

Higher B-cell activating factor levels at birth are positively associated with maternal dairy farm exposure and negatively related to allergy development

Anna-Carin Lundell, PhD,^a Bill Hesselmar, MD, PhD,^c Inger Nordström, BSc,^a Ingegerd Adlerberth, MD, PhD,^b Agnes E. Wold, MD, PhD,^b and Anna Rudin, MD, PhD^a Gothenburg, Sweden

Background: A high proportion of circulating immature/naive CD5⁺ B cells during early infancy is a risk factor for allergy development. B-cell activating factor (BAFF) is an important cytokine for B-cell maturation.

Objective: We sought to investigate whether BAFF levels are related to environmental exposures during pregnancy and early childhood and whether BAFF levels are associated with postnatal B-cell maturation and allergic disease.

Methods: In the FARMFLORA study, including both farming and nonfarming families, we measured BAFF levels in plasma from mothers and their children at birth and at 1, 4, 18, and 36 months of age. Infants' blood samples were also analyzed for B-cell numbers and proportions of CD5⁺ and CD27⁺ B cells. Allergic disease was clinically evaluated at 18 and 36 months of age.

Results: Circulating BAFF levels were maximal at birth, and farmers' children had higher BAFF levels than nonfarmers' children. Higher BAFF levels at birth were positively associated with proportions of CD27⁺ memory B cells among farmers' children and inversely related to proportions of CD5⁺ immature/naive B cells among nonfarmers' children. Children with allergic disease at 18 months of age had lower cord blood BAFF levels than nonallergic children. At birth, girls had higher BAFF levels and lower proportions of CD5⁺ B cells than boys.

Conclusions: Farm exposure during pregnancy appears to induce BAFF production in the newborn child, and high neonatal BAFF levels were associated with more accelerated postnatal B-cell maturation, which lend further strength to the role of B cells in the hygiene hypothesis. (J Allergy Clin Immunol 2015;■■■:■■■-■■■.)

Key words: Prospective birth cohort, B-cell activating factor, dairy farm, pregnancy, immature/naive B cells, memory B cells, hygiene hypothesis, allergy, sex

B cells enter the circulation from the bone marrow as CD24^{hi}CD38^{hi} immature transitional cells, and the vast majority of these cells express CD5 in human subjects.¹ At birth, approximately half of the blood B-cell pool consists of transitional cells, whereas these cells represent about 5% of total cells in adults.^{1,2} The immature transitional B cells mature into naive cells, and when they encounter their cognate antigens in secondary lymphoid organs, they can become activated and mature into memory B cells and immunoglobulin-secreting plasma cells.³ During the progressive development from recent bone marrow emigrants to antigen-stimulated B cells, surface CD27 levels gradually increase, whereas CD38 and CD5 expression decreases.^{1,2,4} In children circulating B-cell numbers and proportions of CD5⁺ cells among CD20⁺ B cells increase considerably from birth up to 4 months of age,⁵ which might indicate augmented export of immature/naive B cells from the bone marrow. Thereafter, the proportion of CD5⁺ B cells decreases with age while the proportion of CD27⁺ memory B cells increases,⁵ suggesting accumulation of B cells that have undergone antigen-dependent expansion in secondary lymphoid organs.

The hygiene hypothesis proposes that delayed maturation of the immune system caused by reduced exposure to microbes during early life is a risk factor for allergy development.⁶ Accordingly, we recently showed that a high proportion of circulating immature/naive CD5⁺ B cells during the first month of life predicts development of allergic disease later in childhood.⁴ The identity of the factors regulating CD5⁺ B-cell subset size in human subjects is unclear.

B-cell activating factor (BAFF) is a member of the TNF family that is produced by both innate immune cells and nonhematopoietic cells, including airway epithelial cells.⁷⁻⁹ Signals induced when BAFF interacts with its main receptor, BAFF-R, on B cells are pivotal for the survival and differentiation of immature transitional B cells into mature naive cells.¹⁰ Mice that lack BAFF or express a mutant BAFF-R display normal B-cell development up to the transitional stage but cannot complete further maturation in the spleen.^{11,12}

From the Departments of ^aRheumatology and Inflammation Research, ^bInfectious Diseases, and ^cPediatrics, Sahlgrenska Academy, University of Gothenburg.

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Corresponding author: Anna-Carin Lundell, PhD, Department of Rheumatology and Inflammation Research, Sahlgrenska Academy at University of Gothenburg, Box 480, S-405 30 Göteborg, Sweden. E-mail: anna-carin.lundell@rheuma.gu.se.

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Abbreviations used

APC: Allophycocyanin
 BAFF: B-cell activating factor
 FITC: Fluorescein isothiocyanate
 OPLS: Orthogonal projection to latent structures by means of partial least squares
 TLR: Toll-like receptor
 VIP: Variable influence on projection

BAFF-deficient mice also exhibit a profound reduction in total immunoglobulin levels.¹¹ In line with this, 2 patients with common variable immunodeficiency and BAFF-R deficiency have been reported to display arrested B-cell development at the stage of transitional B cells and a reduction in numbers of cells at all subsequent B-cell maturational stages.¹³ It has also been suggested that BAFF-binding receptors on B cells regulate BAFF levels in the circulation because subjects with low B-cell numbers have higher circulating BAFF levels than those with normal B-cell numbers.¹⁴

Longitudinal data regarding circulating BAFF levels in the first years of life are lacking, and their relation to infant B-cell maturation is unclear. Furthermore, the role of environmental exposures and BAFF levels in the circulation have not been examined. To address these questions, we measured BAFF levels in plasma over the first 3 years of life in the FARMFLORA study, which includes both farming and nonfarming families from the same geographic area. We also investigated the relation between BAFF levels at birth and subsequent B-cell maturation with the use of multivariate factor analysis. We demonstrate that BAFF levels were maximal at birth and that farmers' children displayed higher levels of this cytokine in cord blood compared with nonfarmers' children. We also found that BAFF levels at birth were related to higher proportions of memory B cells among farmers' children and lower proportions of immature/naïve B cells among nonfarmers' children later in childhood. Finally, children with an allergic disease at 18 months of age had significantly lower cord blood BAFF levels compared with children who remained nonallergic.

METHODS**Study design**

In the prospective FARMFLORA study farming and nonfarming families from rural areas in the Skaraborg region in southwest Sweden were enrolled at maternity care clinics. Rural areas in this region are fairly similar with respect to population density and farming characteristics. The inclusion criteria for the farming group were that the families should live on small family-owned dairy farms with cows. In Sweden cows are kept in a barn separated from the dwelling house during the winter period. Families included in the nonfarming group should live in the same rural geographic region but not on a farm. Sixty-five healthy infants born at term (≥ 38 gestational weeks) were included in the study and have been followed up to 3 years of age. Twenty-eight of the children were included in the farming group, whereas 37 were included in the control group. Blood samples were obtained from the umbilical cord at delivery (6 mL), and venous blood was sampled at 3 to 5 days (1 mL) and 1 (3 mL), 4 (3 mL), 18 (6 mL), and 36 (6 mL) months of age. Venous blood was also drawn from mothers either before ($n = 10$; median, 19 days; range, 1–60 days) or after ($n = 42$; median, 30 days; range, 3–480 days) delivery. For flow cytometric analyses of CD24 and CD38 expression within the CD5⁺ or CD5[−] B-cell populations (see Fig E1 in this article's Online Repository at www.jacionline.org), cord blood samples ($n = 4$) were collected from

unselected healthy newborn infants born at term (≥ 38 gestational weeks) at the Sahlgrenska University Hospital, and venous blood was obtained from healthy 8-year-old children not included in the FARMFLORA study ($n = 4$). Informed written consent was obtained from parents, 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 diagnosis

Children were examined for sensitization and allergic disease by a pediatrician at 18 and 36 months of age, as previously described.⁴ Briefly, venous blood was collected for measurement of total IgE and the presence of specific IgE against food (6-mix food test; Phadia/Pharmacia Diagnostics, Uppsala, Sweden) and inhalant (Phadiatop, Phadia/Pharmacia Diagnostics) allergens. 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 of 0.35 kU/L or greater was considered positive. Skin prick tests were performed in accordance with European guidelines by using standard allergen extracts (Soluprick SQ; ALK-Abelló AS, Hørsholm, Denmark), allergen diluents as the negative control, and histamine (10 mg/mL) as the positive control.

On the basis of clinical examination and laboratory test results, the following diagnostic groups were defined. *Food allergy* was defined as 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 6-mix food ImmunoCAP test result and/or verification by an open food challenge test. *Eczema* was diagnosed according to the criteria of Williams.¹⁵ Eczema at 18 months denoted diagnosis at any time before or at 18 months of age, whereas eczema at 36 months required symptoms to be present after 24 months of age. *Asthma* was defined as persistent wheezing for 4 or more weeks or 3 or more episodes of wheezing combined with 1 or more minor criteria (symptoms between colds, eczema, allergic rhinoconjunctivitis, or food allergy). For asthma at 36 months, 1 or more wheezing episodes should have occurred after 24 months of age, and response to inhaled glucocorticoids or leukotriene antagonists was added to the minor criteria above. *Allergic rhinoconjunctivitis* was defined as symptoms in the eyes, nose, or both on exposure to pollen or animal dander, together with a positive allergen-specific IgE test result directed against a corresponding allergen. Table E1 in this article's Online Repository at www.jacionline.org describes children with allergic disorders, sensitization, or both at 18 and 36 months of age.

Quantification of BAFF and immunoglobulins in plasma

Plasma was prepared from collected venous blood samples and stored at -80°C . BAFF levels were determined by using human BAFF/BLyS/TNFSF13B Quantikine ELISA Kits (R&D Systems, Minneapolis, Minn), according to the manufacturer's instructions. Total IgM, IgG, and IgA levels were measured by coating plates (Nunc, Roskilde, Denmark) with goat anti-human IgM, IgG, or IgA (Jackson ImmunoResearch, Suffolk, United Kingdom), after which diluted plasma and standards (polyclonal IgM, IgG, or IgA from human plasma; Calbiochem, Darmstadt, Germany) were added. Detection was performed by using horseradish peroxidase-conjugated goat anti-human IgM, IgG, or IgA (Jackson ImmunoResearch), followed by treatment with O-phenylenediamine dihydrochloride (Sigma-Aldrich, St Louis, Mo) and addition of H_2O_2 as a substrate. Total IgE levels were measured with ImmunoCAP 250 (Phadia, Uppsala, Sweden). Because of the limited volume of blood obtained from each child, measurement of BAFF and immunoglobulin levels could not be performed for all children at all time points.

Phenotypic characterization of B cells in the FARMFLORA study

The flow cytometric procedure for phenotypic characterization of B cells in the FARMFLORA study was performed, as previously described in detail.⁵

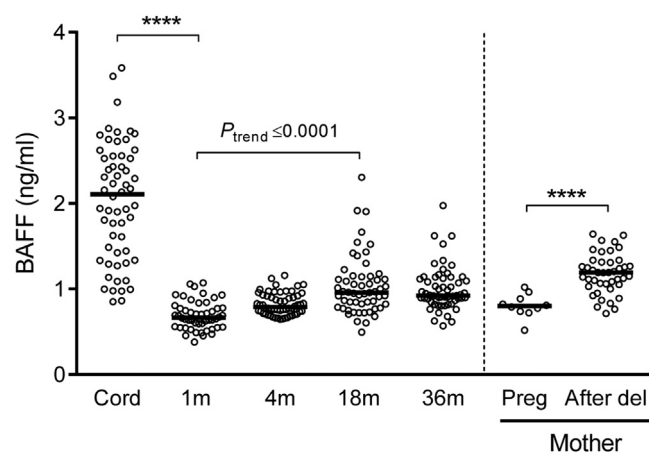


FIG 1. Circulating BAFF levels are maximal at birth. BAFF levels in plasma at birth and at 1, 4, 18, and 36 months of age and in maternal plasma. Maternal plasma was obtained either during pregnancy or after delivery (median values: 19 days before and 30 days after giving birth, respectively). Horizontal bars indicate medians. **** $P \leq .0001$, Mann-Whitney U test and ANOVA posttest for linear trend.

Briefly, the following mAbs were used: peridinin-chlorophyll-protein complex–conjugated anti-CD20 (clone L27; BD Biosciences, Erembodegem, Belgium), allophycocyanin (APC)–conjugated anti-CD5 (clone UCHT2; PharMingen, San Diego, Calif), and fluorescein isothiocyanate (FITC)–conjugated anti-CD27 (clone L128, BD Biosciences). APC- and FITC-conjugated mouse IgG₁ (clone X40, APC IgG₁ and FITC IgG₁; BD Biosciences) were used as controls when gating on the CD5⁺ and CD27⁺ populations, respectively. For absolute cell numbers, the Trucount assay was used (BD Biosciences), as described in detail elsewhere.⁵ Samples were run in a FACSCalibur (BD Biosciences) equipped with CellQuest Pro software, or in a FACSCanto II (BD Biosciences) equipped with FACSDiva software and analyzed with FlowJo software (TreeStar, Ashland, Ore).

Statistical analysis

To examine the relation between BAFF levels at birth (Y-variables) and various B-cell parameters, as well as environmental and genetic factors (X-variables), multivariate factor analysis (SIMCA-P+ software; Umetrics, Umeå, Sweden) was used. Orthogonal projection to latent structures by means of partial least squares (OPLS) was implemented to correlate a selected Y-variable and X-variables with each other in linear multivariate models. OPLS discriminant analysis was implemented to examine whether male and female sex could be discriminated based on the various B-cell parameters examined. 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 (ie, how well the variation of the variables is explained by the model) and Q^2 (ie, how well a variable can be predicted by the model). OPLS plots are based on X-variables with variable influence on projection (VIP) values of greater than 0.9 and greater than 1.1, respectively. 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 the Y-variable is represented by column bars. Jackknifing was used to calculate SEs displayed as an error bar on each column (representing the 95% CI). Univariate analyses were performed exclusively on the X-variables that contributed most to the respective multivariate model to avoid mass significance. Univariate analyses were performed with the 2-tailed Mann-Whitney U test, the Spearman rank correlation test, the ANOVA posttest for linear trend, or the Fisher exact test, as described in the figure legends and in Table I (GraphPad Prism; GraphPad Software, La Jolla, Calif). A P value of .05 or less was regarded as being statistically significant.

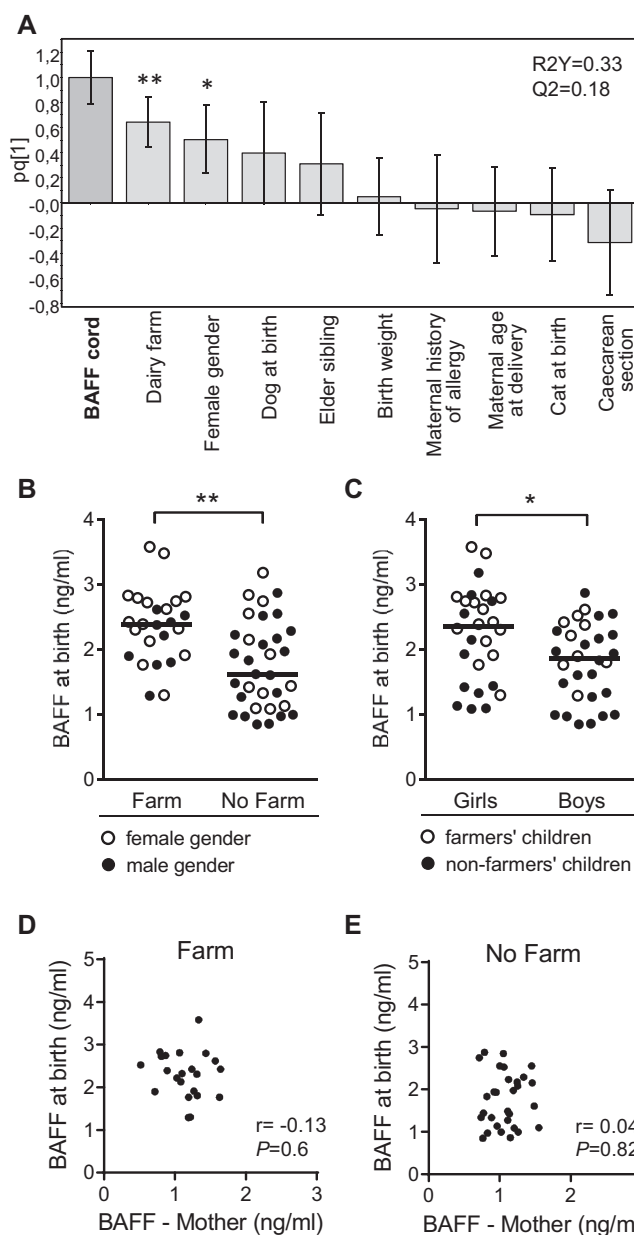


FIG 2. Farmers' children have higher BAFF levels at birth than nonfarmers' children. **A**, OPLS column loading plots depicting association between BAFF levels at birth (Y-variable) in relation to X-variables, including farming environment, sex, elder sibling, pets at home, delivery mode, birth weight, maternal age at delivery, and maternal history of allergy. R^2Y indicates how well the variation of the Y-variable is explained, and Q^2 indicates how well the Y-variable can be predicted. **B**, BAFF levels in plasma at birth among farmers' and nonfarmers' children. Open and solid symbols indicate female and male sex, respectively. **C**, BAFF levels in cord blood from girls and boys. Open and solid symbols indicate farmers' and nonfarmers' children, respectively. **D** and **E**, Correlations between maternal and infant BAFF levels among farming and nonfarming families. Horizontal bars indicate medians. * $P \leq .05$ and ** $P \leq .01$, Mann-Whitney U test (Fig 2, B and C) and Spearman correlation test (Fig 2, D-E).

RESULTS

Circulating BAFF levels are maximal at birth

We determined BAFF levels in plasma from children on repeated occasions from birth to 36 months of age and in the mothers' plasma. The highest BAFF levels were found in cord

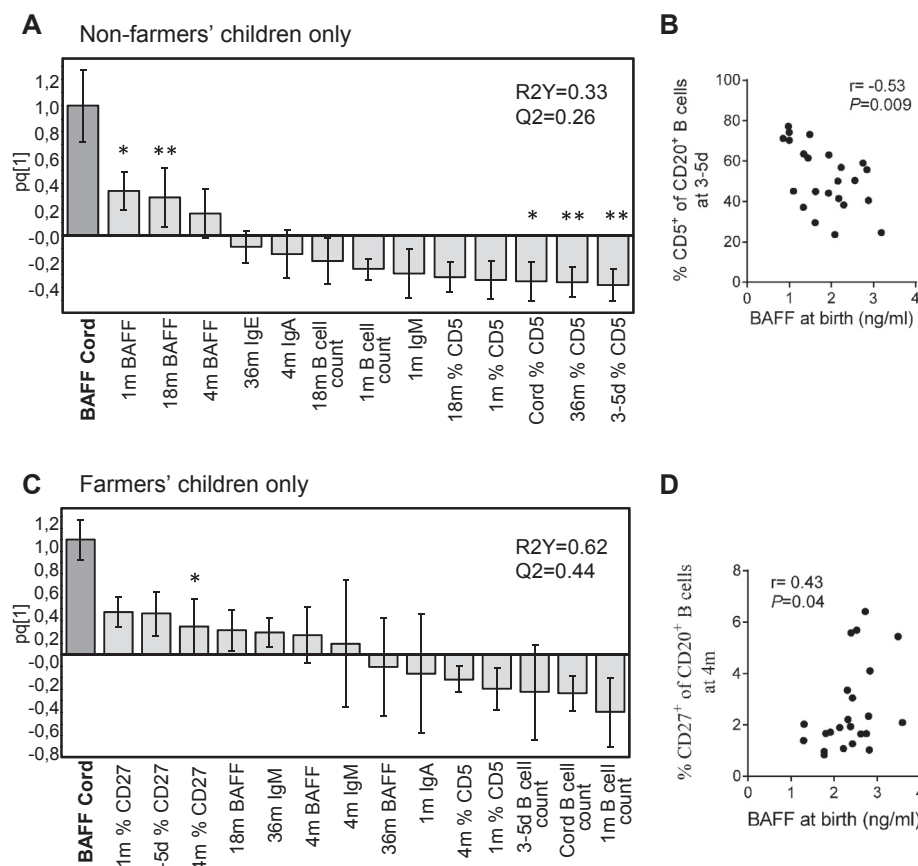


FIG 3. Higher BAFF levels at birth are associated with a higher degree of B-cell maturation later in childhood. **A** and **C**, OPLS column loading plots depicting association between BAFF levels at birth among nonfarmers' children (Fig 3, A) or farmers' children (Y-variable; Fig 3, C) in relation to X-variables, including numbers of CD20⁺ B cells, proportions of immature/naive CD5⁺ or memory CD27⁺ B cells, and total levels of IgM, IgG, IgA, and BAFF measured in blood on repeated occasions from birth up to 36 months of age. The OPLS column plots are based on B-cell variables with VIP values of greater than 0.9. R²Y indicates how well the variation of the Y-variable is explained, and Q² indicates how well the Y-variable can be predicted. **B**, Correlation between BAFF levels at birth and proportions of CD5⁺ B cells at 3 to 5 days of age among nonfarmers' children. **D**, Correlation between BAFF levels at birth and proportions of CD27⁺ B cells at 4 months of age among farmers' children. Fig 3, B and D, Spearman correlation test.

blood (Fig 1). At 1 month of age, these levels had decreased drastically. Thereafter, BAFF levels increased slowly over the first 18 months. Maternal BAFF levels were measured in plasma obtained either during pregnancy or after delivery (median values, 19 days before or 30 days after giving birth, respectively). BAFF levels were higher when measured after than before delivery, but irrespective of this, the mothers had lower BAFF levels than the newborn infants ($P \leq .0001$ for both analyses).

High BAFF levels in cord blood of farmers' children

BAFF is produced by innate immune cells and also by airway epithelial cells,⁷⁻⁹ but the signals that trigger their production *in vivo* are not known. We investigated the relation of BAFF levels in cord blood with a range of potential explanatory maternal factors by using the multivariate factor analysis method OPLS. Fig 2, A, shows which factors are positively or negatively associated with BAFF levels in cord blood: factors represented by a bar pointing in the same direction as BAFF at birth are associated with higher BAFF levels, whereas factors pointing in

the opposite direction are related to lower BAFF levels at birth. Accordingly, farming environment and female sex of the infant were associated with higher BAFF levels in cord blood (Fig 2, A). Univariate analysis confirmed that farmers' children had significantly higher BAFF levels at birth compared with nonfarmers' children (Fig 2, B). This difference was also evident at 4 months of age but not at any other time point examined ($P = .005$ at 4 months). Girls had significantly higher BAFF levels in cord blood relative to boys (Fig 2, C). All statistically significant differences in univariate analyses are indicated with asterisks in Fig 2, A. However, there was no difference in circulating BAFF levels between farming and nonfarming mothers ($P = .32$) or between mothers giving birth to a girl or a boy ($P = .67$). Female sex was overrepresented among farming families (Table I). To resolve these factors, we performed multiple regression analysis. As shown in Table II, farming environment and female sex both contributed independently to BAFF levels at birth.

We examined the relation between mother and infant BAFF levels. Because the farming environment was related to higher

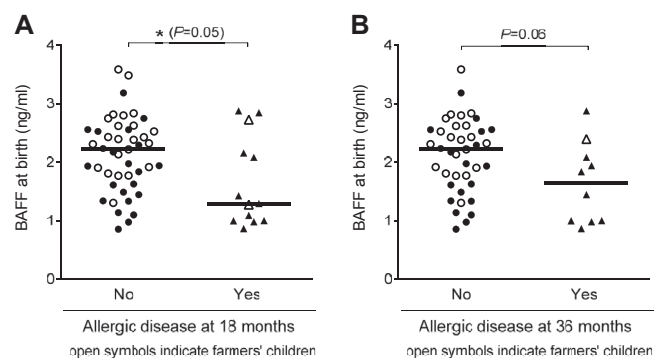


FIG 4. Cord blood BAFF levels and development of allergic disease. BAFF levels at birth in nonallergic children and children given a diagnosis of any allergic disease (ie, food allergy, eczema, asthma, and/or allergic rhinoconjunctivitis) at 18 (A) and 36 (B) months of age, respectively. Open symbols indicate farmers' children. Horizontal bars show medians. $*P \leq .05$, Mann-Whitney *U* test.

BAFF levels in cord blood, we performed separate correlation analyses for mother-infant pairs among farming and nonfarming families, respectively. Cord blood BAFF levels were unrelated to maternal BAFF levels both within the farming and nonfarming groups (Fig 2, D and E). In conclusion, exposure to a dairy farm environment during pregnancy appears to stimulate higher BAFF levels only in the fetal circulation.

BAFF levels at birth and subsequent B-cell maturation

BAFF signaling is important for differentiation of immature transitional B cells into mature naive cells.¹¹⁻¹³ We investigated whether BAFF levels in cord blood were associated with B-cell maturation, measured as B-cell numbers, proportions of immature/naive CD5⁺ and memory CD27⁺ B cells, and total IgM, IgG, IgA, IgE, and BAFF levels, analyzed in blood on repeated occasions from birth to 36 months of age. Fig E1 demonstrates that the vast majority of all circulating CD5⁺ B cells at birth and in 8-year-old children are of an CD24⁺CD38⁺CD38⁺ immature/mature naive phenotype, whereas the majority of the CD27⁺ B cells in 8-year-old children are of an CD24⁺CD38^{low/-} memory phenotype. Analysis of CD24 and CD38 was not used in this study because the combination of these 2 markers to distinguish different human B-cell maturation stages in blood was acknowledged after the FARMFLORA study was initiated.^{3,16}

The B-cell maturation variables that displayed the strongest association (positive or negative) with BAFF levels at birth were identified by means of OPLS: B-cell variables represented by a bar pointing in the same direction as BAFF at birth are associated with higher BAFF levels, whereas variables pointing in the opposite direction are related to lower BAFF levels at birth. Variables with the largest contribution to the model were selected (ie, only variables with VIP values >0.9) and presented in Fig 3, A and C. Among nonfarmers' children, higher BAFF levels in cord blood were inversely related to the proportions of immature/naive CD5⁺ B cells at several time points during the first 36 months of life (Fig 3, A). This was confirmed by means of correlation analysis; we found a significant moderate inverse correlation between BAFF levels at birth and proportions of CD5⁺ B cells at 3 to 5 days (Fig 3, B), at 36 months ($r = -0.47$, $P = .01$),

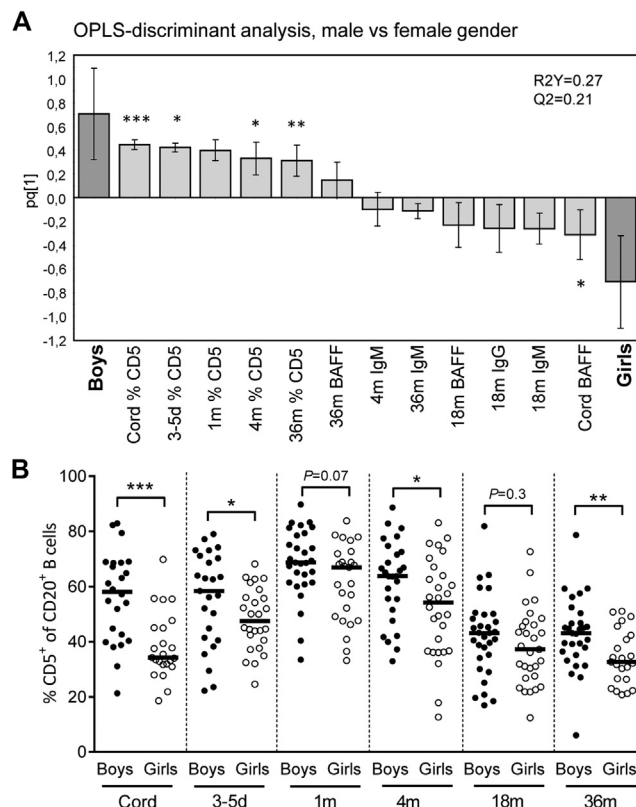


FIG 5. Boys and girls differ with respect to postnatal B-cell maturation. A, OPLS discriminant analysis column loading plot depicting associations between sexes and B-cell variables. The OPLS discriminant analysis column plot is based on variables with VIP values of greater than 1.1. R^2Y indicates how well the variation of the Y variable is explained, and Q^2 indicates how well the Y variable can be predicted. B, Proportion of CD5⁺ B cells within CD20⁺ total B cells in blood from boys and girls at several time points in the first 36 months of life. Horizontal bars indicate median. $*P \leq .05$, $**P \leq .01$, and $***P \leq .001$, Mann-Whitney *U* test.

and at birth ($r = -0.42$, $P = .03$). BAFF levels at birth were also positively related to BAFF levels at later time points, which was also confirmed by means of correlation analysis (at 1 month: $r = 0.4$, $P = .04$; at 18 months: $r = 0.54$, $P = .002$). Among farmers' children, BAFF levels in cord blood were inversely associated with proportions of CD5⁺ B cells at 1 and 4 months of age and with B-cell numbers at birth and 1 month of age (Fig 3, C). In this group we also found a positive association between cord blood BAFF levels and proportions of CD27⁺ memory B cells at 3 to 5 days and at 1 and 4 months of age (Fig 3, C). However, only BAFF levels at birth and the proportion of CD27⁺ B cells at 4 months of age correlated significantly in univariate analysis (Fig 3, D). All significant univariate correlations are indicated with asterisks in Fig 3, A and C.

Taken together, higher BAFF levels at birth were related to lower proportions of immature/naive B cells among nonfarmers' children and to higher proportions of memory B cells among farmers' children. Although there are somewhat different association patterns between BAFF levels in cord blood and subsequent B-cell maturation, these results indicate that higher BAFF levels at birth are related to a more accelerated B-cell maturation in both groups of children.

It is known that B cells bind BAFF and reduce circulating levels of this cytokine.^{13,14} The highest BAFF levels were observed

TABLE I. Demographic data

	No. (%) of children			<i>P</i> value
	All (n = 65)	Dairy farm (n = 28)	No farm (n = 37)	
Girls	32 (49)	18 (64)	14 (38)	.05
Cesarean section	10 (15)	3 (11)	7 (19)	.49
Birth weight (g)	3545 (2440-4830)	3525 (2780-4740)	3618 (2440-4830)	.61
Mother with history of allergy*	18 (28)	7 (25)	11 (30)	.78
Age of mother at delivery	32 (21-42)	33 (21-42)	32 (22-41)	.47
Sibling at birth	35 (54)	18 (64)	17 (46)	.21
Dog at birth†	22 (35)†	13 (46)	9 (26)†	.11
Cat at birth‡	26 (41)‡	14 (50)	12 (33)‡	.21

The statistical difference between farmers' and nonfarmers' children is shown as the *P* value (Fisher exact test or Mann-Whitney *U* test). Boldface value indicates statistical significance.

*Mother with a history of doctor-diagnosed asthma, allergic rhinoconjunctivitis, or eczema.

†Data obtained from 35 nonfarming families.

‡Data obtained from 36 nonfarming families.

TABLE II. Effect of lifestyle factors on cord blood BAFF levels determined by using multiple linear regression analysis

Variable	B	<i>P</i> value
Intercept	1.67	
Dairy farm	0.39	.04
Female sex	0.38	.04
Dog at birth	0.17	.36
Cesarean section	−0.11	.63
<i>R</i> ²	0.26	

Boldface values indicate statistical significance.

B, Unstandardized regression coefficient; *Intercept*, point at which the curve intersects the y-axis (BAFF in nanograms per milliliter); *R*² (× 100), percentage of BAFF variation explained by the model.

when the numbers of circulating CD20⁺ B cells were lowest (ie, at birth; see Fig E2, A, in this article's Online Repository at www.jacionline.org; all data regarding B-cell numbers have been published previously by Lundell et al⁵). Furthermore, the striking decrease in BAFF levels in the first month of life occurred in parallel with a significant increase in B-cell numbers in the periphery (see Fig E2, A). We examined the relationship between BAFF levels and B-cell numbers at different time points. There was a weak inverse correlation between BAFF levels and B-cell numbers at birth and 36 months of age (see Fig E2, B and C) but not at any other time points, which indicates that the postnatal homeostatic expansion of B cells is not solely regulated by BAFF.

BAFF levels at birth in relation to development of allergic disease

Next, we examined the association between cord blood BAFF levels and subsequent development of allergic disease at 18 and 36 months of age. Children who were given a diagnosis of food allergy, eczema, asthma, and/or allergic rhinoconjunctivitis are presented in Table E1. We compared children who were given a diagnosis of any allergic disease at 18 months of age with those who were not. Children who were given a diagnosis of any allergic disease at 36 months were compared with those who were nonallergic at both 18 and 36 months of age. As shown in Fig 4, A, children who were allergic at 18 months of age had significantly lower BAFF levels at birth compared with nonallergic children. There was a similar trend at 36 months of age, although it was not statistically significant (Fig 4, B).

Separate analyses for farmers' and nonfarmers' children could not be performed because of the low number of children with allergic disease among farming families. Thus children who have allergic disease in the first 3 years of life seem to have lower BAFF levels at birth compared with children who remain nonallergic.

Boys and girls differ with respect to postnatal B-cell maturation

Girls had higher BAFF levels in cord blood than boys (Fig 2, C), an observation that prompted us to examine whether B-cell maturation differed between the sexes by using OPLS discriminant analysis. Variables with the largest contribution to the model were selected (ie, only variables with VIP values >1.1). Variables represented by a bar pointing in the same direction as boys are positively associated with male sex, whereas bars pointing in the same direction as girls are positively related to female sex (Fig 5, A). Male sex was related to higher proportions of immature/naïve CD5⁺ B cells at several time points over the first 3 years of life (Fig 5, A). These multivariate associations were confirmed in univariate analysis: boys had significantly higher proportions of immature/naïve CD5⁺ B cells at birth, 3 to 5 days of age, and 4 and 36 months of age relative to girls (Fig 5, B). Girls had higher BAFF levels in cord blood, as previously shown (Fig 2, C). There were no significant differences between girls and boys with respect to IgM or IgG levels at 18 months of age. All statistically significant differences in univariate analyses are indicated with asterisks in Fig 5, A.

DISCUSSION

We have recently shown that a high proportion of immature/naïve CD5⁺ B cells in early infancy is a predictor for development of allergic disease later in childhood.⁴ Previous work has also shown that delayed secretory IgA^{17,18} or serum IgA¹⁹ production is a risk factor for allergy development. This prompted us to study BAFF, an important cytokine for maturation of immature transitional B cells, and to investigate environmental factors that could stimulate its production in a prospective study. Here we show that circulating BAFF levels were maximal at birth when measured on repeated occasions over the first 3 years of life. Farmers' children had increased concentrations of BAFF in cord blood compared with nonfarmers' children living in the

same geographic area, and higher BAFF levels at birth were associated with a more rapid B-cell maturation later during childhood. Moreover, cord blood BAFF levels were lower among children with an allergic disease at 18 months of age compared with those who were nonallergic. We also found that boys had lower BAFF levels in cord blood than girls, as well as higher proportions of immature/naive CD5⁺ B cells. Hence delayed B-cell maturation might contribute to the higher prevalence of allergic disease among boys during early childhood.^{20,21}

Longitudinal data regarding circulating BAFF concentrations over the first years of life have been lacking. Here we show that the highest BAFF levels were found already at birth. Previous cross-sectional data demonstrate that circulating BAFF levels decrease with age, but relatively few subjects were included in each age group.²² Thus the drastic decrease in BAFF levels in the first month of life followed by a slow increase over the first 18 months of life that we observed in our cohort is a novel finding. We also demonstrate that newborn children had significantly higher BAFF levels in the circulation compared with their mothers, a finding also observed by others.^{14,23} It has been shown that BAFF is secreted by monocyte-derived macrophages and dendritic cells in response to IFN- γ and IL-10 and by airway epithelial cells when stimulated with double-stranded RNA.^{7,9} However, which factors trigger BAFF production *in vivo* is not known.

For the first time, to our knowledge, we demonstrate that a farming lifestyle is associated with BAFF protein levels in children. Infants born of mothers who lived on a dairy farm during pregnancy had significantly higher concentrations of BAFF in cord blood compared with children born of nonfarming mothers. One plausible explanation for higher BAFF levels in blood at birth among farmers' children could be that these families are exposed to a higher degree and variety of microbes and microbial compounds. For example, farming families have higher levels of endotoxin, an intrinsic part of the outer membrane of gram-negative bacteria; mold β (1,3)-glucans; and fungal extracellular polysaccharides in their homes compared with nonfarming families.^{24,25} Later in childhood, farming status might not be associated with circulating BAFF levels. It was recently shown that white blood cells from farmers' and nonfarmers' children aged 5 to 13 years do not differ in gene expression of BAFF when using quantitative real-time PCR.²⁶

Because the difference in BAFF levels in relation to farming status was found already at birth, microbes or their products might reach the placenta, where BAFF can be produced in response to these stimuli. Bacterial cells, bacterial fragments, or both have been demonstrated by using histology and DNA-based analysis in human placentas obtained from normal full-term pregnancies.^{27,28} It has also been shown that *in utero* priming of the fetal immune system in response to maternal parasitic infections occurs because newborn children born to schistosome-infected mothers have enhanced levels of schistosome egg-specific IgE in cord blood.²⁹

Toll-like receptors (TLRs) are expressed by various cells of the human placenta. Explants obtained from tissue beneath the decidua basalis of the maternal side of the placenta express transcripts for TLR1 to TLR10, and macrophages and natural killer (NK) cells isolated from the decidua basalis express transcripts for TLR1 to TLR9.^{30,31} Regarding fetal cells of the placenta, primary first-trimester trophoblasts display gene expression of TLR1 to TLR10 when examined by means of

quantitative RT-PCR.³² Both decidua-isolated immune cells and first-trimester trophoblasts produce proinflammatory cytokines in response to several TLR ligands.^{31,32}

Interestingly, BAFF mRNA, as well as BAFF protein, is expressed by both maternal decidual cells and fetal trophoblasts,³³ and fetal mesenchymal cells from the placenta contain high levels of BAFF transcripts.³⁴ Thus placenta-derived cells could be a potential source for microbially triggered production of BAFF measured in cord blood. Also, because newborn children had higher BAFF levels compared with their mothers, cells from the fetal side of the placenta, including trophoblasts and mesenchymal cells, may produce BAFF that is transported into the circulation of the fetus.

In mice BAFF plays a well-known role for B-cell maturation at the immature transitional stage. In cell-culture systems BAFF stimulates the differentiation of CD23⁻ immature transitional cells into a more mature B-cell phenotype that express CD23.^{35,36} Accordingly, BAFF^{-/-} and wild-type mice have similar numbers of CD23⁻ immature transitional B cells in the spleen, whereas BAFF^{-/-} mice present with a marked reduction in numbers of more mature CD23⁺ B cells.¹¹ Human BAFF-R deficiency resembles the phenotype found in BAFF mutant mice because they exhibit a much higher proportion of circulating CD10⁺ transitional cells and a lower percentage of more mature CD27⁺ B-cell subsets compared with healthy control subjects.¹³ Among nonfarmers' children, we found that BAFF levels at birth inversely correlated with proportions of immature/naive CD5⁺ B cells at birth and also later during childhood. Among farmers' children, on the other hand, higher cord blood BAFF levels correlated positively with the proportions of CD27⁺ memory B cells at 4 months of age. Although somewhat different association patterns exist between BAFF levels in cord blood and subsequent B-cell maturation, our results indicate that higher BAFF levels at birth are related to a more rapid subsequent B-cell maturation in both groups of children. Additionally, because moderate, but not strong, inverse correlations were found between BAFF levels and proportions of immature/naive B cells among nonfarmers' children, our results also suggest that BAFF is one, but not the only, factor that influences proportions of immature/naive B cells. Indeed, we have previously shown that proportions of CD5⁺ B cells do not differ between farmers' and nonfarmers' children.⁴

We also examined the association between cord blood BAFF levels and subsequent development of allergic disease because we and others have demonstrated a lower prevalence of allergic disease among farmers' children^{4,37,38} and because a high proportion of immature/naive B cells in early infancy is a risk factor for allergic disease.⁴ We found that children who were given a diagnosis of an allergic disease (ie, food allergy, eczema, asthma, and/or allergic rhinoconjunctivitis) at 18 months of age had significantly lower BAFF levels at birth compared with nonallergic children. At 36 months of age, there was a similar non-statistically significant trend. Although the cohort is small and these results do not demonstrate a causal relationship between BAFF levels at birth and allergy outcome, they still suggest that higher cord blood BAFF levels are associated with a lower risk of allergic disease in early childhood.

We also found that boys had significantly lower BAFF levels at birth compared with girls and that boys had strikingly higher proportions of immature/naive CD5⁺ B cells at this time point but also at several time points later in childhood. In fact, among the

B-cell variables assessed here, the proportion of CD5⁺ B cells was the most strongly positively associated variable with male sex. We have previously reported from the FARMFLORA study that there was a significantly higher prevalence of allergic disease among boys compared with girls at 36 months of age,⁴ a finding that is in accordance with other studies.^{20,21} It has been suggested that higher prevalence of asthma in boys could be a result of their smaller airways relative to lung size compared with girls.³⁹ However, because a high proportion of CD5⁺ B cells in infants is a risk factor for allergic disease,⁴ we propose that the high percentage of this cell subset in combination with lower BAFF levels might be a marker of a more immature/naive immune system in general that could contribute to the higher prevalence of allergic diseases among boys in early childhood.

One limitation of the present study is that the combination of CD24 and CD38 was not used in the flow cytometric panel. These markers, now commonly used to distinguish different peripheral B-cell maturational stages in human subjects, were acknowledged after this prospective study was initiated in 2004.¹⁶ However, with the use of blood samples from healthy unselected children, we show that the majority of the CD5⁺ B-cell population at birth and at 8 years of age is of a CD24^{hi/+}CD38^{hi/+} immature/naive phenotype. Moreover, the relatively small cohort might be a limitation. Yet our data clearly demonstrate significant differences among the immunologic molecules investigated. The relatively small cohort also permitted detailed and structured examinations by a study pediatrician at 18 and 36 months of age and also between follow-ups if symptoms suggesting commencement of allergic disease occurred. Although the criteria used for an asthma diagnosis are not the same as those used in older children and adults, our criteria are probably the best available without using lung function tests. In a future follow-up of children aged 7 to 9 years in this cohort, we will be able to examine the relationship between neonatal BAFF levels and allergic disease with even more strict criteria, especially for asthma. Another strength of the present study is that all flow cytometric analyses were performed blindly because all demographic and clinical data were obtained after cell data were compiled.

In conclusion, for the first time, we demonstrate that circulating BAFF concentrations are maximal at birth and that higher neonatal levels of this cytokine were related to a more accelerated B-cell maturation later in childhood. We also show that maternal dairy farm exposure during pregnancy appears to stimulate increased BAFF levels in the newborn child. These findings in combination with the fact that children with an allergic disease at 18 months of age had lower cord blood BAFF levels compared with children who remained nonallergic add further evidence to the role of B cells in the hygiene hypothesis. Additional studies are now required to identify the cell source for cord blood BAFF and to identify specific factors associated with a farming environment that trigger production of this cytokine.

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Key messages

- Children born of mothers who are exposed to a dairy farm environment during pregnancy have increased BAFF levels in cord blood.
- Higher neonatal BAFF levels are associated with more rapid B-cell maturation later in childhood.
- Boys have lower BAFF levels in cord blood than girls, as well as higher proportions of immature/naive CD5⁺ B cells.

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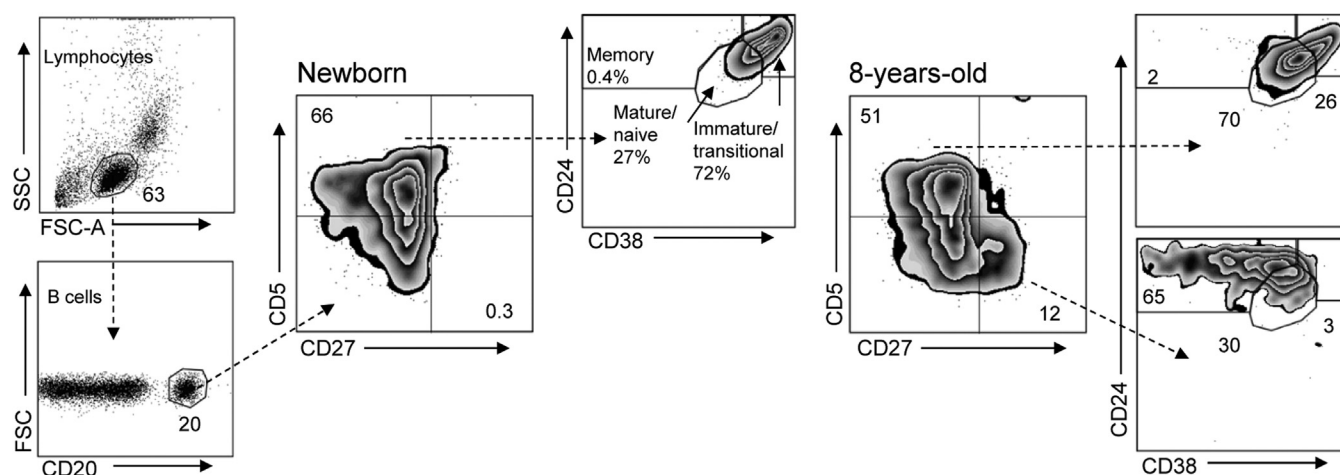


FIG E1. Gating strategy for lymphocytes and CD20⁺ B cells, with zebra plots demonstrating the proportion of immature transitional, mature naive, or memory B cells based on CD24 and CD38 expression within the CD5⁺ or CD5⁻ B-cell subsets in blood from 1 newborn child and 1 8-year-old child. The following mAbs were used: APC-H7-conjugated anti-CD20 (clone L27, BD Biosciences), phycoerythrin-Cy7-conjugated anti-CD24 (clone ML5, BD Biosciences), phycoerythrin-conjugated anti-CD38 (clone HB7, BD Biosciences), APC-conjugated CD5 (clone UCHT2; PharMingen, San Diego, Calif), and FITC-conjugated anti-CD27 (clone L128, BD Biosciences). All samples were run in a FACSCanto II (BD Biosciences) equipped with FACSDiva software and analyzed with FlowJo software. FSC, Forward scatter; SSC, side scatter.

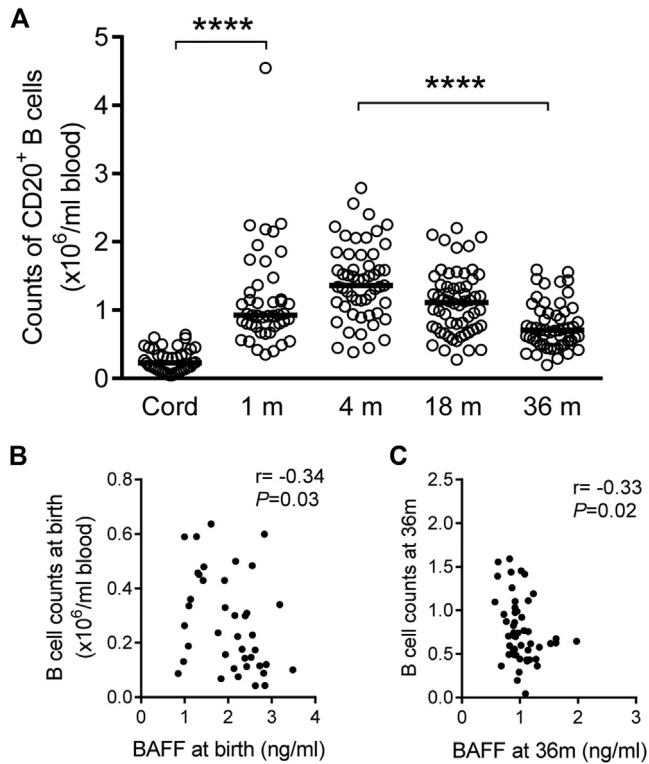


FIG E2. **A**, Numbers of circulating B cells within the lymphocyte gate at birth and at 1, 4, 18, and 36 months of age. **B** and **C**, Correlation plots depicting BAFF levels in relation to B-cell counts at birth and 36 months of age, respectively. Fig E2, **A**, Horizontal bars indicate medians. **** $P \leq .0001$, Kruskal-Wallis test, followed by the Dunn multiple-comparison test. Fig E2, **B** and **C**, Spearman correlation test. Fig E2, **A**, All data regarding B-cell numbers have been published previously by Lundell et al.⁵

TABLE E1. Allergic disease and sensitization in children at 18 and 36 months of age

Child ID	Age 18 mo					Age 36 mo				
	Food allergy	Eczema	ARC	Asthma	Sensitization	Food allergy	Eczema	ARC	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 mo				
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

ARC, Allergic rhinoconjunctivitis.