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

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Higher BAFF levels at birth are positively associated with maternal dairy farm exposure and negatively related to allergy development

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Abstract

BACKGROUND: A high proportion of circulating immature/naïve $CD5^+$ B cells during early infancy is a risk factor for allergy development. BAFF is an important cytokine for B cell maturation.

OBJECTIVE: To investigate whether BAFF levels are related to environmental exposures during pregnancy and early childhood, and if BAFF levels are associated with postnatal B cell maturation and allergic disease.

METHODS: In the FARMFLORA-study, including farming and non-farming families, we measured BAFF levels in plasma from mothers and their children at birth, and at 1, 4, 18 and 36 months of age. Infant 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 non-farmers' 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/naïve B cells among non-farmers' children. Children who had developed allergic disease at 18 months of age had lower cord blood BAFF levels than non-allergic 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 a more accelerated postnatal B cell maturation, which lend further strength to the role of B cells in the hygiene hypothesis.

Key messages

- Children born by mothers who are exposed to a dairy farm environment during pregnancy have elevated BAFF levels in cord blood.
- Higher neonatal BAFF levels are associated with a 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/naïve $CD5^+$ B cells.

Capsule summary

BAFF is important for differentiation of immature to mature B cells. Farm exposure during pregnancy induces elevated BAFF levels in the newborn child, and high neonatal BAFF levels are associated with an accelerated B cell maturation.

Key words

prospective birth-cohort, BAFF, dairy farm, pregnancy, immature/naïve B cells, memory B cells, hygiene hypothesis, allergy, gender

Abbreviations

BAFF, B cell activating factor

OPLS, orthogonal projection to latent structures by means of partial least squares

VIP, variable influence on projection

Introduction

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 humans (1). At birth approximately half of the blood B cell pool consists of transitional cells, while they represent about 5% in adults (1, 2). The immature transitional B cells mature into naïve cells and when they encounter their cognate antigens in secondary lymphoid organs, they may 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 gradually increases, while CD38 and CD5 expression decreases (1, 2, 4). In children, circulating B cell numbers and the proportion of $CD5^+$ cells among the $CD20^+$ B cells rise considerably from birth up to four months of age (5), which might indicate augmented export of immature/naïve 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), suggestedly due to 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 due to reduced exposure to microbes during early life is a risk factor for development of allergy (6). Accordingly, we recently showed that a high proportion of circulating immature/naïve $CD5^+$ B cells during the first month of life predicts development of allergic disease later in childhood (4). Which factors that regulate the size of the $CD5^+$ B cell subset in humans are unclear. BAFF, B cell activating factor, is a member of the TNF family that is produced by both innate immune cells and non-hematopoeitic cells, including airway epithelial cells (7-9). Signals induced when BAFF interacts with its main receptor BAFF-R on B cells are pivotal for survival and differentiation of immature transitional B cells into mature naïve 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). BAFF-deficient mice also exhibit a profound reduction in total immunoglobulin levels (11). In line with this, two CVID patients with BAFF-R-deficiency have been reported to display arrested B cell development at the stage of transitional B cells and reduction in the numbers of all subsequent B cell maturational stages (13). It has also been suggested that BAFF-binding receptors on B cells regulate the BAFF levels in the circulation, since individuals 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 are unclear. Further, the role of environmental exposures and the levels of BAFF in the circulation has not been examined. To address these questions, we measured BAFF levels in plasma over the first three years of life in the FARMFLORA study, including farming and non-farming families from the same geographical 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 farmers' children displayed higher levels of this cytokine in cord blood compared to non-farmers' children. We also found that BAFF levels at birth were related to higher proportions of memory B cells among farmers' children, and to lower proportions of immature/naïve B cells among non-farmers' children later in childhood. Finally, children who had developed an allergic disease at 18 months of age had significantly lower cord blood BAFF levels compared to children who remained non-allergic.

Material and Methods

Study design

In the prospective FARMFLORA study, farming and non-farming families from rural areas in the Skaraborg region in South-West 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 into the non-faming group should live in the same rural geographical region, but not on a farm. Sixty-five healthy infants born at term (≥38 gestational weeks) were included into the study and have hitherto been followed up to 3 years of age. Twenty-eight of the children were included into the farming group, while 37 were included into the control group. Blood samples were obtained from the umbilical cord at delivery (6ml) and venous blood was sampled at 3-5 days (1ml), and at 1 (3ml), 4 (3ml), 18 (6ml) and 36 (6ml) months of age. Venous blood was also drawn from the mothers, either before delivery (n=10, median and range: 19 days: 1-60 days) or after delivery (n=42, 30 days: 3-480 days). For flow cytometric analyses of CD24 and CD38 expression within the $CD5^+$ or $CD5^{neg}$ B cell populations (Repository Figure 1), 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 the 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 diagnoses

The children were examined for sensitization and allergic disease by a paediatrician at 18 and 36 months of age as previously described (4). Briefly, venous blood was collected for total IgE and presence of specific IgE against food (6-mix food test, Phadia/Pharmacia Diagnostics, Uppsala, Sweden) and inhalant allergens (Phadiatop®, Phadia/Pharmacia Diagnostics). Positive samples were further analysed 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 was considered positive. 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.

Based on clinical examination and results of laboratory tests, the following diagnostic groups were defined: *Food allergy*, 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 and/or verified by an open food challenge test; *Eczema*, diagnosed according to Williams' criteria (15). Eczema at 18 months denoted diagnosis at any time before or at 18 months, while eczema at 36 months required symptoms to be present after 24 months of age; *Asthma*, persistent wheezing for ≥4 weeks or ≥3 episodes of wheezing combined with ≥1 minor criterion (symptoms between colds, eczema, allergic rhinoconjuctivitis, or food allergy). For asthma at 36 months, ≥1 wheezing episode 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*, symptoms in the eyes and/or nose upon exposure to pollen or animal dander, together with a positive allergen-specific IgE test directed against a corresponding allergen. Repository table 1 displays children with allergic disorders and/or sensitization 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. The levels of BAFF were determined using human BAFF/BLyS/TNFSF13B Quantikine ELISA Kits according to the manufacturer's instructions (R&D Systems, Minneapolis, MN, USA). 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, England), whereafter diluted plasma and standards (polyclonal IgM, IgG or IgA from human plasma, Calbiochem, Darmstadt, Germany) were added. Detection was performed by horseradish peroxidase (HRP)-conjugated goat anti-human IgM, IgG or IgA (Jackson), followed by treatment with O-phenylenediamine dihydrochloride (OPD; Sigma-Aldrich, St. Louis, USA) and addition of H_2O_2 as substrate. Total IgE levels were measured with ImmunoCAP®250 (Phadia, Uppsala, Sweden). Owing to a limited volume of blood obtained from each child, measurement of BAFF and immunoglobulins could not be performed for all children at all time points.

Phenotypic characterization of B cells 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 (5). Briefly, the following monoclonal antibodies were used: PerCP-conjugated anti-CD20 (clone L27, BD Bioscience, Erembodegem, Belgium), APC-conjugated anti-CD5 (clone UCHT2, Pharmingen, San Diego, California), FITC-conjugated anti-CD27 (clone L128, BD Bioscience). APC- and FITC-conjugated mouse immunoglobulin (Ig)G1 (clone X40, APC IgG_1 and FITC IgG_1, BD Bioscience) were used as controls when gating on the $CD5^+$ and

CD27$^+$ populations, respectively. For absolute cell numbers, the TruCOUNT assay was employed (BD Bioscience), also described in detail elsewhere (5). Samples were run in a FACS-Calibur (BD Bioscience) equipped with CellQuestPro software, or in a FACS-Canto II (BD Bioscience) equipped with FACS-Diva software and analyzed with FlowJo (TreeStar, Ashland, Oregon).

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 to each other in linear multivariate models (Fig. 2A and 3A-B). OPLS-discriminant analysis (DA) was implemented to examine if male and female sex could be discriminated based on the various B cell parameters examined (Fig. 5A). All data were scaled to unit variance by dividing each variable by 1/(SD), where SD represents the standard deviation 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 R2, i.e., how well the variation of the variables is explained by the model, and Q2, i.e. how well a variable can be predicted by the model. OPLS plots in Fig. 3 and 5 are based on X-variables with VIP-values >0.9 and >1.1, respectively. Variable of importance (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 model to avoid mass significance. Univariate analyses were performed with two-tailed Mann-Whitney U test, Spearman's rank correlation test, ANOVA post test for linear trend, or Fisher's exact test as described in the figure legends and in Table 1 (GraphPad Prism; GraphPad Software, La Jolla, CA). A *P*-value $\leq$0.05 was regarded as being statistically significant.

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 plasma from the mothers. The highest levels of BAFF were found in cord blood (Fig. 1). At one month of age, these levels had dropped 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 ($P \leq 0.0001$ 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 which signals that trigger their production *in vivo* is not known. We investigated the relation of BAFF levels in cord blood with a range of potential explanatory maternal factors using the multivariate factor analysis method orthogonal projection to latent structures by means of partial least squares (OPLS). Figure 2A shows which factors that are positively or negatively associated with BAFF levels in cord blood, i.e. 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. 2A). Univariate analysis confirmed that farmers' children had significantly higher BAFF levels at birth compared to non-farmers' children (Fig. 2B). This difference was also evident at 4 months of age, but not at any other time point examined (P=0.005 at 4 months). Girls had significantly higher BAFF levels in cord blood relative to boys (Fig. 2C).

All statistically significant differences in univariate analyses are indicated with asterisks in Fig. 2A. There was, however, no difference in circulating BAFF levels between farming and non-farming mothers (*P*=0.32), or between mothers giving birth to a girl or a boy (*P*=0.67). Female gender was overrepresented among farming families (Table 1). To resolve these factors, we performed multiple regression analysis. As shown in Table 2, farming environment and female gender both contributed independently to BAFF levels at birth.

We examined the relation between mother and infant BAFF levels. Since farming environment was related to higher BAFF levels in cord blood, we performed separate correlation analyses for mother-infant pairs among farming and non-farming families, respectively. Cord blood levels of BAFF were unrelated to maternal BAFF levels, both within the farming and the non-farming group (Fig. 2D-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 if BAFF levels in cord blood were associated with B cell maturation, measured as B cell numbers, proportions of immature/naïve $CD5^+$ and memory $CD27^+$ B cells, and total levels of IgM, IgG, IgA, IgE and BAFF, analyzed in blood on repeated occasions from birth to 36 months of age. Repository Fig. 1 demonstrates that the vast majority of all circulating $CD5^+$ B cells at birth and in 8-years-old children are of an $CD24^{+/hi}CD38^{+/hi}$ immature/mature naïve phenotype, whereas the majority of the $CD27^+$ B cells in 8-years-old children are of an $CD24^{+/hi}CD38^{low/neg}$ memory phenotype. Analysis of CD24 and CD38 was not used in this study since the combination of these two markers to distinguish different human B cell maturational 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 OPLS, i.e. 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, i.e. only variables with VIP-values >0.9 (variable influence on projection), and presented in Fig. 3A and C. Among non-farmers' children, higher BAFF levels in cord blood were inversely related to the proportions of immature/naïve $CD5^+$ B cells at several time points during the first 36 months of life (Fig. 3A). This was confirmed by correlation analysis, we found a significant moderate inverse correlation between BAFF levels at birth and the proportions of $CD5^+$ B cells at 3-5 days (Fig. 3B), at 36 months (r=-0.47, *P*=0.01) and at birth (r=-0.42, *P*=0.03). BAFF levels at birth were also positively related to BAFF levels at later time points, which was also confirmed by correlation analysis (at 1 month r=0.4, *P*=0.04 and at 18 months r=0.54, *P*=0.002). Among farmers' children, BAFF levels in cord blood were inversely associated with the proportions of $CD5^+$ B cells at 1 and 4 months of age and with B cell numbers at birth and at 1 month of age (Fig. 3C). In this group, we also found a positive association between cord blood BAFF levels and the proportions of $CD27^+$ memory B cells at 3-5 days, at 1 and at 4 months of age (Fig. 3C). However, only BAFF levels at birth and the proportion of $CD27^+$ B cells at 4 months of age correlated significantly in univariate analysis (Fig. 3D). All significant univariate correlations are indicated with asterisks in Fig. 3A and C. Taken together, higher BAFF levels at birth were related to lower proportions of immature/naïve B cells among non-farmers' children, and to higher proportions of memory B cells among farmers' children. Although 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 levels of BAFF were observed when the numbers of circulating $CD20^{+}$ B cells were lowest, i.e. at birth (Repository Fig. 2A, all data regarding B cell numbers have been published previously by Lundell et al (5)). Further, 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 (Repository Fig. 2A). 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 at 36 months of age (Repository Fig. 2B-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 diagnosed with food allergy, eczema, asthma and/or allergic rhinoconjuctivitis are presented in Repository table 1.We compared children who were diagnosed with any allergic disease at 18 months of age with those who were not. Children who were diagnosed with any allergic disease at 36 months were compared to those who were non-allergic at both 18 and 36 months of age. As shown in Fig. 4A, children who were allergic at 18 months of age had significantly lower BAFF levels at birth compared to non-allergic children. There was a similar trend at 36 months of age, although not statistically significant (Fig. 4B). Separate analyses for farmers' and non-farmers' children could not be performed due to the low number of children with allergic disease among farming families. Open symbols indicate farmers' children in Fig. 4A-B. Thus, children who develop allergic disease in the first three years of life seem to have lower BAFF levels at birth compared to children who remain non-allergic.

Boys and girls differ with respect to postnatal B cell maturation

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

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 have also shown that delayed secretory IgA (17, 18) or serum IgA (19) production are risk factors 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. We here show that circulating BAFF levels were maximal at birth when measured on repeated occasions over the first three years of life. Farmers' children had elevated concentrations of BAFF in cord blood compared to non-farmers' children living in the same geographical 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 non-allergic. We also found that boys had lower BAFF levels in cord blood than girls, as well as higher proportions of immature/naïve $CD5^+$ B cells. Delayed B cell maturation may, hence, 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. We here 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 drop 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 therefore a novel finding. We also demonstrate that newborn children had significantly higher BAFF levels in the circulation compared to their mothers, a finding also observed by others (14, 23). It has been shown that BAFF is secreted by monocyte-derived macrophages and DCs in response to IFN-γ and IL-10, and by airway epithelial cells when stimulated with dsRNA (7, 9). Yet, which factors that trigger BAFF production *in vivo* is not known.

For the first time, to our knowledge, we here demonstrate that a farming lifestyle was associated with BAFF protein levels in children. Infants born by mothers who lived on a dairy farm during pregnancy had significantly higher concentrations of BAFF in cord blood compared to children born by non-farming 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, mould β(1,3)-glucans and fungal extracellular polysaccharides in their homes compared to non-farming families (24, 25). Later in childhood farming status may not be associated with circulating BAFF levels. It was recently shown that white blood cells from farmers' and non-farmers' children, aged 5-13 years, do not differ in gene expression of BAFF when using quantitative real-time PCR (26).

Since 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 may be produced in response to these stimuli. Bacterial cells and/or bacterial fragments have been demonstrated in the human placenta obtained from normal full term pregnancies by histology and by DNA-based analysis (27, 28). It has also been shown that *in utero* priming of the fetal immune system in response to maternal parasitic infections occurs as newborn children born to schistosome-infected mothers have enhanced levels of schistosome egg (SEA)-specific IgE in cord blood (29). 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-TLR10, and macrophages and NK-cells isolated from the decidua basalis express transcripts for TLR1-TLR9 (30, 31). Regarding fetal cells of the placenta, primary first-trimester trophoblasts display gene expression of TLR1-TLR10 when examined by RT-qPCR (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 the 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 microbial-triggered production of BAFF measured in cord blood. Also, since newborn children had higher BAFF levels compared to 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 $CD23^{neg}$ 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 $CD23^{neg}$ immature transitional B cells in the spleen, while $BAFF^{-/-}$ 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 as they exhibit a much higher proportion of circulating $CD10^{+}$ transitional cells, and lower percentage of more mature $CD27^{+}$ B cell subsets compared to healthy controls (13). Among non-farmers' children, we found that BAFF levels at birth inversely correlated with the proportions of immature/naïve $CD5^{+}$ 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 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, as moderate, but not strong, inverse correlations were found between BAFF levels the proportions of immature/naïve B cells among non-farmers' children, our results also suggest that BAFF is one, but not the only, factor that influence the proportions of immature/naïve B cells. Indeed, we have previously shown that the proportions of $CD5^+$ B cells do not differ between farmers' and non-farmers' children (4).

We also examined the association between cord blood BAFF levels and subsequent development of allergic disease since we and others have demonstrated a lower prevalence of allergic disease among farmers' children (4, 37, 38), and since a high proportion of immature/naïve B cells in early infancy is a risk factor for allergic disease (4). We found that children who were diagnosed with an allergic disease, i.e. food allergy, eczema, asthma and/or allergic rhinoconjunctivitis, at 18 months of age had significantly lower BAFF levels at birth compared to non-allergic 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 developing allergic disease in early childhood.

We also found that boys had significantly lower BAFF levels at birth compared to girls, and boys had strikingly higher proportions of immature/naïve $CD5^+$ 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 gender. We have previously reported from the FARMFLORA study that there was a significantly higher prevalence of allergic disease among boys compared to 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 to girls (39). However, since 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, may be a marker of a more immature/naïve 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 cytometry panel. These markers, now commonly used to distinguish different peripheral B cell maturational stages in humans, 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/naïve phenotype. Moreover, the relatively small cohort may be a limitation. Yet, our data clearly demonstrate significant differences among the immunological 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 7-9 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 cytometry analyses were performed blindly as all demographic and clinical data were obtained after the cell data was compiled.

In conclusion, we here demonstrate for the first time that circulating BAFF concentrations are maximal at birth, and 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 elevated BAFF levels in the newborn child. These findings in combination with the fact that children who had developed an allergic disease at 18 months of age had lower cord blood BAFF levels compared to children who remained non-allergic 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 levels, and to identify specific factors associated with a farming environment that triggers the production of this cytokine.

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Conflict of interest

The authors declare no conflict of interest

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Figure legends

Figure 1. Circulating BAFF levels are maximal at birth. BAFF levels in plasma at birth, at 1, 4, 18 and 36 months of age and in plasma from the mothers. 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 the median and **** $P \leq 0.0001$ (Mann-Whitney U-test and ANOVA post test for linear trend).

Figure 2. Farmers' children have higher BAFF levels at birth than non-famers' children. **(A)** OPLS column loadings plots depicting the association between BAFF levels at birth (Y) in relation to X-variables including: farming environment, gender, elder sibling, pets at home, delivery mode, birth weight, maternal age at delivery and maternal history of allergy. R2Y indicates how well the variation of Y is explained, while Q2 indicates how well Y can be predicted. **(B)** BAFF levels in plasma at birth among farmers' and non-farmers' children. Open and filled symbols indicate female and male gender, respectively. **(C)** BAFF levels in cord blood from girls and boys. Open and filled symbols indicate farmers' and non-farmers' children, respectively. **(D-E)** Correlations between maternal and infant BAFF levels among farming and non-faming families. **(B-C)** Horizontal bars indicate the median and *$P \leq 0.05$ and **$P \leq 0.01$ (Mann-Whitney U-test) and **(D-E)** Spearman's correlation test.

Figure 3. Higher BAFF levels at birth are associated with a higher degree of B cell maturation in the circulation later in childhood. **(A and C)** OPLS column loadings plots depicting the association between BAFF levels at birth among non-farmers' children **(A)** or farmers' children **(C)** (Y-variables) in relation to X-variables including: numbers of $CD20^+$ B cells, the proportions of immature/naïve $CD5^+$ 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 >0.9. R2Y indicates how well the variation of Y is explained, while Q2 indicates how well Y can be predicted. **(B)** Correlation between BAFF levels at birth and the proportions of $CD5^+$ B cells at 3-5 days of age among non-farmers' children. **(D)** Correlation between BAFF levels at birth and the proportions of $CD27^+$ B cells at 4 months of age among farmers' children. **(B and D)** Spearman's correlation test.

Figure 4. Cord blood BAFF levels and development of allergic disease. **(A-B)** BAFF levels at birth in non-allergic children and in children diagnosed with any allergic disease, i.e. food allergy, eczema, asthma and/or allergic rhinoconjuctivitis, at 18 and 36 months of age, respectively. Open symbols indicate farmers' children. Horizontal bars show the median and $^*P \leq 0.05$ (Mann-Whitney U-test).

Figure 5. Boys and girls differ with respect to postnatal B cell maturation. **(A)** OPLS-DA column loadings plot depicting the associations between the genders and B cell variables. The OPLS-DA column plot is based on variables with VIP-values >1.1. R2Y indicates how well the variation of Y is explained, while Q2 indicates how well Y can be predicted. **(B)** The 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 the median and $^*P \leq 0.05$, $^{**}P \leq 0.01$ and $^{***}P \leq 0.001$ (Mann-Whitney U-test).

Figure 1

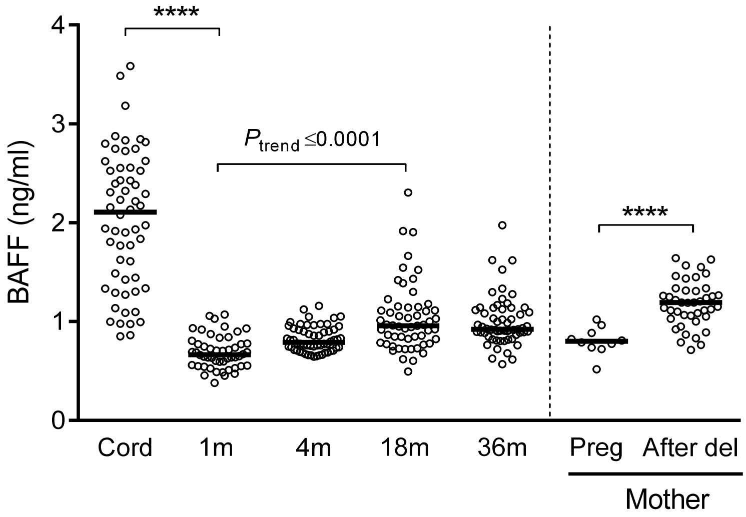
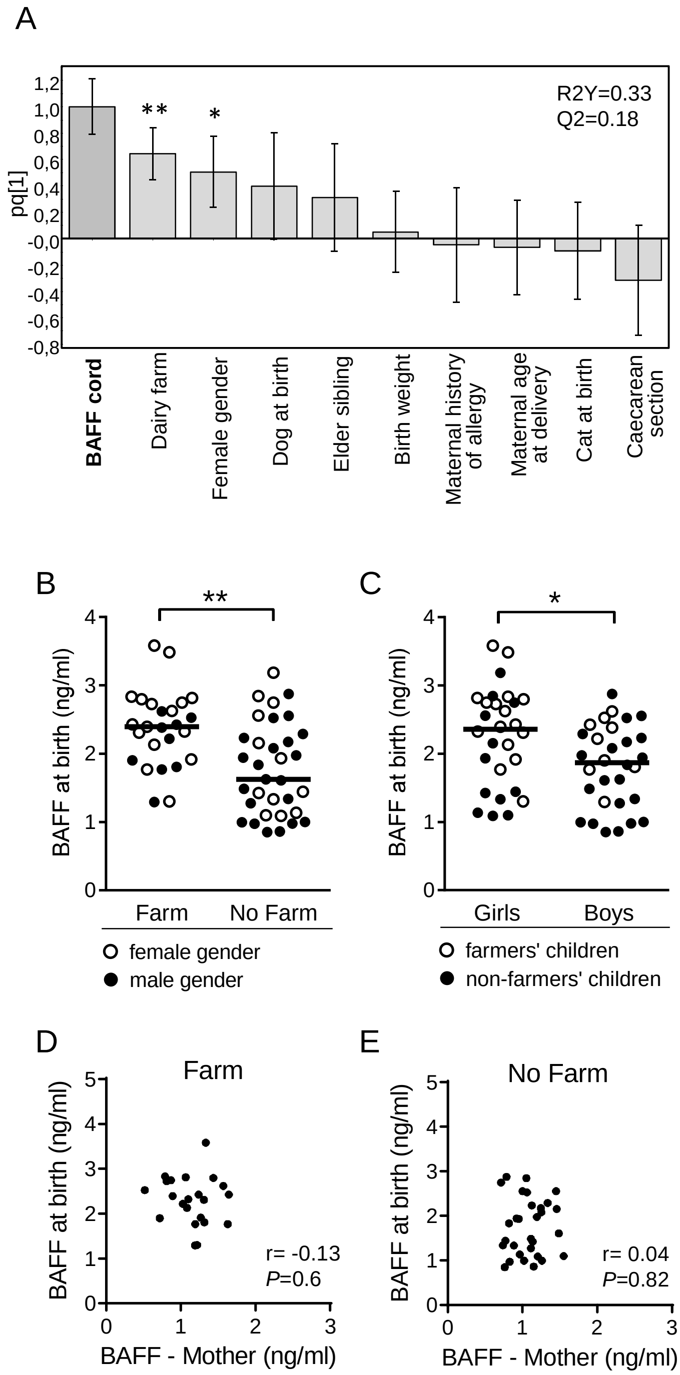

****
4
3
2
1
0
BAFF (ng/ml)
P_{trend} ≤0.0001
****
Cord
1m
4m
18m
36m
Preg
After del
Mother

Figure 2



A
R2Y=0.33
Q2=0.18
pq[1]
1,2
1,0
0,8
0,6
0,4
0,2
-0,0
-0,2
-0,4
-0,6
-0,8
**
*
BAFF cord
Dairy farm
Female gender
Dog at birth
Elder sibling
Birth weight
Maternal history of allergy
Maternal age at delivery
Cat at birth
Caecarean section
B
**
BAFF at birth (ng/ml)
Farm
No Farm
female gender
male gender
C
*
BAFF at birth (ng/ml)
Girls
Boys
farmers' children
non-farmers' children
D
Farm
BAFF at birth (ng/ml)
r= -0.13
P=0.6
BAFF - Mother (ng/ml)
E
No Farm
BAFF at birth (ng/ml)
r= 0.04
P=0.82
BAFF - Mother (ng/ml)

Figure 3

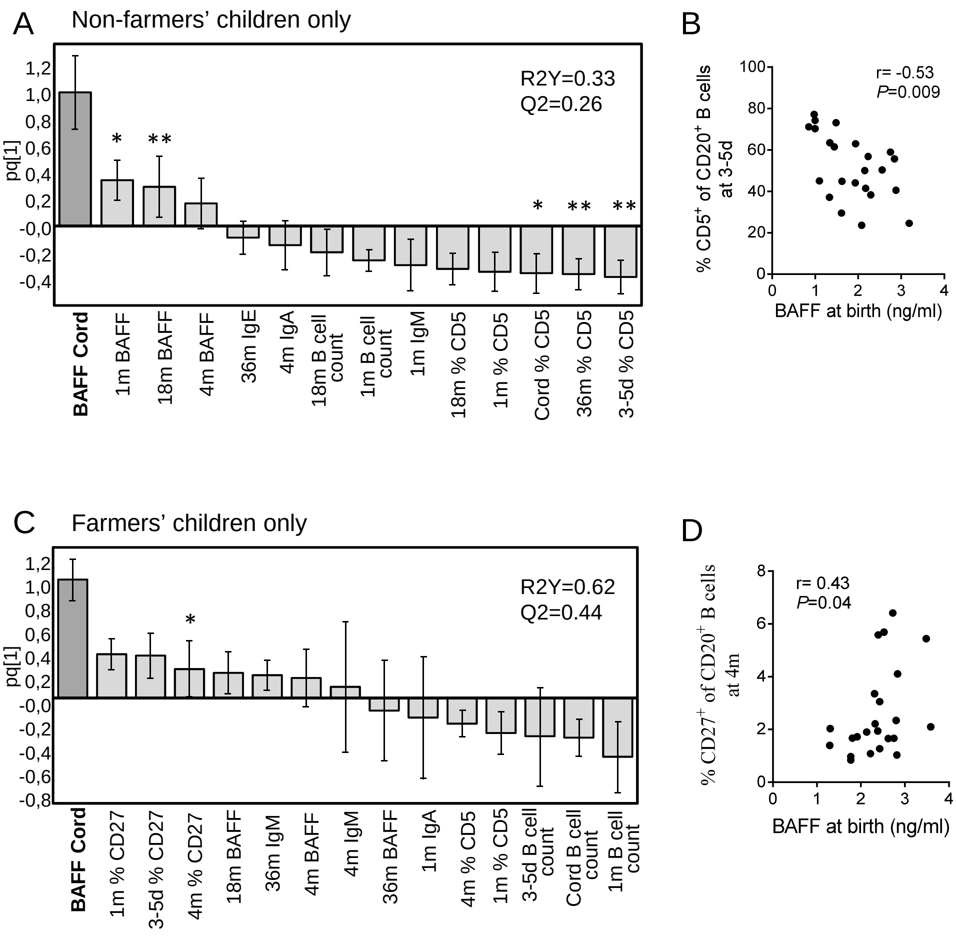
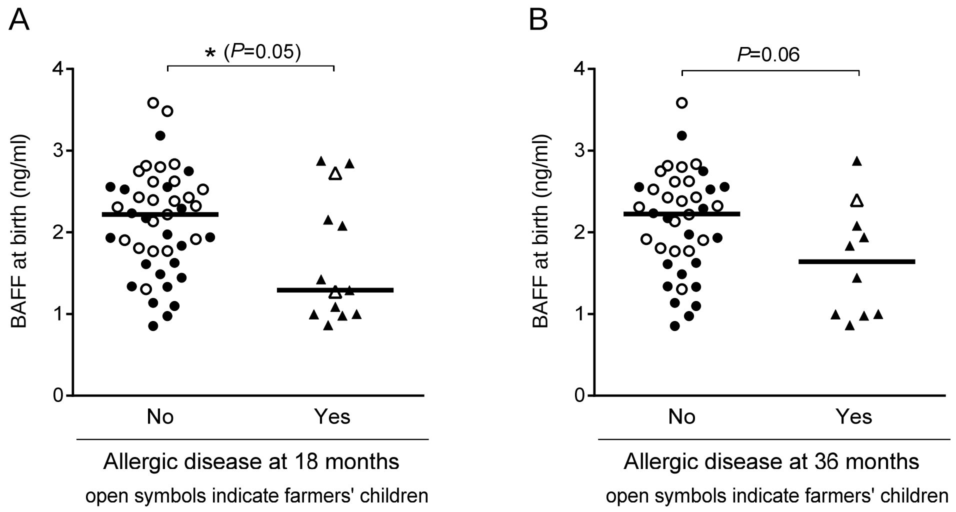

A
Non-farmers' children only
R2Y=0.33
Q2=0.26
pq[1]
1,2
1,0
0,8
0,6
0,4
0,2
-0,0
-0,2
-0,4
*
**
*
**
**
BAFF Cord
1m BAFF
18m BAFF
4m BAFF
36m IgE
4m IgA
18m B cell count
1m B cell count
1m IgM
18m % CD5
1m % CD5
Cord % CD5
36m % CD5
3-5d % CD5
B
r= -0.53
P=0.009
% CD5+ of CD20+ B cells at 3-5d
BAFF at birth (ng/ml)
C
Farmers' children only
R2Y=0.62
Q2=0.44
pq[1]
1,2
1,0
0,8
0,6
0,4
0,2
-0,0
-0,2
-0,4
-0,6
-0,8
*
BAFF Cord
1m % CD27
3-5d % CD27
4m % CD27
18m BAFF
36m IgM
4m BAFF
4m IgM
36m BAFF
1m IgA
4m % CD5
1m % CD5
3-5d B cell count
Cord B cell count
1m B cell count
D
r= 0.43
P=0.04
% CD27+ of CD20+ B cells at 4m
BAFF at birth (ng/ml)

Figure 4



A
* (P=0.05)
BAFF at birth (ng/ml)
4
3
2
1
0
No
Yes
Allergic disease at 18 months
open symbols indicate farmers' children
B
P=0.06
BAFF at birth (ng/ml)
4
3
2
1
0
No
Yes
Allergic disease at 36 months
open symbols indicate farmers' children

Figure 5

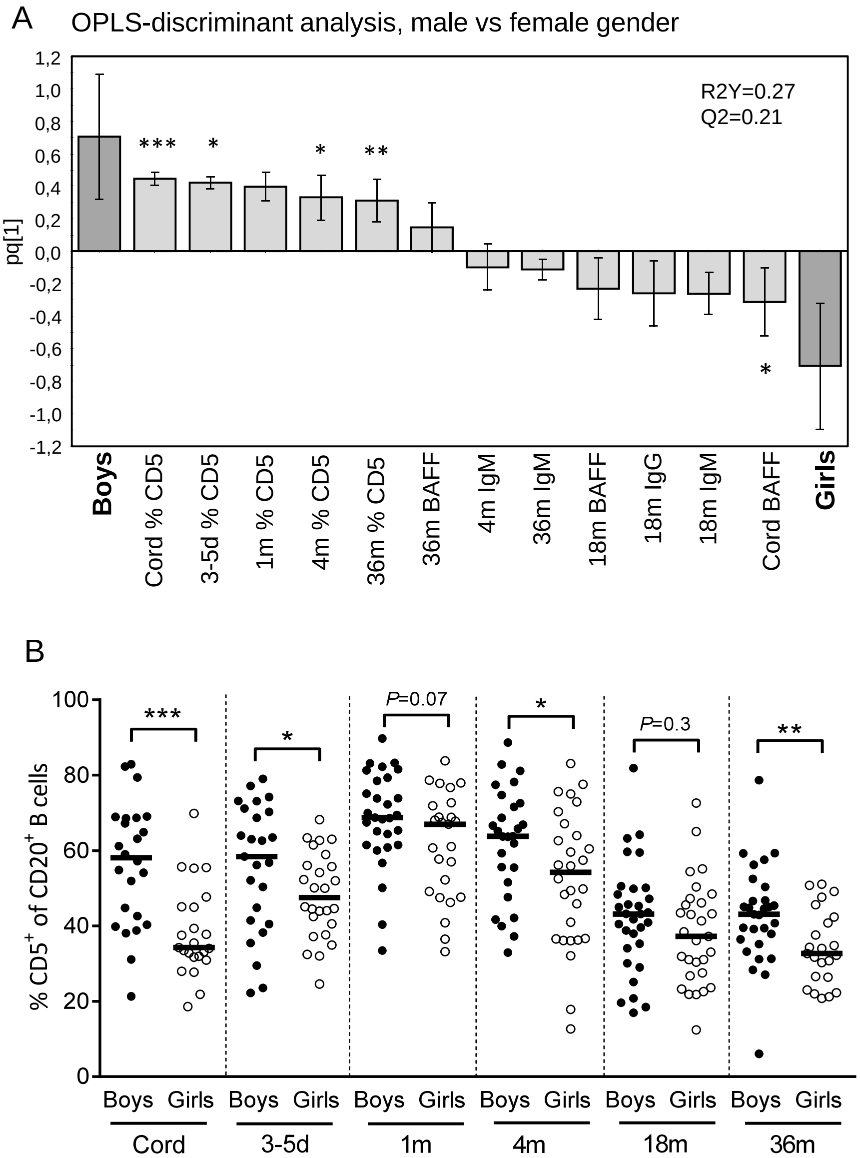

A
OPLS-discriminant analysis, male vs female gender
R2Y=0.27
Q2=0.21
pq[1]
1,2
1,0
0,8
0,6
0,4
0,2
0,0
-0,2
-0,4
-0,6
-0,8
-1,0
-1,2
***
*
*
**
*
Boys
Cord % CD5
3-5d % CD5
1m % CD5
4m % CD5
36m % CD5
36m BAFF
4m IgM
36m IgM
18m BAFF
18m IgG
18m IgM
Cord BAFF
Girls
B
% CD5+ of CD20+ B cells
100
80
60
40
20
0
***
*
P=0.07
*
P=0.3
**
Boys Girls
Cord
Boys Girls
3-5d
Boys Girls
1m
Boys Girls
4m
Boys Girls
18m
Boys Girls
36m

Table 1. Demographic data

	No. of children (%)			
	All (n=65)	Dairy Farm (n=28)	No Farm (n=37)	
Girls	32 (49)	18 (64)	14 (38)	**0.05**
Caecarean section	10 (15)	3 (11)	7 (19)	0.49
Birth weight (g)	3545 (2440-4830)	3525 (2780-4740)	3618 (2440-4830)	0.61
Mother with a history of allergy[a]	18 (28)	7 (25)	11 (30)	0.78
Age of the mother at delivery	32 (21-42)	33 (21-42)	32 (22-41)	0.47
Sibling at birth	35 (54)	18 (64)	17 (46)	0.21
Dog at birth[b]	22 (35)[b]	13 (46)	9 (26)[b]	0.11
Cat at birth[c]	26 (41)[c]	14 (50)	12 (33)[c]	0.21

[a] Mother with a history of doctor-diagnosed asthma, allergic rhinoconjunctivitis or eczema.
[b] data obtained from 35 non-farming families
[c] data obtained from 36 non-farming families
[*] Statistical difference between farmers' and non-farmers' children (Fisher's exact test or Mann-Whitney U-test).

Table 2. The effect of life-style factors on cord blood BAFF levels determined by multiple linear regression analysis.

Variable	**B**	***P*-value**
Intercept	1.67	
Dairy farm	0.39	**0.04**
Female gender	0.38	**0.04**
Dog at birth	0.17	0.36
Caecarean section	-0.11	0.63
R^2	0.26	

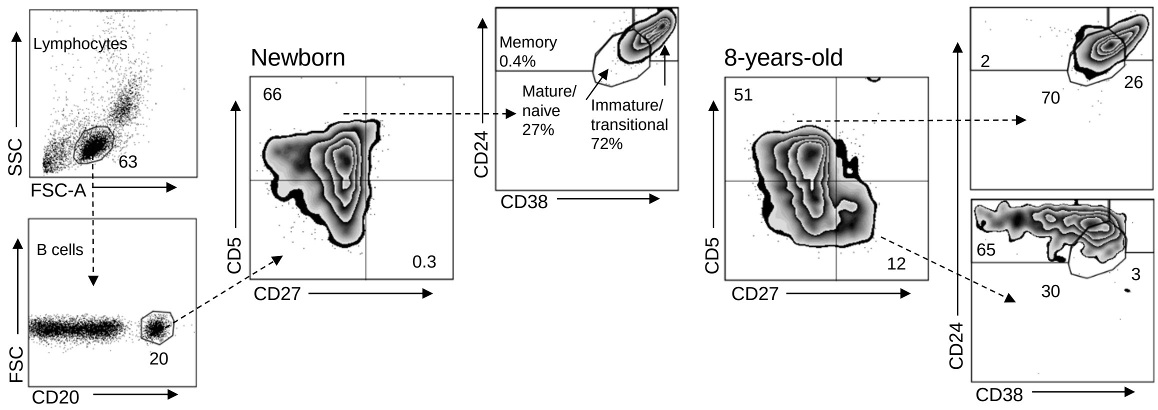
Intercept = The point at which the curve intersects the Y-axis (BAFF ng/ml)
B = Unstandardized regression coefficient
R^2 x 100 = Percent of BAFF variation explained by the model

Supplemental figure legends

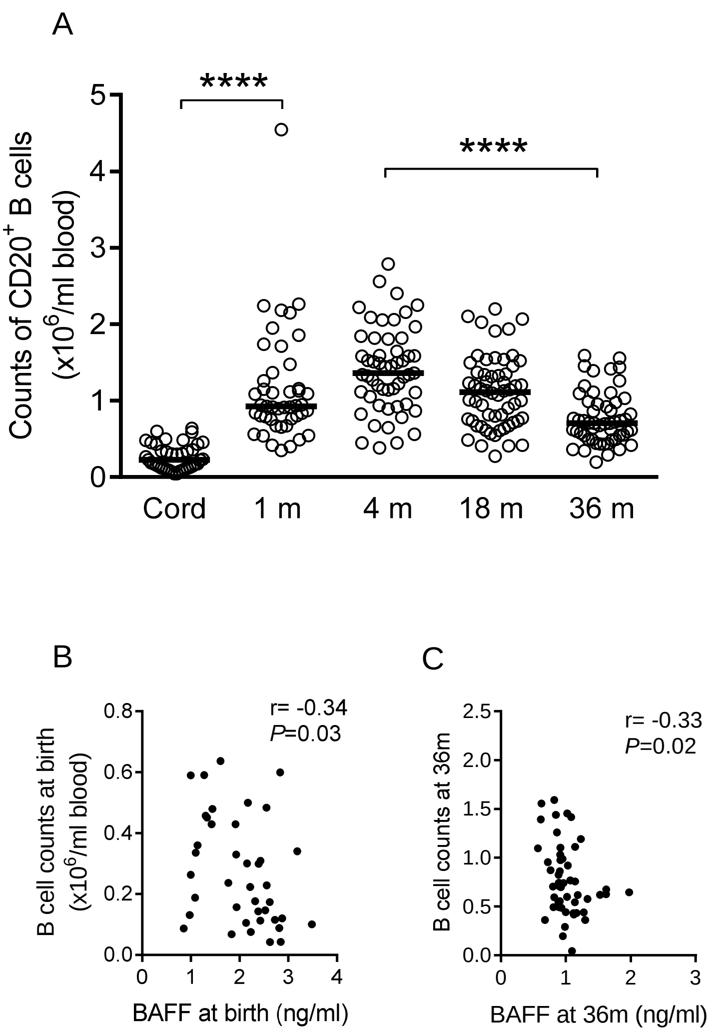
Figure E1. Gating strategy for lymphocytes and $CD20^+$ B cells, and zebra plots demonstrate the proportion of immature transitional, mature naïve or memory B cells based on CD24 and CD38 expression within the $CD5^+$ or $CD5^{neg}$ B cell subsets in blood from one newborn child and one 8-years-old child. The following monoclonal antibodies were used: APC-H7-conjugated anti-CD20 (clone L27, BD Bioscience), PE-Cy7-conjugated anti-CD24 (clone ML5, BD Bioscience), PE-conjugated anti-CD38 (clone HB7, BD Bioscience), APC-conjugated CD5 (clone UCHT2, Pharmingen, San Diego, California) and FITC-conjugated anti-CD27 (clone L128, BD Bioscience). All samples were run in a FACS-Canto II (BD Bioscience) equipped with FACS-Diva software and analyzed with FlowJo software (TreeStar, Ashland, Oregon).

Figure E2. Numbers of circulating B cells within the lymphocyte gate **(A)** at birth, and at 1, 4, 18 and 36 months of age. **(B-C)** Correlation plots depicting BAFF levels in relation to B cell counts at birth and at 36 months of age, respectively. **(A)** Horizontal bars indicate the median and **** ≤ 0.0001 (Kruskal-Wallis test followed by Dunn's multiple-comparison test). **(B-C)** Spearman's correlation test. **(A)** All data regarding B cell numbers have been published previously by Lundell et al (J Immunol, 2012, 188: 4315-4322)

Repository figure 1



Repository figure 2



Repository Table 1. Allergic disease and sensitization in children at 18 and 36 months of age

	at 18 months					**at 36 months**				
Child ID	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