

ORIGINAL ARTICLE

Basic Mechanisms in Allergic Disease

Allergic disease in 8-year-old children is preceded by delayed B cell maturation

A. Strömbeck¹ | I. Nordström¹ | K. Andersson¹ | H. Andersson² | S. Johansen² |
 C. Maglio¹ | H. Rabe³ | I. Adlerberth³ | A. E. Wold³ | B. Hesselmar⁴ | A. Rudin¹ |
 A-C. Lundell¹ 

¹Department of Rheumatology and Inflammation Research, Sahlgrenska Academy, University of Gothenburg, Gothenburg, Sweden

²Pediatric Clinic, Skaraborg Hospital, Lidköping, Sweden

³Department of Infectious Diseases, University of Gothenburg, Gothenburg, Sweden

⁴Department of Pediatrics, Sahlgrenska Academy, University of Gothenburg, Gothenburg, Sweden

Correspondence

Anna-Carin Lundell, Department of Rheumatology and Inflammation Research, Sahlgrenska Academy, University of Gothenburg, Gothenburg, Sweden.
 Email: anna-carin.lundell@rheuma.gu.se

Funding information

The Sahlgrenska Academy; Gothenburg University; the Region Västra Götaland; the Swedish Research Council, Grant/Award Number: K2012-57X-22047-01-6; the Health and Medical Care Committee of the Regional Executive Board; Torsten and Ragnar Söderberg's Foundation; IngaBritt and Arne Lundberg's Foundation; Queen Silvia of Sweden Tercentenary Foundation; the Magnus Bergvall Foundation; the Ellen, Walter and Lennart Hesselmanns foundation; the Sahlgrenska Hospital Foundations.

Summary

Background: We previously reported that exposure to a farming environment is allergy-protective, while high proportions of neonatal immature/naïve CD5⁺ B cells and putative regulatory T cells (Tregs) are risk factors for development of allergic disease and sensitization up to 3 years of age.

Objective: To examine if B and T cell maturation are associated with allergic disease and farming environment over the first 8 years in life.

Methods: In the prospective FARMFLORA study, including both farming and non-farming families, 48 of 65 children took part in the 8-year follow-up study. Various B and T cell maturation variables were examined in blood samples obtained at several occasions from birth to 8 years of age and related to doctors' diagnosed allergic disease and sensitization, and to farming environment.

Results: We found that the incidence of allergic disease was lower among farmers' compared to non-farmers' children during the 8-year follow-up period, and that farmers' children had higher proportions of memory B cells at 8 years of age. Moreover, a high proportion of neonatal CD5⁺ B cells was a risk factor for and may predict development of allergic disease at 8 years of age. A high proportion of Tregs was not protective against development of these conditions.

Conclusion and Clinical Relevance: High proportions of neonatal naïve B cells remained as a risk factor for allergic disease in school-aged children. Thus, the accelerated B cell maturation observed among farmers' children may be crucial for the allergy-protective effect of a farming environment.

KEYWORDS

basic immunology, basic mechanisms, clinical immunology, environment and hygiene hypothesis, farm, B cells, T cells, allergic disease, pediatrics

1 | INTRODUCTION

Allergic diseases, such as eczema, asthma, allergic rhinoconjunctivitis (ARC) and food allergy, result from failure to develop immune tolerance to innocuous inhaled or ingested allergens. Instead, allergens

may trigger an adaptive immune response with production of allergen-specific IgE antibodies, that is, sensitization. In some individuals, these antibodies can trigger inflammation upon renewed exposure to the allergen, giving rise to immune-mediated hypersensitivity, that is, allergy. Although sensitization can occur without symptoms of

allergic disease, sensitization in early childhood is a risk factor for development of allergic disease.¹ The cumulative incidence of allergic disease is 29% and 27% among Swedish and Danish 5-year-old children, respectively.² The dramatic increase in the prevalence of allergic diseases during the second half of the twentieth century points to an essential role of environmental factors in development of these conditions.^{3–6} Several studies show that a traditional dairy farming lifestyle is associated with protection from allergic disease in school-aged children.^{7–9} Still, the impact of a farming environment on adaptive immunity remains elusive.

Although B cells are the precursors of IgE-producing plasma cells, and hence, key players in allergic disease, few studies have examined postnatal B cell maturation in relation to development of allergic disease. In the rural prospective FARMFLORA birth-cohort study, we have demonstrated that children with a high proportion of immature/naïve CD5⁺ B cells during early infancy had a higher risk of developing allergic disease up to 3 years of age.¹⁰ In accordance, adults with allergic asthma have higher proportions of immature/transitional B cells (CD24^{hi}CD38^{hi}) compared to healthy subjects.¹¹ We further showed that allergic disease is associated with lower BAFF levels at birth,¹² which is a pivotal cytokine for differentiation of immature/transitional B cells into mature/naïve cells.¹³ Moreover, we have also shown that children who developed sensitization in the first 3 years of life had higher proportions of putative regulatory T cells (Tregs) in early infancy.¹⁴ As Tregs have suppressive effects on both T and B cell activation,^{15–19} a high proportion of these cells early in life may impede adaptive immune maturation. However, whether a delay in postnatal adaptive immune maturation is still relevant for allergic disease and/or sensitization later in childhood is not known.

To address these gaps in knowledge, we performed a detailed immunological follow-up of the FARMFLORA study when the children had reached school age. The incidence of allergic disease was lower among farmers' compared with non-farmers' children during the 8-year follow-up period, and farmers' children had higher proportions of memory B cells at 8 years of age. Further, a high proportion of neonatal CD5⁺ B cells remained as a risk factor for development of allergic disease in school-aged children, and high proportions of Tregs were not protective against development of these conditions.

2 | MATERIALS AND METHODS

2.1 | Subjects and collection of blood samples

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. In this region, rural areas 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. Families included in the non-farming group should live in the same rural geographical region, but not on a farm. Sixty-five infants (28 farmers' and 37 non-farmers' children) born at term were included in the study and have been followed up in detail with respect to adaptive immune maturation and development of allergic disease and sensitization up to 3 years of age.^{10,12,14} In the 8-year follow-up study, 48 children (74%; median age; 8.3 years, range 6.5–9.4) and their parents agreed to participate as shown in the participation flow diagram (Figure 1). The children who were included in the 8-year follow-up were representative of the whole cohort regarding demographic data (Table S1). All

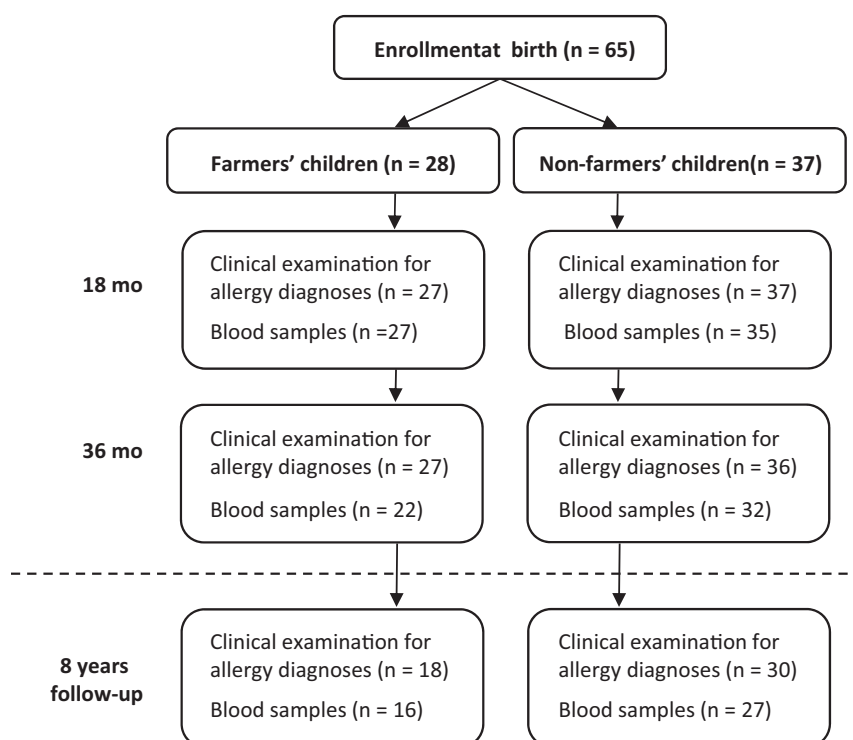


FIGURE 1 Participation flow diagram from birth to 8 y of age in the FARMFLORA birth cohort study.

children and parents provided written informed consent and the study protocol was approved by the Human Research Ethics Committee of the Medical Faculty, University of Gothenburg, Sweden.

2.2 | Laboratory tests and clinical examination at 8 years of age

Venous blood was collected for measurement 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 analysed for specific IgE against birch, timothy, grass, 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. Skin prick tests were performed in accordance with European guidelines using standard allergen extracts (birch, grass, mugwort, cat, dog, horse, rabbit, *Dermatophagoides pteronyssinus*, *Dermatophagoides farinae* and *Cladosporium*, (Soluprick SQ; ALK Abello AS, Hørsholm, Denmark), allergen diluents as negative control and histamine (10 mg/mL) as positive control. A wheal diameter ≥ 3 mm was considered positive. Sensitization was defined as a positive Phadiatop test or a positive 6-food mix test (ImmunoCAP ≥ 0.35 kU/L). Eczema was diagnosed according to Williams' criteria or based on at least three major Hanifin and Rajka criteria.²⁰ Asthma was defined as wheeze or heavy breathing in the last year together with bronchial obstruction reversible to β_2 -agonist ($\geq 12\%$), bronchial hyperresponsiveness on methacholine challenge (PD20 < 0.6 mg) or ongoing preventive asthma treatment with inhaled corticosteroids. The diagnosis of ARC included symptoms from eyes and/or nose upon exposure to pollens or animal dander, together with a positive allergen-specific IgE test or positive skin prick test directed against a corresponding allergen. 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 six-mix food test. In case, a child was diagnosed with food allergy it was verified by an open food challenge test. Allergic disease was defined as one or more of the diagnoses above.

2.3 | Flow cytometry

The phenotypic characterization of B and T cells up to 36 months of age has previously been described in detail.^{21,22} Briefly, immature/naïve and memory B cells were identified by CD5 and CD27 expression, respectively. Naïve and memory CD4⁺ T cells were identified by CD45RA and CD45RO expression, respectively. Putative Tregs were identified by CD25 in combination with FOXP3 or CTLA-4 expression. At 8 years of age, additional markers were included as follows: CD24 and CD38 to distinguish immature/transitional (CD24^{hi}CD38^{hi}), mature/naïve (CD24^{int}CD38^{int}), memory (CD24^{hi}CD38^{lo/neg}) B cells²³ and CD127 to identify CD4⁺CD25⁺CD127^{lo/neg} putative Tregs.²⁴ We also identified proportions of CD45RA⁺ and CD45RO⁺ putative Tregs. All anti-human monoclonal antibodies are listed in Table S2. The

FOXP3-staining set was purchased from eBiosciences (San Diego, USA) and the Cytofix/Cytoperm kit (BD Biosciences, Erembodegem, Belgium) was used for detection of CTLA-4. To assess total B and T cell numbers, the TruCOUNT assay was used (BD Biosciences). Up to 36 months, samples were run in a FACSCalibur (BD Biosciences) equipped with CellQuestPro software. At 8 years, samples were run in a FACSCanto II (BD Biosciences) equipped with FACSDiva software (BD Biosciences). All samples were analysed with FlowJo software (TreeStar, Ashland, OR, USA).

2.4 | Quantifications of BAFF and immunoglobulins in plasma

BAFF levels were determined using the human BAFF/BLyS/TNFSF13B Quantikine ELISA Kit (R&D Systems, Minneapolis, MN, USA). Total IgM, IgG and IgA levels were determined by ELISA as previously described in detail.¹² Total IgE levels were measured with ImmunoCAP[®]250 (Phadia, Uppsala, Sweden).

2.5 | Statistical analysis

OPLS with discriminant analysis (DA) was used to obtain a maximum separation of X-variables, that is, B and T cell maturation variables, based on class information, for example, allergic disease (yes/no) and farm (yes/no). OPLS analysis was implemented to investigate associations between a selected Y-variable and a set of X-variables (SIMCA 13.0; Umetrics, Umeå, Sweden). The contribution of each X-variable to the model, that is the VIP value, was calculated for models in Figures 2C, 4C and 6A. X-variables with a VIP-value below 0.97, 1.03 and 1.2, respectively, were discarded, and new models were generated based on the remaining variables. The scale presented on the Y-axis of the OPLS plot is a dimensionless scale, the loading vector is normalized to unit length. The quality of OPLS analyses is based on R², how well the variation of the variables is explained by the model, and Q², an estimate of the predictive ability of the model. To avoid biased preselection and mass significance, two-tailed Mann-Whitney U-test, Spearman's rank test and Fischer's exact test (GraphPad Software; La Jolla, CA, USA) were performed exclusively on the X-variables that contributed most to the respective multivariate model. Additional analyses include Kaplan-Meier survival analysis with log-rank test, hazard ratios (HR) using Cox proportional hazard models, receiver operating characteristics curves (ROC) and multiple linear regression (SPSS version 19.0.0; Chicago, IL, USA, and GraphPad software). A P-value of .05 or less was regarded as statistically significant. Significant differences or correlations are indicated with asterisks (added in retrospect) in OPLS-plots.

3 | RESULTS

3.1 | Allergic disease and sensitization at 8 years of life

By the age of 8 years, 10 of 48 children (21%) were diagnosed with allergic disease, five of whom had eczema, four asthma, three ARC

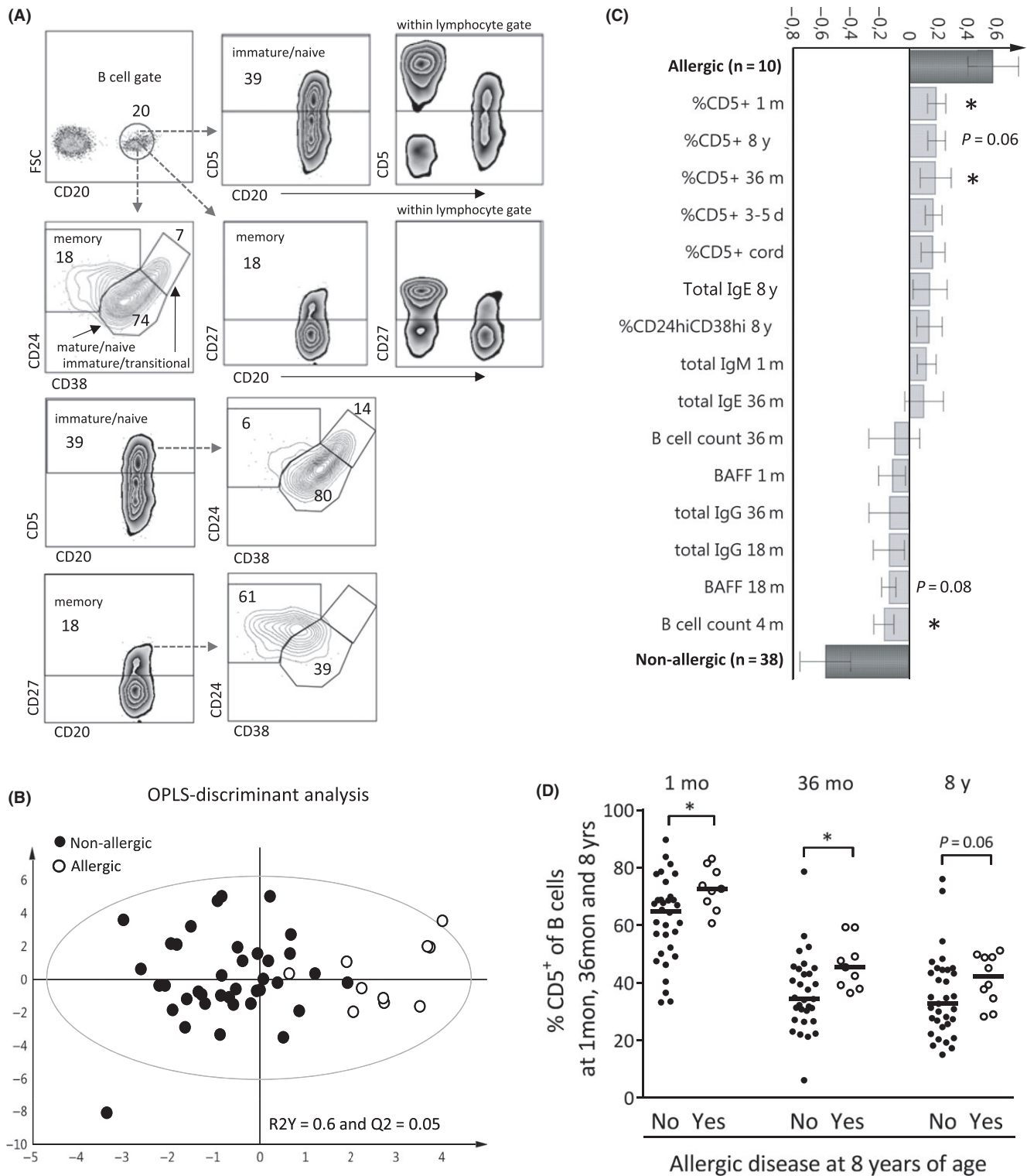


FIGURE 2 Allergic and non-allergic children differ in postnatal B cell maturation. (A) B cell gating strategy at 8 y. (B) OPLS-DA-plot displaying a separation between allergic and non-allergic children. (C) OPLS-plot depicting associations between allergy and B cell maturation variables assessed. (D) Proportions of CD5⁺ B cells at 1 mo, 36 mo and 8 y in allergic and non-allergic children. Horizontal bars indicate the median values. * $P \leq 0.05$ (Mann-Whitney *U*-test).

and none of the children were diagnosed with food allergy (Table 1). Blood samples were obtained from 43 children for detection of allergen-specific IgE (Figure 1), and 11 of these children were sensitized

to one or more of the allergens included in the ImmunoCAP panel (26%; Table 1). Among the sensitized children, four were also diagnosed with allergic disease. The prevalence of allergic disease and

TABLE 1 Clinical diagnosis of allergic disease and sensitization at 8 y of life

	n=48	
	n	%
Any allergy ^a	10	21
Eczema	5	10
Asthma	4	8
Allergic rhinoconjunctivitis	3	6
Food allergy	0	0
Sensitization ^b	11	26 ^c

^aOne or more of the following diagnoses: eczema, asthma, allergic rhinoconjunctivitis or food allergy

^bDefined as positive 6-mix food test or Phadiatop. Screening for allergen-specific IgE, ie, 6-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, grass, dog, cat, horse and house dust mite

^cn=43, blood samples not available for all 48 children

sensitization at 18 and 36 months of age in this cohort has been published previously.^{10,14}

3.2 | Delayed B cell maturation precedes allergic disease in 8-year-old children

By multivariate analysis, we investigated whether postnatal B cell maturation differed between children with and without allergic at 8 years of age. The B cell gating strategy is presented in Figure 2A, which also shows that the vast majority of the CD5⁺ B cells reside within the CD24^{hi/int}CD38^{hi/int} immature/naïve gates, while the majority of CD27⁺ B cells are found in the CD24^{hi}CD38^{lo/neg} memory gate. OPLS-DA demonstrated a separation between these two groups of children based on the B cell maturation variables assessed (Figure 2B). Allergic disease was associated with higher proportions of immature/naïve CD5⁺ B cells from birth to 8 years of age (Figure 2C), also corroborated by univariate analysis for several time points (Figure 2D). Conversely, allergic disease associated with lower B cell counts as well as with lower BAFF and total IgG levels during early childhood (Figure 2C). Higher proportions of CD5⁺ B cells at 1 month was further positively associated and highly correlated with higher proportions of CD5⁺ as well as with CD24^{hi}CD38^{hi} (immature/transitional) B cells at 8 years (Figure S1A-C).

OPLS-analysis shows that sensitization was unrelated to B cell maturation (Figure S2A). In all, allergic disease at 8 years of life is associated with higher fractions of immature/naïve CD5⁺ B cells throughout childhood.

3.3 | High proportions of CD5⁺ B cells is a risk factor for allergic disease

During the 8-year follow-up, there was a significantly higher incidence of allergic disease among children with a proportion of CD5⁺ B cells above the median at 1 month of age compared to children with CD5⁺ B cells below the median (log-rank $P=0.007$, unadjusted HR 3.6, 95% CI: 1.2

to 11.3, $P=0.026$; Figure 3A). The analysis was restricted to 41 children from whom data regarding the proportion of CD5⁺ B cells at 1 month as well as clinical evaluation at all time points were available. Among these children, 12 were diagnosed with allergic disease at 18 months, 7 at 36 months and 9 at 8 years of age. After adjustment for sex (female/male) and being raised on a farm (yes/no), a higher proportion of these cells remained as a risk factor for development of allergic disease at 8 years of age (adjusted HR 3.7, 95% CI: 1.2 to 11.7, $P=0.024$). By ROC analysis, we evaluated the diagnostic value of high proportions of CD5⁺ B cells for predicting development of allergic disease. The area under the curve for the fraction of CD5⁺ B cells at 1 month of age was 0.74 ($P=0.03$), while the corresponding value for the fraction of CD5⁺ B cells at 36 months was 0.76 ($P=0.02$, Figure 3B). In conclusion, a high proportion of immature/naïve CD5⁺ B cells in infancy is a risk factor for and may predict allergic disease in school-aged children.

3.4 | Higher proportions of putative Tregs do not protect against allergic disease

We have previously shown that a high proportion of putative Tregs in infancy is related to sensitization up to 3 years of age.¹⁴ Here, we examined if CD4⁺ T cell maturation, including putative Tregs, was related to sensitization and allergic disease at 8 years of age. The T cell gating strategy is presented in Figure 4A. OPLS-DA demonstrated a polarization between allergic and non-allergic children based on the T cell variables included in the analysis (Figure 4B). Allergic disease at 8 years of age was associated with higher proportions of putative Tregs, defined as CD25⁺CD127^{low/neg}, FOXP3⁺CD25⁺ or CTLA-4⁺CD25⁺ within the CD4⁺ T cell population during infancy and childhood (Figure 4C). Indeed, children with allergic disease had significantly higher proportions of FOXP3⁺CD25⁺ and CD25⁺CD127^{low/neg} putative Tregs at 36 months and at 8 years of age, respectively (Figure 4D and E). Further, allergic disease was associated with higher proportions of naïve CD4⁺CD45RA⁺ T cells and accordingly with lower proportions of CD4⁺CD45RO⁺ memory T cells in infancy. Sensitization was unrelated to the T cell maturation variables examined (Figure S2B). These results suggest that high proportions of circulating putative Tregs are not protective against development of allergic disease or sensitization during the first 8 years of life.

3.5 | Lower incidence of allergic disease among farmers' children

In the FARMFLORA study, we have previously shown an allergy-protective effect of farm at 18 and 36 months of age.^{10,14} Thus, we next examined the incidence of allergic disease over the entire 8-year follow-up. As shown in Figure 5, the incidence was significantly lower among farmers' compared with non-farmers' children (log-rank $P=0.021$), and the corresponding HR was borderline significant (unadjusted HR 0.3, 95% CI: 0.08 to 1.0, $P=0.051$). Delivery mode could be a potential confounder, as there was a trend towards a higher rate of caesarean section deliveries among children with allergic disease at 8 years of age (Table 2). When adjusting for

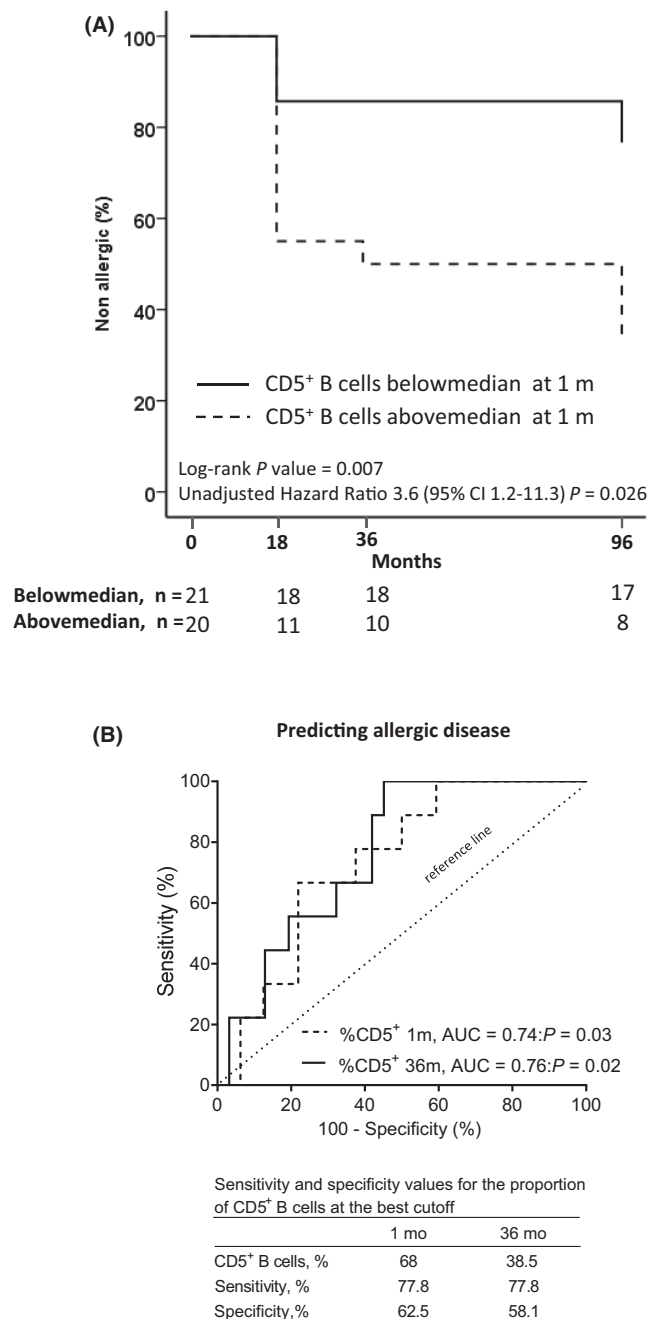


FIGURE 3 High proportions of CD5⁺ B cells is a risk factor for allergy. (A) Incidence of allergy over 8 y in children with a proportion of CD5⁺ B cells above (dotted line) or below (solid line) the median at 1 mo of age (Kaplan-Meier survival analysis with log-rank test). (B) ROC analysis of proportions of CD5⁺ B cells at 1 (dashed line) and 36 (solid line) mo of age in predicting allergy at 8 y.

delivery mode, the HR remained borderline significant (0.30, 95% CI: 0.09 to 1.0, *P* = .056). There were no differences in incidence of allergic disease between children born by caesarean section or vaginally (log-rank *P* = .206, unadjusted HR 1.8, 95% CI: 0.6-5.1, *P* = .266). Despite that the prevalence of allergic disease did not differ between farmers' and non-farmers' children at 8 years of age

(Table 2), a farming environment was associated with a lower incidence of allergic disease during the first 8 years of life.

3.6 | Farmers' children have higher proportions of memory B cells at 8 years of age

We also examined the impact of a farming environment on adaptive immune maturation assessed by flow cytometry over the first 8 years of age. OPLS-DA analysis demonstrate that a farming environment was associated with higher proportions of memory B cells, defined as CD24^{hi}CD38^{lo/neg} or CD27⁺ cells, at 8 years of age (Figure 6A). A non-farming environment was associated with higher proportions of mature/naïve (CD24^{int}CD38^{int}) and immature/naïve CD5⁺ B cells at 8 years of age, and with higher proportions of FOXP3⁺ CD25⁺ putative Tregs at 36 months of age. Differences between farmers' and non-farmers' children that were statistically significant in univariate analyses are shown in Figure 6B-D. Despite an overrepresentation of girls among farmers' children, 67% compared with 43% among non-farmers' children (Table S1), farming environment contributed independently to higher proportions of CD24^{hi}CD38^{lo/neg} and CD27⁺ memory B cells in multiple regression analysis (*P* = .03 and *P* = .01, respectively).

4 | DISCUSSION

Despite the hypothesis that delayed immune maturation is a risk factor for development of allergic disease in children, the relationship between post-natal adaptive immune maturation and allergic disease has not yet been established. For the first time, we here show that high proportions of naïve CD5⁺ B cells in early infancy is a risk factor for development of allergic disease in school-age children and that high proportions of putative Tregs do not protect against development of these conditions. We also demonstrate accelerated B cell maturation among farmers' children, which may be crucial for the allergy-protective effect of a farming environment.

We have previously demonstrated that children with high proportions of immature/naïve CD5⁺ B cells in infancy have a higher risk of developing allergic disease up to 3 years of age.¹⁰ In this follow-up study, a high proportion of these cells early in life were confirmed as a risk factor for allergic disease also in school-aged children. We further show that children with higher proportions of CD5⁺ B cells as neonates have higher proportions of immature/naïve B cells at 8 years of age, which suggests that a compromised infantile adaptive immune maturation may have effects beyond the first years in life. In line with our results, allergic adults have higher proportions of immature/transitional B cells compared to non-allergic subjects.^{11,25} There is also a strong association between higher proportions of transitional B cells during pregnancy and allergy development in the progeny at the age of 6 months.²⁵ Moreover, it was recently shown that the proportion of IgE-expressing transitional B cells was higher in both young children and adults with atopic dermatitis compared to healthy controls.²⁶ Per definition transitional

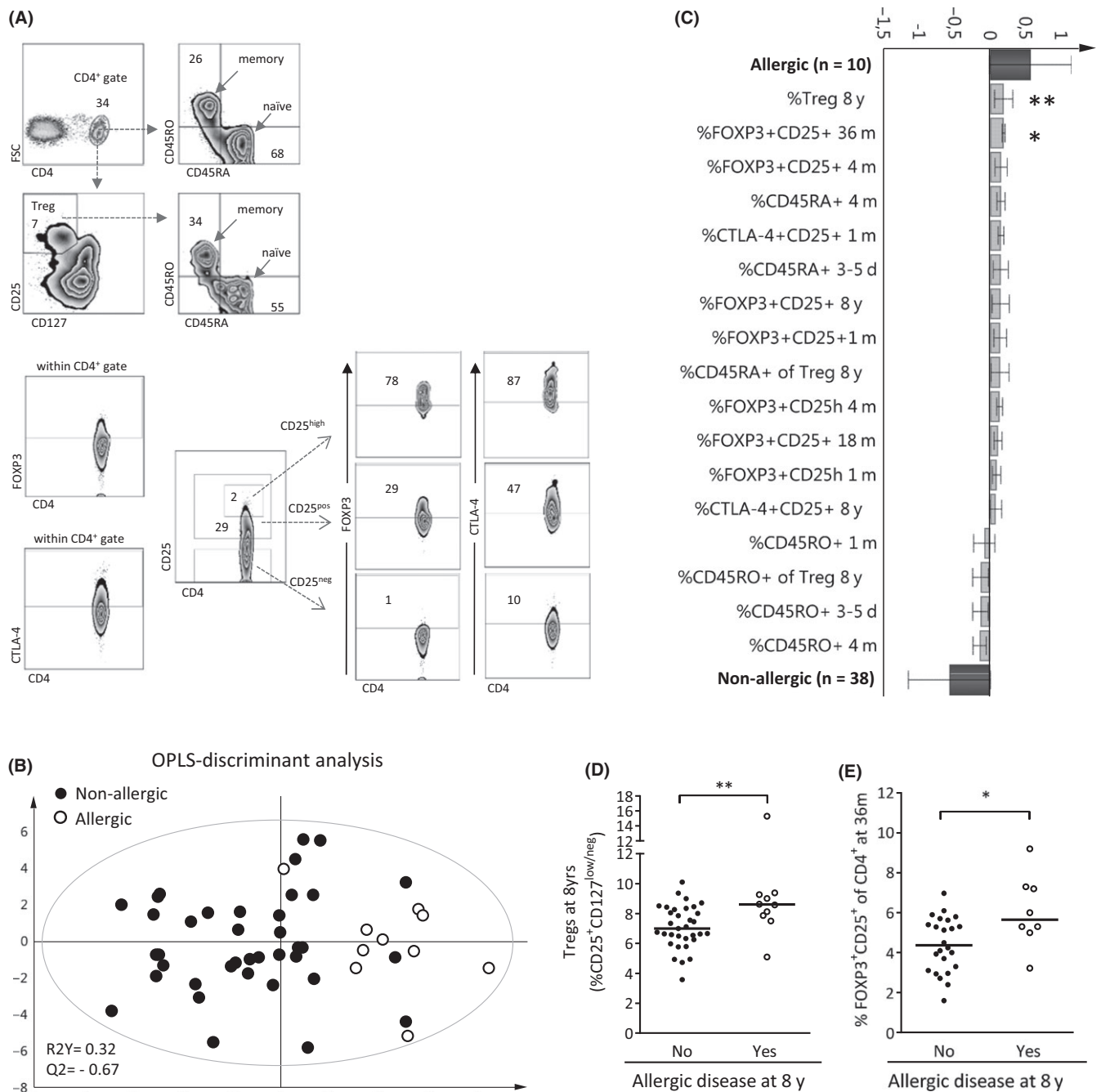


FIGURE 4 Allergic and non-allergic children differ in postnatal T cell maturation. (A) T cell gating strategy at 8 y. (B) OPLS-DA plot displaying a polarization between allergic and non-allergic children. (C) OPLS-plot depicting associations between allergic disease and T cell variables. (D) Proportions of Treg (CD25⁺CD127^{low/neg}) at 8 y and (E) FOXP3⁺CD25⁺ T cells at 1 mo in allergic and non-allergic children. Horizontal bars indicate the median values. * $P \leq 0.05$ and ** $P < 0.01$ (Mann-Whitney U-test).

cells are naïve, and IgE⁺ transitional cells could represent cells with IgE bound to CD23, which is the low-affinity receptor for IgE. Interestingly, however, a role for peripheral B cell maturity has been reported as an intrinsic B cell property that affects the class switch recombination choice of IgE vs IgG in mice. Splenic immature/transitional B cells stimulated with anti-CD40 and IL-4 preferentially switch directly to IgE, while most mature/naïve B cells instead undergo sequential class switch to IgE, that is, from IgM via IgG to

IgE.²⁷ Recently, it was further shown that lower levels of activation signals favour early emergence of IgE-producing cells via direct switching, while IgG-secreting cells appear at later stages along with an amplification of the Th2 response.²⁸ Thus, although immature/naïve B cells per se may not initiate development of allergic disease, a high proportion of these cells may predispose allergic disorders by their intrinsic propensity to directly switch to IgE secretion.

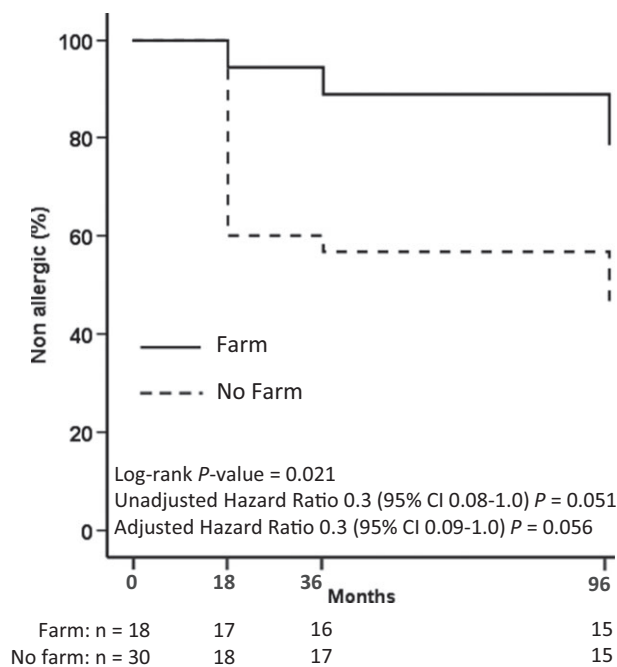


FIGURE 5 Lower incidence of allergic disease among farmers' children. The incidence of allergic disease over the 8-y follow-up among farmers' (solid line) and non-farmers' (dotted line) children (Kaplan-Meier survival analysis with log-rank test, unadjusted and adjusted for and caesarean section).

TABLE 2 Demographic data

	All n=48 (%)	Allergic disease at 8 y		P-value*
		No n=38 (%)	Yes n=10 (%)	
Girls	25 (52)	21 (55)	4 (40)	.49
Parental history of allergy ^a	20 (42)	15 (39)	5 (50)	.72
Caesarean section	9 (19)	5 (13)	4 (40)	.08
Elder sibling(s)	24 (50)	21 (55)	3 (30)	.29
Farm	18 (38)	15 (39)	3 (30)	.72
Cat at birth ^b	19 (40)	15 (39)	4 (44) ^b	.74
Dog at birth ^c	13 (28)	12 (32)	1 (13) ^c	.41
Birthweight, kg (range)	3.6 (2.7-4.8)	3.6 (2.7-4.8)	3.3 (2.7-4.4)	.28

^aMaternal or paternal history of doctor-diagnosed asthma, allergic rhinoconjunctivitis or eczema.

^bMissing data from one child.

^cMissing data from two children.

*Statistical difference between non-allergic and allergic children (Fisher's exact test or Mann-Whitney U-test).

It is generally accepted that Tregs impede proliferation and cytokine production of other T cells and have suppressive effects on B cells.¹⁵⁻¹⁹ In children, substantial efforts have been made to explore the role by Tregs in the development of sensitization and allergic disease, but the results are inconsistent. It has been demonstrated

that lower number of Tregs in cord blood is associated with a higher risk of developing eczema and sensitization at 1 year of age,²⁹ but there are also studies showing that the proportions of Tregs in cord blood do not differ between children who develop sensitization or allergic disease, and children who do not.^{30,31} We previously demonstrated that children who became sensitized in the first 3 years of life had higher proportions of putative Tregs as neonates compared to non-sensitized children.¹⁴ In the same cohort, we now show that allergic disease at 8 years of age is preceded by higher proportions of putative Tregs and lower proportions of memory T cells. Our observations are in accordance with results from another prospective cohort, which demonstrate that sensitization and/or allergic disease at 2 years of age are preceded by higher proportions of circulating Tregs.³² The discrepancies in the results may in part be due to differences in phenotypic characterization of Tregs, and proof of Treg function by suppression assays are often an obstacle due to limiting blood volume from young children. Still, high proportions of putative Tregs in childhood do not seem to prevent the atopic march.

In the present study, we also found that children with current allergic disease had higher proportions of total putative Tregs at 8 years of age, which could be due to expansion of Tregs as a protective mechanism induced by the allergic inflammatory response.^{30,33} In adults with established allergic disease, a comprehensive characterization of antigen-specific Treg responses was recently performed for the first time.³⁴ They show that the frequency of birch allergen-specific Tregs do not differ between allergic and non-allergic individuals. Their findings also suggest that allergic subjects have intact Treg responses to birch, but that the Th2 cells escape the Treg control because of their divergent antigen specificity compared to the Tregs.³⁴

The traditional farming milieu is the most potent allergy-protective environmental factor known in affluent industrialized countries. Numerous studies have shown a lower prevalence of allergic disease among children who grow up on dairy farms than among non-farmers' children.⁷⁻⁹ In agreement, the incidence of allergic disease over the first 8 years of life was lower among farmers' compared with non-farmers' children in the present FARMFLORA study. This finding adds further evidence to a specific allergy-protective effect of a farming environment since our cohort is relatively small and farmers' and non-farmers' children lived in the same rural area.

Despite the known allergy-protective effect of growing up on farms, the impact of farm exposure on adaptive immune maturation remains unclear. For the first time, we demonstrate that farmers' children present with significantly higher fractions of memory B cells, defined as CD24^{hi}CD38^{low/neg} or CD27⁺ cells, and lower proportions of naïve B cells at 8 years of age than non-farmers' children. We have previously shown that the proportions of CD5⁺ B cells do not differ between these two groups of children up to 3 years of age.¹⁰ However, if CD24 and CD38 had been included in the flow cytometry panel when the study was initiated, B cells in different maturational stages could have been distinguished,^{23,35} and possible associations with the farming environment also before 8 years of age might have been revealed. As impeded B cell maturation is

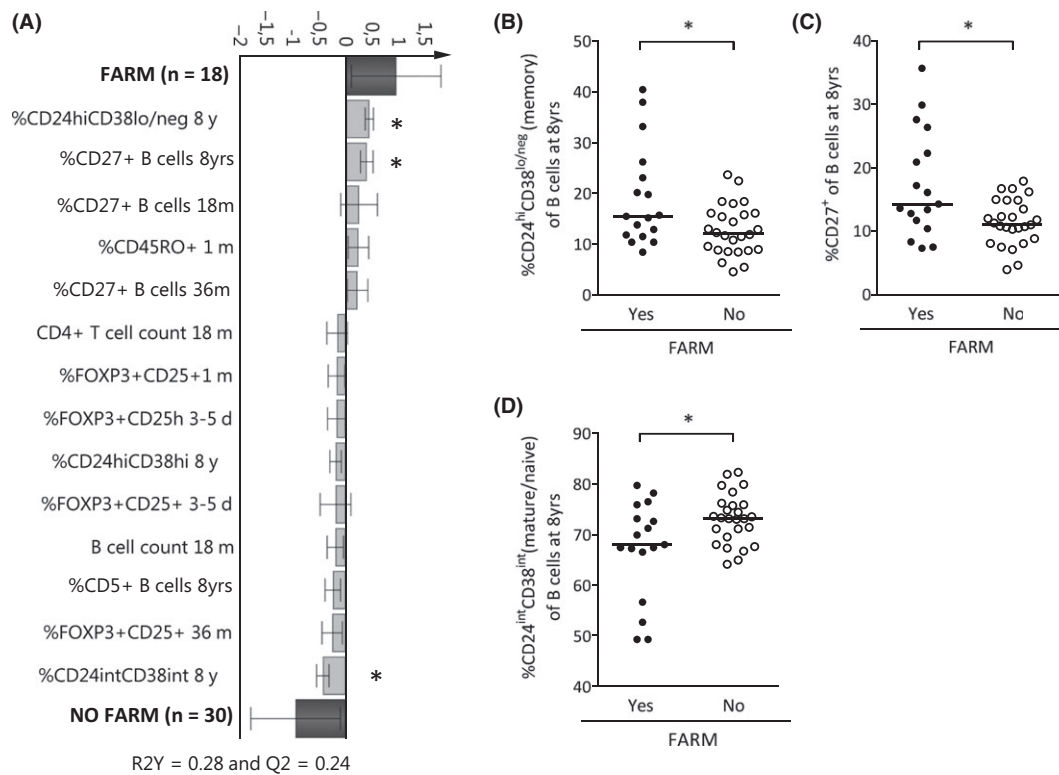


FIGURE 6 Farmers' children have higher proportions of memory B cells at 8 y. (A) OPLS-plot depicting associations between adaptive immune maturation during 8 y among farmer' and non-farmers' children. Associations up to 36 mo of age have been published previously.^{10,14} Proportions of (B) CD24^{hi}CD38^{lo/neg}, (C) CD27⁺ and (D) CD24^{int}CD38^{int} B cells in farmers' and non-farmers' children. Horizontal bars indicate the median values. * $P \leq 0.05$ (Mann-Whitney U-test).

associated with subsequent development of allergic disease, this may be counteracted in children who are exposed to a farming environment during childhood.

A more accelerated B cell maturation among farmers' children could partly be explained by exposure to higher levels of microbial compounds that trigger innate immunity, which, in turn, may promote maturation of the adaptive immune system. Indeed, higher levels of endotoxins, mould $\beta(1,3)$ -glucans and fungal extracellular polysaccharides have been measured in house dust of farming families.^{36,37} It is likely that the gut microbiota also provides important stimuli for adaptive immune maturation. We have previously shown that early gut colonization by *Escherichia coli* and bifidobacteria is associated with higher numbers of memory CD27⁺ B cells up to 18 months of age.²¹ These bacteria are recognized as early gut colonizers in classical studies of the infantile gut colonization pattern³⁸ and, at least the acquisition of *E. coli* is often delayed in modern western societies.³⁹ A reduced diversity of the early gut microbiota is also associated with an increased risk for allergic disease later in childhood.^{40,41} Moreover, it was recently shown that children with allergic disease up to 7 years of age had a lower bacterial load in stool samples at 12 months of age compared with children who remained non-allergic.⁴² The same study also demonstrates that development of allergic disease was associated with a reduced proportion of IgA bound to fecal bacteria at 12 months of age.⁴² However, how a farming environment impact the establishment of the

gut microbiota and maturation of the secretory IgA system during infancy remains to be determined.

An advantage of this study is that it is prospective and that multivariate analyses were employed to investigate longitudinal associations between adaptive immune maturation and clinical outcome as well as with dairy farm environment over the first 8 years of life. This method enables discovery of patterns and trends and prevents biased preselection of X-variables for univariate analysis. A limitation is the small number of participants, which excluded the possibility to analyse the immune maturation in relation to eczema, asthma, ARC and food allergy separately. However, regardless of differences in pathogenesis, a strong association was observed between delayed adaptive immune maturation and allergic disease. The relatively low number of children may also be reflected in the lack of significance in some of the analyses.

This is, to the best of our knowledge, the first study where B cell maturation and T cell maturation have been studied in detail prospectively throughout childhood and related to allergic disease and exposure to farming environment in school-aged children. Still, additional prospective studies in larger birth-cohorts are required to confirm that delayed adaptive immune maturation is a risk factor for allergic disease in school-aged children. Moreover, whether delayed adaptive immune maturation in childhood is a result of lifestyle and environmental settings or genetic programming remains to be resolved. A deeper understanding of relevant stimuli to boost

immune maturation in early childhood may ultimately result in development of effective strategies to reduce the risk of allergic disease in children.

ACKNOWLEDGEMENTS

We are grateful to the staff at the Clinical Immunology Laboratory of the Sahlgrenska University Hospital for technical assistance in collecting flow cytometry data. We thank the study nurse Anders Nordberg at Skaraborgs hospital, Skövde, Sweden and all the families who took part in the study. We also thank Lill Mårtensson-Bopp for critical reading of the manuscript. This work was funded by: The Sahlgrenska Academy, Gothenburg University, the Region Västra Götaland (agreement concerning research and education of doctors; ALF), the Swedish Research Council (Grant K2012-57X-22047-01-6), the Health and Medical Care Committee of the Regional Executive Board, Region Västra Götaland, the Torsten and Ragnar Söderberg's Foundation, the IngaBritt and Arne Lundberg's Foundation, Queen Silvia of Sweden Tercentenary Foundation, the Magnus Bergvall Foundation, the Ellen, Walter and Lennart Hesselmanns foundation and by the Sahlgrenska Hospital Foundations.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

REFERENCES

- Almqvist C, Li Q, Britton WJ, et al. Early predictors for developing allergic disease and asthma: examining separate steps in the 'allergic march'. *Clin Exp Allergy*. 2007;37:1296-1302.
- Henriksen L, Simonsen J, Haerskjold A, et al. Incidence rates of atopic dermatitis, asthma, and allergic rhinoconjunctivitis in Danish and Swedish children. *J Allergy Clin Immunol*. 2015;136: 360-366 e2.
- Bach JF. The effect of infections on susceptibility to autoimmune and allergic diseases. *N Eng J Med*. 2002;347:911-920.
- Asher MI, Montefort S, Björkstén B, et al. Worldwide time trends in the prevalence of symptoms of asthma, allergic rhinoconjunctivitis, and eczema in childhood: ISAAC Phases One and Three repeat multi-country cross-sectional surveys. *Lancet*. 2006;368:733-743.
- Schernhammer ES, Vutuc C, Waldhor T, Haidinger G. Time trends of the prevalence of asthma and allergic disease in Austrian children. *Ped Allergy Immunol*. 2008;19:125-131.
- Hansen TE, Evjenth B, Holt J. Increasing prevalence of asthma, allergic rhinoconjunctivitis and eczema among schoolchildren: three surveys during the period 1985-2008. *Acta Paediatr*. 2013;102:47-52.
- Riedler J, Braun-Fahrlander C, Eder W, et al. Exposure to farming in early life and development of asthma and allergy: a cross-sectional survey. *Lancet*. 2001;358:1129-1133.
- Braun-Fahrlander C, Riedler J, Herz U, et al. Environmental exposure to endotoxin and its relation to asthma in school-age children. *N Eng J Med*. 2002;347:869-877.
- Ege MJ, Frei R, Bieli C, et al. Not all farming environments protect against the development of asthma and wheeze in children. *J Allergy Clin Immunol*. 2007;119:1140-1147.
- Lundell AC, Johansen S, Adlerberth I, Wold AE, Hesselmar B, Rudin A. High proportion of CD5+ B cells in infants predicts development of allergic disease. *J Immunol*. 2014;193:510-518.
- van der Vlugt LE, Mlejnek E, Ozir-Fazalalikhani A, et al. CD24 CD27 B cells from allergic asthma patients have impaired regulatory activity in response to LPS. *Clin Exp Allergy*. 2013;44:517-528.
- Lundell AC, Hesselmar B, Nordstrom I, Adlerberth I, Wold AE, Rudin A. Higher B-cell activating factor levels at birth are positively associated with maternal dairy farm exposure and negatively related to allergy development. *J Allergy Clin Immunol*. 2015;136:e3.
- Batten M, Groom J, Cachero TG, et al. BAFF mediates survival of peripheral immature B lymphocytes. *J Exp Med*. 2000;192:1453-1466.
- Strombeck A, Rabe H, Lundell AC, et al. High proportions of FOXP3 (+) CD25(high) T cells in neonates are positively associated with allergic sensitization later in childhood. *Clin Exp Allergy*. 2014;44:940-952.
- Sakaguchi S, Sakaguchi N, Asano M, Itoh M, Toda M. Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. *J Immunol*. 1995;155:1151-1164.
- Baecher-Allan C, Brown JA, Freeman GJ, Hafler DA. CD4+CD25 high regulatory cells in human peripheral blood. *J Immunol*. 2001;167:1245-1253.
- Wing K, Larsson P, Sandstrom K, Lundin SB, Suri-Payer E, Rudin A. CD4+ CD25+ FOXP3+ regulatory T cells from human thymus and cord blood suppress antigen-specific T cell responses. *Immunology*. 2005;115:516-525.
- Zhao DM, Thornton AM, DiPaolo RJ, Shevach EM. Activated CD4+CD25+ T cells selectively kill B lymphocytes. *Blood*. 2006;107:3925-3932.
- Lim HW, Hillsamer P, Banham AH, Kim CH. Cutting edge: direct suppression of B cells by CD4+ CD25+ regulatory T cells. *J Immunol*. 2005;175:4180-4183.
- Williams HC. Diagnostic criteria for atopic dermatitis. *Lancet*. 1996;348:1391-1392.
- Lundell AC, Björnsen V, Ljung A, et al. Infant B cell memory differentiation and early gut bacterial colonization. *J Immunol*. 2012;188:4315-4322.
- Rabe H, Lundell AC, Andersson K, Adlerberth I, Wold AE, Rudin A. Higher proportions of circulating FOXP3+ and CTLA-4+ regulatory T cells are associated with lower fractions of memory CD4+ T cells in infants. *J Leuk Biol*. 2011;90:1133-1140.
- Carsetti R, Rosado MM, Wardmann H. Peripheral development of B cells in mouse and man. *Immunol Rev*. 2004;197:179-191.
- Seddiki N, Santner-Nanan B, Martinson J, et al. Expression of interleukin (IL)-2 and IL-7 receptors discriminates between human regulatory and activated T cells. *J Exp Med*. 2006;203:1693-1700.
- Martins C, Lima J, Nunes G, Borrego LM. Pregnancy alters the circulating B cell compartment in atopic asthmatic women, and transitional B cells are positively associated with the development of allergy manifestations in their progeny. *Am J Reprod Immunol*. 2016;76:465-474.
- Czarnowicki T, Esaki H, Gonzalez J, et al. Alterations in B-cell subsets in pediatric patients with early atopic dermatitis. *J Allergy Clin Immunol*. 2016; In Press. doi:10.1016/j.jaci.2016.09.060.
- Wesemann DR, Magee JM, Boboila C, et al. Immature B cells preferentially switch to IgE with increased direct Smu to Sepsilon recombination. *J Exp Med*. 2011;208:2733-2746.
- Wu YL, Stubbington MJ, Daly M, Teichmann SA, Rada C. Intrinsic transcriptional heterogeneity in B cells controls early class switching to IgE. *J Exp Med*. 2017;214:183-196.
- Hinz D, Bauer M, Roder S, et al. Cord blood Tregs with stable FOXP3 expression are influenced by prenatal environment and associated with atopic dermatitis at the age of one year. *Allergy*. 2012;67:380-389.

30. McLoughlin RM, Calatroni A, Visness CM, et al. Longitudinal relationship of early life immunomodulatory T cell phenotype and function to development of allergic sensitization in an urban cohort. *Clin Exp Allergy*. 2012;42:392-404.
31. Smith M, Tourigny MR, Noakes P, Thornton CA, Tulic MK, Prescott SL. Children with egg allergy have evidence of reduced neonatal CD4(+)CD25(+)CD127(lo/-) regulatory T cell function. *J Allergy Clin Immunol* 2008;121:1460-1466. e1-7.
32. Prescott SL, Wiltschut J, Taylor A, et al. Early markers of allergic disease in a primary prevention study using probiotics: 2.5-year follow-up phase. *Allergy*. 2008;63:1481-1490.
33. Lluís A, Depner M, Gaugler B, et al. Increased regulatory T-cell numbers are associated with farm milk exposure and lower atopic sensitization and asthma in childhood. *J Allergy Clin Immunol*. 2014;133:551-559.
34. Bacher P, Heinrich F, Stervbo U, et al. Regulatory T cell specificity directs tolerance versus allergy against Aeroantigens in humans. *Cell*. 2016;167:e16.
35. Bemark M, Holmqvist J, Abrahamsson J, Mellgren K. Translational Mini-Review Series on B cell subsets in disease. Reconstitution after haematopoietic stem cell transplantation - revelation of B cell developmental pathways and lineage phenotypes. *Clin Exp Immunol*. 2012;167:15-25.
36. Schram D, Doekes G, Boeve M, et al. Bacterial and fungal components in house dust of farm children, Rudolf Steiner school children and reference children—the PARSIFAL Study. *Allergy*. 2005;60:611-618.
37. von Mutius E, Braun-Fahrlander C, Schierl R, et al. Exposure to endotoxin or other bacterial components might protect against the development of atopy. *Clin Exp Allergy*. 2000;30:1230-1234.
38. Rotimi VO, Duerden BI. The development of the bacterial flora in normal neonates. *J Med Microbiol*. 1981;14:51-62.
39. Adlerberth I, Wold AE. Establishment of the gut microbiota in Western infants. *Acta Paediatr*. 2009;98:229-238.
40. Wang M, Karlsson C, Olsson C, et al. Reduced diversity in the early fecal microbiota of infants with atopic eczema. *J Allergy Clin Immunol*. 2008;121:129-134.
41. Bisgaard H, Li N, Bonnelykke K, et al. Reduced diversity of the intestinal microbiota during infancy is associated with increased risk of allergic disease at school age. *J Allergy Clin Immunol*. 2011;128:e1-e5.
42. Dzidic M, Abrahamsson TR, Artacho A, et al. Aberrant IgA responses to the gut microbiota during infancy precede asthma and allergy development. *J Allergy Clin Immunol*. 2017;139:1017-1025.e14.

SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

How to cite this article: Strömbeck A, Nordström I, Andersson K, et al. Allergic disease in 8-year-old children is preceded by delayed B cell maturation. *Clin Exp Allergy*. 2017;00:1-11. <https://doi.org/10.1111/cea.12922>

Supplementary Fig. 1

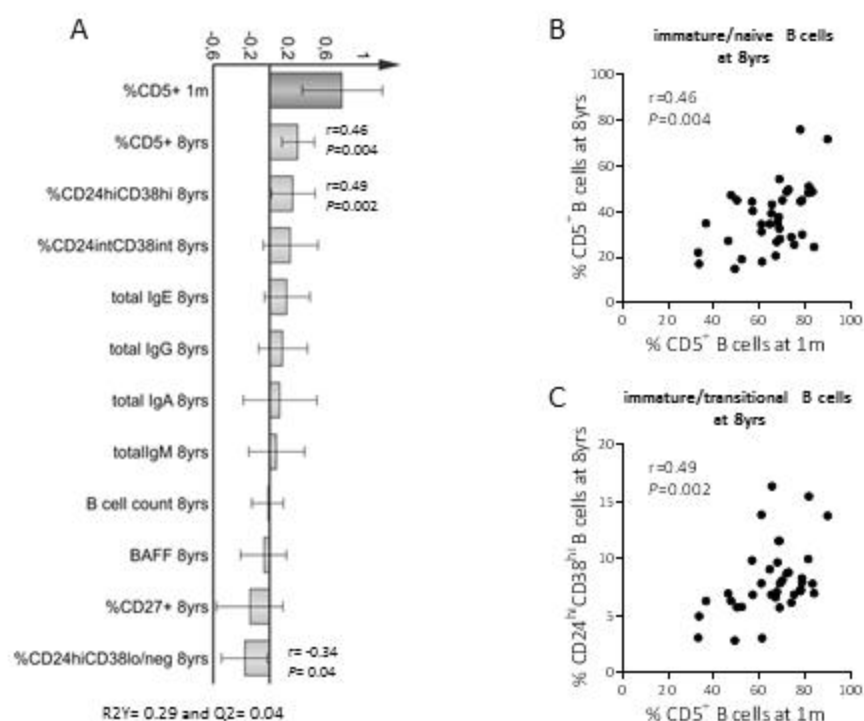


Figure S1. Children with high proportions of immature/naïve B cells in infancy have higher fractions of these cells also at 8 years. **(A)** OPLS plot showing the associations between proportion of CD5⁺ B cells at 1 month and B-cell maturation variables at 8 years of age. **(B-C)** Correlations between proportions of CD5⁺ B cells at 1 month and proportions of CD5⁺ or CD24^{hi}CD38^{hi} B cells at 8 years, respectively. * $p \leq 0.05$ (Spearman's rank correlation test).

Supplementary Fig. 2

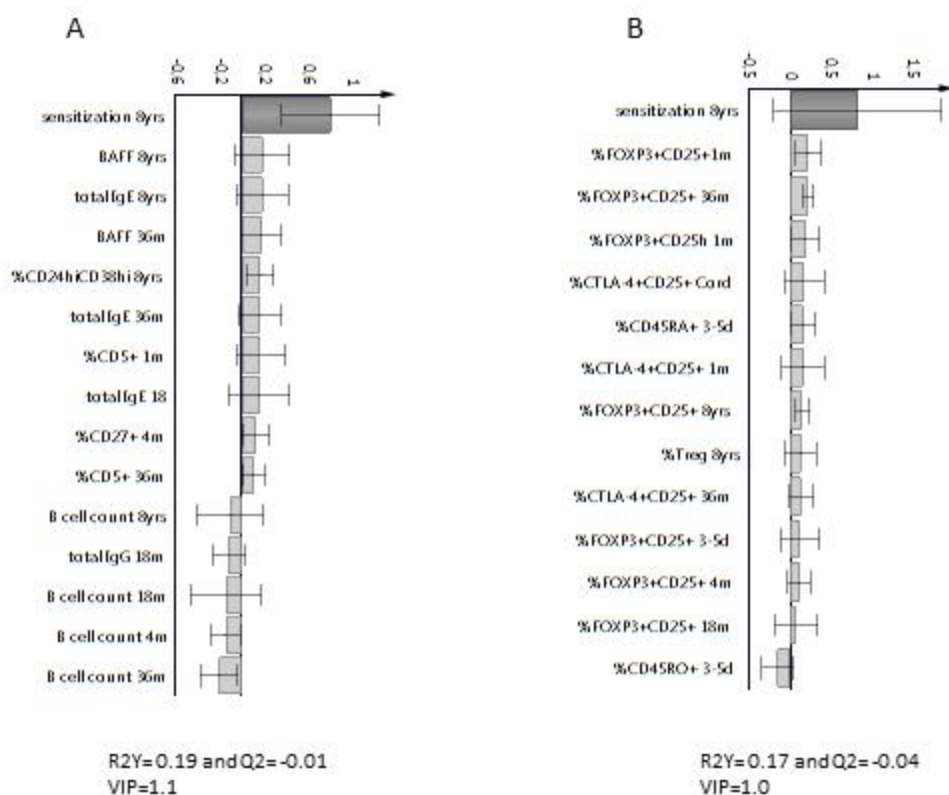


Figure S2. OPLS loadings plots depicting associations between sensitization at 8 years of life and (A) B-cell maturation variables and (B) T-cell maturation variables.

Supplementary Table 1. Demographic data for the children in the FARMFLORA study at birth and for the children who took part in the 8-year follow-up

	Enrollment at birth n=65 (%)	8-years follow-up n=48 (%)	P-value*
Girls	32 (49)	25 (52)	0.85
Parental history of allergy ^a	27 (42)	20 (42)	>0.99
Cesarean section	10 (15)	9 (19)	0.8
Elder sibling(s)	35 (54)	24 (50)	0.71
Farm	28 (43)	18 (38)	0.57
Cat at birth ^b	26 (41) ^b	19 (40) ^b	>0.99
Dog at birth ^c	22 (35) ^c	13 (28) ^c	0.54
Birth weight, kg (range)	3.5 (2.4-4.8)	3.6 (2.7-4.8)	0.91

^aMaternal or paternal history of doctor-diagnosed asthma, allergic rhinoconjunctivitis or eczema

^bMissing data from one child

^cMissing data from two children

*Statistical difference (Fisher's exact test or Mann-Whitney U -test)

Supplementary Table 2. Antibodies used for characterization of B and T cells

Monoclonal antibodies	Fluorochrome	Clone	Company
CD4 (0-36m)	PerCP	SK3	BD Biosciences, Erembodegem, Belgium
CD4 (8yrs)	APC-H7	SK3	BD Biosciences
CD25 (0-8yrs)	APC	2A3	BD Biosciences
CD127 (8yrs)	FITC	HIL-7R-M21	BD
CD127 (8yrs)	Alexa Fluor 647	HIL-7R-M21	BD
CD45RO (0-8yrs)	PE	UCHL-1	BD Biosciences
FOXP3 (0-8yrs)	PE	PCH101	eBioscience, San Diego, USA
biotin-CTLA-4 (0-8yrs)	PE-streptavidin	BNI3	BD Biosciences
CD20 (0-36m)	PerCP	L27	BD Biosciences
CD20 (8yrs)	APC-H7	L27	BD Biosciences
CD24 (8yrs)	Alexa Fluor 647	ML5	BD
CD38 (8yrs)	PE	HB7	BD
CD5 (0-36m)	APC	UCHT2	BD Biosciences
CD5 (8yrs)	Brilliant Violet 421	UCHT2	Biolegend, San Diego, USA
CD27	FITC	L128	BD Biosciences