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High Proportion of CD5⁺ B Cells in Infants Predicts Development of Allergic Disease

Anna-Carin Lundell,* Susanne Johansen,[†] Ingegerd Adlerberth,[‡] Agnes E. Wold,[‡] Bill Hesselmar,[§] and Anna Rudin*

Delayed maturation of the immune system has been proposed to be a risk factor for development of allergy, but B cell maturation in relation to allergic disease has not been examined. B cells lose CD5 and acquire CD27 during maturation from immature via mature/naïve to Ig-secreting cells and memory cells. We sought to investigate B cell maturation in relation to development of allergic disease and sensitization in the FARMFLORA birth cohort including 65 Swedish children. Total B cell numbers, proportions of CD5⁺ and CD27⁺ B cells, and levels of IgM, IgG, IgA, and IgE were measured in blood on repeated occasions from birth to 36 mo of age, and related to allergic disease and sensitization at 18 and 36 mo of age with multivariate discriminant analysis. We also compared the expression of CD24 and CD38 within CD5⁺ and CD5^{neg} B cells in children and in adults. We found that infants with a high proportion of CD5⁺ B cells at birth and at 1 mo of age had an increased risk for having allergic disease at 18 and 36 mo of life. Further, the proportions of CD5⁺ B cells at 1 mo of age were inversely correlated with total IgG levels at 18 and 36 mo of age. The majority of the CD5⁺ B cells were of a CD24^{hi}/CD38^{hi} immature/naïve phenotype at birth (97%), 7 y of age (95%), and in adults (86%). These results suggest that development of allergic disease is preceded by an immaturity in neonatal B cell phenotype. *The Journal of Immunology*, 2014, 193: 000–000.

The prevalence of allergic diseases has increased in economically developed countries over the last 100 y, and they are now the major chronic disorders in young individuals in countries with a Western lifestyle (1). According to the “hygiene hypothesis” proposed by Strachan (2) in 1989, reduced exposure to microbes early in life may lead to delayed immune maturation and, as a consequence, development of allergic disease. Allergic disease results from the failure to develop immune tolerance to innocuous inhaled or ingested Ags, referred to as allergens. Instead, an immune response with production of allergen-specific IgE Abs occurs, that is, sensitization. It has been shown that transient low IgE Ab responses to food proteins appear relatively often in infants who remain healthy, whereas high concentrations of food protein-specific IgE are almost exclusively seen in infants with allergic disease (3, 4). Although sensitization can occur without symptoms of allergic disease, children who are sensitized at 18 mo

of age without allergic symptoms are more likely than others to experience development of asthma and rhinitis at 5 y of age (5). In some children, however, eczema has been found to precede and predict sensitization (5, 6). Strangely, although B cells are of importance for sensitization and allergic disease to develop, it is currently unknown whether the postnatal B cell maturation in the periphery during the first years of life differs between children who become sensitized and/or develop allergic disease and those who remain healthy.

The current model for human peripheral B cell development involves five major stages: immature transitional B cells that are recent bone marrow emigrants, mature naïve B cells, actively engaged germinal center B cells, memory B cells, and Ig-secreting plasma cells (7). Expression of CD24 in combination with CD38 is now acknowledged to distinguish human peripheral B cell maturational stages, that is, CD24^{hi}CD38^{hi} immature transitional cells, CD24⁺CD38⁺ naïve cells, and CD24^{hi}CD38^{neg} memory cells (8–10). Transitional cells represent ~4% of B cells in healthy adult peripheral blood, but in cord blood they constitute more than half of the B cell pool (11). The vast majority of the transitional B cells are positive for CD5 in both newborns and adults (10–12). In mice, the expression of CD5 defines a specific B cell lineage, referred to as B-1a cells, that are predominantly found in the peritoneal cavity (13). However, because there is a gradual decrease in CD5 expression during maturation from immature transitional via mature naïve to memory B cells (10–12), this marker does not identify a specific B cell subset in humans. Instead, as the peripheral proportion of CD5⁺ B cells progressively decreases in an age-dependent manner (14, 15), similar to that of CD24^{hi}CD38^{hi} B cells (11), CD5 expression likely represents immature/naïve B cells. In line with this, CD5⁺ B cells have fewer somatic hypermutations than CD5^{neg} B cells and predominantly produce spontaneous low-affinity IgM Abs (16, 17). Still, if delayed B cell maturation in early infancy, that is, high proportions of peripheral immature/naïve CD5⁺ B cells, are associated with an increased risk for development of sensitization and/or allergic disease later in childhood is unknown.

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Abbreviations used in this article: DA, discriminant analysis; OPLS, orthogonal partial least squares; OPLS-DA, orthogonal partial least squares discriminant analysis; VIP, variable influence on projection.

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Naive B cells that recognize their specific Ag become activated and may mature to memory B cells, which involves upregulation of CD27 (18). Accordingly, memory B cells defined by the CD24⁺ CD38^{neg} phenotype express CD27 (10). Interaction with T cells in germinal centers in the lymphoid organs also leads to class-switch from IgM to IgA, IgG, or IgE and mutations in the Ag-binding region. In humans, CD27 expression correlates with the presence of somatic hypermutations in the variable (V) region of the Ig genes (19). However, memory B cells also include CD27⁺ class-switched cells (20, 21), as well as CD27⁺ IgM-expressing B cells (22). Whether high proportions of Ag-experienced memory B cells early in life reduce the risk for development of allergic disease and/or sensitization later in childhood has not been examined.

In the FARMFLORA birth cohort, we investigated the relation between blood B cell numbers, the proportions of CD5⁺ and CD27⁺ B cells, Ig levels, and development of allergic disease and sensitization over the first 3 y of life. With the use of multivariate discriminant analysis (DA), we found that children who develop allergic disease differ from children who remain healthy regarding the early peripheral B cell maturation. In particular, infants with a high proportion of CD5⁺ B cells early in life had an increased risk for allergic disease within the first 18 or 36 mo of life. Moreover, the majority of the CD5⁺ B cells were of a CD24^{hi/+}CD38^{hi/+} immature/naive phenotype in both children and adults. Thus, our results suggest that B cell immaturity may predispose to allergic disease.

Materials and Methods

Subjects and collection of blood samples

Sixty-five healthy Swedish infants born at term (≥ 38 gestational weeks) in rural areas in the Skaraborg region in South-West Sweden were included in the prospective FARMFLORA study. Twenty-eight of the children were raised on small dairy farms, whereas 37 lived on the countryside, but not on a farm. Blood samples were obtained from the umbilical cord at the delivery ($n = 48$), and peripheral blood was sampled at 3–5 d ($n = 59$), 1 mo ($n = 59$), 4 mo ($n = 58$), 18 mo ($n = 63$), and 36 mo ($n = 50$) of age. The present cohort is similar to the Immunoflora cohort (23) but includes children from the countryside, whereas the latter comprises children from urban areas. Cord blood samples ($n = 5$) were also collected from unselected healthy newborn infants born at term (≥ 38 gestational weeks) at the Sahlgrenska University Hospital, and peripheral blood was obtained from healthy 7-y-old children ($n = 5$) and adult volunteers ($n = 5$) with no relation to the newborns. Informed consent was obtained from the parents and the volunteers, and the study protocol was approved by the Human Research Ethics Committee of the Medical Faculty, University of Gothenburg, Sweden.

Clinical and laboratory examinations for allergy diagnoses

The children were examined by a pediatrician at 18 and 36 mo of age. The children were also clinically examined between follow-ups in case they experienced signs and symptoms suggestive of allergic disease. Venous blood was collected for total IgE and presence of specific IgE against food (milk, egg, soy, fish, wheat, and peanut; six-mix food test; Phadia/Pharmacia Diagnostics, Uppsala, Sweden) and inhalant allergens (birch, timothy grass, mugwort, cat, dog, horse, and house dust mite; Phadiatop; Phadia/Pharmacia Diagnostics). Positive samples were further analyzed for specific IgE against birch, timothy, mugwort, dog, cat, horse, house dust mite, cow's milk, hen's egg, fish, wheat, soy, and peanut (ImmunoCAP; Phadia/Pharmacia Diagnostics). An allergen-specific IgE level ≥ 0.35 kU/l was considered positive. In the evaluation of children who were referred to the study pediatrician before 18 mo of age because of symptoms, allergen-specific IgE tests (ImmunoCAP) or skin prick tests were performed guided by symptoms. Skin prick tests were performed in accordance with European guidelines using standard allergen extracts (Soluprick SQ; ALK-Abello AS, Hørsholm, Denmark), allergen diluents as the negative control and histamine (10 mg/ml) as positive control.

Diagnostic groups

Based on clinical examination by the pediatrician and the results of the laboratory tests, the following diagnostic groups were defined: *eczema* was

diagnosed according to Williams' criteria (24). Eczema at 18 mo denoted diagnosis at any time before or at 18 mo, whereas eczema at 36 mo required symptoms to be present after 24 mo of age. *Asthma* is persistent wheezing for ≥ 4 wk or ≥ 3 episodes of wheezing combined with ≥ 1 minor criterion (symptoms between colds, eczema, allergic rhinoconjunctivitis, or food allergy). For asthma at 36 mo, ≥ 1 wheezing episode should have occurred after 24 mo of age, and response to inhaled glucocorticoids or leukotriene antagonists was included among the minor criteria. *Food allergy* is an immediate or late-onset reaction after ingestion of the specific food, followed by a clear and prompt clinical improvement once the food allergen was eliminated, together with a positive six-mix food ImmunoCAP test and/or verified by an open food challenge test. *Allergic rhinoconjunctivitis* is symptoms in the eyes and/or nose upon exposure to pollens or animal dander, together with a positive allergen-specific IgE test directed against a corresponding allergen. *Sensitization* is a positive six-mix food and/or Phadiatop test.

Flow cytometry analysis in the FARMFLORA study

The flow cytometry procedure for phenotypic characterization of B cells in the FARMFLORA study was performed as previously described in detail (15). The following mAbs were used in the flow cytometry panel: PerCP-conjugated anti-CD20 (clone L27; BD Bioscience, Erembodegem, Belgium), allophycocyanin-conjugated anti-CD5 (clone UCHT2; Pharmingen, San Diego, CA), FITC-conjugated anti-CD27 (clone L128; BD Biosciences), and allophycocyanin- and FITC-conjugated mouse IgG₁ (clone X40; allophycocyanin IgG₁ and FITC IgG₁; BD Biosciences) were used as controls when gating on the CD5⁺ and CD27⁺ populations. For absolute cell numbers, the TruCOUNT assay was used (BD Biosciences), also described in detail elsewhere (15). Samples were run in a FACSCalibur (BD Biosciences) equipped with CellQuestPro software, or in a FACS-Canto II (BD Biosciences) equipped with FACSDiva software and analyzed with FlowJo software (Tree Star, Ashland, OR).

Analysis of CD24 and CD38 expression within the CD5⁺ and CD5^{neg} B cell populations

After the FARMFLORA study was initiated, expression of CD24 in combination with CD38 has been recognized to distinguish peripheral human immature transitional, mature naïve, and memory B cells. For analysis of CD24 and CD38 expression within the CD5⁺ or CD5^{neg} populations in blood samples from unselected healthy newborns, 7-y-old children, and healthy adults (Fig. 3), the following mAbs were used: allophycocyanin-conjugated anti-CD5 (as described earlier), allophycocyanin-H7-conjugated anti-CD20 (clone L27), PE-Cy7-conjugated anti-CD24 (clone ML5), and PE-conjugated anti-CD38 (clone HB7), all purchased from BD Biosciences. The cutoff for CD5, CD24, and CD38 was determined by the fluorochrome minus one control (25). All samples were run in a FACS-Canto II (BD Biosciences) equipped with FACSDiva software and analyzed with FlowJo software (Tree Star, Ashland, OR).

Quantification of total IgM, IgG, and IgA in plasma

Levels of total IgM, IgG, and IgA in plasma collected in the FARMFLORA study were determined by ELISA as described in detail elsewhere (26). ELISA plates were obtained from Nunc (Roskilde, Denmark); all IgM, IgG, and IgA Abs were obtained from Jackson ImmunoResearch (Baltimore, MD); and standards were obtained from Calbiochem (Darmstadt, Germany).

B cell cultures and analysis of secreted IgE and IgG

Mononuclear cells from cord ($n = 3$) or adult ($n = 3$) peripheral blood were isolated by density gradient centrifugation over Lymphoprep and stained with FITC-conjugated CD19 (clone HIB19; BD Biosciences, San Jose, CA) and Brilliant Violet 421-conjugated CD5 (clone UCHT2; Biolegend, San Diego, CA) for 20 min at 4°C. Thereafter, CD19⁺CD5⁺ and CD19⁺CD5^{neg} B cells were sorted using an iCyt Synergy cell sorter (Sony Biotech, Champaign, IL). The CD5⁺ and the CD5^{neg} B cells (purity after sorting 90–97%) were cultured at a concentration of 10×10^3 cells/well in U-bottom 96-well plates in RPMI 1640 media (PAA Laboratories GmbH, Pasching, Austria) supplemented with 10% heat-inactivated FCS, 1 mM L-glutamine, and 50 μ g/ml gentamicin, and kept in 5% CO₂ at 37°. The purified CD5⁺ and the CD5^{neg} B cells were stimulated with recombinant human CD40L alone (prepared as membranes of insect cells infected with baculovirus expressing CD40L, a generous gift from Dr. Stuart Tangye) (27, 28) or in the presence of IL-4 (100 U/ml; Amoytop Biotech, Xiamen, China) and IL-21 (50 ng/ml; Peprotech, Offenbach, Germany) for 10 d. Secretion of total IgE in the supernatant was analyzed with the use of an ImmunoCAP 250 (Phadia, Uppsala, Sweden), and IgG was determined by nephelometry with the use of

an Image 800 (Beckman Coulter, Bromma, Sweden) at the Clinical Immunology Laboratory of the Sahlgrenska University Hospital.

Statistical analysis

Multivariate factor analysis (SIMCA-P+ software; Umetrics, Umeå, Sweden) was used to examine the relation between allergic disease or sensitization (Y-variables) and various B cell parameters (X-variables). Orthogonal projection to latent structures DAs were implemented to examine whether allergic compared with nonallergic and sensitized compared with nonsensitized children could be discriminated based on the various X-variables examined. Orthogonal partial least squares (OPLS) was also implemented to correlate a selected Y-variable and X-variables to each other in linear multivariate models. All data were scaled to unit variance by dividing each variable by $1/(\text{SD})$, where SD represents the SD value of each variable, so that all the variables were given equal weight regardless of their absolute value. The quality was assessed based on the parameters R^2 , that is, how well the variation of the variables is explained by the model, and Q^2 , that is, how well a variable can be predicted by the model. OPLS plots in the *Results* are based on X-variables with variable influence on projection (VIP) values >0.8 . VIP values can be used to discriminate between important and unimportant predictors for the model. In the OPLS analyses, the importance of each X-variable to Y is represented by column bars. Jackknifing was used to calculate SEs displayed as an error bar on each column (representing the 95% confidence interval). Univariate analyses were performed exclusively on the X-variables that contributed most to the respective multivariate models to avoid mass significance. A receiver operating characteristic curve was used to evaluate diagnostic value of the proportion of CD5⁺ B cells for allergic disease, and the area under the curve was computed. Univariate analyses were performed with two-tailed *t* test, Mann–Whitney *U* test, Pearson correlation test, or χ^2 test for trend as described in the figure legends (GraphPad Prism; GraphPad Software, La Jolla, CA). A *p* value ≤ 0.05 was regarded as being statistically significant ($*p \leq 0.05$ and $**p \leq 0.01$).

Results

Allergic disease and sensitization

By the age of 18 and 36 mo, 23 and 17% of the children, respectively, were diagnosed with an allergic disease, that is, eczema, food allergy, allergic rhinoconjunctivitis, and/or asthma (Table I). Sensitization occurred in 15 and 25% of the children at these time points (Table I). Diagnosing food allergy in young children is a challenge; therefore, we introduced strict criteria in this study. Data regarding in vitro tests, organ(s) involved, and food challenge tests are described in detail for the children who were diagnosed with food allergy in Supplemental Table I. For further analyses, we compared children who were diagnosed with any allergic disease at 18 mo of age ($n = 15$) with those who were not ($n = 49$). Children who were diagnosed with allergic disease at 36 mo ($n = 11$) were compared with those who were healthy at both

18 and 36 mo of age ($n = 44$; Table II). For this analysis, eight children were excluded from comparisons at 36 mo because they were diagnosed with allergic disease at 18 mo, but not at 36 mo of age, and could neither be included in the group of children with allergic disease nor in the group of healthy children. One child did not undergo clinical examination at 18 mo of age, and was hence excluded from the 36-mo analyses. Sensitized and nonsensitized children were compared in the same way; that is, at 18 mo, sensitized children ($n = 9$) were compared with those who were not sensitized ($n = 53$), and at 36 mo, sensitized children ($n = 14$) were compared with those who were nonsensitized at both 18 and 36 mo of age ($n = 39$; Table II). In this cohort, some of the children who were sensitized did not present with allergic disease, and some of the children with allergic disease were not sensitized (Supplemental Table II). Yet, the frequency of sensitization at 18 and 36 mo was higher among children with an allergic disease relative to nonallergic children ($p = 0.03$ and $p = 0.05$, respectively). There was also a higher proportion of boys among those with an allergic disease by 36 mo of age (Table II). The total IgE levels did not differ between children having an allergic disease or not, either at 18 (32 versus 19 kU/l, $p = 0.68$) or 36 mo of age (130 versus 20 kU/l, $p = 0.07$).

High proportions of CD5⁺ B cells are positively associated with allergic disease later in childhood

It is unknown whether the B cell maturation early in infancy is associated with development of allergic disease later in childhood. Thus, we investigated whether the proportions of immature CD5⁺ or memory CD27⁺ B cells, plasma levels of total IgM, IgG, IgA, and IgE, and the absolute numbers of lymphocytes or B cells (X-variables), measured in blood at several time points during the first 36 mo of age, were associated with allergic disease at 18 and 36 mo of age (Y-variables). OPLS-DA demonstrated a separation between children having an allergic disease or not at both 18 and 36 mo of age based on the X-variables described earlier (Fig. 1A, 1B). The majority of the children with an allergic disease appeared in the *two left quadrants*, whereas the majority of the nonallergic children were plotted in the *two right quadrants* in both models (Fig. 1A, 1B). In Fig. 1A, three children diagnosed with allergic disease are located outside the ellipse. These outliers are due to variation or missing data in one or more variables compared with the other observations. The X-variables that displayed the strongest association (positive or negative) with allergic disease at 18 and 36 mo of age are identified in the OPLS-DA column plots in Fig. 1C and 1D, respectively. Fig. 1C and 1D are models based on X-variables with VIP values >0.8 . VIP column plots for all X-variables up to 18 or 36 mo of age, respectively, are found in Supplemental Fig. 1. X-variables represented by a bar pointing in the same direction as allergic disease are positively associated, whereas X-variables pointing in the opposite direction are related to not being allergic. Allergic disease at 18 mo of age was positively associated with high proportions of CD5⁺ B cells at 3–5 d, 1 mo, in cord blood, and at 18 mo of age (Fig. 1C). Conversely, allergic disease at 18 mo of age was negatively related with high numbers of B cells and lymphocytes at 4 mo of age and a high proportion of CD27⁺ B cells at 3–5 d of age (Fig. 1C). Being diagnosed with an allergic disease at 36 mo of age was also positively associated with high proportions of CD5⁺ B cells at several time points (Fig. 1D). Allergic disease at 36 mo of age was negatively associated with high numbers of B cells at both 4 and 36 mo of age, high lymphocyte counts, and high levels of total IgG (Fig. 1D). Thus, these results indicate that children with allergic disease and nonallergic children differ with respect to postnatal B cell maturation.

Table I. Allergic disease and sensitization at 18 and 36 mo of age

	18 mo ($n = 64$) ^a		36 mo ($n = 63$) ^a	
	<i>n</i>	%	<i>n</i>	%
Any allergic disease	15	23	11	17
Food allergy ^b	2	3	2	3
Eczema	13	20	7	11
Allergic rhinoconjunctivitis ^c	1	2	1	2
Asthma	5	8	4	6
Sensitization ^d	9	15 ^e	14	25 ^f

^aOne child did not undergo clinical examination at 18 mo of age ($n = 64$), and two children ended the study after 18 mo of age ($n = 63$).

^bAll children diagnosed with food allergy had a positive six-mix food ImmunoCAP test.

^cAll children diagnosed with allergic rhinoconjunctivitis had a positive Phadiatop test.

^dScreening for allergen-specific IgE, that is, six-mix food test and Phadiatop, followed by analysis for specific IgE against cow's milk, hen's egg, fish, wheat, soy, peanut, birch, timothy, mugwort, dog, cat, horse, and house dust mite (ImmunoCAP).

^e $n = 62$ (blood samples not available for all 64 children).

^f $n = 56$ (blood samples not available for all 63 children).

Table II. Demographic data

	Number of Children (%)													
	Allergic Disease							Sensitization						
	All (<i>n</i> = 65)	At 18 mo			At 36 mo			<i>p</i> ^b	At 18 mo			At 36 mo		<i>p</i> ^b
		No (<i>n</i> = 49)	Yes (<i>n</i> = 15)	<i>p</i> ^b	No (<i>n</i> = 44)	Yes (<i>n</i> = 11)	<i>p</i> ^b		No (<i>n</i> = 53)	Yes (<i>n</i> = 9)	<i>p</i> ^b	No (<i>n</i> = 39)	Yes (<i>n</i> = 14)	
Boys	33 (51)	24 (49)	9 (60)	0.56	22 (50)	9 (82)	0.03	27 (51)	5 (56)	1.00	21 (54)	9 (64)	0.55	
Parental history of allergy ^a	27 (42)	18 (37)	8 (53)	0.37	15 (34)	7 (64)	0.09	23 (43)	2 (22)	0.29	18 (46)	6 (43)	1.0	
Sibling(s)	35 (54)	28 (57)	7 (47)	0.56	23 (52)	6 (55)	1.0	30 (57)	4 (44)	0.72	22 (56)	7 (50)	0.76	
Cesarean section	10 (15)	6 (12)	4 (27)	0.23	5 (11)	4 (36)	0.07	7 (13)	3 (33)	0.15	5 (13)	5 (36)	0.11	

^aAt least one parent with a history of doctor-diagnosed asthma, allergic rhinoconjunctivitis, or eczema.
^bStatistical difference between healthy children and children with allergic disease (Fisher's exact test or Mann-Whitney *U* test).

Children with allergic disease have higher proportions of CD5⁺ B cells in early infancy than children who remain nonallergic

Multivariate findings were corroborated by univariate analyses (Fig. 2). Children with allergic disease at 18 or 36 mo of age had significantly higher proportions of CD5⁺ B cells at birth and at 1 mo of age relative to nonallergic children (Fig. 2A, 2B), whereas children with allergic disease had significantly lower total B cell counts at 4 mo compared with children who remained healthy (allergic versus nonallergic at 18 mo: *p* = 0.03; at 36 mo: *p* = 0.04). The gating strategy for CD5 expression on B cells in the FARMFLORA study cohort is demonstrated in Fig. 2C, which

also shows representative plots on the proportion of CD5⁺ B cells in children with allergic disease or not.

In Fig. 2D, we stratified the infants into quartiles according to percentage of CD5⁺ B cells at 1 mo and compared the prevalence of children with allergic disease at 36 mo of age in these four groups. The first quartile had the lowest prevalence of allergic children (0%), whereas the quartile of infants with the highest percentage of CD5⁺ B cells had the highest prevalence of allergic children (40%; Fig. 2D). A similar trend was observed for allergic disease at 18 mo of age (*p*_{trend} = 0.13). Receiver operating curve analysis was used to evaluate the diagnostic value of the proportion of CD5⁺ B cells in separating children with an allergic disease or not. An

FIGURE 1. Allergic and nonallergic children differ with respect to postnatal B cell maturation. (A and B) OPLS-DA score scatter plot showing the separation of children with allergic disease (black dots) and nonallergic children (gray dots) at 18 and 36 mo of age, respectively, based on the B cell variables examined. (C and D) OPLS-DA column loading plots depicting the associations between allergic disease at (C) 18 and (D) 36 mo of age and B cell variables. X-variables represented by a bar pointing in the same direction as allergic disease (located to the far left) are positively associated, whereas variables in the opposite direction are inversely related to allergic disease. The OPLS-DA column plots are based on B cell variables with VIP-values >0.8. R2Y indicates how well the variation of Y is explained, whereas Q2 indicates how well Y can be predicted.

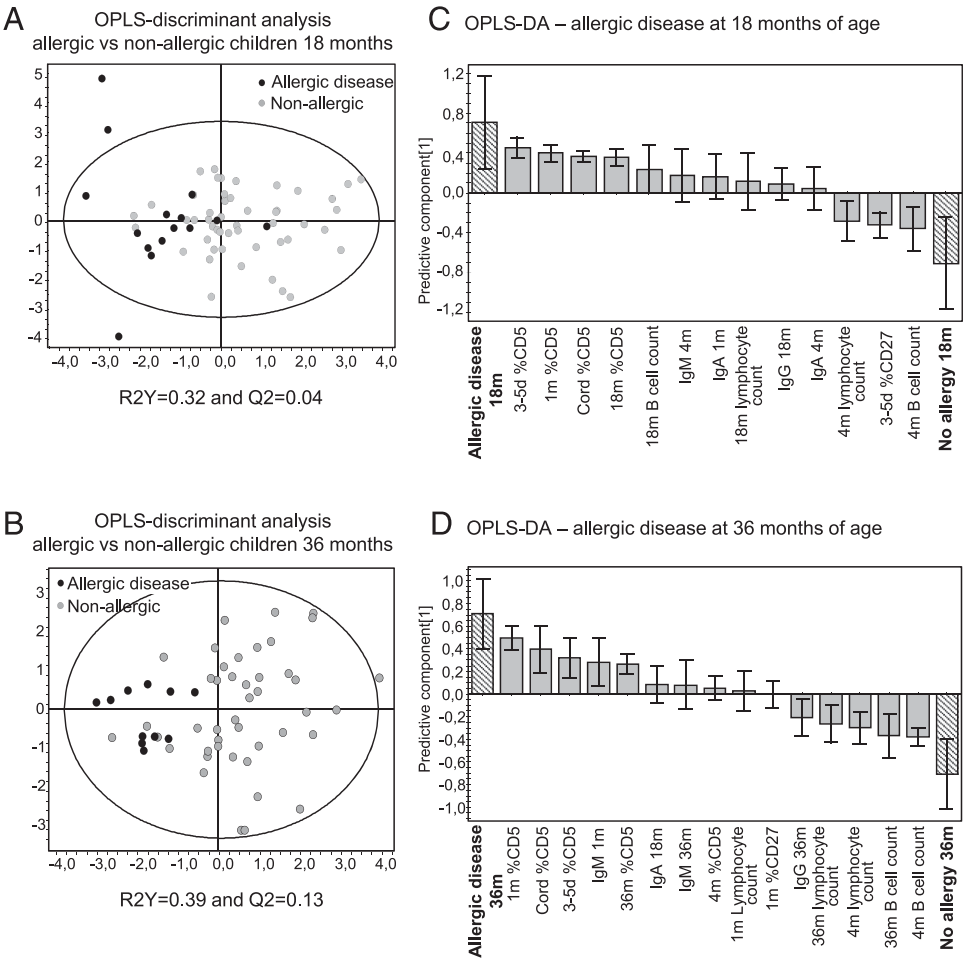
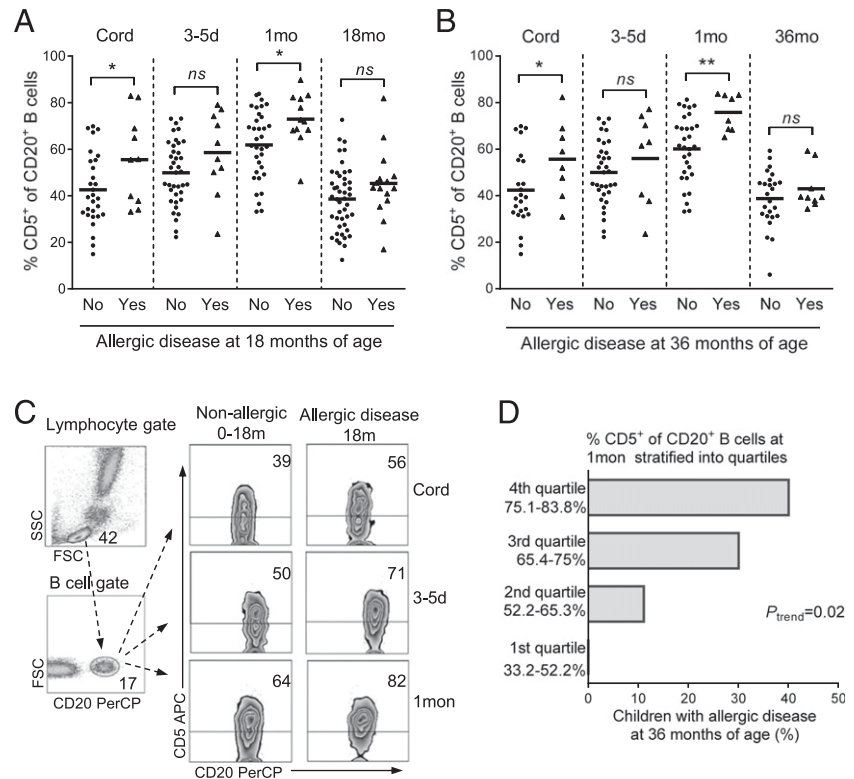


FIGURE 2. Allergic children have higher proportions of CD5⁺ B cells in early infancy than nonallergic children. **(A and B)** The proportions of B cells that express CD5 at different time points in nonallergic children and in children with allergic disease at **(A)** 18 and **(B)** 36 mo of age. **(C)** Representative flow cytometry plots showing our gating strategy in the FARMFLORA study. Lymphocytes were defined in a forward versus side scatter plot. These cells were then presented and gated according to their expression of CD20, and further analyzed according to CD5 expression. **(D)** The percentage of CD5⁺ B cells at 1 mo was stratified into quartiles, and the prevalence of children with allergic disease was compared in these four groups. **(A and B)** Horizontal bars indicate the mean. * $p \leq 0.05$, ** $p \leq 0.01$ (two-tailed t test). **(D)** χ^2 test for trend.



area under the curve of 0.82 ($p = 0.005$, 95% CI 0.67–0.98) was calculated for CD5⁺ B cells at 1 mo of age as a predictor for development of allergic disease at 36 mo of age (Supplemental Fig. 2A).

The prevalence of allergy is reduced in children raised on dairy farms (29, 30). In this study, we found a significantly lower prevalence of allergic disease, but not sensitization, at both 18 and 36 mo of age among farmers' children compared with nonfarmers' children (Supplemental Fig. 2B, 2C). However, the proportions of CD5⁺ cells did not differ between these two groups at any time point (Supplemental Fig. 2D). Thus, being raised on a dairy farm per se was not a confounding factor for the positive association between high proportions of CD5⁺ B cells and development of allergic disease. We also analyzed the relationship between the proportion of CD5⁺ B cells and other possible confounding factors, including sex, parental history of allergy, delivery by caesarean section, sibling(s) at time of birth, or cat and/or dog in home at time of birth. Girls had significantly lower proportions of these cells at 1 mo of age compared with boys ($p = 0.02$), but the fraction of CD5⁺ B cells did not differ among girls and boys who had allergic disease or not.

CD5⁺ B cells are of an immature/naïve phenotype

After the FARMFLORA study was initiated, cell-surface expression of CD24 in combination with CD38 has been acknowledged to distinguish different human B cell maturational stages in the periphery (7, 8). Thus, we examined whether the expression of CD24 and CD38 could verify that the CD5⁺ B cells in blood samples from unselected healthy newborn children, 7-y-old children, and healthy adults were immature/naïve B cells.

The gating strategy for CD5⁺ and CD5^{neg} B cells within the CD20⁺ B cells, and for the expression of CD24 and CD38 within the CD5⁺ and CD5^{neg} B cell populations, are shown in Fig. 3A. Transitional B cells are usually separated from mature naïve B cells based on the expression of CD24 and CD38 (10), but because our aim was to examine whether the CD5⁺ B cells were immature and/or naïve, we used a single gate that included both

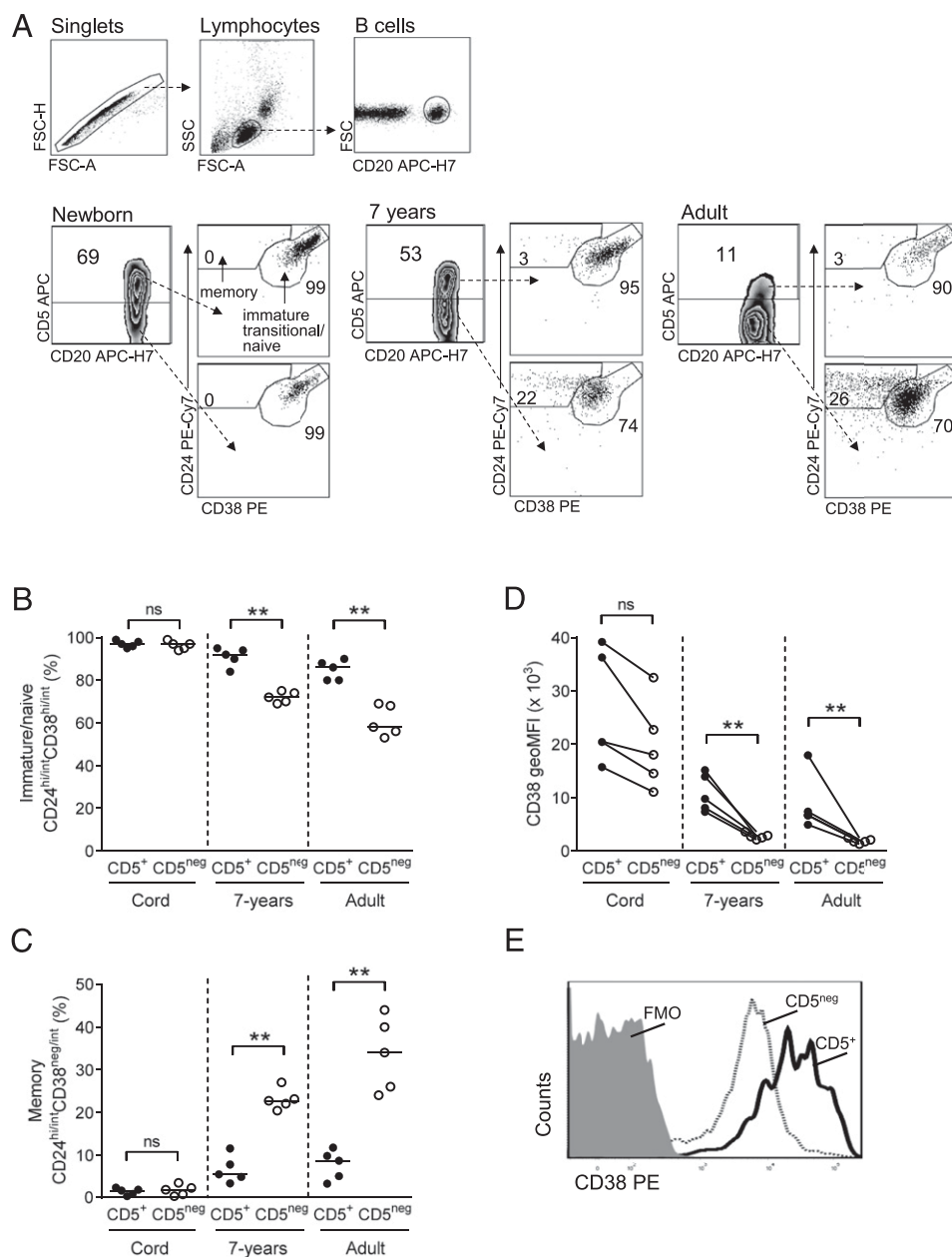
phenotypes (Fig. 3A). As previously described (15), we found that the proportions of CD5⁺ B cells decreased with age. Further, at birth, all circulating CD20⁺ B cells, including both CD5⁺ and CD5^{neg} cells, were of a CD24^{hi/int}CD38^{hi/int} immature transitional/naïve phenotype (Fig. 3A, 3B). The vast majority of the CD5⁺ B cell population in children 7 y of age and in adults was found in the CD24^{hi/int}CD38^{hi/int} gate, whereas only a very small proportion was found in the memory B cell gate (Fig. 3A–C). Accordingly, the CD24^{hi/int}CD38^{neg/int} memory B cells were mainly negative for CD5 in older children and in adults. Still, more than half of the CD5^{neg} population was found within the immature transitional/naïve gate, but the CD5^{neg} cells expressed less CD38 than the CD5⁺ B cells in all paired samples examined (Fig. 3A, 3D, 3E). These results indicate that peripheral CD5⁺ B cells are mainly immature/naïve in both children and adults.

Sensitization in relation to B cell maturation progress

Next, we examined B cell markers that correlated to sensitization with OPLS analysis. High proportions of CD5⁺ B cells at birth, 1 mo, and at 3–5 d of age were positively associated to sensitization at 18 mo, but not at 36 mo of age (Fig. 4A and data not shown), but the difference was not significant in univariate analyses (Fig. 4B). In addition, the strongest B cell variable that was associated to sensitization both at 18 (Fig. 4A) and 36 (data not shown) mo of age was high levels of total IgE ($p \leq 0.0001$ at both time points comparing IgE levels between sensitized and non-sensitized children). These results indicate that the relation between high proportions of neonatal CD5⁺ B cells and sensitization was weaker than that observed for allergic disease.

Because both IgE and the proportion of CD5⁺ B cells were associated to sensitization in multivariate analysis, we next examined whether CD5⁺ and CD5^{neg} B cells differed in their ability to produce IgE. We found that CD5⁺ B cells from one of three newborns produced IgE in response to CD40L in combination with IL-4 and IL-21 (26.6 ng/ml), whereas the CD5^{neg} B cells were unresponsive in all three experiments. Neither CD5⁺ nor

FIGURE 3. CD5⁺ B cells in children and adults are of an immature/naïve phenotype. **(A)** Gating strategy for CD20⁺ B cells and representative flow cytometry plots demonstrating the proportion of immature transitional/naïve CD24^{hi/int}CD38^{hi/int} cells or memory CD24^{hi/int}CD38^{neg/int} cells within the CD5⁺ and the CD5^{neg} CD20⁺ B cell populations at birth, at 7 y of age, and in adults (one representative subject out of five). **(B)** The proportion of transitional/naïve CD24^{hi/int}CD38^{hi/int} cells and **(C)** memory CD24^{hi/int}CD38^{neg/int} cells within the CD5⁺ and the CD5^{neg} CD20⁺ B cell populations at birth, at 7 y of age, and in adults. **(D)** Mean fluorescence intensity (MFI) of CD38 on the CD5⁺ and CD5^{neg} B cell populations in paired samples at birth, 7 y of age, and in adults. **(E)** Representative histogram of CD38 expression on the CD5⁺ and CD5^{neg} B cell populations from one adult subject. The histogram also depicts the fluorescence minus one control. (B and C) Horizontal bars indicate the median. ** $p \leq 0.01$ (two-tailed Mann–Whitney U test).



CD5^{neg} B cells from adults ($n = 3$) responded with IgE secretion. We also examined the production of total IgG and found that only the CD5^{neg} B cells from adults produced detectable levels of this Ab isotype (17.3, 42.7, and 66.7 $\mu\text{g/ml}$).

Proportion of CD5⁺ B cells early in life is inversely correlated to total IgG levels

Next, we examined which B cell variables were linked to the proportion of CD5⁺ B cells at 1 mo of age. As depicted in Fig. 5A, a high proportion of CD5⁺ B cells at 1 mo of age was negatively associated with the total levels of IgG in the circulation at 18 and 36 mo, and with a high number of total B cells at 4 mo of age. These associations could also be seen in correlation plots. Fig. 5B shows the proportion of CD5⁺ B cells at 1 mo in relation to total IgG at 18 mo of age, whereas their relation to the IgG levels at 36 mo of age is shown in Fig. 5C. The proportion of CD5⁺ B cells was unrelated to plasma levels of total IgM, IgA, and IgE. Thus, infants with higher proportions of immature/naïve CD5⁺ B cells had lower levels of total IgG in the circulation later in childhood.

Discussion

Delayed immune maturation early in life has been suggested to be a risk factor for development of allergic disease (2). To our knowledge, this is the first demonstration that children who experience development of allergic disease and those who remain nonallergic differ in postnatal peripheral B cell maturation. In particular, children who were diagnosed with allergic disease at both 18 and 36 mo of age had significantly higher proportions of circulating immature/naïve CD5⁺ B cells at birth and at 1 mo of age compared with children who remained healthy. Sensitization was also positively related to higher proportions of CD5⁺ B cells early in infancy, but these associations were not corroborated by univariate analyses.

Eczema was the dominating allergic disease in this cohort, as often is the case in young children. Indeed, according to the allergic march, eczema is often followed by a typical sequence of food allergy, asthma, and allergic rhinoconjunctivitis (31). Few of the children who were diagnosed with eczema in this study were

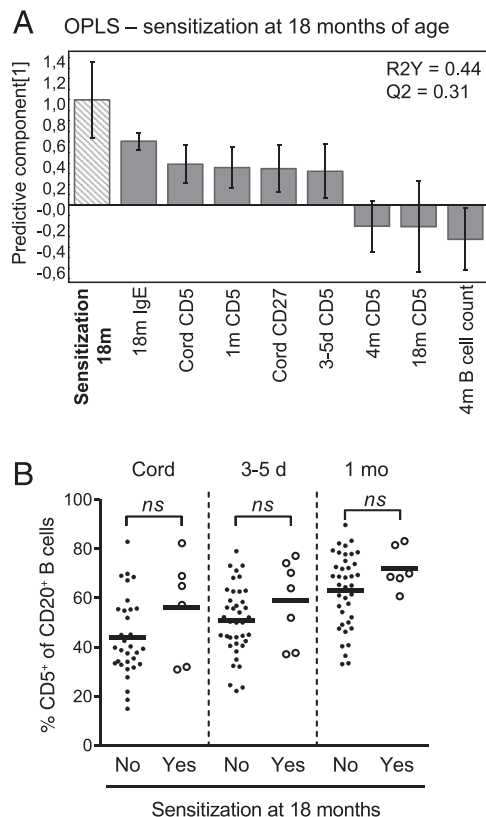


FIGURE 4. Sensitization in relation to B cell maturation progress. **(A)** OPLS plot depicting the associations between sensitization at 18 mo of age (Y) and X-variables including numbers of lymphocytes and B cells, the proportions of CD5⁺ or CD27⁺ B cells, and plasma levels of total IgM, IgG, IgA, and IgE determined at several time points during the first 18 mo of life. X-variables represented by a bar pointing in the same direction as Y are positively associated, whereas variables in the opposite direction are inversely related to Y. The OPLS column plot is based on B cell variables with VIP-values >0.8. R²Y indicates how well the variation of Y is explained, whereas Q² indicates how well Y can be predicted. **(B)** The proportions of CD5⁺ B cells at different time points in nonsensitized and sensitized children at 18 mo of age.

sensitized (15 and 29% at 18 and 36 mo of age, respectively). It should, however, be noted that development of eczema in non-sensitized children in early life has been found to precede and predict the onset of sensitization to allergens later in childhood (5, 6). Thus, a relation between high proportions of neonatal CD5⁺ B cells and sensitization in children with allergic disease who are >3 y old may not be ruled out. Indeed, it was recently shown that sensitized adult subjects with allergic asthma had significantly higher proportions of circulating immature transitional B cells defined as CD24^{hi}CD38^{hi} compared with healthy individuals (32). It should be noted, however, that the absolute numbers of CD24^{hi}CD38^{hi} B cells did not differ between these two groups (32).

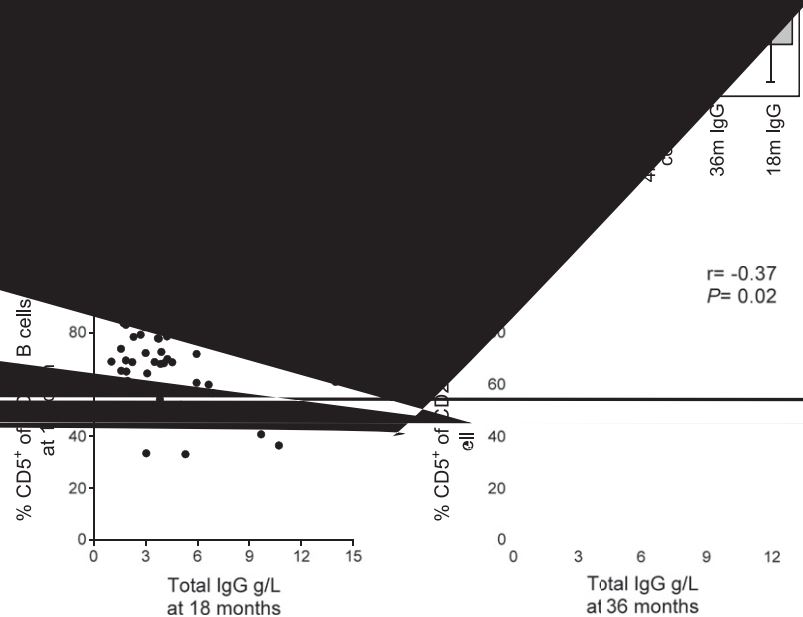
One limitation of this study is that the combination of CD24 and CD38 was not included in the flow cytometry panel, because these markers have been acknowledged to distinguish different peripheral B cell maturational stages in humans after the FARMFLORA study was initiated in 2004 (7, 8). However, with the use blood samples from healthy unselected children and adults, we sought to examine whether CD5 in combination with CD24 and CD38 could verify that CD5⁺ B cells were immature/naive. Indeed, depending on age, 86–97% (median values) of the CD5⁺ B cell population at birth, at 7 y of age, and in adults was of a CD24^{hi}CD38^{hi/+} immature transitional/naive phenotype. In accordance with our results, it has been shown that the vast

majority of peripheral transitional immature B cells express CD5 in both newborns and adults (11, 12). In addition, 2–12 wk after bone marrow transplantation, immature B cells, defined as CD5⁺ or CD24^{hi}CD38^{hi}CD5⁺, respectively, are a major lymphocyte population in peripheral blood and often precede the recovery of CD5^{neg} B cells (11, 33). Moreover, during maturation from immature transitional to mature naive B cells, there is a gradual decrease in the expression of CD5 and CD38 (12). In this study, we found that the CD5⁺ B cell population expressed higher levels of CD38 than the CD5^{neg} B cells at all ages. Therefore, we consider peripheral CD5⁺ B cells to be immature/naive cells.

How various B cell subsets based on degree of maturation contribute to host immunity is of interest in view of the present observations. It has been shown that B cells from newborns, which are mainly immature/naive, secrete very low or undetectable levels of IgE in response to anti-CD40 and IL-4 (34, 35), and that CD27⁺ memory B cells produce somewhat higher levels of IgE than CD27^{neg} immature/naive B cells from adults (34). More recently, however, it has been shown that cord blood B cells produce IgE and IgG levels comparable with naive B cells from adults when stimulated with a CD40L-expressing membrane in combination with IL-4 and IL-21 (28, 36). In this study, we found that CD5⁺ B cells from one out of three newborns produced IgE when using the same stimuli. IgE secretion from more neonates might have been detected if analyzed after a longer cell-culture period. Regarding IgG secretion, it was clear that IgG was detected only in cultures of CD5^{neg} B cells from adults, but not in cultures of adult CD5⁺ B cells, neonatal CD5^{neg} B cells, or CD5⁺ B cells. Although a low number of subjects was examined, our results suggest that under these culture conditions, IgE is predominantly secreted by neonatal naive CD5⁺ B cells, whereas CD5^{neg} B cells from adults are the primary source of IgG. In line with our findings, it has been clearly demonstrated that CD27⁺ memory B cells from adults produced significantly higher levels of IgG in response to *Staphylococcus aureus* and IL-2 compared with adult CD27^{neg} naive B cells and cord blood B cells (34). Regarding T cell activation, it has been found that prenaive B cells have the same ability to induce T cell proliferation as mature naive B cells in adults (37). Because human B cells gradually lose CD5 and acquire CD27 during maturation from immature transitional cells via mature naive cells to memory cells (10), immature/naive B cells per se may not necessarily be involved in the immunological mechanisms that could lead to development of allergic disease. Instead, a high proportion of CD5⁺ B cells could be a marker of a more immature/naive immune system in general that may predispose to allergic disease.

Based on our findings, it would be of interest to find environmental factors that stimulate immune maturation early in life. One hypothesis is that the farming environment has a stimulatory impact on the developing immune system that may confer protection from allergic disease. Allergy-protective effects of a farming lifestyle have previously been reported by large epidemiological questionnaire-based studies regarding clinical diagnosis of allergy (29, 30). Indeed, even though a relatively low number of study subjects were included in this cohort, we show that the prevalence of allergic disease, but not sensitization, is significantly reduced in children raised on a dairy farm compared with nonfarmers' children who lived on the countryside. However, we found no differences with respect to proportions of immature CD5⁺ or memory CD27⁺ B cells among farmers' and nonfarmers' children during their first 3 y of life.

Another hypothesis is that the gut microbiota could have an impact on B cell maturation because the proportions of circulating IgA⁺ and IgG⁺ B cells with a gut-homing phenotype is higher in



early infancy than in adults, which points to activation of naive B cells in the gut in early infancy (38). It was also recently proposed that immature transitional 2 B cells are selectively recruited to the GALT for maturation into mature naive cells in healthy adults (39). However, we recently showed that an early gut flora including *Escherichia coli* and bifidobacteria was associated with higher numbers of CD27⁺ memory B cells later in childhood, but no relationship was found between the levels of CD5⁺ B cells and bacterial colonization (15). Yet, the diversity of the gut flora and/or noncultivable bacteria might have an impact on the proportions of CD5⁺ B cells. Indeed, T-RFLP analysis of fecal samples obtained from vaginally delivered infants showed a high diversity of noncultivable bacteria in the first weeks that are likely to provide important stimuli for the immune system (40). Interestingly, a reduced diversity of the early gut microbiota is associated with an increased risk for allergic disease later in childhood (41, 42).

Genetic factors may also play a role in prenatal and postnatal immune maturation. We found that girls had lower proportions of CD5⁺ B cells at 1 mo of age than boys. However, because the proportions of these cells did not differ among girls and boys who had an allergic disease or not, sex is most likely not a confounding factor for the positive association between high proportions of CD5⁺ B cells and development of allergic disease. Moreover, parental history of allergy was unrelated to the proportions of CD5⁺ B cells in this study. These observations do not exclude that other genetic factors may influence the fractions of these cells early in infancy.

The relatively small scale of this study and accordingly a low number of children diagnosed with allergic disease may be a limitation. Yet, the small size of the study permitted detailed and structured follow-up. All children were examined by a study pediatrician at 18 and 36 mo of age, and also between follow-ups if symptoms suggesting the commencement of allergic disease occurred. Further, all diagnoses were based on strict criteria. For

eczema, we used the validated criteria of Williams (24). Our criteria for a diagnosis of food allergy were based on an immediate or late-onset reaction after ingestion of the specific food, followed by a clear and prompt clinical improvement once the food allergen was eliminated, together with a positive six-mix food test and/or verified by open food challenge test. Although our criteria for an asthma diagnosis is far from the strict asthma criteria used in older children and adults, the criteria used in this study are probably the best available without using infant lung function tests and invasive methods. Moreover, in a coming clinical follow-up of this cohort, at 8 y of age, we will be able to establish whether the children who were diagnosed with eczema at 18 and/or 36 mo of age experience development of food allergy, asthma, or allergic rhinoconjunctivitis later in childhood. Because we observed a decrease in the prevalence of allergic disease between 18 and 36 mo of age, we will also in this follow-up have the opportunity to find out who will have persistent and who will have transient disease. A strength of this study is that all flow cytometry analyses were performed blindly as clinical data on allergic disease or whether they lived on a dairy farm or not were obtained after the cell data were compiled. Still, further studies in other cohorts are required to confirm that a delayed maturation of the peripheral B cells in early infancy may predict development of allergic disease later in childhood, as indicated in our study. Moreover, it remains to be examined whether B cell maturation in early life might depend on genetic factors or reduced immunological stimulation (both prenatal and postnatal), or a combination of these factors.

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Disclosures

The authors have no financial conflicts of interest.

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Supplementary Table 1. Specific details for the children who were diagnosed with food allergy

Child ID	Age of diagnosis	Causative food and organ(s) involved	Total IgE		6-mix-food test ^a	Specific IgE		Provocation ^d
			18 mon	36 mon		18 mon	36 mon	
BF012	18 months	egg (immediate) urticaria	36 kU/L	NA ^b	positive	milk 0.40 egg 0.35 fish 0.35 soy 0.35 wheat 0.35 peanut 0.35		open
BF023	36 months	kiwi (immediate) urticaria	NA	26 kU/L	positive		milk 2.9 egg 0.30 fish 0.31 soy 0.19 wheat 0.52 peanut 0.40 kiwi 0.95 ^c	accidental
BF035	18 and 36 months	milk (delayed) eczema and gastrointestinal	200 kU/L	180 kU/L	positive	milk 0.25 egg 2.4 fish 0.00 soy 0.04 wheat 0.09 peanut 0.03	milk 0.21 egg 0.34 fish 0.00 soy 0.02 wheat 0.08 peanut 0.30	open

^a Screening test for specific IgE against milk, egg, fish, soy wheat and peanut

^b NA= not analyzed

^c Kiwi is not included in the 6-mix-food test, but levels of allergen-specific IgE to kiwi were measured as an immediate food reaction to kiwi was reported by the parents

^d Type of provocation could either be an open food challenge test performed at the pediatric clinic or occur by accidental ingestion of the specific food in their homes

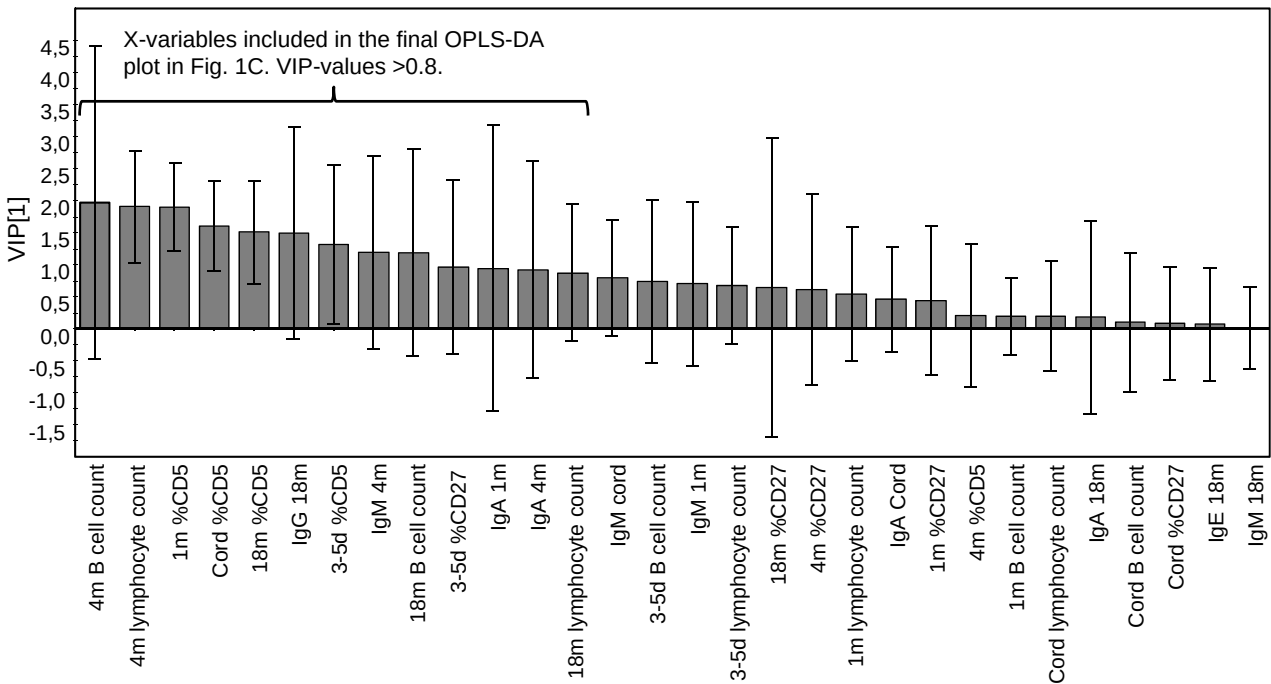
Supplementary Table 2. Allergic disease and sensitization in children at 18 and 36 months of age

Child ID	at 18 months					at 36 months				
	Food allergy	Eczema	ARC ^a	Asthma	Sensitization	Food allergy	Eczema	ARC ^a	Asthma	Sensitization
001					X					X
007										X
008			X		X			X	X	X
009				X	X				X	X
011										X
012	X	X			X	ended the study after 18 months				
014		X								
016							X			
018										X
023		X				X				X
024		X					X			
025									X	X
028		X		X						
029							X		X	
034		X		X						
035	X	X			X	X	X			X
036					X					X
041					X					
045					X		X			X
046		X					X			
049		X		X			X			
050		X								
055										X
058		X								
060		X		X						
062					X					X
064		X								X

^a Allergic rhinoconjunctivitis

Supplementary figure 1

A VIP plot based on OPLS-DA analysis including allergic disease at 18 months (Y) and all X-variables up to 18 months



B VIP plot based on OPLS-DA analysis including allergic disease at 36 months (Y) and all X-variables up to 36 months

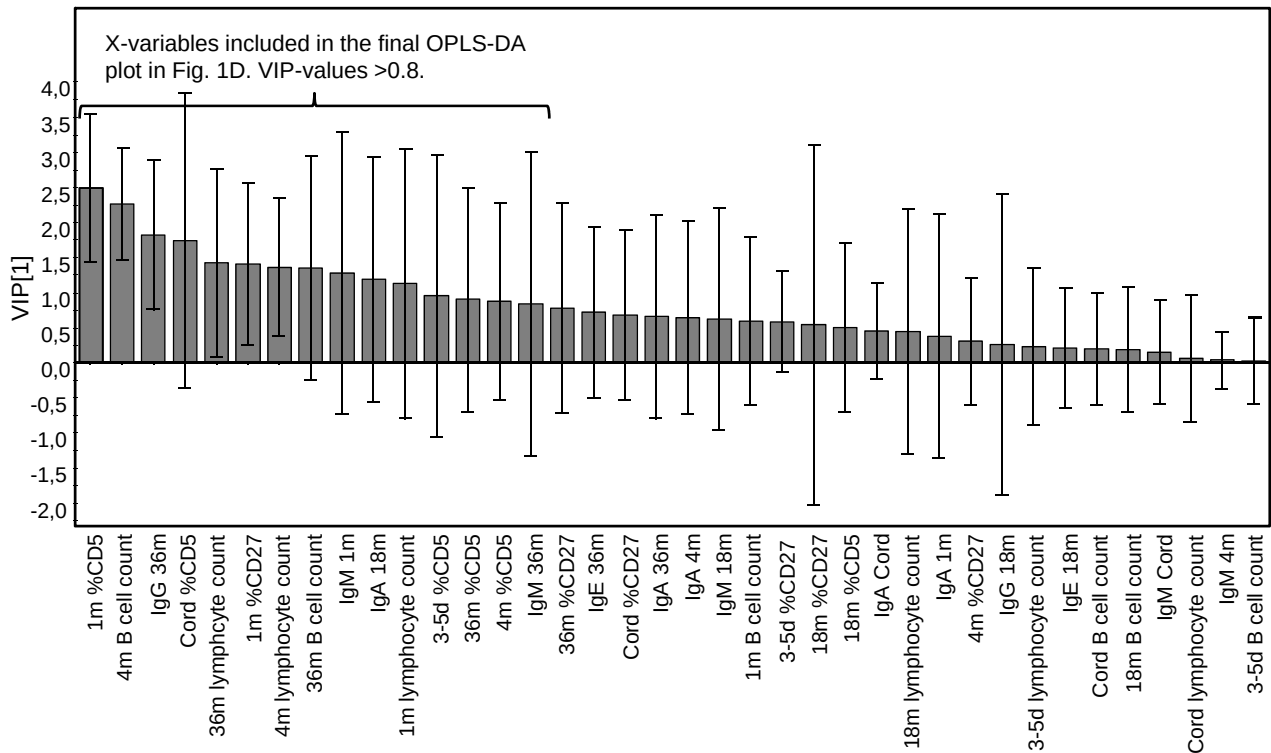


Figure S1. Variable influence on projection (VIP) plots. The VIP plots display the VIP values as a column plot sorted in descending order with confidence intervals derived from jack knifing. VIP summarizing the importance of the X-variables that contribute most (positively or negatively) to allergic disease at **(A)**18 and **(B)** 36 months of age.

Supplementary figure 2

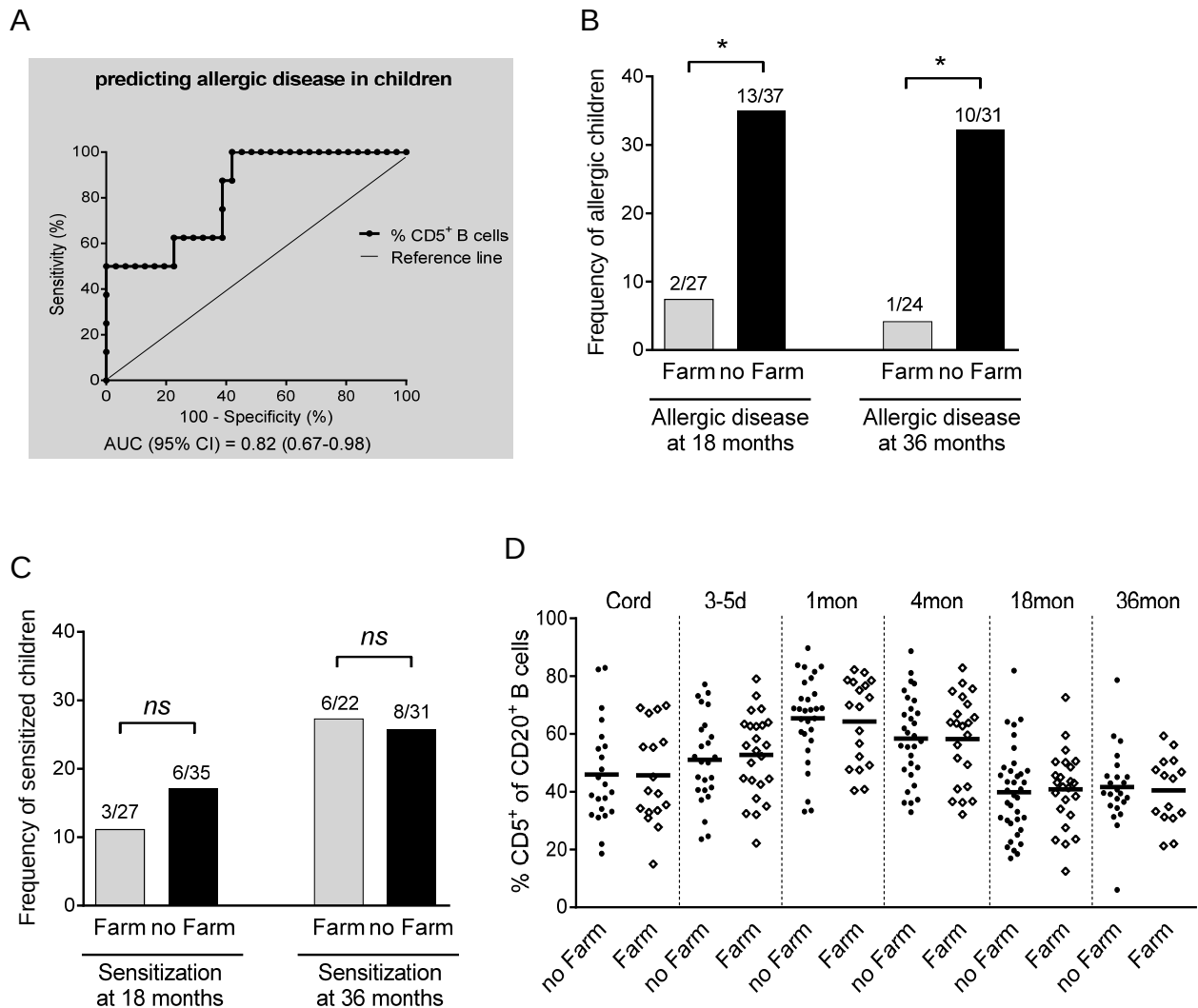


Figure S2. (A) Receiver-operating characteristics curve for the prediction of allergic disease in 36 months old children based on the proportion of CD5⁺ B cells at 1 month of age. The reference line indicates an area under the curve (AUC) = 0.5 (no better than chance alone). 95% CI = 95% confidence interval. The prevalence of allergic disease **(B)** or sensitization **(C)** among children raised on dairy farms or children raised on the countryside, but not on a farm, at 18 or 36 months of age. **(D)** The proportion of B cells that express CD5 during the first 3 years of life among farmer's and non-farmer's children. **(B-C)** Chi-square test * ≤ 0.05 .