

ORIGINAL ARTICLE

Epidemiology and Genetics



WILEY

Associations of Th2, Th17, Treg cells, and IgA⁺ memory B cells with atopic disease in children: The Generation R Study

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Funding information

ZonMW The Netherlands, Grant/Award Number: 529051014; Australian National Health and Medical Research Council, Grant/Award Number: 1117687; European Union's Horizon 2020 research and innovation programme, Grant/Award Number: 733206; European Union's Horizon 2020 co-funded programme ERA-Net on Biomarkers for Nutrition and Health (ERA HDHL), Grant/Award Number: 696295

Abstract

Background: New insights into immune cells could contribute to treatment and monitoring of atopic disease. Because nongenetic factors shape the human immune system, we here studied these immune cells in a large cohort with atopic children with adjustment for prenatal and postnatal confounders.

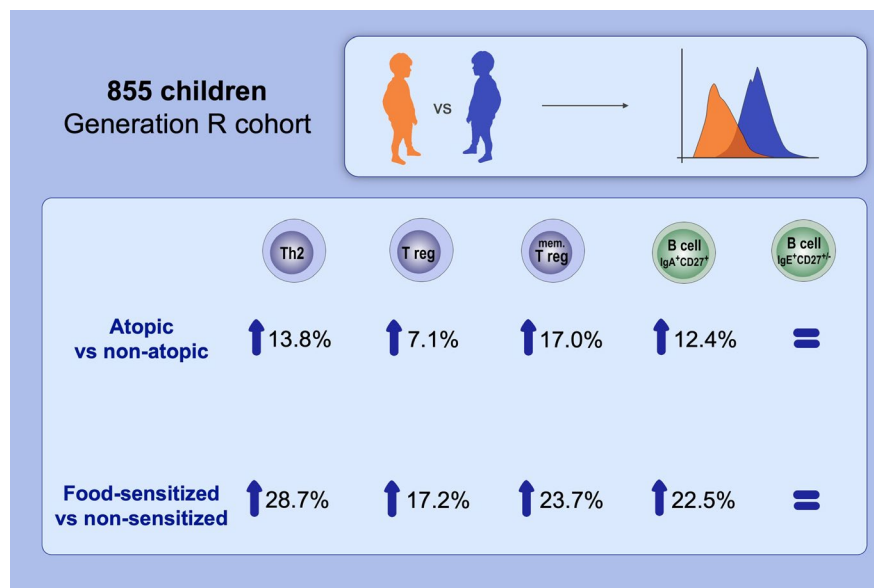
Methods: Information on atopic dermatitis, inhalant- and food-allergic sensitization, asthma lung function scores was obtained from 855 10-year-old children within the Generation R cohort. 11-color flow cytometry was performed to determine CD27⁺ and CD27⁺IgG⁺, IgE⁺ and IgA⁺ memory B cells, Th1, Th2, Th17, and Treg-memory cells from venous blood. Associations between any atopic disease, the individual atopic diseases, and immune cell numbers were determined.

Results: Children with any atopic disease had higher Th2, Treg, Treg-memory, and CD27⁺IgA⁺ memory B-cell numbers compared to children without atopic disease. When studying the individual diseases compared to children without the individual diseases, children with atopic dermatitis, inhalant-, and food-allergic sensitization had higher memory Treg cell numbers 12.3% (95% CI 4.2; 21.0), (11.1% (95% CI 3.0; 19.8), (23.7% (95% CI 7.9; 41.8), respectively. Children with food-allergic sensitization had higher total B and CD27⁺IgA⁺ memory B-cell numbers (15.2% [95% CI 3.2; 28.7], 22.5% [95% CI 3.9; 44.3], respectively). No associations were observed between asthma and B- or T-cell numbers.

Conclusion: Children with any atopic disease and children with inhalant- and food-allergic sensitization or atopic dermatitis had higher circulating memory Treg cells, but not higher IgE⁺B-cell numbers. The associations of higher Treg and CD27⁺IgA⁺ B-cell numbers in children with food-allergic sensitization are suggestive of TGF- β -mediated compensation for chronic inflammation.

KEYWORDS

atopic disease, IgA memory B cell, IgE memory B cell, T helper cell, T regulatory cell

**GRAPHICAL ABSTRACT**

This cross-sectional study including 855 children from the Generation R cohort was performed to determine immune cell numbers by flow cytometry analysis. Increased Th2, Treg, memory Treg, and IgA⁺CD27⁺ B cells were observed in atopic compared to non-atopic and food-sensitized compared to non-sensitized children. The higher IgA⁺CD27⁺ B-cell numbers in food-sensitized children suggests TGF-mediated compensation for chronic inflammation.

1 | INTRODUCTION

Atopic dermatitis, food allergy, allergic rhinitis, and asthma are atopic conditions with a high prevalence in children.¹ These diseases often coexist leading to high comorbidity of disease at young age.² This may be due to a shared immunopathophysiology.¹⁻³ Atopic diseases share a T helper 2 (Th2) cell-driven pathogenesis.⁴⁻⁶ This Th2-driven response is characterized by skewed cytokine responses including IL-4, IL-5, and IL-13, which in turn drive eosinophilic inflammation and immunoglobulin (Ig) class switching to IgE in B cells.⁴⁻⁷ Soluble IgE binds to high-affinity receptors, FcεR1, on mast cells and basophils, and subsequent exposure to the initial allergen will induce IgE-mediated FcεR1 receptor cross-linking and instant release of inflammatory mediators responsible for allergic symptoms present in atopic diseases.^{8,9} Regulatory T (Treg) cells have the ability to suppress this inflammatory response.¹⁰ Treg cells can be subdivided in two distinct subsets: naive and memory

Tregs. While naive Tregs mostly originate from the thymus, memory Tregs are induced in the periphery and display the highest suppressive ability.^{11,12}

Insights into the biology of the cells that produce IgE are limited. In contrast to B cells expressing other Ig isotypes (IgM, IgD, IgA or IgG), IgE-expressing B cells are scarce and surface expression of IgE on these cells is low.^{13,14} Our group has developed a method to enable detection of IgE-expressing B cells and plasma cells.¹³ In addition to the classical CD27⁺ memory B cells and plasma cells that are derived from T-cell-dependent responses in the germinal center, CD27⁺IgE⁺ B cells can be derived independent of cognate T-cell help.^{13,15} These CD27⁺IgE⁺ memory B cells were increased in adults and children with atopic dermatitis and displayed increased levels of somatic hypermutations, a sign of enhanced antibody maturation.¹³⁻¹⁷ However, these previous results did not adjust for confounders and are based on limited numbers of hospital-based patient populations (largest study n = 164).¹⁶

Therefore, we now quantified circulating memory B cell, Treg, and Th cell subsets with 11-color flow cytometry in a large birth cohort of 10-year-old children and assessed the association of atopic dermatitis, food- and inhalant-allergic sensitization, and asthma with these cells following adjustment for confounders.

2 | METHODS

2.1 | Study design

This cross-sectional study was embedded in the Generation R Study, a population-based prospective birth cohort study located in Rotterdam, the Netherlands.¹⁸ The Medical Ethical Committee of the Erasmus University Medical Center in Rotterdam has approved the study protocol (MEC-2012-165). Written informed consent was obtained from parents or legal representatives of all participants. For the present study, we selected 10-year-old children with at least one atopic disease measurement and with at least one immune cell outcome. This resulted in the inclusion of 855 children. The number of children included in the individual analyses is dependent on the combination of exposure and outcome and ranges from 648 to 776 children (Table S1).

2.2 | Atopic dermatitis measurements

Physician-diagnosed atopic dermatitis ever was diagnosed from parental-reported questionnaires obtained at the child's age of 10 years ('Was your child ever diagnosed by a physician with atopic dermatitis', 'yes; no').¹⁹⁻²¹

2.3 | Allergic sensitization measurements

We used skin prick tests using the scanned area method to determine the presence of total, inhalant- and food-allergic sensitization at the age of 10 years.²² The skin prick test included a positive control (histamine dihydrochloride 10 mg/mL) in duplicate and a negative control (NaCl 0.9%). We included house dust mite, 5-grass mixture, birch, cat, and dog (ALK-Abelló B.V., Almere, the Netherlands) as inhalant allergens and hazelnut, cashew nut, peanut, and peach as food allergens (homemade food allergen extracts).²⁰ A positive skin test was defined as an area $\geq 40\%$ of the histamine response.¹⁹ Information on physician-diagnosed allergy was obtained from questions adapted from the International Study on Asthma and Allergy in Childhood (ISAAC).²⁰

2.4 | Asthma and lung function measurements

At 10 years of age, we measured forced expiratory volume in 1 second (FEV₁), FEV₁/forced vital capacity (FVC), and forced expiratory flow after exhaling 75% of the FVC (FEF₇₅) by spirometry (MasterScreen-Pneumo, Jaeger Toennies (Viasys) CareFusion Netherlands), according to ERS/ATS guidelines.²³⁻²⁵ Lung function measurements were converted into z-scores adjusted for sex, age, height, and ethnicity

according to Global Lung Initiative reference data.²⁶ Information on wheezing in the past 12 months and physician-diagnosed asthma ever was obtained from questions adapted from ISAAC.^{23,24} Current asthma was defined as physician-diagnosed asthma ever together with use of asthma medication or wheezing in the past 12 months.

2.5 | Immune cell outcomes

Peripheral blood samples were obtained from children at 10 years. Absolute counts of peripheral blood CD19⁺ B cells and CD3⁺ T cells were obtained using a diagnostic lyse-no-wash protocol (BD Biosciences). In addition, detailed immune phenotyping of B and T cells using 11-color flow cytometry was performed on LSRII Fortessa (BD Biosciences) with standardized configuration according to Euroflow protocols.²⁷

In addition to total CD19⁺ B cells, the following CD19⁺CD38^{dim}IgD⁻ memory B-cell subsets were defined: CD27⁺IgG⁺, CD27⁺IgG⁺, CD27⁺IgE⁺, CD27⁺IgE⁺, CD27⁺IgA⁺, and CD27⁺IgA⁺ memory B cells.¹⁶ Within total CD3⁺ T cells, CD4⁺ and CD8⁺ lineages were defined, as well as CD4⁺CD127^{dim}CD25^{high} Treg cells and their naive (CD45RA⁺) and memory (CD45RA⁻) subsets. After exclusion of Tregs, Th cell subsets within CD4⁺CD45RA⁻ cells were defined as Th1 CCR6⁻CXCR3⁺CCR4⁻, Th2 CCR6⁻CXCR3⁻CCR4⁺, and Th17 CCR6⁺CXCR3⁻CCR4⁺ cells.¹⁶ Finally, within both the CD4⁺ and CD8⁺ lineages, naive (CD45RO⁻CCR7⁺), central memory T cells (Tcm; CD4RO⁺CCR7⁺), effector memory RO-positive T cells (TemRO; CD45RO⁺CCR7⁺), and effector memory RA-positive cells (TemRA; CD45RO⁻CCR7⁺) were defined.²⁸⁻³⁰ The data were analyzed with the software packages FacsDIVA software v8 (BD Biosciences), Infinicyt software (Cytognos, Salamanca, Spain), and FlowJo software v10 (FlowJo LLC). Cell numbers were presented per μ L blood. The gating strategies are represented in Figures S1-S3.

2.6 | Covariates

Information on maternal age, prepregnancy body mass index, smoking during pregnancy, breastfeeding during the first 4 months, family history of atopy and asthma, and net household income was derived from parental questionnaires.^{19,20,23} Child's sex and birth-weight were obtained from midwife and hospital registries.²⁸ Child's weight and height were measured at the research center, and body mass index (BMI) was calculated. Information on child's ethnicity and number of children in household was obtained from parental questionnaires.^{19,20,23,28}

2.7 | Statistical analysis

To obtain a normal distribution, the cell numbers were natural log-transformed. Multivariable linear regression analyses were conducted to determine associations between any atopic condition, atopic dermatitis, allergic sensitization, asthma, lung function, and immune cell numbers to limit bias due to confounding. Covariates were added if they were associated with immune cells based on

TABLE 1 Characteristics of the study population

	(N = 855)	Missing (%)
Child characteristics		
Sex (N, %)		
Girls	460 (53.8)	0.0
Birthweight, g	3510 [3150; 3830]	0.1
BMI at 9 y, kg/m ²	17.08 [15.81; 18.49]	0.0
Ethnicity (N, %)		
Dutch or Western	484 (84.0)	32.6
Siblings in household (N, %)		
0	103 (13.8)	12.9
1	406 (54.5)	
2 or more	194 (26.0)	
Ever diagnosed with atopic dermatitis (N, %)	163 (22.1)	13.7
Ever diagnosed with asthma (physician) (N, %)	63 (8.5)	13.1
Current asthma within the past 12 mo at 9 y (N, %)	34 (4.6)	13.1
Positive allergy skin prick test (N, %)	230 (29.6)	9.2
Positive food allergy skin prick test (N, %)	50 (6.5)	9.4
Positive inhalant skin prick test (N, %)	225 (29.0)	9.1
Maternal characteristics		
Age, years	31.80 [29.12; 34.34]	0.0
Prepregnancy BMI, kg/m ²	22.60 [20.72; 24.91]	24.0
Smoking during pregnancy (N, %)	176 (23.8)	13.6
Breastfeeding during first 4 mo (N, %)	606 (90.3)	21.5
Family history of atopy and asthma (N, %)	397 (49.9)	6.9
Net household income, euro (N, %)		
>2200	525 (73.4)	16.4

Note: Values are based on nonimputed dataset. Values are presented as median [25%-75% range] or numbers (%)

our previous study and if they met the epidemiological criteria for confounders in our study, and finally, if the addition of the covariate resulted in an effect estimate alteration of at least 10%.^{31,32} We adjusted for the following covariates: sex, gestational age, maternal age, maternal prepregnancy BMI, breastfeeding during the first 4 months, number of children in the household, child ethnicity, family history of atopy or asthma, cesarean section, child BMI at 9 years.³² In the nonimputed dataset, percentages of missing values within the covariates ranged from 0% to 24% and for child ethnicity 32.6%. Tenfold multiple imputation was performed. Pooled regression coefficients are presented in our study. We did not adjust for multiple testing because of strong correlations between immune cell numbers. A $P < 0.05$ was considered significant. Statistical analyses were performed using SPSS version 21.0 (IBM Corp.) and R version 3.3.3 (R Foundation for Statistical Computing).

3 | RESULTS

Our study population consisted of 855 children with a median age of 9.8 years (Table 1). Two hundred and thirty (29.6%) children had

allergic sensitization, of which 50 (6.5%) to food allergens and 225 (29.0%) to inhalant allergens. Sixty-three children (8.5%) were ever diagnosed with asthma. A total of 163 (22.1%) had ever atopic dermatitis. The concomitant atopic diseases in the study population are shown in Figure S4.

3.1 | Th and Treg cell subsets

Children with any atopic condition compared to no atopic condition had 13.8% (95% CI 4.1; 24.4) higher Th2, 7.1% (95% CI 1.0; 13.5) higher Treg, and 18.4% (95% CI 5.1; 33.3) higher Th17 cells (Table 2). Next, we studied the T-cell subsets per atopic condition. No associations were observed between atopic conditions and Th1 cell numbers.

14.5% (95% CI 4.4; 25.5) higher Th2 cell numbers were observed in children with allergic sensitization compared to those without allergic sensitization. Specifically, 12.5% (95% CI 2.5; 23.5) and 28.7% (95% CI 8.8; 52.3) higher Th2 cell numbers were observed in inhalant- and food-allergic sensitization, respectively. Th2 cells were not associated with atopic dermatitis, asthma, or lung function (Tables 3 and 4). To further study Th2 cells in relation to intrinsic and extrinsic atopic dermatitis, we stratified for food-allergic sensitization. Although

TABLE 2 Associations between any atopic condition and B- and T-cell subsets

Outcome	Exposure
	Any atopic condition (n = 729; yes = 343, no = 386)
T-cell subsets	
Th1	3.29 (−8.54; 16.64)
Th2	13.82 (4.12; 24.44)
Treg	7.10 (1.03; 13.54)
Treg naive	2.76 (−5.01; 11.16)
Treg memory	17.03 (8.79; 25.91)
Th2/Th1 ratio	10.10 (−4.88; 27.45)
Th17	18.37 (5.11; 33.31)
B-cell subsets	
B total	9.79 (3.60; 16.36)
B naive	9.28 (2.34; 16.69)
IgG ⁺ CD27 [−]	5.52 (−3.64; 15.55)
IgG ⁺ CD27 ⁺	9.21 (−0.94; 20.39)
IgE ⁺ CD27 [−]	−0.93 (−2.25; 2.16)
IgE ⁺ CD27 ⁺	0.11 (−3.56; 3.93)
IgA ⁺ CD27 [−]	14.20 (2.29; 27.49)
IgA ⁺ CD27 ⁺	12.35 (3.15; 22.37)

Note: The numbers represent % cell increase or decrease (95% CI) for the children with any atopic condition compared to children without any atopic condition. Numbers are based on multivariable linear regressions and adjusted for the following covariates: sex, gestational age at birth, maternal age, maternal prepregnancy BMI, breastfeeding during the first 4 mo, number of children in the household, child ethnicity, family history of atopy or asthma, cesarean section, child BMI at 9 y. The bold values represent the statistically significant ($P < .05$) results.

higher effect estimates were observed in children with food-allergic sensitization, no significant associations were observed (Table S3). Children with food-allergic sensitization had 17.2% (95%CI 4.8; 31.1) higher total Tregs. Following separation into naive (CD45RA⁺) and memory (CD45RA[−]) Treg, we found that the difference in total Treg was fully explained by 23.7% (95%CI 7.9; 41.8) higher memory Treg numbers. In addition, memory Tregs were 12.3% (95%CI 4.2; 21.0) and 11.1% (95%CI 3.0; 19.8) higher in children with any or inhalant-allergic sensitization, respectively. Children with atopic dermatitis had 12.0% (95% CI 4.8; 19.8) higher total Tregs, 11.0% (95%CI 1.5; 21.5) higher naive Treg, and 17.2% (95% CI 7.5; 27.8) higher memory Treg cells. In addition, children with 1SD increase in FEV₁ or FEF₇₅ had 3.7% (95% CI 0.3; 7.3) and 3.6% (95% CI 0.1; 7.2) higher total Tregs, respectively. For FEF₇₅, the difference in Tregs was completely dependent on naive Tregs with 4.8% (95%CI 0.1; 9.8) higher naive Tregs for children with 1SD increase in FEF₇₅. T-cell subsets were not associated with asthma. Finally, children with atopic dermatitis had 15% (95% CI 0.2; 32.0) higher Th17 and children with food-allergic sensitization had 26.2% (95% CI 0.7; 58.2) higher Th17 cell numbers, whereas there was no association for any and inhalant-allergic sensitization or asthma (Tables 3 and 4). No associations were observed between physician-diagnosed allergy and T cells (Table S4).

3.2 | Memory B cells

Children with any atopic conditions compared to no atopic condition had 9.8% (95% CI 3.6; 16.4) higher total and 9.3% (95% CI 2.3; 16.7) higher naive B-cell numbers. Furthermore, they had 14.2% (95% CI 2.3; 27.5) higher IgA⁺CD27[−] and 12.4% (95% CI 3.2; 22.4) higher IgA⁺CD27⁺B-cell numbers. No associations were observed for IgE⁺ and IgG⁺ memory B cells. When studying the individual atopic conditions, children with food-allergic sensitization had 15.2% (95% CI 3.2; 28.7) higher total B cells and 16.2% (95% CI 2.6; 31.5) higher naive B cells compared to children without food-allergic sensitization. Children with 1 SD increase in FEV₁ z-score had 3.9% (95% CI 0.6; 7.4) higher total B cells. However, lung function was not associated with B-cell subsets (Tables 2–4). Numbers of CD27⁺ and CD27[−] IgG⁺, IgA⁺, and IgE⁺ memory B cells were not associated with any or inhalant-allergic sensitization, atopic dermatitis, or asthma. Food-allergic sensitization was associated with 22.5% (95% CI 3.9; 44.3) higher CD27⁺IgA⁺ B cells but not with CD27⁺IgE⁺, CD27[−]IgE⁺, CD27⁺IgG⁺, CD27[−]IgG⁺, or CD27⁺IgA⁺ B cells (Table 3). To further analyze the immune cells in children with asthma, we stratified children with information on asthma diagnoses by inhalant-allergic sensitization. However, no differences were observed (data not shown). No associations were observed for physician-diagnosed allergy and B cells (Table S4).

3.3 | Memory T-cell subsets and atopic diseases

Children with atopic dermatitis had 5.8% (95% CI 0.1; 11.7) higher CD4⁺ total and 11.4% (95% CI 1.1; 22.8) higher CD4⁺Tcm cell numbers. Children with any allergic sensitization had 10.9% (95% CI 1.5; 21.2) higher CD4⁺TemRO cell numbers. This was for inhalant-allergic sensitization 10.2% (95% CI 0.8; 20.5) higher and food-allergic sensitization 22.1% (95% CI 3.7; 43.8) higher. Furthermore, children with food-allergic sensitization had 11.2% (95% CI 0.9; 22.4) higher CD4⁺ total cell numbers. No associations were observed between CD8⁺ T-cell subsets and atopic conditions (Table S2).

4 | DISCUSSION

In this population-based cross-sectional study, we demonstrated that children with any atopic disease had higher Th2, Treg, Treg-memory, Th17, and CD27[−] and CD27⁺IgA⁺B-cell numbers. More specifically, children with allergic sensitization had higher Th2 and Th17 cell numbers compared to children without allergic sensitization. Furthermore, we observed that food and inhalant-allergic sensitization and atopic dermatitis were associated with higher Treg numbers. These associations were mainly explained by higher memory Treg numbers, in the presence of normal naive Treg numbers. In addition, we observed that CD27⁺IgE⁺ and CD27[−]IgE⁺ B cells were similar in children with and without atopic conditions. In contrast, children with food-allergic sensitization had higher numbers of CD27⁺IgA⁺ memory B cells than children without. Finally, children with allergic

TABLE 3 Associations between allergic sensitization, atopic dermatitis, asthma, and B- and T-cell subsets

Outcome	Exposure					
	Any allergic sensitization	Inhalant-allergic sensitization	Food-allergic sensitization	Atopic dermatitis	Asthma ever	Asthma current
T-cell subsets						
Th1	-2.11 (-13.64; 10.97)	-3.28 (-14.75; 9.73)	-1.25 (-21.54; 24.27)	11.56 (-2.97; 28.26)	-16.19 (-31.91; 3.15)	-9.40 (-31.19; 19.30)
Th2	14.49 (4.42; 25.53)	12.52 (2.52; 23.49)	28.70 (8.77; 52.27)	5.70 (-4.53; 17.02)	13.05 (-3.07; 31.86)	14.19 (-6.78; 39.89)
Treg	5.89 (-0.41; 12.58)	5.88 (-0.46; 12.63)	17.2 (4.79; 31.07)	12.02 (4.78; 19.76)	4.80 (-5.21; 15.86)	2.13 (-10.43; 16.45)
Treg naive	3.56 (-4.66; 12.48)	4.61 (-3.74; 13.69)	14.28 (-1.72; 32.89)	11.02 (1.45; 21.51)	1.61 (-11.18; 16.25)	1.90 (-14.54; 21.52)
Treg memory	12.27 (4.17; 21.00)	11.06 (2.98; 19.77)	23.70 (7.91; 41.79)	17.17 (7.45; 27.78)	2.30 (-10.31; 16.67)	2.68 (-13.46; 21.83)
Th2/Th1 ratio	16.89 (0.48; 35.97)	16.28 (-0.16; 35.43)	30.28 (-1.29; 71.94)	-5.30 (-20.10; 12.23)	34.84 (4.82; 73.46)	25.97 (-9.78; 75.90)
Th17	12.79 (-0.21; 27.48)	10.90 (-1.94; 25.43)	26.20 (0.68; 58.19)	15.00 (0.22; 31.97)	-6.03 (-23.58; 15.55)	-5.41 (-28.16; 24.55)
B-cell subsets						
B total	4.67 (-1.46; 11.18)	3.66 (-2.46; 10.17)	15.24 (3.16; 28.74)	3.29 (-3.47; 10.52)	2.01 (-7.80; 12.86)	-2.67 (-14.96; 11.39)
B naive	5.24 (-1.64; 12.61)	4.26 (-2.61; 11.63)	16.15 (2.57; 31.54)	1.46 (-6.02; 9.53)	-0.43 (-11.11; 11.52)	-5.99 (-19.22; 9.40)
IgG ⁺ CD27 ⁻	0.55 (-8.50; 10.49)	0.03 (-9.04; 9.99)	5.40 (-11.41; 25.37)	-1.09 (-11.03; 9.94)	-1.89 (-16.48; 15.22)	-6.60 (-24.56; 15.57)
IgG ⁺ CD27 ⁺	4.21 (-5.80; 15.28)	3.34 (-6.65; 14.40)	16.98 (-2.91; 40.95)	0.26 (-10.62; 12.46)	7.14 (-9.82; 27.29)	-3.54 (-23.31; 21.33)
IgE ⁺ CD27 ⁻	-0.54 (-2.75; 1.88)	-0.51 (-2.79; 1.86)	2.48 (-1.80; 6.94)	-0.60 (-2.92; 2.18)	-2.92 (-6.55; -0.84)	-2.18 (-6.99; 2.88)
IgE ⁺ CD27 ⁺	1.01 (-2.83; 5.00)	0.55 (-3.30; 4.55)	6.25 (-1.05; 14.10)	-3.64 (-7.69; 0.58)	-2.97 (-8.98; 3.43)	-2.37 (-10.33; 6.28)
IgA ⁺ CD27 ⁻	4.07 (-7.21; 16.72)	2.65 (-8.55; 15.23)	20.14 (-2.76; 48.44)	6.97 (-5.90; 21.60)	1.04 (-16.80; 22.70)	2.51 (-20.76; 32.62)
IgA ⁺ CD27 ⁺	8.29 (-0.95; 18.40)	6.69 (-2.48; 16.73)	22.47 (3.91; 44.33)	1.40 (-8.23; 12.04)	6.05 (-8.69; 23.17)	-3.76 (-21.14; 17.46)

Note: The numbers represent % cell increase or decrease (95% CI) for children with allergic sensitization, atopic dermatitis, or asthma vs children without these respective diseases. Numbers are based on multivariable linear regressions and adjusted for the following covariates: sex, gestational age at birth, maternal age, maternal prepregnancy BMI, breastfeeding during the first 4 mo, number of children in the household, child ethnicity, family history of atopy or asthma, cesarean section, child BMI at 9 y.

The bold values represent the statistically significant ($P < .05$) results.

TABLE 4 Associations between lung function and B- and T-cell subsets

Outcome	Exposure		
	FEV ₁	FEV ₁ /FVC	FEF ₇₅
T-cell subsets			
Th1	5.50 (−1.28; 12.74)	0.03 (−6.37; 6.88)	2.76 (−3.92; 9.89)
Th2	2.90 (−2.14; 8.19)	1.51 (−3.46; 6.73)	1.91 (−3.15; 7.24)
Treg	3.73 (0.32; 7.26)	2.39 (−0.95; 5.84)	3.59 (0.14; 7.15)
Treg naive	4.51 (−0.20; 9.45)	3.14 (−1.47; 7.97)	4.83 (0.05; 9.83)
Treg memory	1.75 (−2.36; 6.03)	1.23 (−2.87; 5.52)	0.88 (−3.31; 5.25)
Th2/Th1 ratio	−2.49 (−9.96; 5.60)	1.46 (−6.30; 9.87)	−0.80 (−8.51; 7.55)
Th17	4.05 (−2.51; 11.05)	3.04 (−3.43; 9.94)	4.32 (−2.30; 11.38)
B-cell subsets			
B total	3.93 (0.58; 7.40)	−0.24 (−3.46; 3.10)	0.90 (−2.42; 4.33)
B naive	3.40 (−0.35; 7.29)	−0.27 (−3.88; 3.47)	0.66 (−3.05; 4.51)
IgG ⁺ CD27 [−]	1.35 (−3.73; 6.70)	0.11 (−4.90; 5.38)	1.20 (−3.96; 6.63)
IgG ⁺ CD27 ⁺	3.60 (−2.09; 9.62)	0.11 (−5.37; 5.90)	1.22 (−4.44; 7.21)
IgE ⁺ CD27 [−]	−0.65 (−1.59; 0.91)	−0.22 (−2.02; 0.47)	−0.26 (−2.00; 0.53)
IgE ⁺ CD27 ⁺	−0.04 (−2.93; 1.04)	−1.13 (−3.09; 0.87)	−1.20 (−3.19; 0.84)
IgA ⁺ CD27 [−]	1.66 (−4.52; 8.24)	1.09 (−5.03; 7.62)	0.56 (−5.66; 7.19)
IgA ⁺ CD27 ⁺	3.27 (−1.69; 8.47)	−0.86 (−5.60; 4.13)	−0.20 (−5.08; 4.94)

Note: The numbers represent % cell increase or decrease (95% CI) per SD increase in lung function. Numbers are based on multivariable linear regressions and adjusted for the following covariates: sex, gestational age at birth, maternal age, maternal prepregnancy BMI, breastfeeding during the first 4 mo, number of children in the household, child ethnicity, family history of atopy or asthma, cesarean section, child BMI at 9 y.

The bold values represent the statistically significant ($P < .05$) results.

sensitization or atopic dermatitis had higher CD4⁺ memory T-cell numbers but not CD8⁺ memory T-cell numbers. No differences were observed between ever or current asthma diagnosis and B- and T-cell subsets.

4.1 | Previous literature and interpretation

We expanded our previous studies on immunological phenotype in atopic children and extended previous literature by studying memory Treg and IgA⁺ and IgE⁺B cells in a large population-based cohort with adjustment for confounders. In accordance with previous findings, we observed higher Th2 cell numbers in children with inhalant- and food-allergic sensitization compared to children without these conditions.^{4–6} Previous studies showed that this Th2-driven pathogenesis in atopic disease may result in higher Treg cell numbers.^{10,33} On the other hand, Treg activation may skew the Th1/Th2 balance toward Th2.^{33,34} We indeed observed higher memory Treg cell numbers in children with inhalant- and food-allergic sensitization or atopic dermatitis, but not in those with asthma.³⁵ Interestingly, when studying Tregs divided in naive and memory Tregs, the association was mainly explained by memory Treg cells. The naive and memory Tregs have been shown to be two distinct subsets.¹¹ Naive Treg cells derive from the thymus, whereas the production of memory Treg cells is induced peripherally.¹² Therefore, higher memory Treg cell numbers in children

with allergic sensitization or atopic dermatitis may not be due to an intrinsic T-cell abnormality but to peripheral inflammatory responses that result in the induction of Treg differentiation.^{12,36} It has been hypothesized that memory Treg cells are important to reduce proinflammatory immune responses in atopic disease by anti-inflammatory cytokine production such as IL-10 and TGF- β .^{12,36,37} Therefore, higher memory Treg cell numbers in our population may indicate that children with allergic sensitization and atopic dermatitis experience a chronic inflammatory immune response that needs to be suppressed.^{33,38,39} Numbers of Treg cells were highest in food-allergic sensitization. A possible explanation is the production of the cytokine TGF- β . TGF- β is produced within the gut-associated lymphoid tissues, which are important in the intestinal homeostasis and thus most likely involved in food-allergic sensitization.¹⁰ By the induction of TGF- β , conventional T cells differentiate to peripheral Treg cells.¹² Peripheral Treg cells, also known as memory Treg cells, are important in the recognition of non-self-antigens.^{10,12} We speculate that chronic inflammation as seen in atopic diseases contributes to the production of TGF- β and thereby the increase in peripheral Treg cell numbers.^{3,10} Additionally, peripheral Tregs can be produced in specialized non-thymic niches located in the lungs.¹⁰ This could explain our observation of higher Treg cell numbers in inhalant-allergic sensitization.

In addition to higher Treg cell numbers in children with food-allergic sensitization, we observed higher CD27⁺IgA⁺ memory

B cells in this group. CD27⁺IgA⁺ memory B cells develop in germinal center responses following T-cell costimulation, whereas CD27⁺IgA⁺ cells can be produced independently of T-cell responses.^{13,40,41} TGF- β is also essential in IgA production by mediating IgA class switch recombination.⁴² Secretory IgA is important in maintaining the intestinal function.⁴³ Previous studies suggest that IgA contributes to the discrimination of commensal bacteria and food allergens from invading pathogens.^{13,43} Our finding that children with food-allergic sensitization have more IgA⁺ memory B cells may suggest that these children have an increased IgA response compared to children without food-allergic sensitization. In addition, Treg cells have been shown to maintain IgA⁺ B-cell numbers.⁴² This might explain why we specifically observed the association with CD27⁺IgA⁺ memory B cells in food-allergic sensitization.

We observed increased Th17 in food-allergic sensitization and atopic dermatitis but not in asthma. Th17 induces the production of proinflammatory cytokines and chemokines causing acute inflammatory circumstances.^{44,45} In addition, Th17 cell differentiation is dependent on TGF- β produced specifically by T cells.^{44,45} The associations for Treg, CD27⁺IgA⁺ memory B cells and Th17 might indicate that production of TGF- β is enhanced in chronic inflammatory processes of allergic disease.⁴⁴ Possibly, this may not drive the Th2-mediated inflammation, but represents a compensation mechanism to dampen responses.

The findings in the Th and Treg cell subsets are supported by our subanalyses on CD4⁺ and CD8⁺ memory subsets. We observed higher CD4⁺Tcm and CD4⁺TemRO cell numbers in children with inhalant- and food-allergic sensitization. No differences between allergic and nonallergic children were observed in CD8⁺ T-cell subsets. These findings support our observation that the number of Th2 and Treg cells is higher in children with allergic sensitization compared to children without allergic sensitization because both cell types are characterized by CD4⁺ surface molecules.^{12,33}

In contrast with previous studies, we did not observe associations between IgE⁺ B cells and atopic diseases.^{13,14,16,17,46} Similarly, a small study ($n = 23$) did not observe differences in IgE⁺ memory B cells in patients with and without atopic dermatitis.⁴⁷ Most likely, the difference could be explained by differences in study populations: tertiary referral center vs population based.¹⁶ Also, in contrast with our study, these previous studies studied current atopic disease instead of ever atopic disease, which probably is inherent to a more severe and persistent atopic condition. This suggests that IgE⁺ B cells are more involved in severe atopic disease. Also, the lack of associations could be due to an attenuation of disease activity of atopic dermatitis and asthma ever over time.³

We observed higher overall Treg cell numbers in children with higher FEV₁ or FEF₇₅, mainly explained by higher naive Tregs. In children with allergic sensitization or atopic dermatitis, we found higher memory Treg cells. As higher memory Treg cells represent chronic inflammation, naive cells have no memory of sensitization to specific antigens.^{33,48,49} Children with better lung functions are less likely to have asthma,^{50,51} and indeed, high naive Tregs suggest that these

children had no chronic immune activation compared to children with allergic sensitization and atopic dermatitis.³³ However, we did not observe lower Treg-memory cells in children with better lung function. This might be explained by differences in systemic immune responses, but could also be a chance finding.⁶

Eosinophilic asthma is most likely Th2 and IgE mediated.^{3,6} However, other types of asthma such as virally induced asthma or neutrophilic asthma have different underlying pathophysiologies.^{6,52} This might dilute any association between asthma and immune parameters. Also, local immune responses in the airways may not be detectable in peripheral blood.^{53,54}

4.2 | Methodological considerations

A major strength of our study is the detailed, extensive data on determinant and outcome. We used 11-color flow cytometry to obtain detailed information on an extensive set of immune cell numbers. We used skin prick tests and lung function test besides questionnaires to obtain objective data and limit response bias. Furthermore, this study was embedded in a large population-based prospective cohort with detailed data on potential confounders. Previous studies on this topic did not adjust for confounders.^{13,14,38} The addition of this adjustment in a large cohort is crucial to extend unbiased knowledge on immune cell composition in children with atopic disease. However, the following limitations apply. First, because atopic diseases share a common underlying immunopathophysiology,³ we had high percentages of concomitant atopic diseases within the individual atopic disease groups studied. We were unable to perform sensitivity analyses in children with one atopic disease because the exclusion of children with multiple atopic diseases would cause a loss of statistical power. Second, the number of current asthma was low in our population. Therefore, we also studied children with asthma ever and determined lung function at the age of 10 years. Consequently, our study is underpowered to test the underlying hypothesis that IgE⁺ positive B cells may be a feature of more severe persistent conditions, defined as current atopic disease. Also, the definition of the diagnosis asthma did not limit to IgE-mediated asthma. Therefore, we stratified asthma diagnosis by inhalant-allergic sensitization. This led to low subgroup numbers. Third, for measuring allergic sensitization we selected common allergens in 10-year-old children. Consequently, allergens with low sensitization prevalence at the age of 10 years, such as milk and egg, were not included.

5 | CONCLUSIONS

Children with any atopic disease have higher Th2, Treg, Treg memory, and CD27⁺IgA⁺ memory B-cell numbers compared to children without atopic disease. Specifically, food or inhalant-allergic sensitization and atopic dermatitis had CD4⁺ effector memory T-cell numbers, explained by a Th2 and Th17-mediated immune response and higher Treg-memory cell numbers. Furthermore, children with

food-allergic sensitization had higher CD27⁺IgA⁺ memory B cells, suggestive of TGF- β -mediated compensation for chronic inflammation. B and T cells did not differ in children with and without asthma.

ACKNOWLEDGMENTS

We gratefully acknowledge the contribution of children and parents, general practitioners, hospitals and midwives, and pharmacies in Rotterdam. The funders had no role in the design or conduct of the study; the collection of the data and analyses; the interpretation of data; the preparation and review or approval of the manuscript; or the decision to submit the manuscript.

CONFLICT OF INTEREST

The Generation R Study is conducted by Erasmus University Medical Center Rotterdam in close collaboration with the School of Law and Faculty of Social Sciences of Erasmus University Rotterdam, the Municipal Health Service Rotterdam Metropolitan Area, the Rotterdam Homecare Foundation, and the Stichting Trombosedienst & Artsenlaboratorium Rijnmond. The Dept Immunology of the Erasmus MC funded the immunological measurements. LD received funding from the European Union's Horizon 2020 cofunded program ERA-Net on Biomarkers for Nutrition and Health (ERA HDHL) (ALPHABET project (no 696295; 2017), ZonMW The Netherlands (no 529051014; 2017), and the European Union's Horizon 2020 research and innovation program (LIFECYCLE project, grant agreement no 733206; 2016). MCvZ is supported by the Australian National Health and Medical Research Council (NHMRC, Senior Research Fellowship 1117687). All authors declare that no competing interests exist.

AUTHOR CONTRIBUTIONS

MCvZ designed and supervised the flow cytometry experiments; CG, FJMV, and JK performed the flow cytometry experiments. LD and JCDJ designed the lung function experiments and the collection of the data on asthma diagnosis. NWdJ is designed the collection of the allergic sensitization data. SGMAP designed the collection of the atopic dermatitis data. HAM, MCvZ, and KIML designed the study. KIML had full access to all of the data and performed the data analyses. HAM, MCvZ, KIML, LD, ERvM interpreted the data. KIML, MCvZ, and HAM wrote the manuscript, and all authors critically revised and commented on the manuscript. HAM supervised the study.

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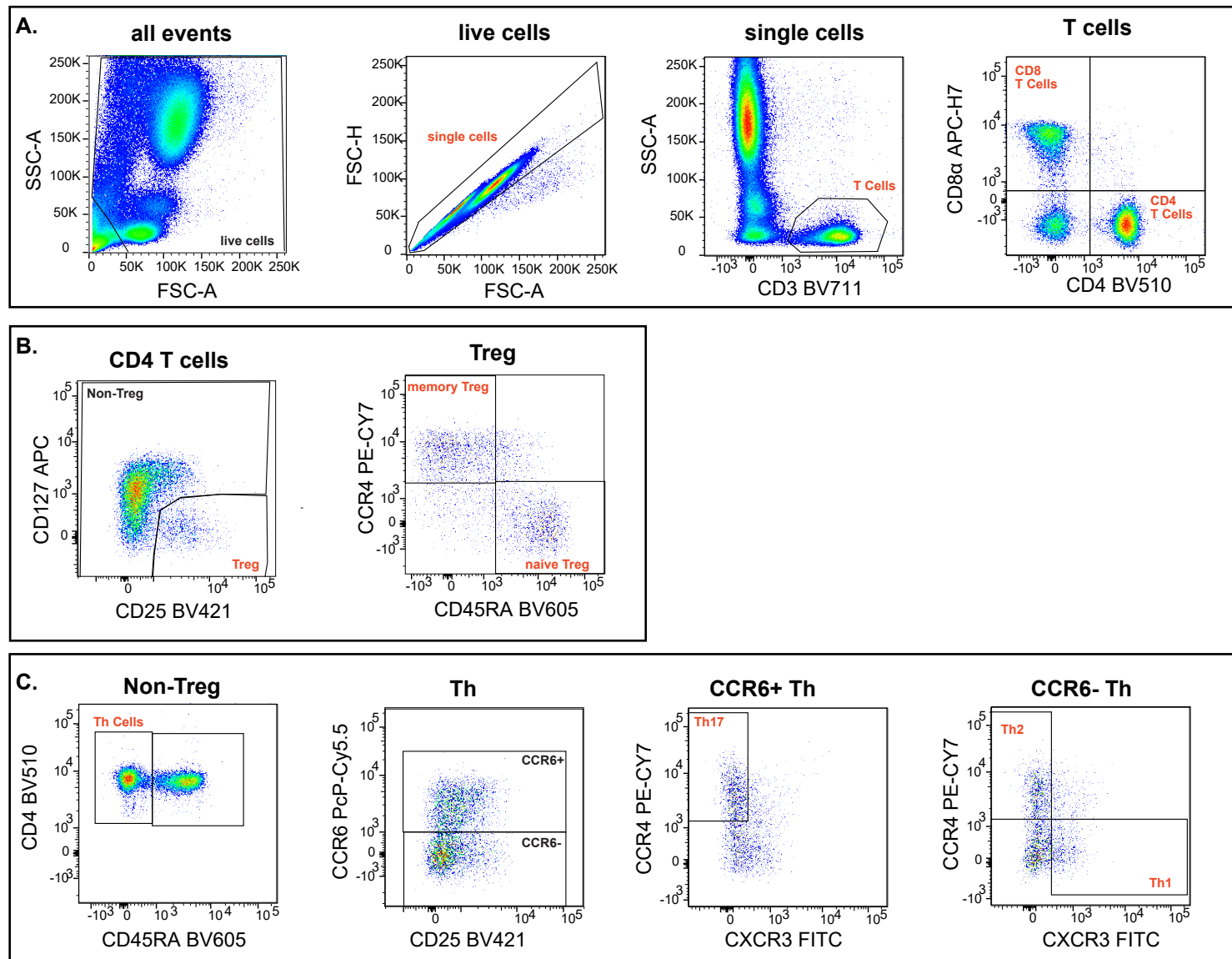
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SUPPORTING INFORMATION

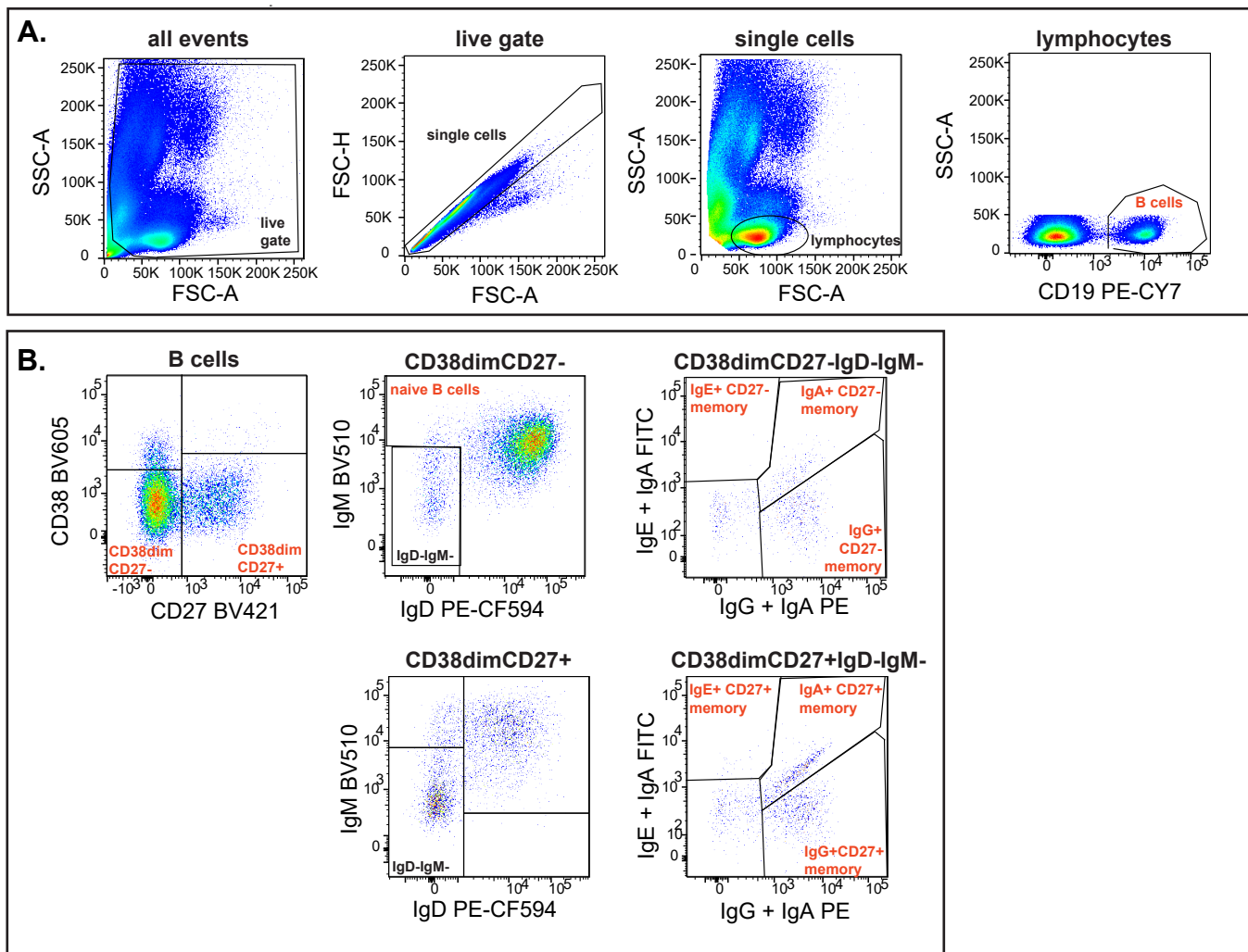
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How to cite this article: Looman KIM, van Meel ER, Grosserichter-Wagener C, et al. Associations of Th2, Th17, Treg cells, and IgA⁺ memory B cells with atopic disease in children: The Generation R Study. *Allergy*. 2019;00:1–10. <https://doi.org/10.1111/all.14010>

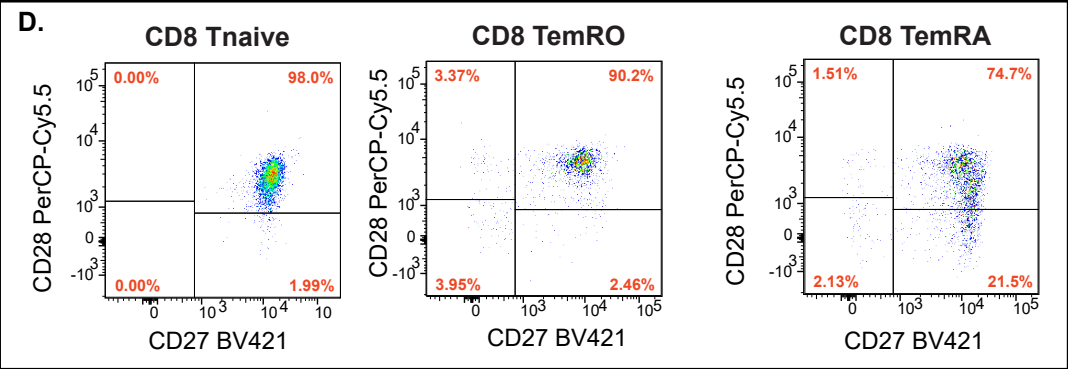
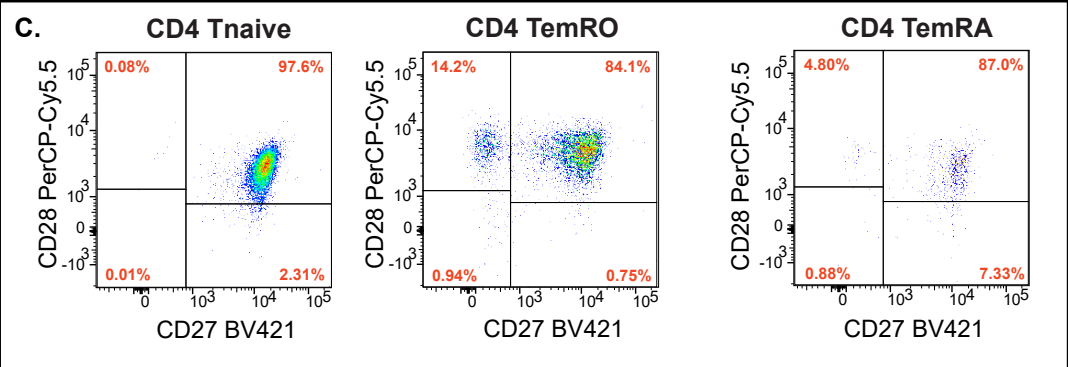
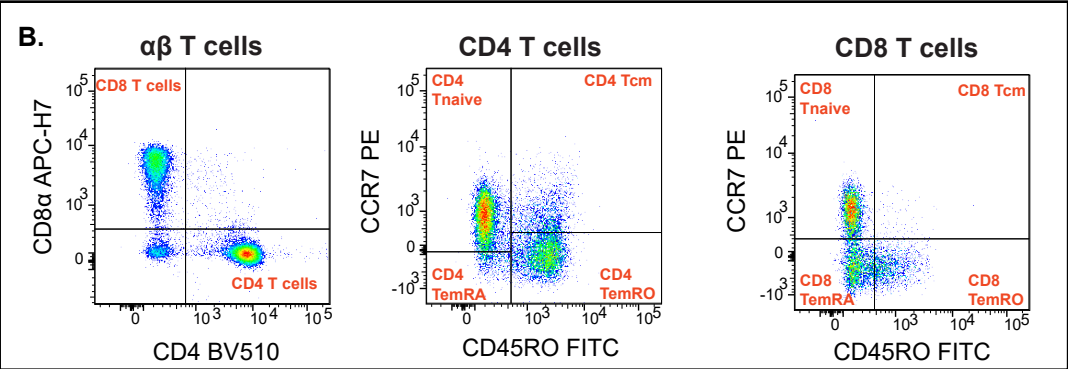
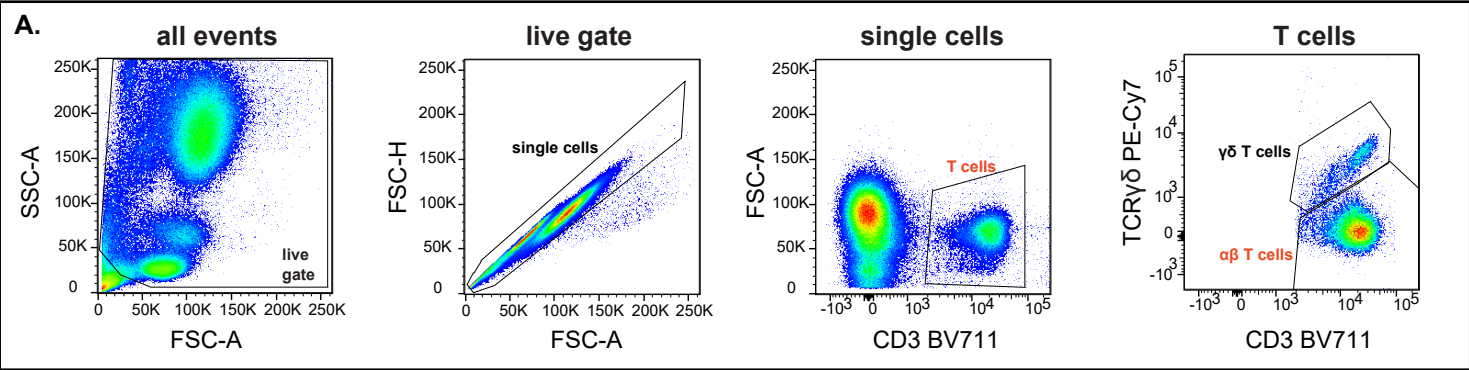
Supplemental Figure 1. Gating strategy T helper subsets



Supplemental Figure 2. Gating strategy B cell subsets



Supplemental Figure 3. Gating strategy naive and memory T cell subsets



Supplemental Figure 4. Overview of concomitant atopic disease in the study population



Supplemental Table 1. Numbers of children included in the individual analyses performed.

Exposure Outcome	Any allergy sensitization	Inhalant allergic sensitization	Food allergic sensitization	Atopic dermatitis	Asthma ever or current	Lung function
T cell subsets						
Th1	747	748	746	709	714	661
Th2	746	747	745	708	713	660
Treg	736	737	735	700	706	651
Treg naive	733	734	732	697	703	648
Treg memory	733	734	732	697	703	648
Th2/Th1 ratio	746	747	745	708	713	656
Th17	742	743	741	705	709	656
B cell subsets						
B total	775	776	774	737	742	689
B naive	768	769	767	729	734	685
IgG ⁺ CD27 ⁻	767	768	766	728	733	684
IgG ⁺ CD27 ⁺	767	768	766	728	733	684
IgE ⁺ CD27 ⁻	767	768	766	728	733	684
IgE ⁺ CD27 ⁺	767	768	766	728	733	684
IgA ⁺ CD27 ⁻	767	768	766	728	733	684
IgA ⁺ CD27 ⁺	767	768	766	728	733	684

The numbers represent the number of children included in the individual analyses. The analyses are based on a total number of 855 different children.

Supplemental Table 2. Associations between allergic sensitization, atopic dermatitis and asthma and memory T cell subsets.

Exposure Outcome	Any allergic sensitization	Inhalant allergic sensitization	Food allergic sensitization	Atopic dermatitis	Asthma ever	Asthma current
CD4 ⁺	2.58 (-2.69;8.13)	1.80 (-3.47;7.37)	11.16 (0.93;22.42)	5.77 (0.11;11.74)	1.38 (-6.63;10.08)	-4.89 (-14.69;6.04)
CD4 ⁺ naive	1.66 (-4.82;8.58)	0.91 (-5.58;7.85)	11.55 (-1.16;25.88)	7.01 (-0.25;14.80)	-0.73 (-10.70;10.36)	-7.98 (-19.99;5.85)
CD4 ⁺ Tcm	3.06 (-5.67;12.59)	1.29 (-7.34;10.73)	4.77 (-11.00;23.34)	11.39 (1.07;22.77)	1.35 (-12.53;17.44)	-6.81 (-23.26;13.16)
CD4 ⁺ TemRA	16.12 (-1.56;36.99)	15.91 (-1.85;36.88)	30.84 (-3.51;77.41)	0.23 (-16.74;20.66)	9.35 (-17.34;44.67)	3.91 (-28.00;49.96)
CD4 ⁺ TemRO	10.89 (1.49;21.15)	10.21 (0.81;20.48)	22.12 (3.74;43.75)	6.19 (-3.69;17.07)	10.38 (-4.98;28.22)	8.42 (-10.91;31.95)
CD8 ⁺	2.41 (-3.53; 8.72)	2.17 (-3.80;8.51)	4.67 (-6.19;16.80)	-0.12 (-6.34;6.51)	5.05 (-4.57;15.63)	1.40 (-10.68;15.12)
CD8 ⁺ naive	5.52 (-2.32;13.98)	4.42 (-3.38;12.86)	10.49 (-4.13;27.34)	2.13 (-6.18;11.18)	6.40 (-6.40;20.96)	4.03 (-12.10;23.11)
CD8 ⁺ Tcm	2.07 (-11.48;17.69)	0.98 (-12.51;16.54)	-10.40 (-31.06;16.47)	1.14 (-13.51;18.29)	-0.94 (-21.77;25.45)	1.31 (-25.67;38.08)
CD8 ⁺ TemRA	-3.28 (-13.57;8.23)	-2.84 (-13.25;8.82)	11.69 (-9.16;37.34)	-4.85 (-16.08;7.88)	15.28 (-4.90;39.74)	16.82 (-9.04;50.02)
CD8 ⁺ TemRO	1.28 (-8.10;11.62)	1.52 (-7.94;11.95)	4.36 (-12.74;24.82)	-0.49 (-10.35;10.46)	2.64 (-12.45;20.34)	-8.02 (-25.33;13.30)

The numbers represent % cell increase or decrease (95%CI) per SD increase in lung function or for the children with asthma compared to children without asthma. Numbers are based on multivariable linear regressions and adjusted for the following covariates: sex, gestational age at birth, maternal age, maternal pre-pregnancy BMI, breastfeeding during the first 4 months, number of children in the household, child ethnicity, family history of atopy or asthma, cesarean section, child BMI at 9 years.

Supplemental Table 3. Associations between atopic dermatitis and immune cells stratified for allergic sensitization and food allergic sensitization.

Exposure Outcome	With allergic sensitization (n _{total} =198, n _{atopicdermatitis} = 70)	Without allergic sensitization (n _{total} =469, n _{atopicdermatitis} = 70)	With food allergic sensitization (n _{total} =43, n _{atopicdermatitis} = 20)	Without food allergic sensitization (n _{total} =623, n _{atopicdermatitis} = 119)
T cell subsets				
Th1	19.17 (-7.05; 52.78)	8.74 (-11.44; 33.53)	26.44 (-34.21; 143.01)	12.73 (-4.0; 32.38)
Th2	-0.45 (-15.91; 17.85)	4.78 (-10.04; 22.05)	-23.31 (-47.50; 12.01)	5.97 (-5.87; 19.29)
Treg	19.08 (6.88; 32.68)	4.41 (-5.45; 15.30)	20.09 (-5.11; 51.97)	10.70 (2.55; 19.50)
Treg naive	27.92 (11.00; 47.41)	-0.08 (-12.86; 14.58)	35.51 (-3.90; 91.09)	9.56 (-1.26; 21.57)
Treg memory	10.32 (-4.14; 26.96)	16.07 (2.37; 31.60)	2.05 (-22.45; 34.29)	16.86 (5.97; 28.87)
Th2/Th1 ratio	-16.47 (-38.62; 13.69)	-3.71 (-24.76; 23.23)	-39.35 (-74.41; 43.76)	-6.04 (-22.62; 14.08)
Th17	-1.10 (-19.42; 21.40)	24.00 (-0.45; 54.46)	112.44 (-11.18; 408.12)	16.78 (-0.91; 37.64)
B cell subsets				

B total	-8.11 (-18.79; 3.98)	11.60 (1.25; 23.01)	43.80 (8.98; 89.74)	3.82 (-3.95; 12.22)
B naive	-11.28 (-22.71; 1.84)	11.85 (0.01; 25.09)	52.99 (7.82; 117.09)	3.02 (-5.68; 12.53)
IgG ⁺ CD27 ⁻	-17.90 (-31.75; -1.25)	6.16 (-8.87; 23.67)	-23.04 (-48.82; 15.74)	-1.22 (-12.42; 11.41)
IgG ⁺ CD27 ⁺	-14.71 (-31.33; 5.92)	-1.21 (-15.90; 16.04)	-6.27 (-39.08; 44.20)	-4.12 (-16.00; 9.45)
IgE ⁺ CD27 ⁻	-0.70 (-5.05; 3.85)	1.82 (-1.88; 5.66)	0.84 (-11.45; 14.83)	0.12 (-2.71; 3.04)
IgE ⁺ CD27 ⁺	-5.60 (-12.82; 2.21)	-0.71 (-6.59; 5.54)	10.37 (-11.24; 37.23)	-4.35 (-8.85; 0.37)
IgA ⁺ CD27 ⁻	-17.78 (-35.31; 4.49)	17.64 (-1.80; 40.92)	-9.08 (-46.43; 54.33)	4.26 (-9.93; 20.67)
IgA ⁺ CD27 ⁺	-12.84 (-26.80; 3.77)	0.61 (-13.02; 16.38)	11.17 (-27.54; 70.55)	-2.56 (-13.04; 9.19)
The numbers represent % cell increase or decrease (95%CI) for children with, atopic dermatitis compared to children without atopic dermatitis stratified for (food) allergic sensitization. Numbers are based on multivariable linear regressions and adjusted for the following covariates: sex, gestational age at birth, maternal age, maternal pre-pregnancy BMI, breastfeeding during the first 4 months, number of children in the household, child ethnicity, family history of atopy or asthma, cesarean section, child BMI at 9 years.				

Supplemental Table 4. Associations between physician-diagnosed allergy and B- and T-cell subsets

Exposure Outcome	Physician-diagnosed allergy (yes, n=79; no, n=649)
T cell subsets	
Th1	13.13 (-6.46 ;36.82)
Th2	12.54 (-2.22; 29.53)
Treg	7.89 (-1.45; 18.12)
Treg naive	5.87 (-6.30; 19.62)
Treg memory	10.73 (-1.58; 24.57)
Th2/Th1 ratio	-0.56 (-21.07; 25.28)
Th17	2.66 (-14.94; 23.90)
B cell subsets	
B total	10.45 (0.82; 21.01)
B naive	10.02 (-0.68; 21.88)
IgG ⁺ CD27 ⁻	0.65 (-12.84; 16.20)
IgG ⁺ CD27 ⁺	10.99 (-4.94; 29.60)
IgE ⁺ CD27 ⁻	-2.84 (-6.08; 0.52)
IgE ⁺ CD27 ⁺	-3.94 (-9.29; 1.73)
IgA ⁺ CD27 ⁻	-2.20 (-17.86; 16.44)
IgA ⁺ CD27 ⁺	12.83 (-1.44; 29.16)
The numbers represent % cell increase or decrease (95%CI) for children with physician-diagnosed allergy versus children without allergy diagnosis. Numbers are based on multivariable linear regressions and adjusted for the following covariates: sex, gestational age at birth, maternal age, maternal pre-pregnancy BMI, breastfeeding during the first 4 months, number of children in the household, child ethnicity, family history of atopy or asthma, cesarean section, child BMI at 9 years.	

