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Increased Th22 cell numbers in a general pediatric population with filaggrin haploinsufficiency: The Generation R Study

Kirsten I. M. Looman[1,2] | Minke M. F. van Mierlo[3] | Menno C. van Zelm[4] | Chen Hu[1,3] | Liesbeth Duijts[5,6] | Johan C. de Jongste[1,5] | Tamar Nijsten[3] | Luba M. Pardo[3] | Jessica C. Kiefte-de Jong[7] | Henriëtte A. Moll[2] | Suzanne G. M. A. Pasmans[3]

[1]The Generation R Study Group, Erasmus MC, University Medical Center Rotterdam, Rotterdam, The Netherlands

[2]Department of General Pediatrics, Erasmus MC, University Medical Center Rotterdam-Sophia Children's Hospital, Rotterdam, The Netherlands

[3]Department of Dermatology, Erasmus MC, University Medical Center Rotterdam-Sophia Children's Hospital-Center of Pediatric Dermatology, Rotterdam, The Netherlands

[4]Department Immunology and Pathology, Central Clinical School, Monash University and The Alfred Hospital, Melbourne, VIC, Australia

[5]Department of Pediatrics, Division of Respiratory Medicine and Allergology, Erasmus MC, University Medical Center Rotterdam, Rotterdam, The Netherlands

[6]Department of Pediatrics, Division of Neonatology, Erasmus MC, University Medical Center Rotterdam, Rotterdam, The Netherlands

[7]Department of Public Health and Primary Care, Leiden University Medical Center/LUMC Campus, Leiden, The Netherlands

Correspondence
Suzanne G. M. A. Pasmans, Department of Dermatology-Center of Pediatric Dermatology, Erasmus MC University Medical Center Rotterdam-Sophia Children's Hospital, Sp-1540, Wytemaweg 80, 3015 CN Rotterdam, The Netherlands.
Email: s.pasmans@erasmusmc.nl

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Abstract

Background: Mutations in the filaggrin gene (*FLG*) affect epidermal barrier function and increase the risk of atopic dermatitis (AD). We hypothesized that *FLG* mutations affect immune cell composition in a general pediatric population. Therefore, we investigated whether school-aged children with and without *FLG* mutations have differences in T- and B-cell subsets.

Methods: This study was embedded in a population-based prospective cohort study, the Generation R Study, and included 523 children of European genetic ancestry aged 10 years. The most common *FLG* mutations in the European population (R501X, S1085CfsX36, R2447X, and S3247X) were genotyped. Additionally, 11-color flow cytometry was performed on peripheral blood samples to determine helper T (Th), regulatory T (Treg), and $CD27^+$ and $CD27^-$ memory B cells. Subset analysis was performed in 358 non-AD and 102 AD cases, assessed by parental questionnaires.

Results: *FLG* mutations were observed in 8.4% of the total population and in 15.7% of the AD cases. Children with any *FLG* mutation had higher Th22 cell numbers compared to *FLG* wild-type children in the general and non-AD population. Children with and without *FLG* mutations had no difference in Th1, Th2, Th17, Treg, or memory

Abbreviations: AD, Atopic dermatitis; FLG, Filaggrin gene; Ig, Immunoglobulin; IL, Interleukin; ILC2, Type 2 innate lymphoid cells; IV, Ichthyosis vulgaris; IQR, Interquartile range; N, Number; OR, Odds ratio; SC, Stratum corneum; Tcm, Central memory T cell; Th, Helper T cell; Tem, Effector memory T cell; TNF, Tumor necrosis factor; Treg, Regulatory T cell.

Kirsten .I M. Looman and Minke M. F. van Mierlo denote equal contribution.

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B-cell numbers. Furthermore, in children with AD, *FLG* mutation carriership was not associated with differences in T- and B-cell subsets.

Conclusions: School-aged children of a general population with *FLG* mutations have higher Th22 cell numbers, which reflects the immunological response to the skin barrier dysfunction. *FLG* mutations did not otherwise affect the composition of the adaptive immunity in this general pediatric population.

KEYWORDS

atopic dermatitis, Filaggrin, IgE memory B cell, T helper cell, T regulatory cell

Key Message

This study in a general pediatric population showed that filaggrin haploinsufficiency is associated with higher Th22, but not with other T and B cells. This might reflect the immunological response to the altered skin barrier in children with filaggrin haploinsufficiency.

1 | INTRODUCTION

Filaggrin is a filament-associated protein that is encoded by the filaggrin gene (*FLG*) and is an important contributor to the preservation of the skin barrier.[1,2] Approximately 10% of the European population is a heterozygote carrier of a disrupting mutation in *FLG*.[3] Both complete loss-of-function and reduced functional activity of filaggrin lead to destruction of the stratum corneum (SC) and consequently skin barrier dysfunction.[2,4] This barrier dysfunction due to *FLG* mutations is presumed to be caused by lower numbers of tight junctions, reduced density of the protein corneodesmosin, and impaired maturation and excretion of lamellar bodies in the epidermis which are important in maintaining cell-to-cell integrity.[1]

Failure in barrier function through mutations in *FLG* results in increased skin permeability for percutaneous transfer of exogenous particles including allergens and pollutants.[1,2,4] Accordingly, *FLG* mutations are the strongest genetic risk factor for atopic dermatitis (AD).[2,3,5] A previous meta-analysis showed that *FLG* haploinsufficiency results in an odds ratio (OR) of 3.12 for the incidence of AD.[6] In addition, *FLG* mutations are associated with a form of AD that starts in early infancy and persists into adulthood, a higher incidence of skin infections, and a higher likelihood of having asthma, inhalant, or food allergies.[1,7-9]

The increased permeability of the skin as a result of *FLG* mutations is thought to affect immune responses and maturation of adaptive immune cells. Filaggrin is also expressed in the thymus, the primary lymphoid organ in which T cells are formed.[10] Hence, *FLG* mutations potentially affect the peripheral immune cell compartment through effects in skin and thymus, and previous studies observed higher γδT17 and T helper (Th) 17 in filaggrin-deficient flaky tail (ft/ft) mice.[10] In addition, a case study reported higher numbers of circulating thymus-emigrated regulatory T (Treg) cells and Th2 in 6 AD patients with a heterozygote FLG mutation.[11] Another study, including 2 heterozygous, 2 homozygous, and 1 compound heterozygous AD patient, showed increased Th17 cells in the *FLG* mutation group.[12] On the other hand, literature on the role of B-cell dysregulation in AD is scarce and conflicting.[13-16] It can be hypothesized that mutations in *FLG* can affect B-cell numbers due to skewing of the Th cell populations.

We hypothesized that *FLG* mutations affect T- and B-cell maturation in children through effects on the skin and thymus. Until now, no studies on this association have been performed in the general pediatric population and only case studies have been performed in AD patients.[11,12] It is of interest to examine the role of *FLG* mutations in adaptive immune maturation in both a general population, a non-AD, and AD population to further understand the role of *FLG* in the immune maturation. Therefore, we here studied the associations between common *FLG* mutations in the European population and immune cell numbers, as determined using 11-color flow cytometry, within a population-based birth cohort study including a subgroup non-AD and AD patients.

2 | METHODS

2.1 | Study design

This study was embedded within the Generation R Study, a prospective birth cohort study conducted in Rotterdam, the Netherlands. The Medical Ethical Committee of the Erasmus MC, University Medical Center Rotterdam approved the study (MEC-2012-165).[17] Written informed consent was obtained from parents or legal representatives of all children. We included all children with European genetic ancestry[18] with information on *FLG* mutation (homozygous, compound heterozygous, heterozygous, or wild type) and information on at least one of the immune cell outcomes. This resulted in a total number of 523 children (Figure 1). AD was defined as physician-diagnosed eczema from parental questionnaires obtained at the child's age of 10 years ('Was your child ever

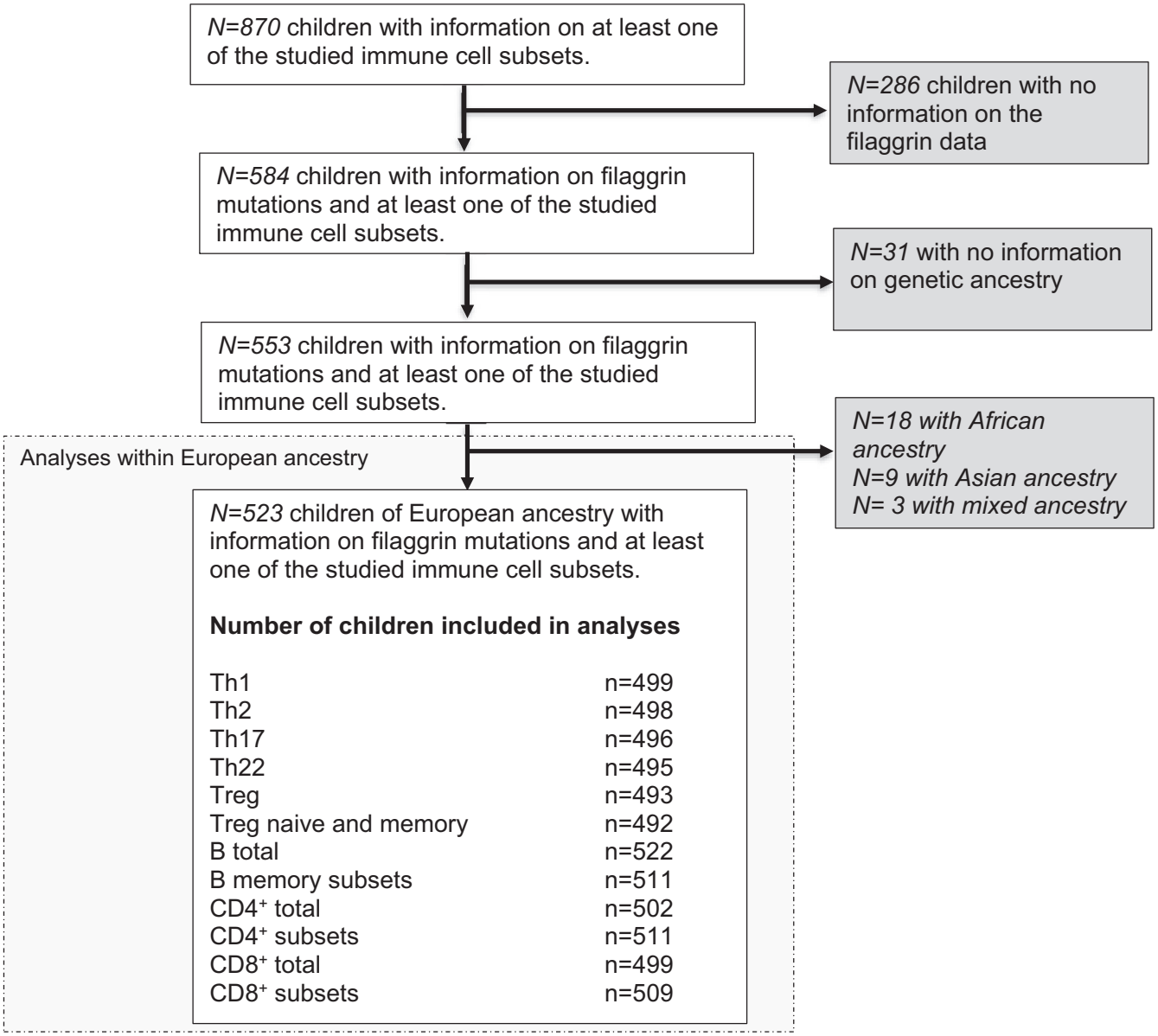

FIGURE 1 Flowchart of participants included in the study. Abbreviations: Th, helper T cell; Treg, regulatory T cell.; Tcm, central memory T lymphocytes; TemRA, effector memory RA-positive T lymphocytes; TemRO, effector memory RO-positive T lymphocytes

diagnosed by a physician with atopic dermatitis', 'yes; no'). This information was available for 470 children, including 102 subjects with AD.[16]

2.2 | *FLG* genotype

DNA samples obtained from umbilical cord blood were genotyped by modified Taqman allelic discrimination assays for common European mutations in *FLG* (R501X (rs61816761), S1085CfsX36 (rs41370446), R2447X (rs138726443), and S3247X (rs150597413) with the use of primers as described previously.[19,20] The distribution of the *FLG* mutations was as follows: 4.2% had R501X (rs61816761), 1.1% had S1085CfsX36 (rs41370446), 3.5% had R2447X (rs138726443), and 0.0% had S3247X (rs150597413). Because S3247X (rs150597413) was not present within our study population, this mutation was not included in the analyses. Children were classified as having a *FLG* mutation if they were homozygous, compound heterozygous, or heterozygous for any of the three mutations that were present in our study population. Children without any of the mutations were classified as wild type.

2.3 | Immune cell numbers

Peripheral blood samples from children were obtained at the age of 10 years.[16] The analyses were performed on fresh blood cells within 24 hours of sampling. Absolute counts of $CD3^+$ T cells

TABLE 1 Details of the study population

Child characteristics	Total (n = 523)	Total population				Subset analyses	
		Wild-type population (n = 479)	*FLG* mutation population (n = 44)	*P*-value	Missing in total study population (N, %)	Atopic dermatitis (n = 102)	Non-atopic dermatitis (n = 358)
Sex (N, %)							
Female	280 (53.5)	256 (53.4)	24 (54.5)	1.0	0.0	48 (47.1)	192 (53.6)
Male	243 (46.5)	223 (46.6)	20 (45.5)			54 (52.9)	166 (46.4)
FLG mutations (N, %)							
Wild type	479 (91.6)	479 (100.0)	-	-	0.0	86 (84.3)	336 (93.9)
1 or more Mutations	44 (8.4)[†]	-	44 (100.0)			16 (15.7)[2]	22 (6.1)[1]
Type *FLG* mutations (N, %)							
S1085CfsX36 (rs41370446)	22 (4.2)	-	22 (4.2)	-	0.0	7 (6.9)	12 (3.4)
R2447X (rs138726443)	6 (1.1)	-	6 (1.1)	-	0.2	1 (1.0)	4 (1.1)
R501X (rs61816761)	18 (3.5)	-	18 (3.5)	-	0.4	9 (9.0)[‡]	7 (2.0)
S3247X (rs150597413)	0 (0.0)	-	0 (0.0)	-	0.4	0 (0.0)	0 (0.0)
Ever physician-diagnosed atopic dermatitis (N, %)[§]	102 (22.2)	86 (20.4)	16 (42.1)	0.004	12.0	102 (100.0)	0 (0.0)

Note: Table 1 represents the child and maternal characteristics for the study population stratified for *FLG* mutations. Values are based on the non-imputed dataset and represented as number (%). Chi-squared tests were conducted to examine possible differences in baseline characteristics between the different genotypes.

FLG, filaggrin gene; N, number;

[1]1 compound heterozygous biallelic mutation,

[2]1 compound heterozygous and 1 homozygous mutation.

[†]Including 3 biallelic mutations (2 compound heterozygous and 1 homozygous),

[‡]Including one biallelic mutations (homozygous),

[§]Based on parental-reported questionnaires obtained at the child's age of 10 years: 'Was your child ever diagnosed by a physician with atopic dermatitis?' (no or yes),

and CD19$^+$ B cells per μL blood were determined with diagnostic lyse-no-wash protocol, and detailed immunophenotyping was performed with 11-color flow cytometry (LSR Fortessa, BD Biosciences). We determined naive (CD45RO$^-$CCR7$^+$), effector memory RO-positive T cells (TemRO; CD45RO$^+$CCR7$^-$), and effector memory RA-positive T cells (TemRA; CD45RO$^-$CCR7$^-$) within CD4$^+$ and CD8$^+$ lineages.[16,21,22] Within Treg cells, the differentiation in naive (CD45RA$^+$) and memory (CD45RA$^-$) was determined.[16] Finally, the following T helper (Th) cell subsets (CD4$^+$CD45RA$^-$) were determined after exclusion of Treg cells on the basis of chemokine receptor profiles as defined previously[16,23-27]: Th1 (CCR6$^-$CXCR3$^+$CCR4$^-$), Th2 (CCR6$^-$CXCR3$^-$CCR4$^+$), Th17 (CCR6$^+$CXCR3$^-$CCR4$^+$CCR10$^-$), Th17.1(CCR6$^+$CXCR3$^+$CCR4$^-$), and Th22 (CCR6$^+$CXCR3$^-$CCR4$^+$CCR10$^+$). In addition, CD27$^+$ and CD27$^-$ IgG$^+$, IgA$^+$, IgE$^+$ CD19$^+$CD38^{dim}IgD$^-$ memory B-cell subsets were defined.[16] Gating strategies for Th cell determination are presented in eFigure 3.

2.4 | Statistical analyses

First, characteristics of the study population were determined, stratified for *FLG* mutation status and AD diagnosis. *P*-values for determining differences between the categorical variables of both groups were calculated with chi-squared tests. Next, median cell numbers with interquartile range (IQR) were determined. Differences in cell numbers between children with and without *FLG* mutations were determined with the non-parametric Mann-Whitney U tests. Subset analyses on the associations of *FLG* genotype with immune cell numbers were performed within non-AD and AD children. The possibility of effect modification by AD diagnosis was tested by performing linear regression analyses between immune cell numbers and *FLG* mutation status with the addition of an interaction term between *FLG* mutation status and AD diagnosis. To assure a normal distribution of the outcome in the linear regression analysis, a natural-log transformation for the immune cell outcomes was used. No adjustment for multiple testing was performed because of strong correlation between the immune cells studied. Statistical analyses were performed with SPSS version 21.0 (IBM Corp.) and R version 3.6.1 (R Foundation for Statistical Computing).

3 | RESULTS

3.1 | Study population

Characteristics of the study population are presented in Table 1. Within the total group of 523 children with European ancestry, *FLG* mutations were detected in 44 (8.4%) children, including 3 biallelic mutations (2 compound heterozygous and 1 homozygous). The proportion of patients with AD was lower in the wild-type group compared to the group with *FLG* mutations (20% versus 42%; $P < .01$). Within the non-AD population, 6.1% of the children had a *FLG* mutation, including one compound heterozygous. Within the AD population, 15.7% of the children had a *FLG* mutation, including one homozygous and one compound heterozygous.

3.2 | Higher Th22 cell counts in children of the general population with *FLG* mutations

Children of the general population with a *FLG* mutation had higher Th22 cell numbers compared to children of the wild-type population (Figure 2A, eTable 1). The median cell number within the *FLG* mutation group was 5.60/μL (IQR 4.04;8.94) and 4.5/μL (IQR 2.5;7.4, $P = .03$)within the wild-type group. To determine whether this association between Th22 and *FLG* was different between children with

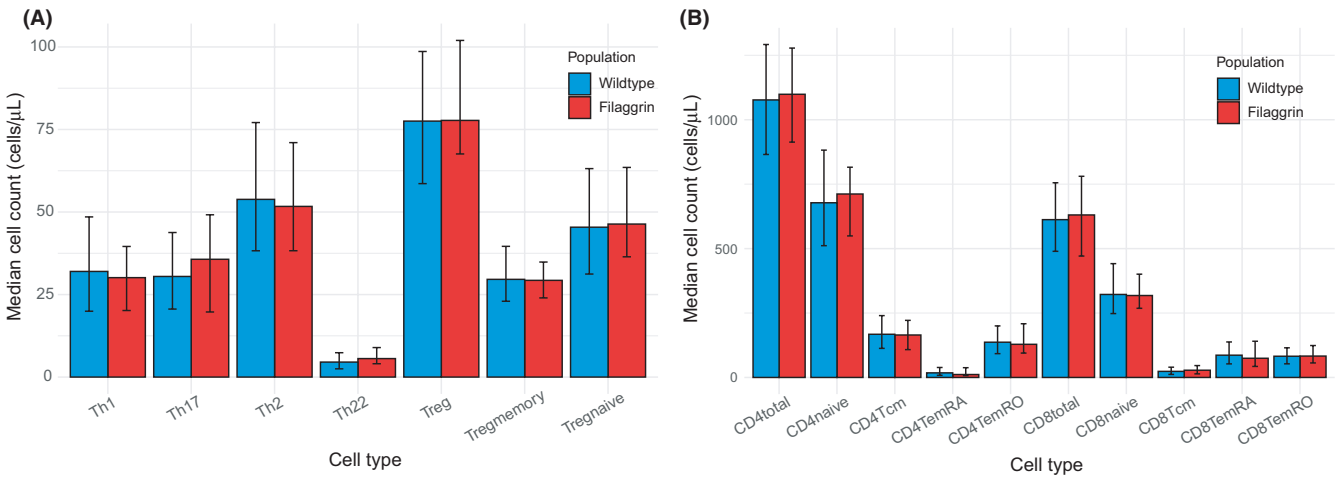

FIGURE 2 Absolute numbers of blood T-cell subsets stratified by *FLG* mutation status. A, The median (IQR) cell count per μL blood for Th and Treg cell numbers stratified for *FLG* mutation. B, The median (IQR) cell count per μL blood for CD4$^+$ and CD8$^+$ effector memory T-cell numbers stratified for *FLG* mutation. Abbreviations: IQR, interquartile range; Tcm, central memory T lymphocytes; TemRA, effector memory RA-positive T lymphocytes; TemRO, effector memory RO-positive T lymphocytes; Th, helper T cell; Treg, regulatory T cell. * denotes a two-sided *P*-value < .05. Supplementary Tables 1 and 3 show absolute numbers and *P*-values

and without AD, we performed a linear regression analysis with the following interaction term: *FLG* mutation status*AD. This interaction term was non-significant ($P = .13$) and therefore effect modification by AD in this association is not likely. However, when the analyses were stratified, the association between *FLG* and Th22 was slightly stronger in the non-AD group with a median cell number of 6.8/μL (IQR 4.9;11.4) in non-AD children with *FLG* mutations compared to 4.5/μL (IQR 2.4;7.5, $P = .006$) in non-AD children without *FLG* mutations (eTable 2). In contrast, when studying the differences in absolute Th22 cell numbers between children with and without *FLG* mutations in the AD population, no significant differences were observed (median 5.0/μL (IQR 3.6;6.4) and 4.6/μL (IQR 2.9;7.4), respectively ($P = .64$, eFigure 1A)).

3.3 | No associations between Th1, Th2, Th17, and Treg and *FLG* mutations

No differences in median cell numbers between *FLG* mutation and wild-type group were observed for Th1, Th2, Th17, and Treg (Figure 2A, eTable 1). This was similar in the subset analyses that were stratified for AD diagnosis (eFigure 1A, eTable 2). No differences in median cell numbers between the *FLG* mutation and the wild-type group were observed for the effector memory $CD4^+$ and $CD8^+$ T-cell subsets: naive, Tcm, TemRA, TemRO (Figure 2B, eFigure 1B).

3.4 | No associations between memory B cells and *FLG* mutations

No differences in median cell numbers between the *FLG* mutation and the wild-type group were observed for total B cells and naive mature B cells (eTable 1). In addition, no associations between *FLG* mutations and the following $CD27^+$ and $CD27^-$ memory B-cell subsets were observed: IgA^+, IgE^+, IgG^+, IgM^+ (Figure 3). Similarly, no changes were observed in the subset analyses that stratified the analyses for AD diagnosis (eFigure 2, eTable 2).

4 | DISCUSSION

In this population-based study among children of European genetic ancestry, we observed a prevalence of 8.4% for *FLG* mutations. In addition, we demonstrated that children with *FLG* mutations had higher Th22 cell numbers than children without *FLG* mutations. In contrast, the Th1, Th2, Th17, Treg, and memory B-cell numbers were comparable between children with and without *FLG* mutations. In addition, among children with AD, those with or without *FLG* mutations had no differences in B- or T-cell subsets.

4.1 | Comparison with literature and interpretation

All previous studies on *FLG* mutations and immune cell numbers have been performed within mice models or smaller numbers of AD patients.[10-12,28] This is the first study that provides insight in the role of *FLG* mutations on immune cell numbers in school-aged children of a general population. The setting of this study within a population-based pediatric cohort study is unique to study the association of *FLG* on immune cell numbers in a general population.

We observed higher Th22 cell numbers in children with *FLG* mutations in the general populations, with a slightly higher median Th22 cell number in non-AD children with a mutation in *FLG*. No previous studies that assess the association between *FLG* mutations and Th22 have been performed within a general population.

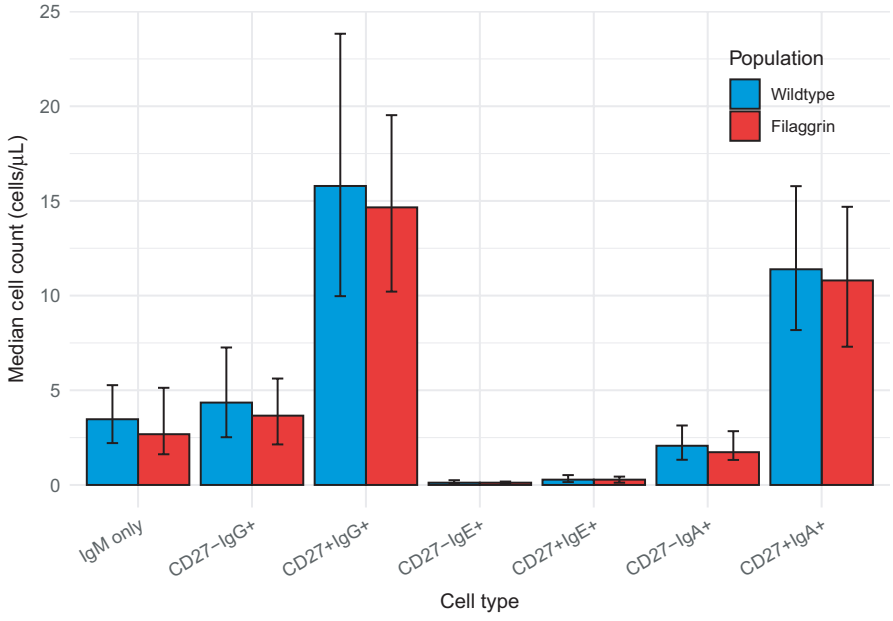

FIGURE 3 Absolute numbers of blood memory B-cell subsets stratified by *FLG* mutation status. The median (IQR) cell count per μL blood for B memory cell numbers stratified for *FLG* mutation is shown. Abbreviations: IQR, interquartile range; Ig, Immunoglobulin. Supplementary Table 2 shows absolute numbers and *P*-values

Interestingly, in contrast to our findings, several studies have observed increased Th22 cell numbers in the skin and circulation of patients with AD.[29,30] Within AD patients, the role of Th22 is still not fully elucidated. Current literature suggests both protective and pro-inflammatory roles for Th22 by the production of IL-22.[31] IL-22 contributes to skin integrity and is known for its role in the defense against different pathogens in the skin by the production of antimicrobial proteins.[32,33] However, the combined secretion of IL- 22 and TNF-α is thought to have an pro-inflammatory effect as observed in AD.[31] Possible explanations for the higher number of Th22 cell numbers in children without AD but with *FLG* mutations could be the following. First, the increase in Th22 could represent some level of inflammation due to *FLG* mutations without apparent clinical symptoms. Second, hypothetically, the increase in Th22 could contribute to skin homeostasis in children without AD to prevent further inflammatory processes leading to AD. However, further studies are needed to elaborate on the Th22 cell function in children with and without AD.

In contrast to previous studies investigating the effect of *FLG* mutation status, we did not observe differences in Th2, Th17, and Treg cell numbers between children with and without *FLG* mutations both in the total study population and in the subgroup of patients with AD.[10-12,28] The discrepancies between previous studies and our current study could be explained by differences in investigated populations and species. Previous mice studies and skin equivalents studied the effect of complete absence of filaggrin, compared to the filaggrin haploinsufficiency in our study population which leads to 50% reduction in filaggrin expression.[1,10,28] In addition, these studies could represent a different immunological setting than is present in human skin.[28] It is also expected that previous results on immune cell numbers in AD populations are affected by disease severity. Namely, the presence of different immune cells is dependent on disease state, including disease flare and chronic AD.[29,30] In turn, immune cells in active AD skin can induce downregulation of filaggrin protein expression in the skin independent of *FLG* mutations, subsequently affecting immune cell composition.[20] Although we do not have information on disease severity in our AD population, this study included a population-based, relatively healthy cohort in which we expect most children to have mild AD. Therefore, alteration in immune cell numbers is probably not only dependent on *FLG* mutation genotype, but also on AD severity and epigenetic and environmental factors.

In addition, we did not observe differences in memory B-cell numbers between children with and without *FLG* mutations. This is in line with our previous study in which we did not observe any association between B cells and AD.[16] No previous studies have investigated B-cell subsets in relation to *FLG* mutations.

Finally, within our study, we studied the associations between *FLG* mutations and the adaptive immunity. It can be speculated that *FLG* mutations cause alterations in the innate immunity such as eosinophilic granulocytes and ILC2 cell numbers. Future studies are needed to determine whether *FLG* mutations are associated with altered innate immunity cell numbers.

4.2 | Methodological considerations

A major strength is that this study investigated the association between *FLG* genotype and a large panel of B and T cells in the general population for the first time. We had detailed and extensive information on immune cell numbers from 11-color flow cytometry and obtained objective information on genetic ancestry. However, the following four limitations need to be addressed. First, we used chemokine receptor profiles which are surrogate markers of Th cells. Due to the large scale of our study and the need to process fresh blood within 24 hours, in vitro activation and cytokine staining were not feasible. Importantly, multiple studies have shown that the use of surface chemokine receptors is a robust approach to define Th subsets with the corresponding cytokine profiles.[23,24] Second, the AD population for the subset analyses was relatively small which could have limited the power in the statistical analyses. Nevertheless, in comparison with previous studies, only including a maximum of 6 AD patients with *FLG* mutations, this is the largest study on *FLG* mutations in both the general population and AD patients. Third, our AD population was defined by ever-having physician-diagnosed AD before or at the age of 10 years and no information on current disease activity was available. Therefore, it is likely that a subset of the children has outgrown AD at the age of 10 and this might affect their immunophenotype. Fourth, as mentioned previously, our study included the four most common *FLG* mutations in the European population. To prevent misclassification, we selected children with genetic European ancestry for the current study. Although the choice for including the most common *FLG* mutations in European populations is in line with previous studies,[11,12] other less frequent *FLG* mutations could exist in low numbers since up to 113 *FLG* mutations resulting in premature protein termination have been described. A recent study including patients with AD and ichthyosis vulgaris (IV) showed that screening the entire encoding region of *FLG* for mutations led to an improvement of the diagnostic yield.[34] As this is the first study in a general cohort addressing the association between *FLG* mutation and immune cell numbers, future studies are needed for validation of our results.

In conclusion, school-aged children of a general population with *FLG* mutations have higher Th22 cell numbers, which might reflect the skin barrier dysfunction that is caused by decreased filaggrin expression in the epidermis. In our study population, *FLG* mutations do not otherwise affect the composition of T and B cells in a general pediatric population, nor in the children with AD.

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CONFLICT OF INTEREST

The Generation R Study is conducted by Erasmus MC 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 research and innovation program (LIFECYCLE, grant agreement no 733206, 2016; EUCAN-Connect grant agreement no 824989; ATHLETE, grant agreement no 874583). 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. The Department of Dermatology of the Erasmus MC University Medical Center Rotterdam received an unrestricted grant from Micreos Human Health, the Netherlands.

AUTHOR CONTRIBUTION

Kirsten IM Looman: Conceptualization (equal); Data curation (lead); Formal analysis (lead); Methodology (equal); Project administration (equal); Visualization (equal); Writing-original draft (equal); Writing-review & editing (equal). **Minke MF van Mierlo:** Conceptualization (equal); Investigation (equal); Methodology (equal); Project administration (equal); Visualization (equal); Writing-original draft (equal); Writing-review & editing (equal). **Menno C. van Zelm:** Methodology (equal); Software (equal); Supervision (equal); Validation (equal). **Chen Hu:** Data curation (supporting); Project administration (supporting). **Liesbeth Duijts:** Conceptualization (equal); Data curation (equal); Funding acquisition (equal); Methodology (equal); Resources (equal). **Johan C de Jongste:** Conceptualization (equal); Data curation (equal); Funding acquisition (equal); Methodology (equal); Resources (equal). **Tamar EC Nijsten:** Conceptualization (equal); Funding acquisition (equal); Methodology (equal). **Luba M. Pardo:** Formal analysis (supporting); Investigation (supporting). **Jessica K. Kiefte:** Conceptualization (supporting); Formal analysis (supporting); Investigation (supporting); Methodology (equal). **Henriëtte A Moll:** Conceptualization (equal); Data curation (supporting); Funding acquisition (equal); Investigation (supporting); Methodology (equal); Supervision (equal). **Suzanne G.M.A. Pasmans:** Conceptualization (equal); Funding acquisition (equal); Methodology (equal); Supervision (lead).

ETHICAL APPROVAL

The study was conducted according to the principles of the Declaration of Helsinki. The Medical Ethical Committee of the Erasmus MC, University Medical Center Rotterdam, approved the study (MEC-2012-165). Written informed consent was obtained from parents or legal representatives of all children.

PEER REVIEW

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ORCID

Kirsten I. M. Looman https://orcid.org/0000-0002-1984-564X
Minke M. F. van Mierlo https://orcid.org/0000-0001-5397-7382
Menno C. van Zelm https://orcid.org/0000-0003-4161-1919
Liesbeth Duijts https://orcid.org/0000-0001-6731-9452
Tamar Nijsten https://orcid.org/0000-0001-9940-2875
Luba M. Pardo https://orcid.org/0000-0003-0684-3175
Jessica C. Kiefte-de Jong https://orcid.org/0000-0002-8136-0918
Henriëtte A. Moll https://orcid.org/0000-0001-9304-3322
Suzanne G. M. A. Pasmans https://orcid.org/0000-0003-1018-4475

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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