} B and T cells are mostly naive in infants, whereas protective immunity is gradually built up in the form of increasing numbers of memory B (Bmem) and memory T (Tmem) cells during the first 5 years of life, after which these numbers stabilize.^{2-4,8-11} Recent studies have found that 25% to 50% of interindividual variation in cellular composition and functional responses were accounted for by genetic factors,^{12,13} indicating that nongenetic determinants underlie a large part of immune trait variance,¹⁴ which will potentially have long-term effects and underlie part of the immune trait variation in adults.

To date, correlations of patterns between different immune cells are incompletely studied because of either the restricted numbers of analyzed subsets, the short-term follow-up, or the lack of longitudinal analyses correcting for intrapersonal correlations between repeated measures. Furthermore, the nongenetic determinants that drive the kinetics of each type of immune cell remain less well studied. It is likely that these involve various environmental determinants, such as maternal lifestyle, maternal immune-mediated diseases, birth characteristics, and bacterial and viral exposure in childhood.¹⁵⁻²⁴ Studying the effect of these nongenetic factors on the dynamics of childhood immune development requires large cohorts of healthy young children, with multiple measurements per subject.

We investigated which nongenetic factors related to the prenatal maternal lifestyle, prenatal maternal immune-mediated diseases, and birth characteristics or bacterial/viral exposure–related characteristics influence the dynamics of blood leukocyte

Abbreviations used

Anti-TPO:	Anti–thyroid peroxidase IgG
Bmem:	Memory B
CMV:	Cytomegalovirus
HSV-1:	Herpes simplex virus 1
NK:	Natural killer
T _{CM} :	central memory T
TCR:	T-cell receptor
T _{EM} :	Effector memory T
Tmem:	Memory T

populations from birth until 6 years of age. This concerned a total of 62 leukocyte populations, including innate leukocyte subsets and naive T- and B-cell and Bmem and Tmem subsets.

METHODS

Study subjects

This study was embedded in the Generation R Study, a prospective population-based cohort study from fetal life until young adulthood.^{25,26} The current study focused on a subgroup of 1182 two-generation Dutch children born between August 2003 and August 2006. Peripheral blood was obtained at birth and a median age of 6, 14, 25, and 72 months. Detailed immunophenotyping was performed at 1 to 5 time points per child, resulting in a total of 2010 data points (details in the [Methods](#) section in this article's Online Repository at www.jacionline.org). Written informed consent was obtained from parents according to the Medical Ethics Committee guidelines of Erasmus MC.

Immunophenotyping

Absolute numbers of leukocytes, NK cells, T cells, and B cells were obtained with a diagnostic lyse-no-wash protocol using a BD FACSCalibur (BD Biosciences, San Jose, Calif). Six-color flow cytometry was performed to quantify 62 well-defined leukocyte populations ([Table I](#)), which included NK cell, monocyte, granulocyte, naive and memory B (Bmem) cell, naive and memory T-cell receptor (TCR) $\alpha\beta$ ⁺ T-cell, and TCR $\gamma\delta$ ⁺ T-cell subsets (see [Table E1](#) in this article's Online Repository at www.jacionline.org). Flow cytometric analyses were performed on an LSRII (BD Biosciences) by using standardized measurement settings (details are provided in the [Methods](#) section in this article's Online Repository).²⁷

Determinants

Information on 22 dichotomized and 4 continuous determinants was obtained ([Table II](#)).^{25,26} Six determinants were related to prenatal maternal lifestyle and evaluated by using questionnaires in the first, second, and third trimesters of pregnancy²⁶: maternal age and body mass index before pregnancy, education, net household income, and smoking or alcohol use during pregnancy. Three determinants were related to prenatal maternal immune-mediated diseases: data on maternal atopy were obtained by means of maternal reported questionnaires during pregnancy,²⁶ and serum anti–thyroid peroxidase IgG (anti-TPO) and anti–tissue transglutaminase IgA antibody levels were measured in the second trimester of pregnancy (mean $\pm$ SD: 13.4 $\pm$ 2.0 and 20.6 $\pm$ 1.2 weeks, respectively).^{28,29} Information on 6 birth-related determinants (ie, sex, gestational age [preterm birth, <37 weeks], birth weight [low birth weight, <2500 g], premature ruptures of the membranes, mode of delivery, and birth season) was obtained from obstetric records assessed in midwife practices and hospitals.^{25,26} Eleven bacterial or viral infection–related determinants were included: breast-feeding and breast-feeding duration, having siblings, antibiotics use, and presence of upper or lower respiratory tract infections in the first year of life (obtained by using questionnaires at child's age of 2, 3, 6, and 12 months),²⁶ *Helicobacter pylori*

TABLE I. Definition of leukocyte subsets

n = 62	n = 31	Population name	Phenotype definition
Innate leukocytes			
1		Granulocytes	SSC ^{high} CD45 ⁺
2		CD15 ⁺ granulocytes	SSC ^{high} CD45 ⁺ CD15 ⁺
3	+	Neutrophils	SSC ^{high} CD45 ⁺ CD16 ⁺
4	+	Eosinophils	SSC ^{high} CD45 ⁺ CD16 ⁻
5		Monocytes	FSC ^{inter} SSC ^{inter} CD45 ⁺
6	+	Classical monocytes	FSC ^{inter} SSC ^{inter} CD45 ⁺ CD14 ⁺ CD16 ⁻
7	+	Intermediate monocytes	FSC ^{inter} SSC ^{inter} CD45 ⁺ CD14 ⁺ CD16 ⁺
8	+	Nonclassical monocytes	FSC ^{inter} SSC ^{inter} CD45 ⁺ CD14 ⁻ CD16 ⁺
9	+	NK cells	SSC ^{low} CD45 ⁺ CD3 ⁻ CD16 ⁺ or CD56 ⁺
Lymphocytes			
10		T cells	SSC ^{low} CD45 ⁺ CD3 ⁺
11		TCRαβ ⁺ T cells	SSC ^{low} CD3 ⁺ TCRγδ ⁻ TCRαβ ⁺
12		└ CD4 ⁺ TCRαβ ⁺ T cells	SSC ^{low} CD3 ⁺ TCRγδ ⁻ TCRαβ ⁺ CD8 ⁻ CD4 ⁺
13		└ CD8 ⁺ TCRαβ ⁺ T cells	SSC ^{low} CD3 ⁺ TCRγδ ⁻ TCRαβ ⁺ CD8 ⁺ CD4 ⁻
14		TCRγδ ⁺ T cells	SSC ^{low} CD3 ⁺ TCRγδ ⁺ TCRαβ ⁻
15		└ CD4 ⁺ TCRγδ ⁺ T cells	SSC ^{low} CD3 ⁺ TCRγδ ⁺ TCRαβ ⁻ CD8 ⁻ CD4 ⁺
16		└ CD8 ⁺ TCRγδ ⁺ T cells	SSC ^{low} CD3 ⁺ TCRγδ ⁺ TCRαβ ⁻ CD8 ⁺ CD4 ⁻
17		└ Vδ1 ⁻ Vδ2 ⁻ TCRαβ ⁻ T cells	SSC ^{low} CD3 ⁺ TCRαβ ⁻ Vδ1 ⁻ Vδ2 ⁻
18		└ Vδ2 ⁺ T cells	SSC ^{low} CD3 ⁺ TCRαβ ⁻ Vδ1 ⁻ Vδ2 ⁺
19	+	└ Vδ1 ⁺ T cells	SSC ^{low} CD3 ⁺ TCRαβ ⁻ Vδ1 ⁺ Vδ2 ⁻
20		└ Vγ9 ⁺ T cells	FSC ^{low} SSC ^{low} CD3 ⁺ TCRαβ ⁻ Vγ9 ⁺
21	+	└ Vδ2 ⁺ Vγ9 ⁺ T cells	FSC ^{low} SSC ^{low} CD3 ⁺ TCRαβ ⁻ Vδ2 ⁺ Vγ9 ⁺
22		CD4 ⁺ T cells	SSC ^{low} CD45 ⁺ CD3 ⁺ CD4 ⁺ CD8 ⁻
23	+	└ CD4 ⁺ T naive	FSC ^{low} SSC ^{low} CD3 ⁺ CD8 ⁻ CD197 ⁺ CD45RO ⁻ CD27 ⁺ CD28 ⁺
24		└ CD4 ⁺ Tmem	FSC ^{low} SSC ^{low} CD3 ⁺ CD8 ⁻ CD197 ⁻ and CD197 ⁺ CD45RO ⁺
25	+	└ CD4 ⁺ T _{CM}	FSC ^{low} SSC ^{low} CD3 ⁺ CD8 ⁻ CD197 ⁺ CD45RO ⁺ CD27 ⁺ CD28 ⁺
26		└ CD4 ⁺ T _{EMRO}	FSC ^{low} SSC ^{low} CD3 ⁺ CD8 ⁻ CD197 ⁻ CD45RO ⁺
27	+	└ CD4 ⁺ early T _{EMRO}	FSC ^{low} SSC ^{low} CD3 ⁺ CD8 ⁻ CD197 ⁻ CD45RO ⁺ CD27 ⁺ CD28 ⁺
28	+	└ CD4 ⁺ intermediate T _{EMRO}	FSC ^{low} SSC ^{low} CD3 ⁺ CD8 ⁻ CD197 ⁻ CD45RO ⁺ CD27 ⁻ CD28 ⁺
29	+	└ CD4 ⁺ late T _{EMRO}	FSC ^{low} SSC ^{low} CD3 ⁺ CD8 ⁻ CD197 ⁻ CD45RO ⁺ CD27 ⁻ CD28 ⁻
30		└ CD4 ⁺ T _{EMRA}	FSC ^{low} SSC ^{low} CD3 ⁺ CD8 ⁻ CD197 ⁻ CD45RO ⁻
31	+	└ CD4 ⁺ early T _{EMRA}	FSC ^{low} SSC ^{low} CD3 ⁺ CD8 ⁻ CD197 ⁻ CD45RO ⁻ CD27 ⁺ CD28 ⁺
32	+	└ CD4 ⁺ intermediate T _{EMRA}	FSC ^{low} SSC ^{low} CD3 ⁺ CD8 ⁻ CD197 ⁻ CD45RO ⁻ CD27 ⁻ CD28 ⁺
33	+	└ CD4 ⁺ late T _{EMRA}	FSC ^{low} SSC ^{low} CD3 ⁺ CD8 ⁻ CD197 ⁻ CD45RO ⁻ CD27 ⁻ CD28 ⁻
34		CD8 ⁺ T cells	SSC ^{low} CD45 ⁺ CD3 ⁺ CD4 ⁻ CD8 ⁺
35	+	└ CD8 ⁺ T naive	FSC ^{low} SSC ^{low} CD3 ⁺ CD8 ⁺ CD197 ⁺ CD45RO ⁻ CD27 ⁺ CD28 ⁺
36		└ CD8 ⁺ Tmem	FSC ^{low} SSC ^{low} CD3 ⁺ CD8 ⁺ CD197 ⁻ and CD197 ⁺ CD45RO ⁺
37	+	└ CD8 ⁺ T _{CM}	FSC ^{low} SSC ^{low} CD3 ⁺ CD8 ⁺ CD197 ⁺ CD45RO ⁺ CD27 ⁺ CD28 ⁺
38		└ CD8 ⁺ T _{EMRO}	FSC ^{low} SSC ^{low} CD3 ⁺ CD8 ⁺ CD197 ⁻ CD45RO ⁺
39	+	└ CD8 ⁺ early T _{EMRO}	FSC ^{low} SSC ^{low} CD3 ⁺ CD8 ⁺ CD197 ⁻ CD45RO ⁺ CD27 ⁺ CD28 ⁺
40	+	└ CD8 ⁺ intermediate T _{EMRO}	FSC ^{low} SSC ^{low} CD3 ⁺ CD8 ⁺ CD197 ⁻ CD45RO ⁺ CD27 ⁻ CD28 ⁻
41	+	└ CD8 ⁺ late T _{EMRO}	FSC ^{low} SSC ^{low} CD3 ⁺ CD8 ⁺ CD197 ⁻ CD45RO ⁺ CD27 ⁻ CD28 ⁻
42		└ CD8 ⁺ T _{EMRA}	FSC ^{low} SSC ^{low} CD3 ⁺ CD8 ⁺ CD197 ⁻ CD45RO ⁻
43	+	└ CD8 ⁺ early T _{EMRA}	FSC ^{low} SSC ^{low} CD3 ⁺ CD8 ⁺ CD197 ⁻ CD45RO ⁻ CD27 ⁺ CD28 ⁺
44	+	└ CD8 ⁺ intermediate T _{EMRA}	FSC ^{low} SSC ^{low} CD3 ⁺ CD8 ⁺ CD197 ⁻ CD45RO ⁻ CD27 ⁻ CD28 ⁻
45	+	└ CD8 ⁺ late T _{EMRA}	FSC ^{low} SSC ^{low} CD3 ⁺ CD8 ⁺ CD197 ⁻ CD45RO ⁻ CD27 ⁻ CD28 ⁻
46		B cells	SSC ^{low} CD45 ⁺ CD19 ⁺
47	+	B naive	SSC ^{low} CD19 ⁺ CD27 ⁻ IgD ⁺
48		Bmem	SSC ^{low} CD19 ⁺ IgD ⁻ and CD27 ⁺ IgD ⁺
49		└ CD27 ⁻ Bmem	SSC ^{low} CD19 ⁺ CD27 ⁻ IgD ⁻
50		└ CD27 ⁺ Bmem	SSC ^{low} CD19 ⁺ CD27 ⁺ IgD ⁻
51		└ IgM ⁺ Bmem	SSC ^{low} CD19 ⁺ CD27 ⁺ IgM ⁺
52	+	└ Natural effector	SSC ^{low} CD19 ⁺ CD27 ⁺ IgM ⁺ IgD ⁺
53	+	└ IgM only	SSC ^{low} CD19 ⁺ CD27 ⁺ IgM ⁺ IgD ⁻
54		└ IgA ⁺ Bmem	SSC ^{low} CD19 ⁺ IgA ⁺
55	+	└ CD27 ⁺ IgA ⁺	SSC ^{low} CD19 ⁺ CD27 ⁺ IgA ⁺
56	+	└ CD27 ⁺ IgA ⁺	SSC ^{low} CD19 ⁺ CD27 ⁺ IgA ⁺
57		└ IgG ⁺ Bmem	SSC ^{low} CD19 ⁺ IgG ⁺
58	+	└ CD27 ⁺ IgG ⁺	SSC ^{low} CD19 ⁺ CD27 ⁺ IgG ⁺
59	+	└ CD27 ⁺ IgG ⁺	SSC ^{low} CD19 ⁺ CD27 ⁺ IgG ⁺
60		CD21 ^{low} B cells	SSC ^{low} CD19 ⁺ CD38 ^{low} CD21 ^{low}
61		Igλ ⁺ B cells	SSC ^{low} CD19 ⁺ Igκ ⁻ Igλ ⁺
62		Igκ ⁺ B cells	SSC ^{low} CD19 ⁺ Igκ ⁺ Igλ ⁻

FSC, Forward scatter; SSC, side scatter.

TABLE II. Characteristics of nongenetic factors

	Negative subjects (%)	Positive subjects (%)	Missing (%)	Significant effect on cluster*
Prenatal maternal lifestyle				
Maternal age (y)	Continuous determinant		0 (0)	—
Low maternal educational level	400 (33.8)	760 (64.3)	22 (1.9)	1, 2, 3, 4
Net household income per month >€2200	242 (20.5)	847 (71.7)	93 (7.9)	—
Smoking during pregnancy	815 (69)	256 (21.7)	111 (9.4)	—
Alcohol use continued during pregnancy	299 (25.3)	750 (63.5)	133 (11.3)	—
Body mass index before pregnancy (kg/m ²)	Continuous determinant		181 (15.3)	—
Prenatal maternal immune-mediated diseases				
Maternal atopy (eczema, allergy HDM, hay fever)	683 (57.8)	383 (32.4)	116 (9.8)	—
Anti-TPO (before 18 wk of pregnancy [mU/mL])	Continuous determinant		278 (23.5)	2
Maternal TG2A level during pregnancy (U/mL)	Continuous determinant		208 (17.6)	—
Birth characteristics				
Sex (girl, yes/no)	600 (50.8)	582 (49.2)	0 (0)	1, 2, 3, 4
Preterm birth <37 wk	76 (6.4)	1,106 (93.6)	0 (0)	—
Low birth weight <2500 g	1119 (94.7)	63 (5.3)	0 (0)	—
Premature rupture of membranes	1089 (92.1)	53 (4.5)	40 (3.4)	4
Cesarean section versus vaginal/forceps/vacuum-assisted birth	903 (76.4)	154 (13)	125 (10.6)	3, 4
Birth season (born in fall/winter)	682 (57.7)	500 (42.3)	0 (0)	—
Bacterial/viral exposure-related characteristics				
Breast-feeding ever	109 (9.2)	969 (82)	104 (8.8)	—
Breast-feeding at age 6 mo	754 (63.8)	299 (25.3)	129 (10.9)	2, 4
Siblings >1	687 (58.1)	480 (40.6)	15 (1.3)	1
Antibiotics/penicillin use in first year	636 (53.8)	342 (28.9)	204 (17.3)	1, 3
Upper respiratory tract infections in first year	547 (46.3)	475 (40.2)	160 (13.5)	—
Lower respiratory tract infections in first year (doctor attended)	819 (69.3)	131 (11.1)	232 (19.6)	—
Carrier of <i>H pylori</i> within 6 y	887 (75)	49 (4.1)	246 (20.8)	2, 3
Seropositivity for CMV at age 6 y	665 (56.3)	269 (22.8)	248 (21)	1, 2, 3, 4
Seropositivity for EBV at age 6 y	534 (45.2)	400 (33.8)	248 (21)	2, 3, 4
Seropositivity for HSV-1 at age 6 y	810 (68.5)	124 (10.5)	248 (21)	2
Seropositivity for varicella zoster virus at age 6 y	73 (6.2)	861 (72.8)	248 (21)	—

HDM, House dust mite; TG2A, anti-tissue transglutaminase IgA antibody.

*First, each determinant was added to the linear mixed-effects model univariably to test for a significant effect (significance was defined as $P < .0125$). Subsequently, per leukocyte population, all determinants with a significant individual effect were added in a multivariable model to correct for confounding effects. Up to 3 determinants were added per multivariable model. Significance was defined as a P value of less than .05. No significant confounding effect was observed.

carriership at 6 years of age, and seropositivity (IgG) for cytomegalovirus (CMV), EBV, herpes simplex virus 1 (HSV-1), and varicella zoster virus at the age of 6 years.^{22,30,31}

Statistical modeling

Linear mixed-effects analyses were performed to model leukocyte dynamics between birth and age 6 years. By including random effects in the model, this approach enabled modeling of cross-sectional data, with further improvement in accuracy by incorporating longitudinal follow-up data from individual children. To capture the trend in the data more precisely, the age of the children was included as a natural spline with different knots (0–3 knots) into the models. Basically, the number of knots is inversely related to the smoothness of the curve. Positions of the knots were defined as the 50th percentile (25.5 months) in the 1-knot model and the 33rd and 66th percentiles (14.1 and 70 months) in the 2-knot model. The knots in the 3-knot model were defined manually at 6, 14, and 24 months, focusing around the time points of data measurement. Model selection was performed by using the likelihood ratio test.

Next, for each leukocyte population, the effect of all 26 determinants on the models was assessed by first adding each determinant univariably into the model and analyzing fixed-effects estimates. To correct for potential multiple testing errors, a correction for the 4 groups of determinants was performed, and consequently, only determinants with an effect P value of less than .0125 were defined as significant. Subsequently, for each leukocyte population, the determinants with a significant effect (≤ 3 determinants were significant per model) were combined in a multivariable model to correct for possible confounding effects. Finally, to test whether changes in leukocyte dynamics over time (from birth until 6 years) induced by the determinant of interest

was most noticeable at a specific age interval, the individual determinants were tested in relation to the age of the children in a multivariable model. The P value for significance for the multivariable analyses was less than .05. Statistical analyses were performed with R software (version R-3.2.1; details are provided in the [Methods](#) section in this article's Online Repository).³²

Clustering analyses

The modeled data of each population were normalized into zero means and unit SDs (z score) to cluster patterns of leukocyte subset kinetics by using the following calculation:

$$z \text{ Score} = \frac{\text{Population size (at month } x) - \text{Average population size (at months 0–76)}}{\text{SD of population size (at months 0–76)}}$$

The normalized leukocyte models were subsequently clustered by using agglomerative (bottom-up) Ward hierarchic clustering at each step, clustering 2 clusters with minimum between-cluster distance by using the Euclidean distance measure.

RESULTS

Linear mixed-effects modeling of leukocyte subset cell numbers versus child's age

To study the dynamics of blood immune cells in young children, we quantified cell numbers of 11 leukocyte subsets in

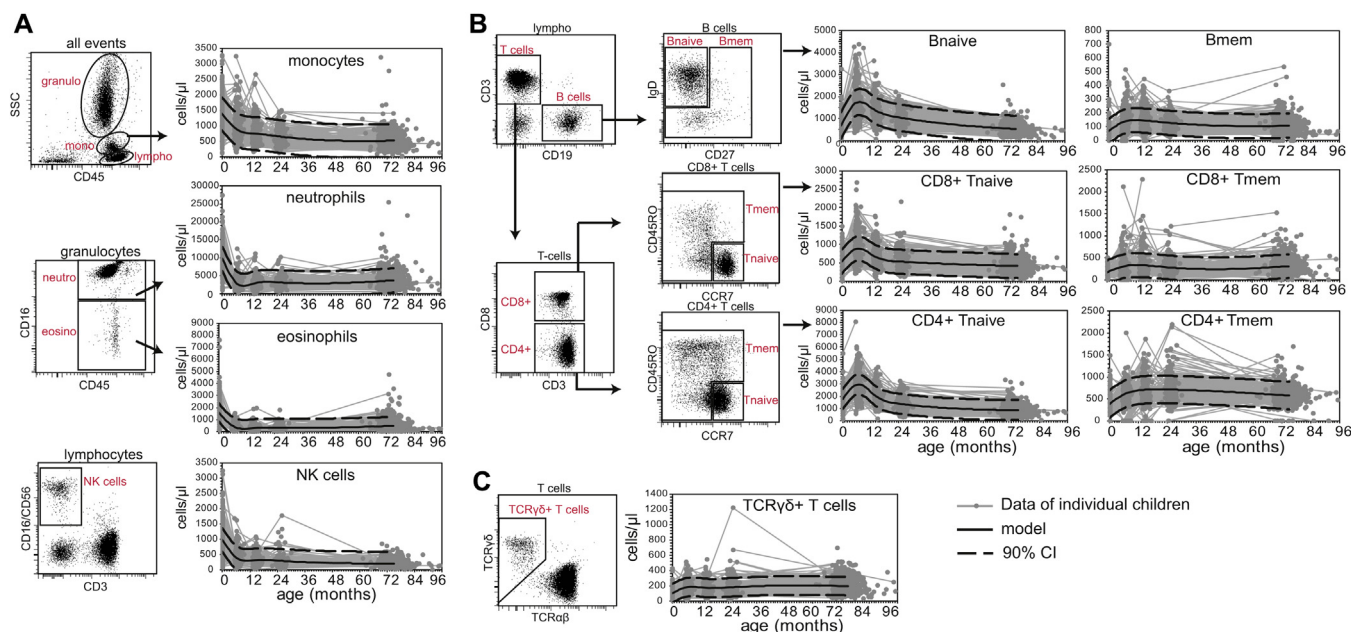


FIG 1. Dynamics of innate leukocyte and naive and memory lymphocyte populations in children between birth and 6 years of age. Dynamics of monocytes, neutrophils, eosinophils, and NK cells (**A**); naive B- and T-cell and Bmem and Tmem subsets (**B**); and TCR $\gamma\delta^+$ T cells (**C**). Flow cytometric plots depict population definitions in 1 representative 6-year-old subject. Graphs depict absolute numbers of cells in blood of 1182 children with a total of 2010 measurements (gray dots). Linear mixed-effects models are depicted by a solid black line for each population, and the 90% CI of the model is depicted by dashed black lines. For clarity, only direct follow-up time points of a subject were connected with gray lines (ie, 0–6 months, 6–14 months, 14–25 months, or 25–76 months).

1182 children between birth and 76 months (6 years) of age. Statistical modeling showed that the numbers of 4 innate leukocyte subsets (ie, monocytes, neutrophils, eosinophils, and NK cells) were high at birth, quickly decreased within the first 6 months of age, and subsequently remained stable (Fig 1, A). Naive B- and T-cell numbers strongly increased after birth, peaked between 6 and 14 months of age, and subsequently decreased to stable levels around the age of 2 to 6 years (Fig 1, B). Bmem and Tmem cell numbers slowly increased within the first 6 to 14 months of life, after which numbers decreased marginally and stabilized from approximately 3 years onward (Fig 1, B). Total TCR $\gamma\delta^+$ T-cell numbers increased until 6 months and remained quite stable at these levels (Fig 1, C).

Distinct dynamics of innate leukocytes and naive and Bmem and Tmem cells

To evaluate whether functionally related immune populations would follow similar dynamics with increasing age, we extended our analysis in the same children to 62 leukocyte subsets based on recent insights into naive B- and T-cell and Bmem and Tmem subsets (Table I and see Figs E1–E5 in this article's Online Repository at www.jacionline.org; reference values per age category are presented in Table E2 in this article's Online Repository at www.jacionline.org).^{24,33}

Hierarchical clustering of all 62 leukocyte populations resulted in 4 distinct clusters of leukocyte dynamics (Fig 2, A). Cell numbers of all populations in the first cluster were high at birth, followed by a sharp decrease within the first 6 months of life, after which they stabilized (Fig 2, B). This cluster exclusively

contained innate leukocyte subsets. All defined innate leukocyte subsets clustered within cluster 1, except for the intermediate monocytes.

The populations in cluster 2 strongly increased shortly after birth and peaked before 14 months of age, followed by a long-term gradual decrease. Cluster 2 included the 3 major naive lymphocyte subsets (CD4 $^+$ T-naive, CD8 $^+$ T-naive and B-naive subsets), as well as 5 memory populations (CD4 $^+$ central memory T [T_{CM}] cells, CD8 $^+$ T_{CM} cells, early differentiated CD8 $^+$ CD45RO $^-$ effector memory T cells [CD8 $^+$ early T_{EMRA}], CD27 $^+$ IgA $^+$ Bmem cells, and natural effector Bmem cells). Also, the total B-cell, CD8 $^+$ T-cell, CD4 $^+$ T-cell, and total T-cell subsets clustered in cluster 2, as did the intermediate monocytes and V δ 1 $^+$ T cells.

The populations in clusters 3 and 4 gradually increased in cell number and peaked either at 14 months (cluster 3) or after approximately 4 years (cluster 4). Clusters 3 and 4 contained only Bmem and Tmem subsets and the TCR $\gamma\delta^+$ T-cell subsets. Cluster 3 contained the total memory lymphocyte populations: Bmem, CD4 $^+$ Tmem, and CD8 $^+$ Tmem cells. In particular, these memory populations included the early, intermediate, and late CD8 $^+$ T_{EMRO} subpopulations, late CD8 $^+$ T_{EMRA} cells, early CD4 $^+$ T_{EMRA} cells, and the IgM-only, CD27 $^+$ IgA $^+$ and CD27 $^+$ IgG $^+$ Bmem cell subsets. Cluster 4 contained total TCR $\gamma\delta^+$ T cells and the V δ 2 $^+$ and the V γ 9 $^+$ T-cell subsets, as well as the early, intermediate, and late CD4 $^+$ T_{EMRO} subpopulations; late CD4 $^+$ T_{EMRA} cells; intermediate CD8 $^+$ T_{EMRA} cells; and CD27 $^+$ IgG $^+$ Bmem cells.

An additional clustering was performed on a selection of 31 nonoverlapping populations to test the effect of including

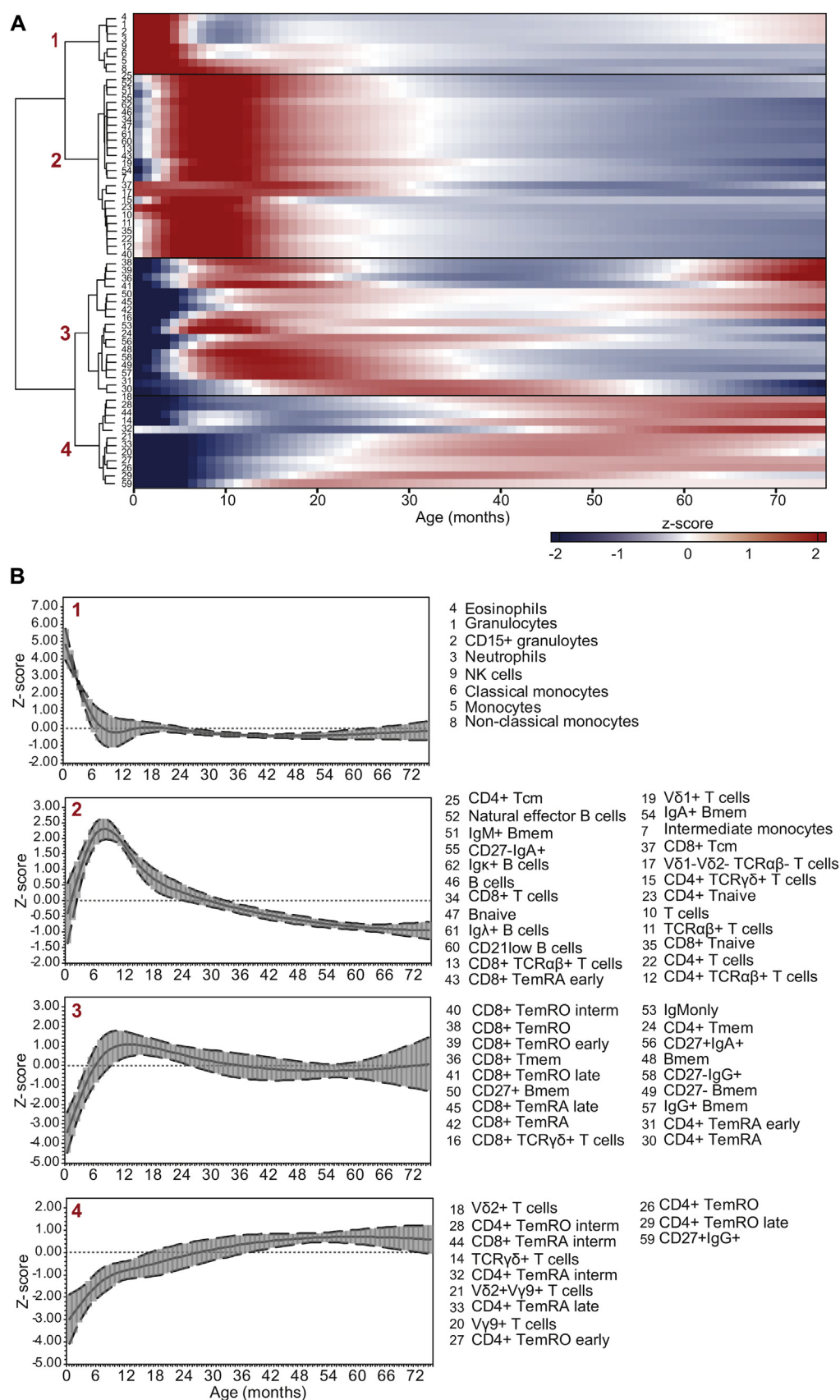


FIG 2. Hierarchical clustering of leukocyte subset dynamics in early childhood. **A**, Ward hierarchical clustering of the cellularity of the 62 populations derived from linear mixed models. Cellularity between 0 and 76 months of age was converted to zero mean and unit SD values (z score). **B**, Average patterns $\pm$ 1 SD of the subsets in each of the 4 major clusters. Populations are numbered according to their order in Table I.

populations with overlapping population definitions on clustering (Table I). The resulting clusters showed similar dynamics (Fig 2, B, and see Fig E6, B, in this article's Online Repository at www.jacionline.org), with only 3 populations (nonclassical monocytes, CD8⁺ late T_{EMRO} cells, and CD8⁺ late T_{EMRA} cells) assigned to a different cluster, indicating that the overlapping populations did not overtly skew our clustering approach. Thus functionally related populations, as well as populations with phenotypic overlapping definitions, displayed similar dynamics in early childhood.

Associations between nongenetic factors and leukocyte subset dynamics in the 4 clusters

The Bmem and Tmem subsets showed large interindividual variation in dynamics of cell numbers (Fig 1). To study the effects of external factors, we analyzed 6 maternal lifestyle-related and 3 maternal immune-related determinants, 6 birth characteristics, and 11 bacterial/viral exposure-related determinants (Table II). Twelve of these 26 determinants showed a significant association with the dynamics of 1 or more of the 62 leukocyte populations after multivariable correction (Table II). Bacterial/viral exposure-related determinants were more frequently found to affect cell numbers in clusters 2, 3, and 4 than in cluster 1 (Fig 3, A and B).

In cluster 1 a low maternal education level was associated with a reduction in the patterns of eosinophils and classical monocytes (Fig 4). Female sex was associated with a significant increase in the pattern of neutrophils and the phenotypically overlapping CD15⁺ granulocyte population. Having more than 1 sibling was associated with a reduction in NK cell numbers, and CMV infection was associated with an increase in NK cell numbers. Antibiotic use in the first year of life was associated with an increase in nonclassical monocytes. Overall effect estimates and associations of effects with specific age intervals are presented in Table E3 in this article's Online Repository at www.jacionline.org.

In cluster 2 none of the determinants included in our study affected the patterns of more than 25% of the populations (Fig 3, C and D). However, female sex was associated with an increase in the patterns of CD4⁺ naive and T_{CM} cells and, consequently, in the phenotypically overlapping CD4⁺TCRαβ⁺ and total CD4⁺ T cells. Although breast-feeding for more than 6 months was associated with a reduction in the pattern of total IgA⁺ and CD27⁺IgA⁺ Bmem cells, *H pylori* carriage was associated with an increase in these populations. Furthermore, *H pylori* carriage was associated with an increase in total Igκ⁺ B-cell and TCRαβ⁺ T-cell counts. CMV and EBV were both associated with an increase in the strongly related total and TCRαβ⁺CD8⁺ T-cell numbers, and CMV was associated with an increase in Vδ1⁺ T-cell numbers. HSV-1 seropositivity was associated with a decrease in the large population of naive B cells and, consequently, with total B cells and Igκ⁺ and Igλ⁺ B-cell subsets.

In cluster 3 CMV and EBV seropositivity were significantly associated with changes in dynamics of greater than 40% of subsets (Fig 3, C, and see Table E3). Both viruses were associated with an increase in CD8⁺ intermediate and late T_{EMRO} cell numbers and the phenotypically related total CD8⁺ T_{EMRO} and total CD8⁺ Tmem populations. In addition, CMV was associated with an increase in numbers of CD8⁺TCRγδ⁺ T cells and CD8⁺

late T_{EMRA} cells and the phenotypically related total CD8⁺ T_{EMRA} population. EBV was associated with an increase in numbers of CD8⁺ early T_{EMRO} cells and a decrease in numbers of total Bmem cells and the CD27⁺ and CD27⁺ Bmem subsets. The effects on CD8⁺ late T_{EMRO} and T_{EMRA} cells were still present in the analysis of 31 nonoverlapping population, even though these were now included in cluster 4 (Fig 3, D).

In cluster 4 sex, premature rupture of membranes, CMV, and EBV were significantly associated with changes in dynamics of 40% of the subsets (Fig 3, C and D, and see Table E3). Female sex was associated with an increase in the patterns of CD27⁺IgG⁺ Bmem cells and CD4⁺ early T_{EMRO} cells and with a reduction in total numbers of TCRγδ⁺ T cells and the CD4⁺ intermediate and late T_{EMRA} populations. Premature rupture of membranes was associated with an increase in numbers of CD4⁺ late T_{EMRO} cells, CD4⁺ intermediate T_{EMRA} cells, and Vδ2⁺ and Vγ9⁺ T-cell subsets. CMV and EBV seropositivity were both associated with an increase in numbers of total CD4⁺ T_{EMRO} and CD4⁺ late T_{EMRO} and T_{EMRA} cells, as well as CD8⁺ intermediate T_{EMRA} cells. In addition, CMV was associated with increased numbers of CD4⁺ intermediate T_{EMRO} cells, and EBV with an increase in CD4⁺ early T_{EMRO} cell numbers.

Altogether, we identified multiple determinants that had a significant effect on the dynamics of leukocyte subset numbers within 1 or more of the 4 distinct age-related patterns. Still, considerable variation of effects could be observed within individual clusters.

Effects of nongenetic factors on leukocyte dynamics within distinct leukocyte lineages

Because clusters 2, 3, and 4 each contained various B- and T-cell populations, each with distinct humoral or cellular functions, we next studied the effects of the 26 determinants on patterns of individual leukocyte lineages.

Within the B-cell lineage, low maternal educational level was associated with a reduction in the patterns of total Bmem cells (Fig 4). Female sex was associated with an increase in the patterns of CD27⁺IgA⁺ and CD27⁺IgG⁺ Bmem cells, although these effects were not reflected in the total Bmem cell populations. Breast-feeding for more than 6 months was associated with a selective reduction in the pattern of CD27⁺IgA⁺ Bmem cells, antibiotics use in the first year of life was associated with a selective increase in the pattern of CD27⁺IgG⁺ Bmem cells, and *H pylori* carriage was associated with an increase in the patterns of both CD27⁺IgA⁺ and CD27⁺IgG⁺ Bmem cells. EBV infection was associated with a reduction in the pattern of total Bmem cells, although not with a specific Bmem cell subset, and HSV-1 infection was associated with a selective reduction in the pattern of naive B cells.

Within the CD4⁺ and CD8⁺ T-cell lineages, increased serum anti-TPO levels were associated with an increase in CD8⁺ T_{CM} cell numbers. Female sex was associated with an increase in the patterns of CD4⁺ naive, T_{CM}, and early T_{EMRO} cells, although these effects were not reflected in the total CD4⁺ Tmem cell populations. In contrast, female sex was associated with a decrease in the patterns of CD4⁺ intermediate and late T_{EMRA} and CD8⁺ late T_{EMRA} cells. Whereas premature rupture of membranes was associated with an increase in CD4⁺ late T_{EMRO} and CD4⁺ intermediate T_{EMRA} cell numbers, child birth

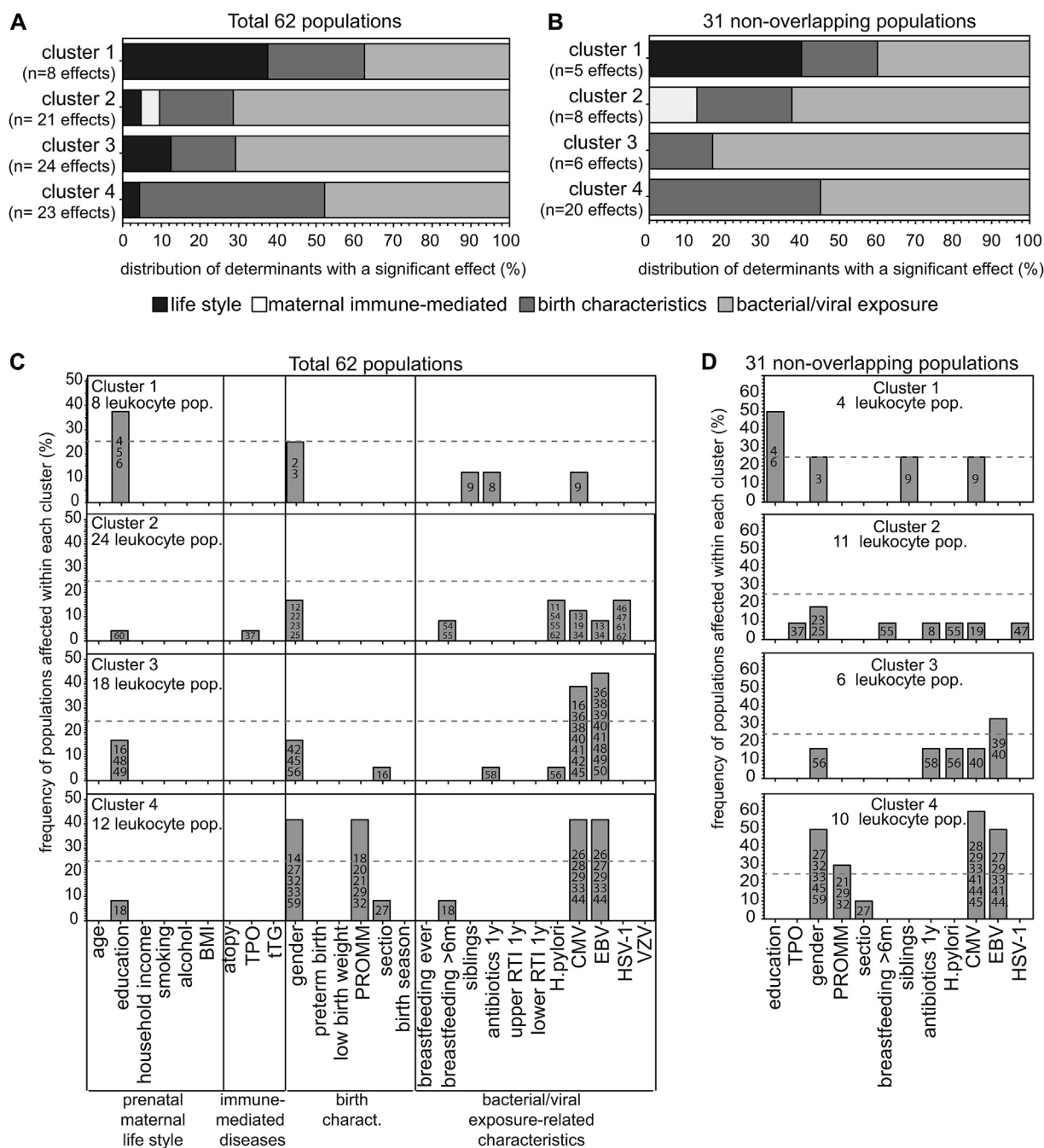


FIG 3. Nongenetic factors associated with leukocyte dynamics in the 4 clusters. Significant associations of the 26 determinants with leukocyte subsets in clusters 1 to 4 based on the clustering of 62 leukocyte populations as in Fig 2, A and C, or of the 31 nonoverlapping subsets as in Fig E6, B and D, corrected for confounding effects are shown. A and B, Relative distribution of types of determinants that significantly affect leukocyte kinetics. C and D, Frequencies of populations within a cluster that were affected by individual determinants. PROMM, Premature rupture of membranes; RTI, respiratory tract infection. Numbers in each bar refer to the populations as defined in Table I.

through cesarean section was associated with a reduction in the pattern of CD4⁺ early T_{EMRO} cells. Persistent viral infection with CMV and EBV significantly affected CD4⁺ and CD8⁺ T-cell dynamics in contrast to breast-feeding, antibiotic use, *H pylori*, HSV-1, and varicella zoster virus carriage. CMV infection associated exclusively with an increase in the patterns of intermediate and late differentiated CD4⁺ and CD8⁺ T_{EMRO}

and T_{EMRA} cells. Associations with EBV were slightly more variable, also including early differentiated CD4⁺ and CD8⁺ T_{EMRO} cells.

Within the TCRγδ⁺ T-cell lineage, premature rupture of membranes was associated with an increase in Vδ2⁺Vγ9⁺ T-cell counts, and CMV infection was associated with an increase in the pattern of Vδ1⁺ T cells.

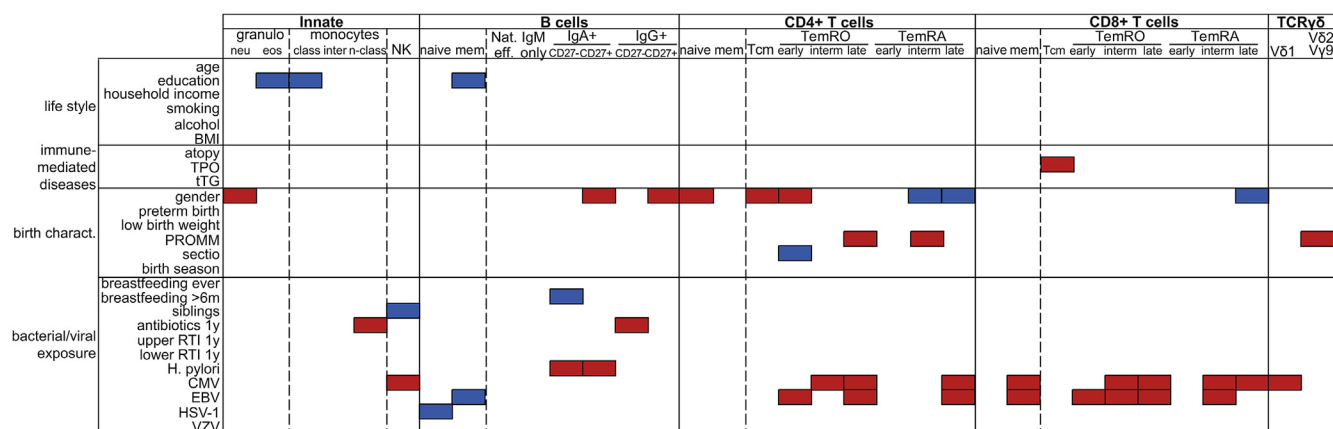


FIG 4. Effects of nongenetic factors on the dynamics of innate leukocytes and B- and T-cell subsets. Significant effects are shown derived from multivariable linear mixed-effects analysis of all 26 determinants on the 31 nonoverlapping populations and the total memory subsets ("mem") within the B-cell, CD4⁺ T-cell, and CD8⁺ T-cell lineages. Blue boxes represent a reduction, and red boxes represent an increase in the pattern of the indicated population. Effect sizes are presented in Table E3, and details of each determinant are presented in Table E2. class, Classical monocytes; eos, eosinophils; inter, intermediate monocytes; n-class, nonclassical monocytes; neu, neutrophils; PROMM, Premature rupture of membranes; RTI, respiratory tract infection.

DISCUSSION

Here we modeled the kinetics of 62 leukocyte subsets and identified distinct patterns of cell numbers in the first 6 years of life for innate leukocytes, naive B and T cells, and Bmem and Tmem cells. Unsupervised clustering revealed that leukocyte dynamics between birth and age 6 years could be summarized into 4 major profiles, with either (1) early predominance and fast decrease to stable numbers, (2) gradual increase in the first year followed by a gradual decrease, or (3 and 4) a slow increase in the first 1 to 2 years, followed by stabilization.

Consistent with their early predominance, innate leukocyte kinetics were affected by maternal education level, which might already influence the fetus prenatally. Low maternal education level is strongly related to a less healthy lifestyle.³⁴ These observations suggest that maternal lifestyle is especially important for shaping of innate leukocyte populations, although the exact mediator remains to be determined.

The nonclassical and intermediate monocytes did not consistently cluster with the innate leukocyte subsets because of a later peak in numbers or a slower decrease. These altered kinetics could be the result of these being derived from classical monocytes and not directly from precursors in bone marrow.³⁵ Still, the various subsets of monocytes express distinct levels of proteins involved in HLA class II-dependent antigen presentation and CD40–CD40 ligand costimulation³⁵ and differ in parasite pattern recognition.^{35–38} Thus our data support the need to discriminate intermediate and nonclassical monocytes from the dominant population of classical monocytes in kinetics studies.

All naive B- and T-cell populations followed profile 2, with a gradual increase in the first 14 months followed by a more gradual decrease. After the first 1 to 2 years of life, B-lymphocyte production in bone marrow decreases,^{39,40} and the thymus starts to involute.⁴¹ These processes are likely causes of the decrease we observed.

Bmem cell populations showed different patterns. Natural effector and CD27⁺ IgA⁺ Bmem cell kinetics were similar to those of naive B cells. Both Bmem cell populations are suggested to derive, at least in part, from germinal center-independent

responses without T-cell help in the splenic marginal zone and the intestinal lamina propria.^{33,42,43} The relatively fast increase numbers of these cells within the first 6 months of life contrasts with the kinetics of the other T cell–dependent, Bmem cell subsets. IgM-only, CD27⁺ IgG⁺, and CD27⁺ IgA⁺ Bmem cells followed profile 3, representing gradual memory formation. The CD27⁺ IgG⁺ population followed profile 4 and developed slightly slower than the other Bmem cell populations. These observations supports the concept of rapid generation of T cell–independent Bmem cells in the absence of the extensive proliferation and selection processes of the germinal center,³³ whereas CD27⁺ IgG⁺ Bmem cells might represent the most mature Bmem cell population. In adults this subset shows a higher level of affinity maturation, a more extensive replication history, and more frequent development through consecutive class-switching than CD27⁺ IgG⁺ Bmem cells.^{33,44} It is perceivable that many of these CD27⁺ IgG⁺ cells are generated from CD27⁺ IgG⁺ Bmem cells in secondary responses, which could explain the gradual decrease in CD27⁺ IgG⁺ B-cell numbers after 14 months of age. Together, the different clustering of the Bmem cell populations thereby follows their increasing functional maturity (see Fig E5, I).

Several external determinants significantly affected the B-cell kinetics. First, we confirmed our previous observations regarding lower numbers of Bmem cells in breast-fed children.^{20,22} However, in the current analysis we could not reproduce the previously reported association between breast-feeding duration and germinal center–dependent Bmem cell numbers at 6 months of age.²² The difference is likely due to our current analysis in which duration of breast-feeding was not included and the overall pattern between birth and 6 years of age was studied.

Numbers of IgA⁺ Bmem cells were significantly increased in *H pylori*–positive children. The colonization of the gastric mucus by *H pylori* was found to correlate with an increase in total blood B-cell counts⁴⁵ on top of the local expansion of antibody-secreting IgA⁺ cells,⁴⁶ which are important for protection against *H pylori* infection.⁴⁷ Our observed expansion of circulating IgA⁺ Bmem cells included both the CD27⁺ IgA⁺ and mucosa-derived CD27⁺ IgA⁺ subsets. This would suggest that the presence of

H pylori results in not only a local expansion of plasma cells but also a systemic expansion of Bmem cells in otherwise asymptomatic carriers. It remains to be determined whether this expansion is beneficial for the host as the bacterial protein CagA inhibits B-cell apoptosis and thereby increases the risk for mucosa-associated B-cell malignancies.^{48,49}

Tmem cell subsets were found in clusters 2, 3, and 4. CD4⁺ and CD8⁺ T_{CM} (cluster 2) express lymph node homing markers and are the presumed precursors of T_{EM} cells.^{24,50,51} The early peak in T_{CM} numbers before those of T_{EM} cells in young children would fit with this function. Similarly, CD8⁺ early T_{EMRA} cells might be precursors for further differentiated T_{EMRA} subsets. Vδ1⁺ T-cell numbers (cluster 2) peaked before the Vδ2⁺ and Vγ9⁺ subsets (cluster 4), thereby confirming the previously observed early shift from Vδ1⁺ to Vδ2⁺Vγ9⁺ predominance in children.^{52,53}

In line with previous observations in both children and elderly,^{22,54-58} both CD4⁺ and CD8⁺ Tmem cell dynamics were predominantly affected by CMV and/or EBV seropositivity. This concerned mostly late T_{EM} cell numbers for CMV and early T_{EM} cell subsets for EBV.^{24,59} HSV-1 seropositivity did not affect CD4⁺ and CD8⁺ T-cell populations^{22,58} but was associated with a loss of naive B cells. To our knowledge, this association has not been described before. EBV infection was associated with a reduction in Bmem cells, likely because of the selective EBV persistence in these cells.^{60,61} Our large-scale analysis allowed us to separate the herpesvirus-associated effects, with CMV and EBV being associated with Tmem cell expansions and HSV-1 and EBV being associated with a decrease in naive or Bmem cell numbers, respectively.

Sex had a widespread effect on 14 subsets within multiple leukocyte lineages. Interestingly, girls showed a skewing of humoral and early differentiated CD4⁺ T-cell responses over cellular cytotoxic responses in contrast to boys. These effects might be associated with differences in sex hormone levels (testosterone, estradiol), which are already detectable during early infancy, as well as with genetic differences between female and male subjects.⁶² These insights can be especially important for dissection of autoimmune diseases, which are much more prevalent in female than male subjects, although predisposition for allergic diseases seems to be opposite in infancy.^{62,63}

Methodological considerations

The strength of this study is its prospective longitudinal population-based design with more than 1000 children and the possibility to study 26 external determinants in 4 subgroups with adjustments for major confounders. The linear mixed-model approach enabled modeling of cross-sectional data, with further improvement of the accuracy by incorporating additionally available longitudinal follow-up data. Furthermore, we included only children with a 2-generation Dutch ancestry, which prevented interference of our analyses by strong ethnic and cultural influences. However, extrapolation of our findings to different ethnic and cultural populations might be limited and would require additional analysis of ethnically different population cohorts.

The inclusion of 62 leukocyte populations allowed for large-scale analysis of the effect of external determinants on both total cell lineages and small subsets defined by extensive and detailed phenotypic definition. The overlap in some populations could

have skewed the hierarchical clustering. However, our selection of 31 phenotypically nonoverlapping populations resulted in clusters with similar patterns, indicating the robustness of the 4 major patterns of leukocyte dynamics and the observed effects of external determinants.

Our study was primarily explorative, with a focus on the identification of determinants that affected leukocyte dynamics between birth and 6 years of age. Although we defined whether determinants had a positive or negative association with leukocyte numbers, we were unable to identify the exact nature of the effect (ie, exactly when these effects presented and whether these effects will be transient or persistent or potentially even increase over time). Consequently, more research into individual determinants will be needed to extend our observations by specifying these effects.

Finally, special consideration needs to be taken for serology of infectious agents that were measured at the age of 6 years. We cannot determine the exact timing of primary infections, and this can consequently be during the whole period preceding age 6 years. Still, most *H pylori* infections already occur in early childhood, and IgG seropositivity to herpesviruses only appears several weeks to 3 months after infection. Thus these determinants were present already during or before the sixth year of life.

Conclusions

With our unbiased approach, for the first time, we classified leukocyte populations according to their dynamics between birth and 6 years of age. Moreover, we identified nongenetic factors associated with the dynamics of cell lineages or specific leukocyte subsets and that underlie, at least in part, human immunologic diversity. These newly identified determinants can provide new targets for studies on the molecular processes that regulate leukocyte development and immune responses and that together underlie formation of long-lasting immunity without inducing destructive, excessive, or insufficient immune responses.

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Key messages

- Absolute numbers of leukocyte populations differ between subjects, but functionally related leukocyte populations follow similar age-related leukocyte dynamics between birth and 6 years of age.
- We identified nongenetic factors associated with the dynamics of innate leukocyte, B-cell, and CD4⁺ and CD8⁺ T-cell lineages (as well as specific leukocyte subsets) and that underlie, at least in part, human immunologic diversity.

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METHODS

Study subjects

Included in our study are 1182 two-generation Dutch children. Peripheral blood was obtained from 220 children at birth, 376 children at age 5 to 9 months, 241 children at age 13 to 17 months, 257 children at age 22 to 30 months, and 916 children at age 61 to 95 months. Of 237 children, detailed immunophenotyping data were available at 3 or more follow-up time points, and of 90 children, detailed immunophenotyping data were available at 4 or more time points. The complete follow-up at 5 time points was available for 12 children.

Sample preparation

Approximately 4 mL of blood was collected in EDTA anticoagulant tubes by means of venous puncture. Blood was kept at room temperature and processed within 24 hours after sampling. Fifty microliters of full blood was mixed with BD Trucount beads (BD Biosciences) and stained for CD3, CD16, CD56, CD19, and CD45 in a diagnostic lyse-no-wash approach to obtain absolute cell counts. Subsequently, red blood cells were lysed by adding NH_4Cl . Absolute numbers of leukocytes, NK cells, T cells, and B cells were obtained without a wash step on a BD FACSCalibur (BD Biosciences).

Two milliliters of full blood was subjected to bulk lysis of red blood cells by means of 10 minutes of incubation with NH_4Cl . After wash steps, the remaining white blood cells were divided over 10 tubes for 6-color flow cytometric analyses with the antibodies listed in Table E1. After 10 minutes of incubation at room temperature, cells were washed, and immunophenotypic measurements were performed on a 3-laser BD LSR II (BD Biosciences). The 488-nm laser was used to excite the fluorescein isothiocyanate (FITC) (530/30-nm filter), phycoerythrin (575/26 nm), peridinin-chlorophyll-protein complex (PerCP)-Cy5.5 (695/40 nm), and phycoerythrin-Cy7 (780/60 nm) fluorophores and the 633-nm laser for allophycocyanin (APC) (660/20 nm) and APC-Cy7 (780/60 nm). PMT voltages were set by using 8-peak rainbow beads based on target values for the seventh peak, as established within the EuroFlow consortium.^{E1} Absolute cells per microliter of blood of leukocyte subpopulations were recalculated by using the total leukocyte, NK cell, T-cell, and B-cell numbers obtained from the Trucount analysis.

Statistical modeling

Our data set included 62 leukocyte populations and 26 determinants for 1182 children with a total of 2010 data points for each leukocyte population (defined as population “i”).

Linear mixed-effects modeling of leukocyte kinetics.

For each leukocyte population “i” in our data set, the following 4 linear mixed effect models were tested:

$\text{lmR_0knots} <-$

$\text{lmer}(\text{dataset}[i] \sim \text{ns}(\text{Age}, 1) + (1|\text{Id}), \text{REML} = \text{FALSE}, \text{data} = \text{data})$

$\text{lmR_1knots} <-$

$\text{lmer}(\text{dataset}[i] \sim \text{ns}(\text{Age}, 2, \text{knots} = \text{c}(25.5)) + (1|\text{Id}), \text{REML} = \text{FALSE}, \text{data} = \text{dataset})$

$\text{lmR_2knots} <-$

$\text{lmer}(\text{dataset}[i] \sim \text{ns}(\text{Age}, 3, \text{knots} = \text{c}(14.1, 70)) + (1|\text{Id}), \text{REML} = \text{FALSE}, \text{data} = \text{dataset})$

$\text{lmR_3knots} <-$

$\text{lmer}(\text{dataset}[i] \sim \text{ns}(\text{Age}, 4, \text{knots} = \text{c}(6, 14, 24)) + (1|\text{Id}), \text{REML} = \text{FALSE}, \text{data} = \text{dataset}),$

where $\text{ns}(\text{Age}, \dots)$ defines the natural spline of the age of the children, with indicated knots and $(1|\text{Id})$ defines the child's ID number as a random effect in the model, to include longitudinal measurements.

The optimal model was selected by using the likelihood ratio test:

```
anov[1]<-ifelse(anova(lmR_0knots,lmR_1knots)[[8]][2]<0.05,2,0)
anov[2]<-ifelse(anova(lmR_1knots,lmR_2knots)[[8]][2]<0.05,3,0)
anov[3]<-ifelse(anova(lmR_2knots,lmR_3knots)[[8]][2]<0.05,4,0)
selected.lmR<-max(anov).
```

Univariate analyses of the effect of nongenetic determinants on leukocyte kinetics.

Each determinant “j” in our data set was included independently as a fixed effect to the optimal linear mixed effect model of individual leukocyte populations (below an example for a leukocyte population modeled by model lmR_3knots):

```
lmer(dataset[i] ~ ns(Age,4, knots =
c(6,14,24))+determinant[j]+(1|Id),REML=FALSE,data=dataset).
```

Determinants with an effect of a P value of less than .0125 were defined as significant.

Multivariable analyses of the effect of nongenetic determinants on leukocyte kinetics.

All determinants with a significant univariate effect on a leukocyte population (≤ 3 determinants per leukocyte population) were combined and included as fixed effects to the optimal linear mixed-effects model of individual leukocyte populations to correct for confounding effects. See below for an example of a leukocyte population modeled by using model lmR_3knots :

```
lmer(dataset[i] ~ ns(Age,4, knots = c(6,14,24))+significant determinant
1+ significant determinant 2+ etc +(1|Id),REML=FALSE,data=dataset).
```

Determinants with an effect of a P value of less than .05 were defined as significant after multivariable correction.

Define whether a determinant has an age-associated effect on a leukocyte population.

For determinants that still affected leukocyte kinetics after multivariable correction, we subsequently defined whether this effect was significantly stronger within a selective age period (either between the age of 0 months and knot1, knot1 and knot2, knot2 and knot3, or knot3 and 76 months). To test this, the models for multivariable analyses were adjusted, testing individual determinants in relation to the age in the children:

eg. Test the age-associated effect of determinant 1:

```
lmer(dataset[i] ~ ns(Age,4, knots = c(6,14,24))* significant determinant 1 +
significant determinant 2+ etc +(1|Id),REML=FALSE,data=dataset).
```

where “*” indicates a test of the natural spline of the age of the children in relation to determinant 1. The other determinants significantly affecting this leukocyte population were still included as fixed effects to correct for potential confounding effects.

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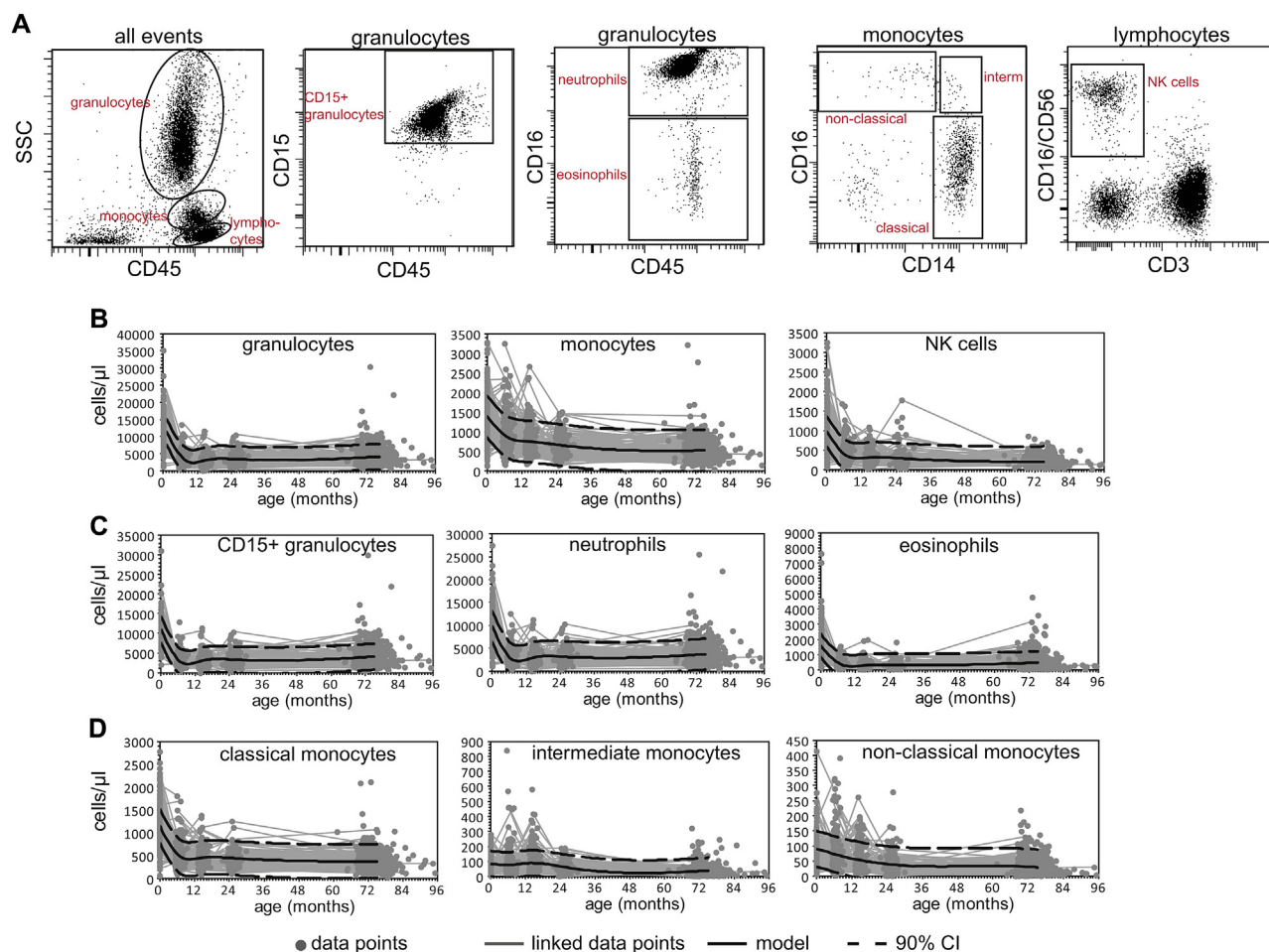


FIG E1. Dynamics of innate leukocyte subsets in children between birth and 6 years of age. **A**, Gating strategy used to identify innate leukocyte subsets. Plots depict data of a representative 61- to 95-month-old child. SSC, Side scatter. **B-D**, Absolute numbers and modeled dynamics of granulocytes, monocytes, and NK cells (Fig E1, B); CD15⁺, neutrophilic, and eosinophilic granulocytes (Fig E1, C); and classical, intermediate, and nonclassical monocyte subsets (Fig E1, D) from birth until 6 years of age. Linear mixed-effects models were generated for each population (solid black line) and represented with the 90% CI of the model (dashed black lines). For clarity of the graphs, gray lines connect only consecutive time points within 1 subject (ie, 0-6 months, 6-14 months, 14-25 months, or 25-76 months).

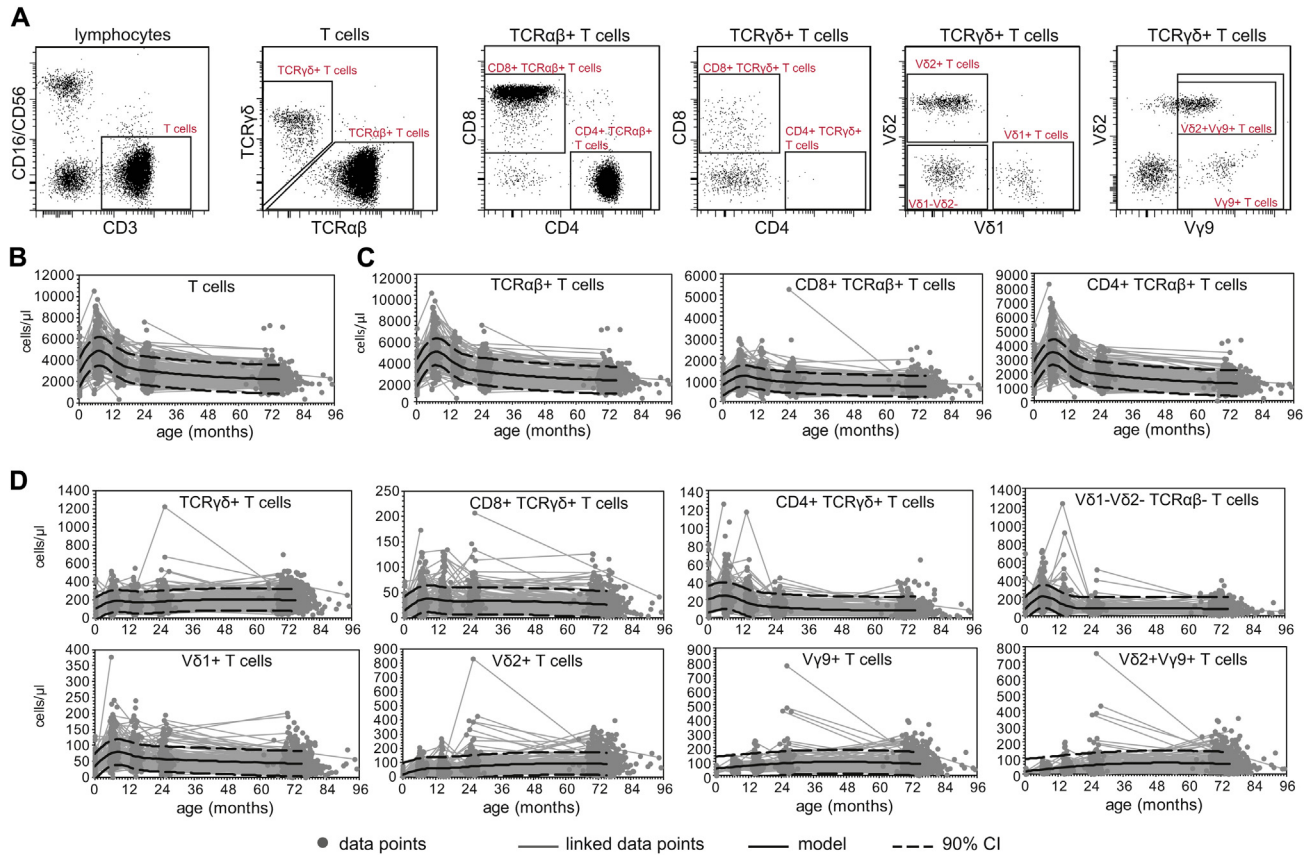


FIG E2. Dynamics of TCRαβ⁺ and TCRγδ⁺ T-cell subsets in children between birth and 6 years of age. **A**, Gating strategy used to identify TCRαβ⁺ and TCRγδ⁺ T-cell subsets. Plots depict data of a representative 61- to 95-month-old child. **B-D**, Absolute numbers and modeled dynamics of T cells (Fig E2, B), TCRαβ⁺ T-cell populations (Fig E2, C), and TCRγδ⁺ T-cell populations (Fig E2, D) from birth until 6 years of age. Linear mixed-effects models were generated for each population (solid black line) and represented with the 90% CI of the model (dashed black lines). For clarity of the graphs, gray lines connect only consecutive time points within 1 subject (ie, 0-6 months, 6-14 months, 14-25 months, or 25-76 months).

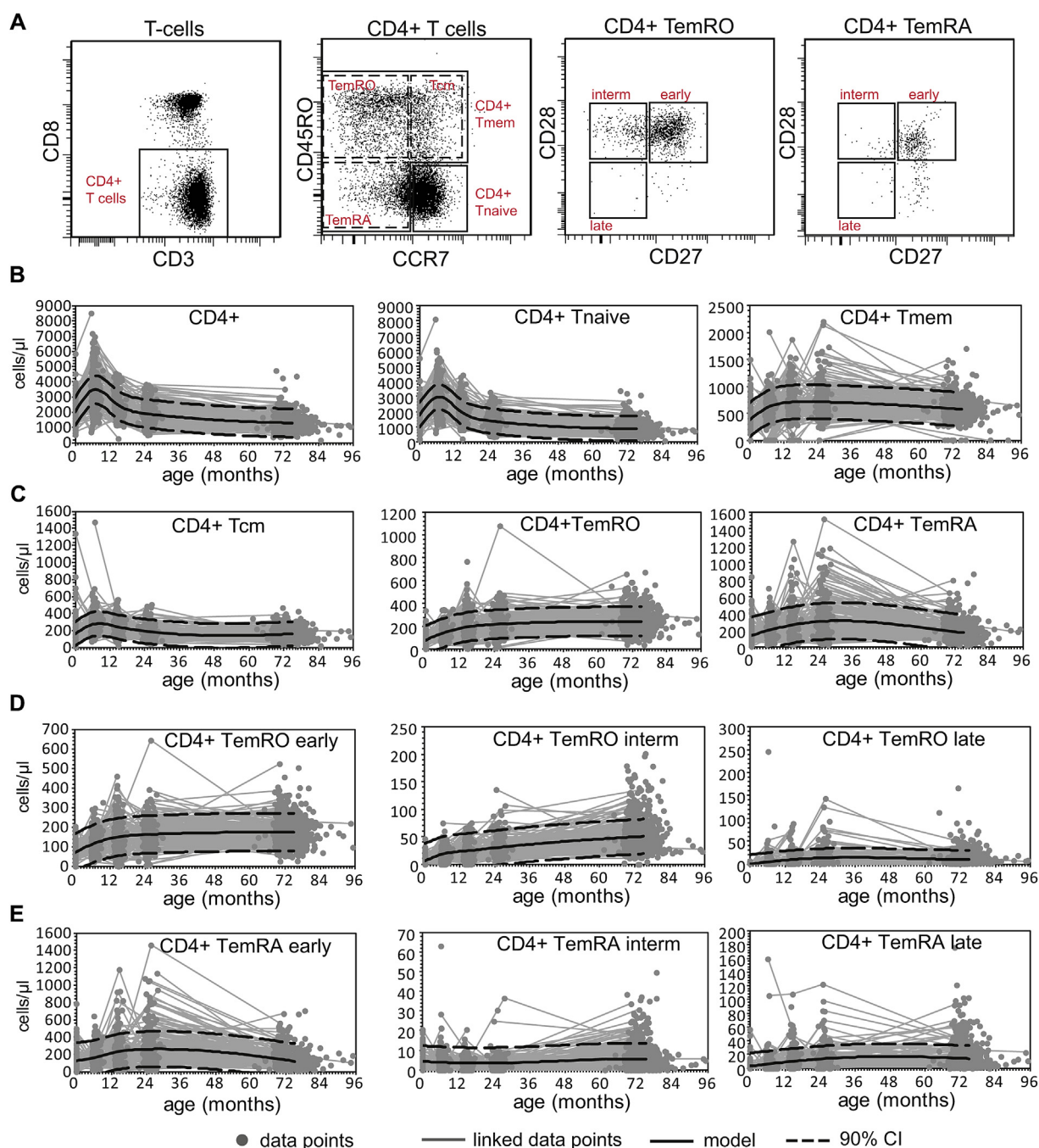


FIG E3. Dynamics of CD4⁺ T-cell subsets in children between birth and 6 years of age. **A**, Gating strategy used to identify CD4⁺ T-cell subsets. Plots depict data of a representative 61- to 95-month-old child. **B-E**, Absolute numbers and modeled dynamics of total, naive, and memory CD4⁺ T cells (Fig E3, **B**); CD4⁺ T_{CM}, CD45RO⁺ effector memory (T_{EMRO}) and CD45RO⁻ effector memory (T_{EMRA}) T-cell subsets (Fig E3, **C**); early, intermediate, or late differentiated T_{EMRO} subsets (Fig E3, **D**); or early, intermediate, or late differentiated T_{EMRA} subsets (Fig E3, **E**) between birth and 6 years of age. Linear mixed-effects models were generated for each population (solid black line) and represented with the 90% CI of the model (dashed black lines). For clarity of the graphs, gray lines connect only consecutive time points within 1 subject (ie, 0-6 months, 6-14 months, 14-25 months, or 25-76 months).

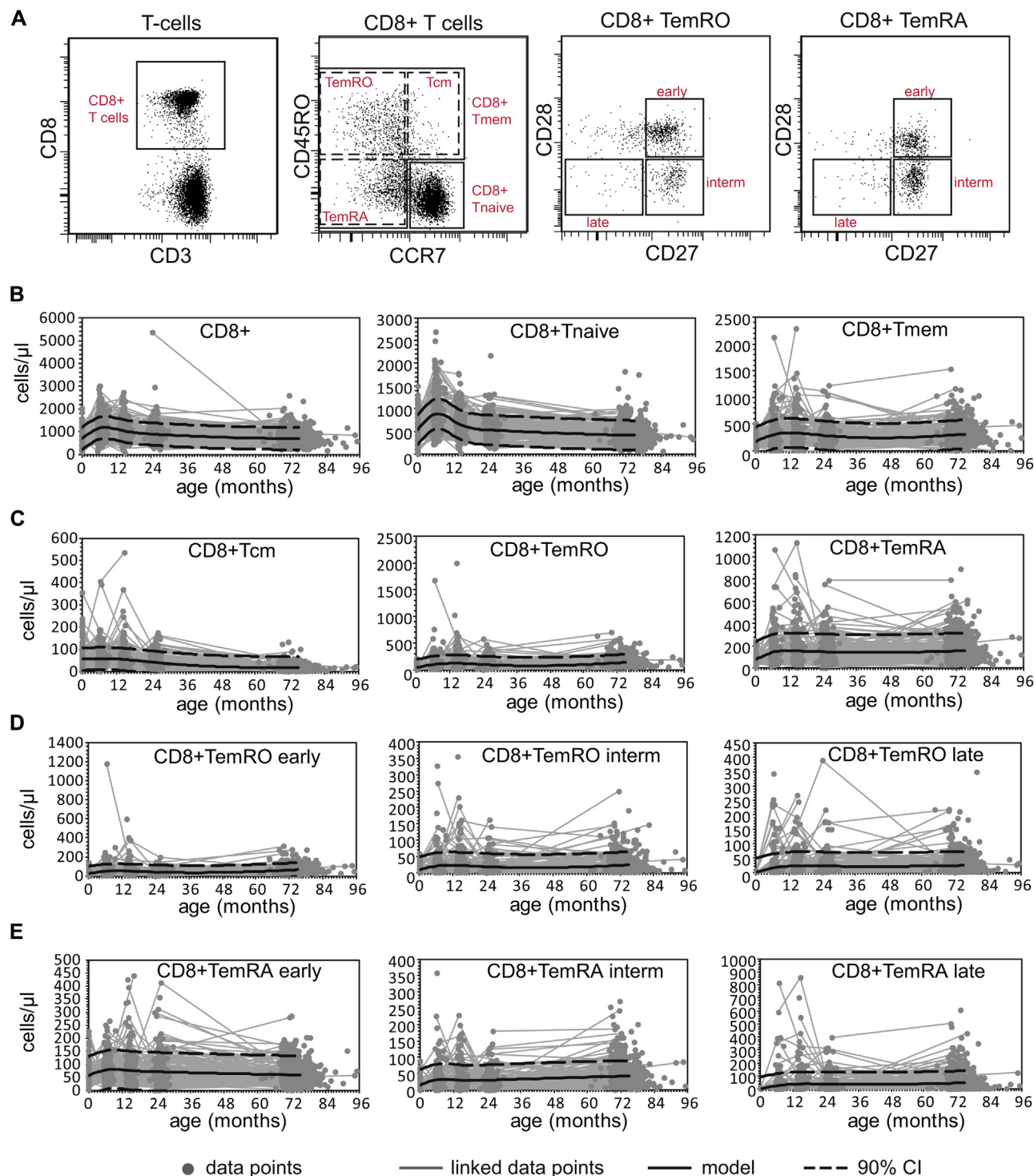


FIG E4. Dynamics of CD8⁺ T-cell subsets in children between birth and 6 years of age. **A**, Gating strategy used to identify CD8⁺ T-cell subsets. Plots depict data of a representative 61- to 95-month-old child. **B-E**, Absolute numbers and modeled dynamics of total, naive, and memory CD8⁺ T cells (Fig E4, B); CD8⁺ T_{CM}, CD45RO⁺ effector memory (T_{EMRO}), and CD45RO⁻ effector memory (T_{EMRA}) T-cell subsets (Fig E4, C); early, intermediate, or late differentiated T_{EMRO} subsets (Fig E4, D); or early, intermediate, or late differentiated T_{EMRA} subsets (Fig E4, E) between birth and 6 years of age. Linear mixed-effects models were generated for each population (solid black line) and represented with the 90% CI of the model (dashed black lines). For clarity of the graphs, gray lines connect only consecutive time points within 1 subject (ie, 0-6 months, 6-14 months, 14-25 months, or 25-76 months).

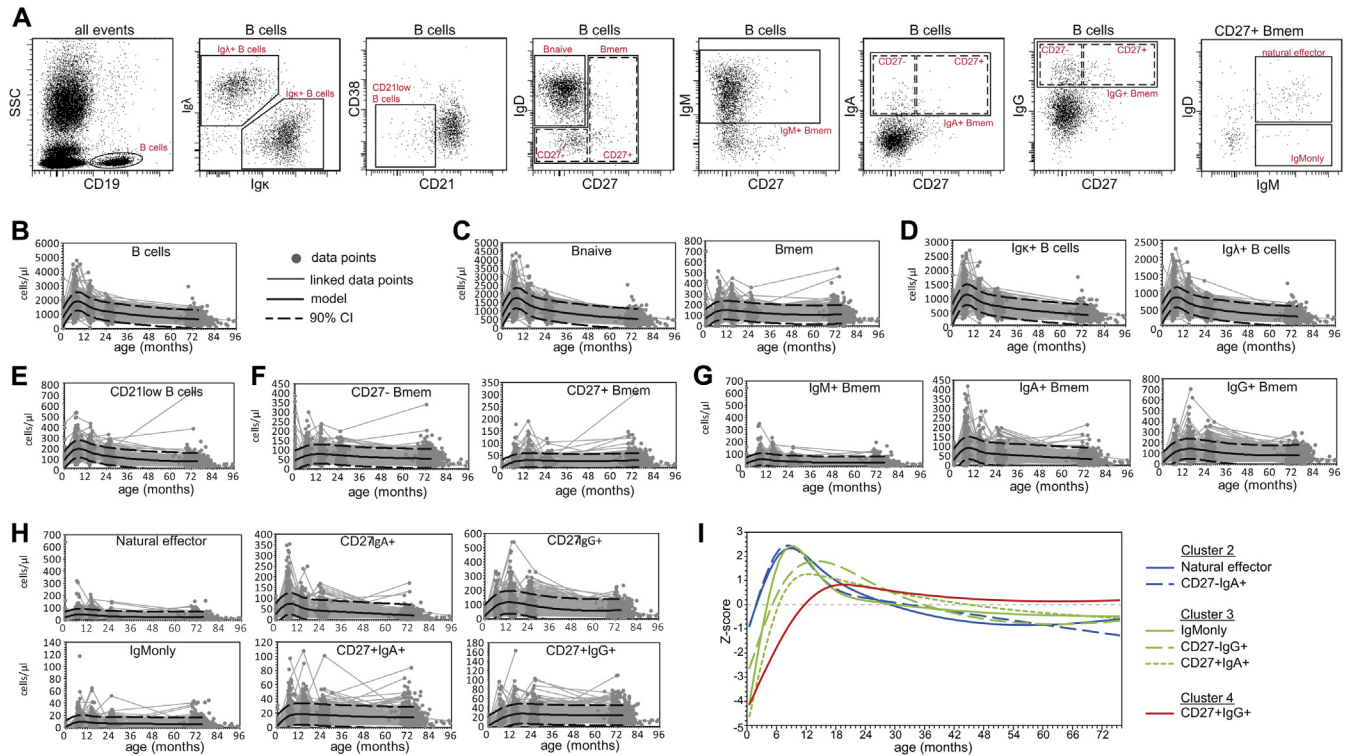


FIG E5. Dynamics of B-cell subsets in children between birth and 6 years of age. **A**, Gating strategy used to identify B-cell subsets. Plots depict data of a representative 61- to 95 month-old child. SSC, Side scatter. **B-H**, Absolute numbers and modeled dynamics of total B cells (Fig E5, **B**); naive and total Bmem cells (Fig E5, **C**); total IgG⁺ and IgA⁺ B-cell populations (Fig E5, **D**); CD21^{low} B cells (Fig E5, **E**); CD27⁻ and CD27⁺ Bmem cells (Fig E5, **F**); total IgM⁺, IgA⁺, or IgG⁺ Bmem cells (Fig E5, **G**); IgM⁺, IgA⁺, or IgG⁺ Bmem cell subsets (Fig E5, **H**) between birth and 6 years of age. Linear mixed-effects models were generated for each population (solid black line) and represented with the 90% CI of the model (dashed black lines). For clarity of the graphs, gray lines connect only consecutive time points within 1 subject (ie, 0-6 months, 6-14 months, 14-25 months, or 25-76 months). **I**, Overlay of the normalized (zero mean; unit SD) linear mixed-effects models of the Bmem cell populations as in Fig E5, **H**.

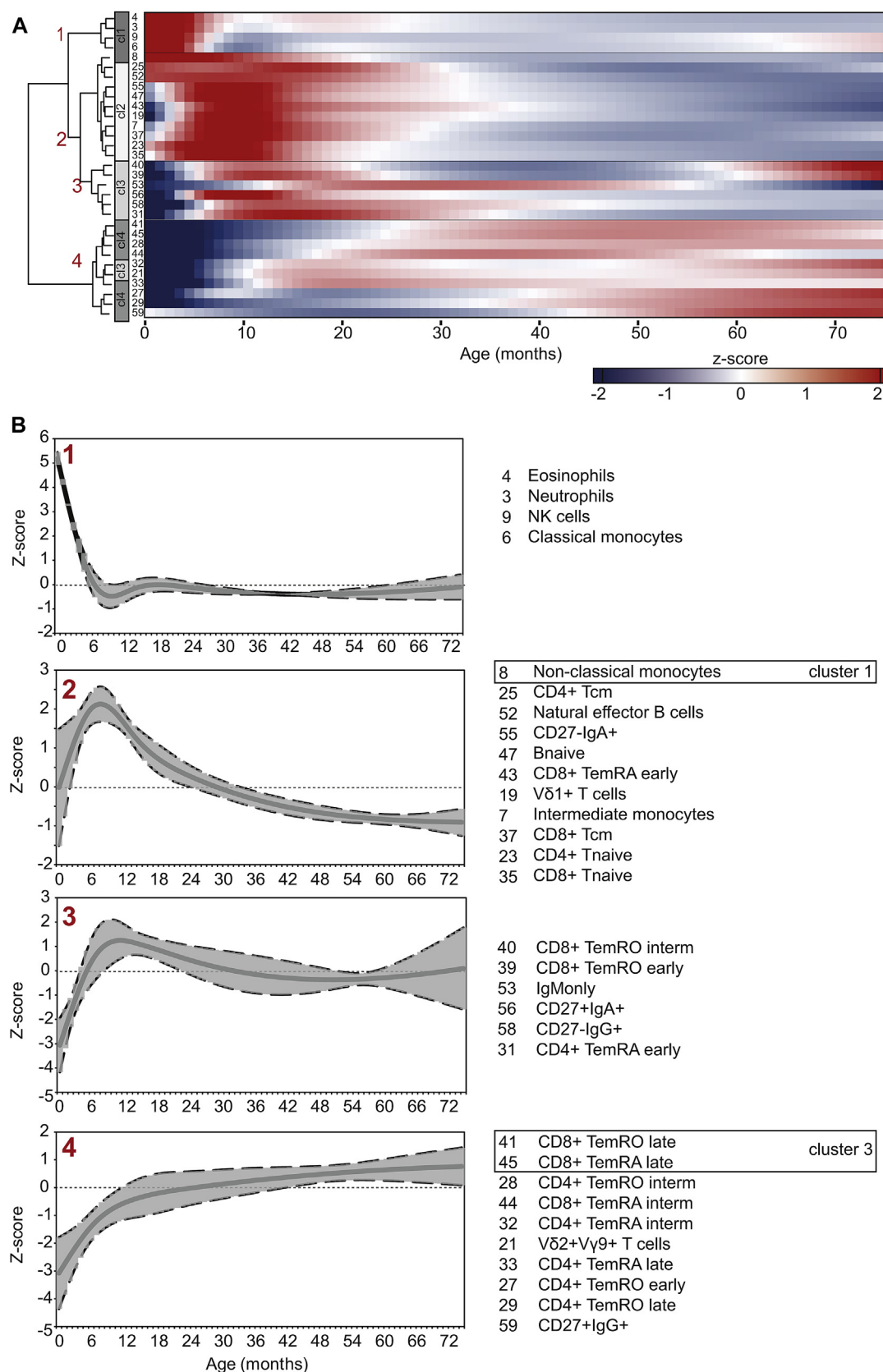


FIG E6. Hierarchical clustering of the dynamics of 31 nonoverlapping leukocyte subsets in early childhood. **A**, Ward hierarchical clustering was performed as in Fig 2, including only the 31 nonoverlapping leukocyte subsets. Indicated in gray squares in front of the heat map is the cluster to which each population was assigned in the analysis of the total 62 leukocyte subsets in Fig 2. **B**, Average patterns ± 1 SD of the subsets in each of the 4 major clusters. Indicated in black squares are 3 subsets that were assigned to different clusters than in Fig 2.

TABLE E1. Antibody details

Tube		Fluorochrome					
		FITC	PE	PerCP-Cy5.5	PE-Cy7	APC	APC-Cy7
1	Antibody	CD3	CD16+CD56	CD45	CD4	CD19	CD8
	Clone	SK7	B73.1+C5.9	2D1	SK3	SJ25C1	SK1
	Manufacturer	BD	BD+Dako	BD	BD	BD	BD
2	Antibody	CD15	—	CD45	CD16	—	CD14
	Clone	MMA	—	2D1	3G8	—	MO-P9
	Manufacturer	BD	—	BD	BD	—	BD
3	Antibody	Igκ	Igλ	CD19	CD21	—	—
	Clone	Polyclonal	Polyclonal	SJ25C1	B-ly-4	—	—
	Manufacturer	Dako	Southern Biotech	BD	BD	—	—
4	Antibody	CD38	—	CD19	CD21	—	—
	Clone	HB7	—	SJ25C1	B-ly-4	—	—
	Manufacturer	BD	—	BD	BD	—	—
5	Antibody	IgD	—	CD19	—	IgM	CD27
	Clone	Polyclonal	—	SJ25C1	—	Polyclonal	L128
	Manufacturer	Southern Biotech	—	BD	—	BD	BD
6	Antibody	IgA	IgG	CD19	—	IgM	CD27
	Clone	Polyclonal	Polyclonal	SJ25C1	—	Polyclonal	L128
	Manufacturer	Kallestad	Southern Biotech	BD	—	BD	BD
7	Antibody	TCRαβ	TCRγδ	CD3	CD4	CD8	—
	Clone	WT31	11F2	SK7	SK3	SK1	—
	Manufacturer	BD	BD	BD	BD	BD	—
8	Antibody	Vδ2	Vδ1*	CD3	CD4	TCRαβ	CD8
	Clone	B6.1	R9.12	SK7	SK3	IP26	SK1
	Manufacturer	BD	Beckman Coulter	BD	BD	eBioscience	BD
9	Antibody	CD28	CD197	CD3	CD8	CD45RO	CD27
	Clone	CD28.2	3D13	SK7	SK1	UCHL-1	L128
	Manufacturer	BD	eBioscience	BD	BD	BD	BD
10	Antibody	Vδ2	Vγ9	CD3	CD4	TCRαβ	CD8
	Clone	B6.1	B3.1	SK7	SK3	IP26	SK1
	Manufacturer	BD	BD	BD	BD	eBioscience	BD

Manufacturers listed are BD Biosciences, San Jose, Calif; DakoCytomation, Glostrup, Denmark; Southern Biotech, Birmingham, Ala; Kallestad Diagnostics, Chaska, Minn; Beckman Coulter, Indianapolis, Ind; and eBioscience, San Diego, Calif.

APC, Allophycocyanin; FITC, fluorescein isothiocyanate; PE, phycoerythrin; PerCP, peridinin-chlorophyll-protein complex.

*Unconjugated antibody detected with goat anti-mouse IgG PE (polyclonal; Invitrogen, Waltham, Mass).

TABLE E2. Age-associated reference values of leukocyte populations

	Percentile	Age category (mo)				
		0	6	14	25	72
No. of children	—	220	376	241	257	916
Age (mo)	5th	0.0	5.5	13.6	23.6	69.3
	25th	0.0	6.0	14.1	24.4	70.6
	50th	0.0	6.2	14.4	25.1	72.4
	75th	0.0	6.7	14.8	25.8	75.0
	95th	0.0	7.7	16.1	27.3	79.0
Innate Leukocytes						
Granulocytes	5th	4,884.2	1,026.1	1,196.3	1,352.5	1,562.4
	25th	8,263.0	2,055.0	2,250.4	1,990.9	2,840.2
	50th	1,0572.7	2,850.0	3,074.1	2,822.9	3,715.0
	75th	13,727.9	4,167.8	4,143.0	3,832.6	5,041.0
	95th	18,784.8	6,519.8	6,056.8	6,467.5	7,597.8
CD15 ⁺ granulocytes	5th	5,161.2	871.6	1,138.2	1,310.8	1,521.0
	25th	8,010.4	1,735.8	2,072.7	1,931.9	2,736.1
	50th	10,397.8	2,617.3	2,973.2	2,690.5	3,608.8
	75th	13,564.7	3,789.1	4,066.6	3,746.3	4,896.7
	95th	17,933.3	5,913.7	5,633.9	6,194.1	7,527.4
Neutrophils	5th	4,433.3	824.4	1,026.1	1,116.3	1,321.7
	25th	7,064.5	1,668.6	19,26.4	1,840.5	2,414.2
	50th	9,091.8	2,497.6	2,750.9	2,533.5	3,274.0
	75th	11,634.0	3,671.1	3,795.5	3,665.9	4,451.2
	95th	16,483.1	5,653.1	5,506.1	5,983.6	6,810.9
Eosinophils	5th	489.5	155.2	84.5	83.9	109.4
	25th	891.3	253.1	178.1	142.2	199.1
	50th	1,340.0	351.6	248.0	203.8	356.4
	75th	1,999.2	486.7	373.2	315.6	598.1
	95th	3,721.9	856.0	763.6	617.5	1,261.5
Monocytes	5th	496.1	360.1	320.6	272.8	233.9
	25th	1,002.2	582.8	504.0	433.1	370.4
	50th	1,346.5	789.6	691.6	577.4	490.9
	75th	1,749.6	1,065.8	962.7	729.6	623.7
	95th	2,429.0	1,755.0	1,510.6	1,066.6	924.1
Classical monocytes	5th	378.1	220.4	192.7	190.0	168.8
	25th	829.5	338.6	301.7	304.0	267.4
	50th	1,114.3	461.1	425.3	402.6	349.6
	75th	1,425.4	623.9	572.6	522.9	455.9
	95th	2,125.5	992.3	849.1	714.1	676.4
Intermediate monocytes	5th	20.6	19.4	26.1	11.6	10.1
	25th	46.6	36.0	42.4	27.9	21.0
	50th	72.3	55.1	70.3	43.7	31.0
	75th	112.1	87.0	116.7	72.0	44.8
	95th	170.0	202.7	243.3	124.3	89.8
Nonclassical monocytes	5th	24.0	16.2	13.4	4.2	4.8
	25th	50.6	39.3	24.3	12.6	11.7
	50th	77.3	60.8	41.3	26.8	22.0
	75th	116.0	92.6	66.1	48.9	41.2
	95th	207.5	162.2	139.2	93.4	80.3
NK cells	5th	229.5	160.0	130.0	120.0	90.0
	25th	460.0	250.0	210.0	180.0	140.0
	50th	840.0	330.0	280.0	240.0	190.0
	75th	1,350.0	450.0	400.0	320.0	250.3
	95th	2,100.5	752.5	690.0	536.0	420.0
Lymphocytes						
T cells	5th	1,478.5	2,587.5	2,180.0	1,586.0	1,329.0
	25th	2,187.5	3,740.0	3,080.0	2,220.0	1,770.0
	50th	2,695.0	4,620.0	3,700.0	2,830.0	2,102.0
	75th	3,457.5	5,690.0	4,610.0	3,650.0	2,571.8
	95th	4,745.5	7,722.5	6,010.0	4,956.0	3,560.0

(Continued)

TABLE E2. (Continued)

		Age category (mo)				
		0	6	14	25	72
TCR $\alpha\beta^+$ T cells	5th	1,403.3	2,509.1	2,063.6	1,461.9	1,170.2
	25th	2,130.3	3,680.3	2,934.0	2,064.0	1,593.1
	50th	2,607.5	4,477.9	3,534.7	2,691.3	1,916.9
	75th	3,318.6	5,426.5	4,401.4	3,345.1	2,328.9
	95th	4,598.5	7,552.0	5,697.5	4,565.6	3,295.1
CD4 $^+$ TCR $\alpha\beta^+$ T cells	5th	957.5	1,788.3	1,388.1	896.6	685.2
	25th	1,446.6	2,679.3	1,926.2	1,338.0	949.5
	50th	1,848.8	3,295.1	2,401.3	1,739.1	1,177.0
	75th	2,338.7	3,994.7	3,023.6	2,233.5	1,450.3
	95th	3,085.2	5,597.1	4,019.3	3,167.0	2,067.5
CD8 $^+$ TCR $\alpha\beta^+$ T cells	5th	351.4	575.1	503.3	411.9	364.1
	25th	542.7	856.5	796.1	630.5	537.4
	50th	736.4	1,101.7	1,013.3	813.2	669.9
	75th	957.6	1,427.4	1,291.6	1,053.7	857.0
	95th	1,454.5	2,112.0	1,894.5	1,582.7	1,227.1
TCR $\gamma\delta^+$ T cells	5th	31.5	69.1	68.8	63.7	82.3
	25th	63.1	123.3	106.5	109.3	132.2
	50th	87.7	164.0	162.8	163.1	178.2
	75th	126.0	226.5	213.2	219.8	248.2
	95th	225.1	333.2	332.6	352.8	377.7
CD4 $^+$ TCR $\gamma\delta^+$ T cells	5th	5.4	6.6	4.8	3.4	2.1
	25th	11.5	13.7	9.1	6.6	4.4
	50th	17.6	20.4	13.3	10.4	6.9
	75th	26.0	31.6	19.3	14.7	10.4
	95th	43.5	50.8	36.0	25.2	18.5
CD8 $^+$ TCR $\gamma\delta^+$ T cells	5th	3.2	8.5	7.2	6.7	6.9
	25th	7.6	18.2	15.7	14.3	14.3
	50th	12.6	27.7	28.3	25.7	22.3
	75th	19.4	44.4	42.6	41.2	33.7
	95th	39.8	81.0	92.5	75.4	62.2
V δ 1 $^-$ V δ 2 $^-$ TCR $\alpha\beta^-$ T cells	5th	22.9	69.1	28.0	30.6	23.6
	25th	43.1	113.5	53.6	49.0	43.9
	50th	62.5	173.8	89.9	70.7	65.9
	75th	89.7	274.8	130.6	95.9	93.6
	95th	164.1	448.2	288.3	151.5	164.7
V δ 2 $^+$ T cells	5th	4.0	13.1	11.4	12.9	22.8
	25th	8.1	30.4	28.8	30.8	50.9
	50th	12.2	43.5	47.5	53.5	77.1
	75th	19.7	65.5	74.1	90.9	118.4
	95th	35.0	124.4	127.8	177.5	212.9
V δ 1 $^+$ T cells	5th	7.6	29.0	23.4	18.5	13.6
	25th	18.6	48.2	42.2	33.2	25.4
	50th	30.7	65.1	62.8	49.7	37.7
	75th	43.8	96.4	87.4	67.8	54.3
	95th	69.2	163.8	135.5	134.4	94.4
V γ 9 $^+$ T cells	5th	15.1	25.0	28.0	24.7	25.5
	25th	20.1	39.9	43.5	45.1	46.8
	50th	29.7	56.7	62.8	67.1	69.5
	75th	38.7	80.8	100.3	99.2	104.0
	95th	53.6	119.0	181.9	174.8	187.0
V δ 2 $^+$ V γ 9 $^+$ T cells	5th	1.5	5.2	9.0	8.7	13.3
	25th	3.6	11.8	18.3	20.9	31.0
	50th	5.7	22.9	31.7	39.2	49.9
	75th	12.5	38.4	58.0	66.0	83.9
	95th	24.3	71.0	116.5	135.7	163.1
CD4 $^+$ T cells	5th	1,060.7	1,820.5	1,430.2	922.3	737.1
	25th	1,552.3	2,706.7	1,991.4	1,362.5	988.2
	50th	1,972.5	3,366.2	2,457.8	1,782.7	1,240.0
	75th	2,498.9	4,101.9	3,062.0	2,288.7	1,525.3
	95th	3,266.2	5,553.1	4,123.0	3,217.7	2,134.0
CD4 $^+$ T naive	5th	848.7	1,519.9	941.0	575.3	392.9
	25th	1,321.1	2,233.6	1,469.5	916.6	621.4

(Continued)

TABLE E2. (Continued)

		Age category (mo)				
Percentile		0	6	14	25	72
CD4 ⁺ Tmem	50th	1,695.9	2,849.1	1,972.0	1,256.0	821.9
	75th	2,159.7	3,460.9	2,461.5	1,662.9	1,069.7
	95th	2,898.2	4,841.4	3,373.4	2,336.6	1,538.0
	5th	161.0	354.1	331.6	325.1	344.5
	25th	259.6	470.8	526.9	504.6	461.7
CD4 ⁺ T _{CM}	50th	355.9	580.2	669.0	651.4	559.2
	75th	465.1	713.2	834.9	850.9	679.5
	95th	754.6	953.0	1,227.9	1,274.9	878.5
	5th	39.5	134.9	111.0	86.7	74.2
	25th	81.4	202.7	176.0	129.5	112.5
CD4 ⁺ T _{EMRO}	50th	121.3	261.9	242.8	173.1	149.0
	75th	198.3	322.1	313.2	223.6	198.9
	95th	395.6	449.3	427.4	349.3	285.0
	5th	23.4	61.2	31.8	76.9	118.3
	25th	51.5	93.0	123.7	152.4	181.5
CD4 ⁺ early T _{EMRO}	50th	73.8	138.3	184.3	205.4	235.2
	75th	107.0	177.5	254.7	260.3	295.5
	95th	172.6	260.8	387.9	387.4	408.5
	5th	21.0	50.8	20.6	54.6	82.5
	25th	47.6	79.0	95.6	116.7	127.1
CD4 ⁺ intermediate T _{EMRO}	50th	65.0	110.3	148.8	154.8	168.0
	75th	98.9	144.8	199.8	197.5	210.1
	95th	155.8	211.9	301.6	276.4	292.4
	5th	0.0	2.7	2.6	9.7	18.2
	25th	1.8	10.2	14.0	17.7	29.7
CD4 ⁺ late T _{EMRO}	50th	3.8	17.3	23.0	26.2	43.9
	75th	7.0	24.7	33.2	36.4	61.5
	95th	19.6	38.5	53.4	64.0	105.2
	5th	0.0	0.0	0.0	1.4	1.1
	25th	0.0	0.0	2.5	3.8	3.0
CD4 ⁺ T _{EMRA}	50th	0.0	0.0	5.1	7.7	6.3
	75th	0.0	3.8	11.2	17.6	12.9
	95th	3.3	14.3	29.0	43.4	30.1
	5th	35.9	79.9	50.3	58.1	55.8
	25th	89.2	122.0	115.1	144.0	102.4
CD4 ⁺ early T _{EMRA}	50th	126.8	178.0	221.2	245.8	153.6
	75th	181.1	241.2	343.4	415.3	227.8
	95th	312.1	376.8	570.5	812.5	371.7
	5th	27.9	66.7	33.1	40.3	36.3
	25th	74.2	104.3	91.1	114.3	67.6
CD4 ⁺ intermediate T _{EMRA}	50th	113.7	149.9	189.8	213.6	110.7
	75th	162.7	204.6	304.4	356.7	168.4
	95th	287.3	357.8	542.8	782.6	317.4
	5th	0.0	0.0	0.0	0.0	0.8
	25th	0.0	0.0	0.0	1.4	1.8
CD4 ⁺ late T _{EMRA}	50th	2.7	3.6	2.7	2.7	3.6
	75th	5.1	5.7	4.6	4.7	6.9
	95th	13.5	11.0	9.0	7.8	18.2
	5th	0.0	0.0	0.0	1.7	1.7
	25th	0.0	0.0	3.3	4.4	4.7
CD8 ⁺ T cells	50th	1.5	3.6	6.9	8.8	9.4
	75th	2.9	6.6	15.2	16.0	17.4
	95th	10.4	17.8	44.5	42.0	42.6
	5th	316.6	527.3	447.2	389.3	335.8
	25th	493.3	836.6	776.2	619.4	509.0
CD8 ⁺ T naive	50th	655.5	1,093.1	998.8	805.1	649.7
	75th	886.4	1,389.5	1,277.1	1,038.8	841.6
	95th	1,319.3	2,060.9	1,831.8	1,571.6	1,197.8
	5th	182.4	380.4	269.6	205.6	166.1
	25th	351.1	591.2	453.9	363.8	287.2
	50th	487.2	835.1	610.3	465.1	383.6
	75th	695.2	1,081.6	809.5	640.3	513.2

(Continued)

TABLE E2. (Continued)

		Age category (mo)				
		0	6	14	25	72
CD8 ⁺ T _{mem}	95th	1,037.3	1,632.3	1,238.7	1,033.7	768.1
	5th	57.2	98.1	102.0	71.6	122.4
	25th	102.8	171.4	181.4	152.3	199.0
	50th	149.1	236.3	268.9	224.3	261.0
	75th	214.9	343.6	432.3	331.4	365.3
CD8 ⁺ T _{CM}	95th	351.2	674.6	888.7	550.0	597.5
	5th	10.2	16.7	13.5	9.5	5.1
	25th	27.7	32.1	26.1	18.6	9.3
	50th	47.7	49.2	39.8	31.0	14.4
	75th	71.7	72.6	68.8	56.3	21.7
CD8 ⁺ T _{EMRO}	95th	147.6	128.0	140.4	109.2	43.8
	5th	7.9	16.7	17.8	14.0	40.5
	25th	17.3	39.3	43.5	36.0	75.5
	50th	29.6	62.6	74.9	67.5	114.9
	75th	48.4	106.4	151.2	108.1	166.3
CD8 ⁺ early T _{EMRO}	95th	98.7	284.8	362.8	244.3	281.8
	5th	5.5	10.7	7.4	6.4	19.4
	25th	12.2	22.7	22.7	19.6	38.1
	50th	22.2	36.5	38.3	30.5	56.6
	75th	32.9	55.4	68.0	48.7	79.9
CD8 ⁺ intermediate T _{EMRO}	95th	64.3	106.8	145.0	88.0	132.0
	5th	0.3	0.9	0.1	0.5	3.1
	25th	2.1	4.7	4.7	3.4	8.5
	50th	5.8	10.2	11.6	8.2	17.1
	75th	11.3	21.5	29.9	18.1	30.2
CD8 ⁺ late T _{EMRO}	95th	27.7	64.9	98.6	56.1	64.0
	5th	0.0	0.0	0.0	0.8	2.2
	25th	0.0	1.2	3.2	3.7	6.5
	50th	0.0	3.1	8.3	9.2	14.0
	75th	0.0	9.4	22.0	24.5	30.4
CD8 ⁺ T _{EMRA}	95th	0.7	85.0	94.8	73.9	72.6
	5th	18.9	34.4	33.0	14.8	44.5
	25th	43.1	76.8	69.9	53.0	86.0
	50th	62.5	109.4	122.2	102.8	129.4
	75th	96.3	168.9	224.6	180.9	194.2
CD8 ⁺ early T _{EMRA}	95th	165.1	345.1	467.4	367.6	346.9
	5th	11.7	22.3	13.7	5.5	17.9
	25th	29.2	44.5	36.4	28.8	33.5
	50th	48.0	68.3	62.1	54.1	52.1
	75th	75.7	106.0	101.0	89.9	76.4
CD8 ⁺ intermediate T _{EMRA}	95th	123.8	167.0	211.4	169.2	139.8
	5th	1.4	2.6	2.9	1.1	8.9
	25th	6.7	9.8	11.0	6.8	20.4
	50th	11.5	20.8	22.8	17.5	33.8
	75th	18.8	38.9	46.8	34.7	53.4
CD8 ⁺ late T _{EMRA}	95th	40.9	91.3	114.4	79.7	108.0
	5th	0.0	0.0	1.2	1.2	4.4
	25th	0.0	2.8	5.5	5.4	11.9
	50th	0.4	6.3	11.8	12.9	24.6
	75th	1.0	15.6	36.6	33.0	52.2
B cells	95th	2.1	90.4	187.3	151.6	162.6
	5th	240.0	810.0	710.0	520.0	321.5
	25th	467.5	1,310.0	1,150.0	860.0	480.0
	50th	675.0	1,720.0	1,490.0	1,130.0	630.0
	75th	930.0	2,260.0	1,950.0	1,440.0	765.3
B naive	95th	1,663.5	3,277.5	2,590.0	1,890.0	1,110.0
	5th	260.8	733.9	621.0	466.0	250.2
	25th	425.6	1,190.7	1,001.1	772.7	389.5
	50th	616.3	1,609.4	1,339.5	1,019.3	513.6
	75th	854.8	2,104.3	1,757.7	1,292.0	639.9
Bmem	95th	1,500.9	3,030.8	2,310.1	1,731.0	933.7
	5th	16.2	47.2	59.1	48.8	46.0

(Continued)

TABLE E2. (Continued)

		Age category (mo)				
	Percentile	0	6	14	25	72
	25th	31.4	79.3	98.4	78.3	74.1
	50th	48.0	118.1	142.1	108.2	98.3
	75th	81.2	171.6	191.7	151.5	131.2
	95th	168.4	291.3	305.1	212.6	200.0
CD27 ⁻ Bmem	5th	7.7	20.7	27.4	29.4	20.8
	25th	19.3	38.7	52.6	44.2	35.8
	50th	29.6	62.1	72.2	62.8	48.8
	75th	51.7	88.6	98.6	90.5	65.9
	95th	116.2	153.7	157.5	130.0	103.8
CD27 ⁺ Bmem	5th	0.0	4.1	6.2	6.3	9.8
	25th	1.2	10.0	18.4	12.0	17.0
	50th	1.7	16.3	28.1	21.1	25.2
	75th	3.7	27.7	42.5	30.9	37.2
	95th	7.0	65.2	75.3	57.1	65.9
IgM ⁺ Bmem	5th	3.1	11.4	12.2	8.9	8.7
	25th	6.8	24.5	26.0	15.5	16.2
	50th	13.0	41.8	41.2	24.7	25.0
	75th	24.7	65.9	68.0	38.1	36.8
	95th	67.3	131.2	133.8	70.2	61.7
Natural effector	5th	2.7	8.8	9.6	6.9	6.9
	25th	6.3	19.3	20.1	12.3	13.0
	50th	12.5	33.7	33.3	20.4	19.7
	75th	22.3	53.3	55.8	31.3	30.2
	95th	65.2	107.4	113.5	58.3	52.1
IgM only	5th	0.0	1.0	0.8	0.9	1.0
	25th	0.0	2.8	3.6	2.4	2.4
	50th	0.6	5.7	6.0	4.0	4.1
	75th	1.3	10.8	10.1	6.5	7.1
	95th	3.6	27.2	25.2	15.6	15.7
IgA ⁺ Bmem	5th	5.8	16.9	16.4	15.0	11.6
	25th	11.8	41.3	38.0	30.7	20.4
	50th	21.6	70.2	60.8	47.5	28.9
	75th	36.3	117.1	90.9	75.2	40.0
	95th	67.9	225.2	151.6	131.3	68.1
CD27 ⁻ IgA ⁺	5th	4.7	11.3	10.4	10.1	5.0
	25th	10.3	31.2	25.3	20.3	10.2
	50th	19.0	55.8	43.4	32.4	16.0
	75th	31.9	94.5	67.3	55.6	23.8
	95th	62.9	196.7	119.1	101.9	42.6
CD27 ⁺ IgA ⁺	5th	0.0	3.0	3.3	3.5	4.5
	25th	0.9	7.4	9.8	8.1	8.4
	50th	1.7	12.0	16.3	13.2	12.2
	75th	3.1	20.1	26.7	21.6	17.3
	95th	7.9	42.2	44.2	37.1	27.9
IgG ⁺ Bmem	5th	4.2	30.9	33.1	29.7	31.3
	25th	8.5	61.0	74.3	61.6	55.7
	50th	13.3	87.5	116.8	94.0	77.0
	75th	22.4	143.4	187.6	160.8	102.3
	95th	47.9	229.2	318.2	280.1	158.2
CD27 ⁻ IgG ⁺	5th	3.6	23.3	25.9	23.8	19.2
	25th	7.1	48.7	52.6	45.6	36.8
	50th	11.2	76.6	87.7	75.4	53.8
	75th	19.2	124.2	151.0	129.2	74.1
	95th	42.2	200.3	274.1	224.0	119.3
CD27 ⁺ IgG ⁺	5th	0.0	3.4	4.4	5.2	7.7
	25th	0.8	8.1	14.7	12.6	14.7
	50th	1.6	12.3	23.0	19.6	21.1
	75th	3.3	19.2	37.9	33.4	30.1
	95th	7.2	35.3	60.8	58.8	51.0
CD21 ^{low} B cells	5th	22.0	66.2	60.4	43.1	22.0

(Continued)

TABLE E2. (Continued)

		Age category (mo)				
	Percentile	0	6	14	25	72
Igλ ⁺ B cells	25th	38.8	113.7	98.2	74.5	38.4
	50th	57.8	157.2	142.8	100.3	55.2
	75th	91.1	222.4	193.7	134.8	78.1
	95th	166.2	351.1	282.9	220.0	124.0
	5th	115.0	323.7	290.3	203.9	127.0
	25th	220.7	545.5	500.0	351.5	188.8
	50th	313.6	757.4	638.0	476.5	251.3
	75th	417.7	975.7	840.0	636.2	317.4
	95th	798.4	1,486.1	1,186.2	847.7	477.2
	5th	126.1	456.6	404.8	293.4	179.0
Igκ ⁺ B cells	25th	248.8	718.2	635.8	490.5	272.3
	50th	352.2	967.0	813.6	634.5	357.0
	75th	493.0	1,263.5	1,101.9	821.5	439.7
	95th	841.4	1,857.9	1,429.7	1,057.4	621.4

The presented reference values (in cells per microliter of blood) were obtained by using cross-sectional analyses of all 2,010 data points on the indicated time points.

TABLE E3. Overall effect estimates and association with specific age periods

Innate leukocytes								
Models with 3 knots								
Leukocyte population	Determinant	Significance	Effect estimate	SE	Significance: age intervals			
					0-6 mo	6-14 mo	14-24 mo	24-76 mo
Granulocytes	—	—	—	—	—	—	—	—
CD15 ⁺ granulocytes	Sex (girl, yes/no)	**	359.6	111.6	**	*	****	NS
Neutrophils	Sex (girl, yes/no)	**	296.4	106.6	NS	NS	NS	NS
Eosinophils	Low maternal education level	**	−63.1	24.5	NS	NS	NS	NS
Monocytes	Low maternal education level	**	−51.5	18.1	NS	*	**	NS
Classical monocytes	Low maternal education level	**	−34.3	13.0	*	NS	**	NS
Intermediate monocytes	—	—	—	—	—	—	—	—
NK cells	Seropositivity for CMV at age 6 y	**	34.7	12.9	NS	NS	NS	NS
	Siblings >1	****	−53.2	12.1	****	NS	****	NS
Models with 2 knots								
Leukocyte population	Determinant	Significance	Effect estimate	SE	0-14.1 mo	14.1-70 mo	70-76 mo	
Nonclassical monocytes	Antibiotics/penicillin use in first year	**	6.0	2.2	NS	NS	NS	
Lymphocytes								
Models with 3 knots								
Leukocyte population	Determinant	Significance	Effect estimate	SE	0-6 mo	6-14 mo	14-24 mo	24-76 mo
T cells	—	—	—	—	—	—	—	—
TCRαβ ⁺ T cells	Carrier of <i>H pylori</i> within 6 y	*	303.4	120.1	NS	NS	NS	*
CD4 ⁺ TCRαβ ⁺ T cells	Sex (girl, yes/no)	**	96.9	36.1	NS	NS	NS	NS
CD8 ⁺ TCRαβ ⁺ T cells	Seropositivity for CMV at 6 y	***	79.2	22.1	NS	NS	*	NS
	Seropositivity for EBV at 6 y	*	51.4	20.4	NS	*	*	NS
TCRγδ ⁺ T cells	Sex (girl, yes/no)	*	−11.7	4.6	NS	NS	NS	NS
CD4 ⁺ TCRγδ ⁺ T cells	—	—	—	—	—	—	—	—
CD8 ⁺ TCRγδ ⁺ T cells	Low maternal education level	**	3.9	1.3	NS	NS	**	NS
	Seropositivity for CMV at 6 y	***	4.9	1.3	**	NS	NS	NS
	Cesarean section	**	−4.8	1.7	NS	NS	NS	NS
Vδ1 [−] Vδ2 [−] TCRαβ [−] T cells	—	—	—	—	—	—	—	—
Vδ2 ⁺ T cells	Low maternal education level	**	7.9	2.9	NS	NS	NS	NS
	Breast-feeding at 6 mo of age	*	−6.2	3.1	NS	NS	NS	NS
	Premature rupture of membranes	**	19.5	7.2	NS	NS	NS	NS
Vδ1 ⁺ T cells	Seropositivity for CMV at 6 y	****	9.6	1.9	NS	NS	**	NS
CD4 ⁺ T cells	Sex (girl, yes/no)	**	107.9	37.1	NS	NS	NS	NS
CD4 ⁺ T naive	Sex (girl, yes/no)	*	81.3	32.4	NS	NS	NS	NS
CD4 ⁺ Tmem	—	—	—	—	—	—	—	—
CD4 ⁺ T _{CM}	Sex (girl, yes/no)	**	12.1	4.6	NS	NS	**	NS
CD4 ⁺ T _{EMRO}	Seropositivity for CMV at 6 y	***	20.1	5.4	NS	NS	NS	NS
	Seropositivity for EBV at 6 y	**	15.5	5.0	NS	NS	*	NS
CD4 ⁺ early T _{EMRO}	Sex (girl, yes/no)	**	11.2	3.8	NS	NS	NS	NS

(Continued)

TABLE E3. (Continued)

Lymphocytes								
Models with 3 knots								
Leukocyte population	Determinant	Significance	Effect estimate	SE	0-6 mo	6-14 mo	14-24 mo	24-76 mo
CD4 ⁺ intermediate T _{EMRO} CD8 ⁺ T cells	Seropositivity for EBV at 6 y	*	9.8	3.8	NS	NS	*	NS
	Cesarian section	*	-13.1	5.3	NS	NS	NS	NS
	Seropositivity for CMV at 6 y	***	5.4	1.4	NS	NS	NS	NS
	Seropositivity for CMV at 6 y	***	82.3	21.9	NS	NS	**	NS
CD8 ⁺ T naive CD8 ⁺ Tmem	Seropositivity for EBV at 6 y	*	47.1	20.3	NS	*	**	NS
	—	—	—	—	—	—	—	—
CD8 ⁺ T _{CM} CD8 ⁺ T _{EMRO} CD8 ⁺ early T _{EMRO} CD8 ⁺ intermediate T _{EMRO}	Seropositivity for CMV at 6 y	****	104.3	11.0	*	NS	****	NS
	Seropositivity for EBV at 6 y	****	41.2	10.2	NS	NS	*	NS
	Anti-TPO (before 18 wk of pregnancy) [mU/mL])	***	0.02	0.007	****	****	**	****
	Seropositivity for CMV at 6 y	****	41.0	6.2	NS	NS	*	NS
CD8 ⁺ late T _{EMRO} CD8 ⁺ T _{EMRA} CD8 ⁺ early T _{EMRA} CD8 ⁺ intermediate T _{EMRA}	Seropositivity for EBV at 6 y	****	31.3	5.8	NS	NS	*	NS
	Seropositivity for EBV at 6 y	****	14.1	2.6	NS	NS	*	NS
	Seropositivity for CMV at 6 y	****	7.7	1.6	NS	NS	*	NS
	Seropositivity for EBV at 6 y	****	9.2	1.5	NS	NS	**	NS
CD8 ⁺ late T _{EMRA} B cells B naive Bmem	Seropositivity for CMV at 6 y	****	24.8	1.9	**	NS	***	NS
	Seropositivity for EBV at 6 y	**	4.8	1.8	NS	**	NS	NS
	Sex (girl, yes/no)	**	-18.1	6.0	**	NS	NS	NS
	Seropositivity for CMV at 6 y	****	64.9	6.5	NS	NS	****	NS
CD8 ⁺ early T _{EMRA} CD8 ⁺ intermediate T _{EMRA} CD8 ⁺ late T _{EMRA} CD27 ⁻ Bmem	—	—	—	—	—	—	—	—
	Seropositivity for CMV at 6 y	****	8.0	2.0	NS	NS	NS	NS
	Seropositivity for EBV at 6 y	**	5.5	1.9	NS	NS	*	NS
	Sex (girl, yes/no)	***	-13.7	3.6	**	*	NS	NS
CD27 ⁺ Bmem IgM ⁺ Bmem Natural effector IgM only	Seropositivity for CMV at 6 y	****	53.3	3.9	**	NS	****	NS
	Seropositivity for HSV-1 at 6 y	**	-111.2	35.8	NS	NS	NS	NS
	Seropositivity for HSV-1 at 6 y	***	-108.8	33.0	NS	NS	NS	NS
	Low maternal education level	*	-8.4	3.5	NS	NS	NS	NS
CD27 ⁻ Bmem CD27 ⁺ Bmem IgM ⁺ Bmem Natural effector IgM only	Seropositivity for EBV at 6 y	**	-9.3	3.4	NS	NS	NS	NS
	Low maternal education level	*	-4.7	2.0	NS	NS	NS	NS
	Seropositivity for EBV at 6 y	**	-6.2	2.0	NS	NS	*	NS
	Seropositivity for EBV at 6 y	*	-2.7	1.1	*	NS	NS	NS
IgM ⁺ Bmem Natural effector IgM only	—	—	—	—	—	—	—	—
	—	—	—	—	—	—	—	—
	—	—	—	—	—	—	—	—
	—	—	—	—	—	—	—	—

(Continued)

TABLE E3. (Continued)

Lymphocytes								
Models with 3 knots								
Leukocyte population	Determinant	Significance	Effect estimate	SE	0-6 mo	6-14 mo	14-24 mo	24-76 mo
IgA ⁺ Bmem	Breast-feeding at 6 mo of age	*	−5.8	2.3	NS	NS	NS	*
CD27 [−] IgA ⁺	Carrier of <i>H pylori</i> within 6 y	****	22.0	4.9	NS	NS	*	****
	Breast-feeding at 6 mo of age	**	−4.9	1.9	NS	NS	NS	NS
CD27 ⁺ IgA ⁺	Carrier of <i>H pylori</i> within 6 y	****	17.8	4.1	NS	NS	*	****
	Sex (girl, yes/no)	**	1.6	0.6	NS	NS	NS	NS
IgG ⁺ Bmem	Carrier of <i>H pylori</i> within 6 y	*	3.1	1.2	NS	NS	NS	*
	—	—	—	—	—	—	—	—
CD27 [−] IgG ⁺	Antibiotics/penicillin use in first year	*	7.1	2.8	NS	NS	NS	NS
CD27 ⁺ IgG ⁺	Sex (girl, yes/no)	**	2.1	0.7	NS	NS	NS	NS
CD21 ^{low} B cells	Low maternal education level	***	−11.7	3.2	NS	NS	NS	NS
Igλ ⁺ B cells	Seropositivity for HSV-1 at 6 y	**	−53.0	16.6	NS	NS	NS	NS
Igκ ⁺ B cells	Seropositivity for HSV-1 at 6 y	**	−59.5	19.9	NS	NS	NS	NS
	Carrier of <i>H pylori</i> within 6 y	**	82.2	30.9	NS	NS	NS	**
Models with 2 knots								
Leukocyte population	Determinant	Significance	Effect estimate	SE	0-14.1 mo	14.1-70 mo	70-76 mo	
CD4 ⁺ late T _{EMRO}	Seropositivity for CMV at 6 y	****	6.1	0.7	NS	NS	*	
	Seropositivity for EBV at 6 y	***	2.7	0.7	NS	NS	NS	
	Premature rupture of membranes	**	5.0	1.8	NS	****	NS	
CD4 ⁺ T _{EMRA}	—	—	—	—	—	—	—	
CD4 ⁺ early T _{EMRA}	—	—	—	—	—	—	—	
CD4 ⁺ intermediate T _{EMRA}	Sex (girl, yes/no)	**	−0.7	0.2	NS	NS	NS	
	Premature rupture of membranes	**	1.9	0.6	NS	NS	NS	
Models with 1 knot								
Leukocyte population	Determinant	Significance	Effect estimate	SE	0-25.5 mo	25.5-76 mo		
CD4 ⁺ late T _{EMRA}	Sex (girl, yes/no)	***	−3.0	0.9	NS	NS		
	Seropositivity for CMV at 6 y	****	7.4	0.9	****	*		
	Seropositivity for EBV at 6 y	*	1.8	0.9	NS	NS		
Vγ9 ⁺ T cells	Premature rupture of membranes	**	23.1	7.4	*	NS		
Vδ2 ⁺ Vγ9 ⁺ T cells	Premature rupture of membranes	***	24.6	6.6	*	NS		

NS, Not significant.

P* < .05.*P* < .01.****P* < .001.*****P* < .0001.