Household size, T regulatory cell development, and early allergic disease: a birth cohort study

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**Abstract**

**Background:** Children born to larger households have less allergic disease. T regulatory cell (Treg) development may be a relevant mechanism, but this has not been studied longitudinally.

**Objective:** We aim to (i) describe how prenatal and postnatal environmental factors are associated with Treg development and (ii) investigate whether serial Treg measures predict allergic outcomes at 1 year of age.

**Methods:** A birth cohort (n = 1074) with information on prenatal and postnatal early life factors. Both naïve Treg (nTreg) and activated Treg (aTreg) cell populations (as a proportion of CD4+ T cells) were available in 463 infants at birth (cord blood), 600 at 6 months, and 675 at 12 months. 191 infants had serial measures. Measures of allergic status at 12 months were polysensitization (sensitization to 2 or more allergens), clinically proven food allergy, atopic eczema, and atopic wheeze.

**Results:** Infants born to larger households (3 or more residents) had higher longitudinal nTreg proportions over the first postnatal year with a mean difference (MD) of 0.67 (95% CI 0.30–1.04)%). Higher nTreg proportions at birth were associated with a reduced risk of infant allergic outcomes. Childcare attendance and breastfeeding were associated with higher longitudinal nTreg proportions (MD 0.48 (95% CI 0.08–0.80)%).

**Abbreviations:** AOR, adjusted odds ratio; aTreg, activated T regulatory cell; AUC, area under curve; BIS, Barwon Infant Study; CI, confidence interval; IgE, immunoglobulin E; nTreg, naïve T regulatory cell; SPT, skin prick testing; Treg, T regulatory cell.
1 | INTRODUCTION

Childhood allergic disease has increased over time. In 1989, Strachan reported children born to larger households had less allergic disease. This association has been well replicated. The “Hygiene Hypothesis” proposes that a beneficial effect of larger household size is driven by sibling-related and other environmental microbial exposures. As well as larger household size, other candidate environmental microbial factors that may act as a proxy for environmental microbial load external to the child include dogs and farm animals. Mounting evidence also implicates the potential role of the maternal infant gut microbiome. Infant polysensitization, with atopic sensitization to multiple allergens, strongly predicts severe subsequent allergic disease. FoxP3-positive T regulatory (Treg) cells play a role in maintaining immune tolerance to foods and other environmental antigens. In early life, we reported that the CD4+FoxP3+ cells have a predominantly naïve (CD45RA+) phenotype. This CD4+CD45RA+FoxP3low population of Treg were shown by Miyara et al to be fully demethylated for the 5’ flanking region of the FoxP3 gene and potent suppressive. Naïve Treg (nTreg) in circulation can transform into activated Treg (aTreg, CD4+CD45RA−FoxP3high) in response to antigenic immunostimulation. We reported that a deficit of nTreg cells at birth predicts food allergy at 1 year, with gene demethylation at the FOXP3 and TIGIT loci (functional Treg markers) also lower in sorted cord blood CD4+ T cells of food allergic infants. Other studies have shown that low Treg (CD4+FOXP3+CD25+) proportions at birth predict egg allergy, food sensitization, and atopic dermatitis. We recently reported that maternal and cord vitamin D levels were associated with increased proportions of nTreg, and a number of external factors have been shown to upregulate overall number of Treg, including dog ownership and farm milk, but the influence of a full range of prenatal and postnatal environmental microbial factors on infant Treg development over the first postnatal year, and subsequent infant allergic outcomes, is yet to be evaluated.

Here, we aim to (i) describe how early life environmental factors are associated with infant thymic-derived Treg and (ii) investigate whether serial measures of these Tregs predict allergic outcomes at 1 year of age. The emphasis of this paper is on the former, given that we and others have published on the prospective associations between early life Treg proportions and subsequent allergic disease.

2 | MATERIALS AND METHODS

The Barwon Infant Study (BIS) is an Australian birth cohort study of 1074 infants recruited using an unselected antenatal sampling frame. Women were recruited between June 2010 and June 2013. Exclusion criteria included birth before 32 weeks of gestation, major congenital malformation and/or genetically determined disease, and/or serious illness. Prenatal factors were recorded by questionnaire for trimesters 1 and 2 at enrolment (28–32 weeks gestation) and trimester 3 postnatally at 4 weeks. These factors included some likely to be a proxy for environmental microbial exposure, such as pet ownership, household resident number by adult and child number, siblings by date of birth, and antibiotic and disinfectant use. Parent occupational social contact was assessed by the number of well or sick children, adults, or animals contacted daily through work. Perinatal factors were collected from hospital records. Postnatal factors additionally included infant hygiene, breastfeeding, and childcare attendance. Comprehensive questionnaire, clinical, and biological measures were collected postnatally at birth, 4 weeks, and 3, 6, 9, and 12 months with a relevant follow-up at 4 years.
2.1 | Blood sampling and monocyte isolation

Umbilical cord blood (up to 30 ml) was collected by syringe, and venous peripheral blood was collected at 6 and 12 months of age. Mononuclear cells were isolated by density gradient centrifugation (Lymphoprep, Axis-Shield).

2.2 | Measurement of treg subsets by flow cytometry

All mononuclear cell preparations were stained for flow cytometric analysis within 12 h of blood collection. Flow cytometry was used to characterize subpopulations of Treg as previously described.14,15 All samples were analyzed by 3-channel flow cytometry (FACSCalibur, Becton Dickinson). Characterization of the Treg subsets was performed in accordance with the method used by Miyara et al. with the CD4+CD45RA−FoxP3low subset defined as naïve Treg (nTreg) and CD4+CD45RA−FoxP3high defined as activated Treg (aTreg)12 (Figure 1). Treg proportions are presented as a percentage of CD4+T cells unless otherwise stated. We examined a serial Treg cohort (n = 191) and those with all available data (birth (n = 463), 6 months (n = 600), and 12 months (n = 675)). The former enabled comparison of serial Treg measures, and the latter allowed cross-sectional analyses.

2.3 | Infant allergic outcomes at age 1 year: polysensitization, food allergy, atopic eczema, and atopic wheeze

At 12-month postnatal age, skin prick testing (SPT) was performed using Quintip® (Hollister-Stier Laboratories) to the following allergens: cow’s milk, egg white, peanut, sesame, cashew, dust mite (Dermatophagoides pteronyssinus 1), cat, dog, ryegrass, and Alternaria tenuis (Stallergenes®). Sensitization to an allergen was defined as an SPT wheal size 2 mm or greater than the negative control (saline), in the presence of a positive histamine control as previously published.7 Polysensitization was defined as sensitization to two or more allergens at 12 months of age. Immunoglobulin E (IgE)-mediated food allergy was defined as a positive SPT at 1 year plus clinical history of a recent acute allergic reaction and/or formal in-hospital open food challenge using predetermined stopping criteria.7 At each postnatal review, parents were asked about wheeze and eczema symptoms since the last review. Atopic eczema at age one was defined as eczema plus sensitization.7 Atopic wheeze at age one was defined as the presence of parent-reported wheeze during the first year of life plus sensitization.7 Similar outcomes were obtained when the children were aged 4 years.23 The study was approved by Barwon Health Human Research and Ethics Committee (HREC 10/24), and written consent was provided by the parents or guardians for all participants.

2.4 | Statistical analysis

Household sibling age at birth of index child was calculated using birth dates of participants and siblings. Most children had one or no siblings (Table 1), so homes with 3 or more residents during pregnancy were termed “larger household size.” The main forms of analysis were multiple linear or logistic regression.24,25 Hierarchical multiple regression models26 were used to examine how early life exposures related to Treg proportions longitudinally. An indicator for postnatal stage of the Treg measure, as well as a product term between it and the putative exposure, was added to assess trajectory in the first year. We examined how birth nTreg proportions predicted subsequent allergic outcomes at 1 year (polysensitization, food allergy, atopic eczema, and atopic wheeze) using receiver

![Figure 1: Gating strategies for regulatory T cells (Treg) in freshly collected cord blood samples. Umbilical cord blood (up to 30 ml) was collected by syringe and immediately added to 10 ml of RPMI 1640 (Gibco, Life Technologies) containing preservative-free sodium heparin (Pfizer, final concentration of 10 IU/ml). Venous peripheral blood was collected at 6 and 12 months of age and added to a 15-ml tube containing 100 IU preservative-free sodium heparin (Pfizer, final concentration 10 IU/ml). Mononuclear cells (MNC) were isolated by density gradient centrifugation (Lymphoprep, Axis-Shield). The MNC were stained with fluorochrome-labelled monoclonal antibodies to CD4, CD45RA, and FoxP3. Events were gated to the lymphocytes (FSC and SSC plot) and then to the CD4+ T cells (FL-2 and SSC plot). FoxP3+ subsets were then selected on the basis of CD45RA and FoxP3 expression (FL-1 and FL-3 plot). CD45RA−FoxP3low populations were classified as naïve Treg (nTreg); the CD45RA−FoxP3high as activated Treg (aTreg); and CD45RA+FoxP3low as non-suppressive FoxP3+ T-cell; respectively.}
TABLE 1 Characteristics of the Treg cohort sample with longitudinal nTreg measurements, compared with the full cohort

| Characteristic                  | Total Cohort N = 1074 | All with birth Treg measures N = 463 | nTreg at all 3 time points N = 191 |
|--------------------------------|-----------------------|--------------------------------------|-----------------------------------|
| **Prenatal factors**           |                       |                                      |                                   |
| Parental allergy history (% n/N) | 83.9% (870/1037)      | 86.20% (387/449)                     | 91.0% (171/188)                   |
| Number of residents (% n/N)    |                       |                                      |                                   |
| <3                             | 39.3% (422/1074)      | 36.3% (168/463)                      | 29.8% (57/191)                    |
| 3–4                            | 55.2% (558/1074)      | 57.0% (264/463)                      | 65.5% (125/191)                   |
| >4                             | 8.75% (94/1074)       | 6.7% (31/463)                        | 4.71% (9/191)                     |
| Number of older siblings (% n/N) |                        |                                      |                                   |
| 0                              | 45.0% (483/1073)      | 40.0% (185/462)                      | 33.5% (64/191)                    |
| 1                              | 34.9% (374/1073)      | 39.8% (184/462)                      | 43.5% (83/191)                    |
| >1                             | 20.1% (216/1073)      | 20.1% (93/462)                       | 23.0% (44/191)                    |
| Pet dog during pregnancy (% n/N) | 55.4% (595/1074)     | 51.6% (239/463)                      | 47.12% (90/191)                   |
| Maternal history of smoking (% n/N) | 16.0% (167/1043)  | 16.6% (75/452)                       | 14.4% (27/188)                    |
| All 4 grandparents European (% n/N) | 94.7% (1010/1067) | 95.2% (439/461)                      | 96.7% (184/190)                   |
| SEIFA disadvantage*            |                       |                                      |                                   |
| Low (% n/N)                    | 33.3% (347/1041)      | 33.1% (150/453)                      | 27.7% (52/188)                    |
| Medium (% n/N)                 | 33.3% (347/1041)      | 29.8% (135/453)                      | 33.5% (63/188)                    |
| High (% n/N)                   | 33.3% (347/1041)      | 37.1% (168/453)                      | 38.8% (73/188)                    |
| **Perinatal factor**           |                       |                                      |                                   |
| Sex                            |                       |                                      |                                   |
| Male (% n/N)                   | 51.7% (519/1074)      | 50.5% (234/463)                      | 50.8% (97/191)                    |
| Female (% n/N)                 | 48.3% (555/1074)      | 49.5% (229/463)                      | 49.2% (94/191)                    |
| Caesarean section (% n/N)      | 31.0% (333/1074)      | 29.2% (135/463)                      | 30.9% (59/191)                    |
| Gestational age at birth (weeks) | 39.44 (1.52)         | 39.50 (1.34)                        | 39.58 (1.2)                       |
| Labor time in hours            | 6.07 (5.65)           | 5.83 (5.85)                         | 5.72 (6.62)                       |
| Birthweight (kg)               | 3.54 (0.51)           | 3.58 (0.48)                         | 3.55 (0.44)                       |
| Birthweight Z score (unit)     | 0.51 (0.9)            | 0.57 (0.89)                         | 0.50 (0.83)                       |
| **Postnatal factors**          |                       |                                      |                                   |
| Pet dog during infancy (% n/N) | 54.4% (469/863)       | 49.2% (189/384)                      | 48.7% (91/187)                    |
| Breastfeeding                  |                       |                                      |                                   |
| Any breastmilk feeding at 6 months | 60.7% (603/993)       | 60.6% (263/434)                      | 62.3% (119/191)                   |
| Any breastmilk feeding at 12 months | 37.8% (364/964)     | 34.7% (145/418)                      | 32.5% (62/191)                    |
| Any breastfeeding              |                       |                                      |                                   |
| None                           | 2.3% (22/964)         | 2.4% (10/418)                        | 2.1% (4/191)                      |
| <1 week                        | 2.9% (28/964)         | 3.1% (13/418)                        | 3.1% (6/191)                      |
| 1 week to <6 months            | 35.3% (340/964)       | 35.4% (148/418)                      | 32.5% (62/191)                    |
| 6 to <12 months                | 21.8% (210/964)       | 24.4% (102/418)                      | 29.8% (57/191)                    |
| 12 months                      | 37.8% (364/964)       | 34.7% (145/418)                      | 32.5% (62/191)                    |
| Exclusive breastfeeding up to 12 months |               |                                      |                                   |
| None                           | 70.6% (758/1074)      | 68.9% (319/463)                      | 69.6% (133/191)                   |
| 1–4                            | 20.6% (221/1074)      | 22.3% (103/463)                      | 20.4% (39/191)                    |
| 5–8                            | 4.9% (52/1074)        | 4.9% (23/463)                        | 4.2% (8/191)                      |
| 9–12                           | 4.0% (43/1074)        | 3.9% (18/463)                        | 5.8% (11/191)                     |
| Exclusive up to 12 months (completed months) (%n/N) | 18.8% (181/961)     | 16.6% (69/417)                       | 18.9% (35/185)                    |
operating curve (ROC) analysis with Youden Index indicating optimal outcomes. Mediation analysis was undertaken to determine whether cord blood nTreg proportions were a likely intermediate factor in a causal pathway between the selected exposures and allergic outcomes. We calculated E values for key findings to evaluate the potential impact of unmeasured confounding. We conducted propensity weighting analyses to account for differences between those analyzed and non-responders. Analyses were conducted in Stata 16.0 (StataCorp) and R version 3.6.1 (https://www.r-project.org).

3 | RESULTS

3.1 | Characteristics

Household size was generally small with only 10% of families having more than four residents (Table 1). We describe these 191 infants with Treg measures at birth, 6, and 12 months as the serial Treg subcohort. Overall, there was a high correlation between household resident and sibling number (r = 0.76, p < .0001) and prenatal and postnatal dog ownership (r = 0.86, p < .0001).

3.2 | Early life factors and T regulatory cell profile

We examined early life factors associated with Treg proportions at birth, 6, and 12 months for all available Treg measures at each single time point (Figure 2A–C) and also for the serial Treg subcohort (Figure 3 and S1).

3.2.1 | Naïve T regulatory cells (nTreg)

Overall, among infants with Treg measures at birth (n = 463), of the 19 prenatal environmental microbial factors examined, the strongest patterns were evident for larger family size (three or more residents) and siblings (one or more) being associated
(p < .0001) with higher cord blood nTreg proportions (Figure S1). A range of environmental microbial factors were associated with higher nTreg levels at the 6-month (Figure 2B) and 12-month (Figure 2C) time points. Compared with no breastfeeding, exclusive or mixed breastfeeding was associated with higher naïve Treg at 6 months (Table 2).

3.2.2 Activated T regulatory cells (aTreg)

There was little association between environmental microbial factors and aTreg proportions at birth (n = 463) (Figure 2A and Table S1). Larger household size was associated with higher aTreg levels at age 6 months (Figure 2B). Dog ownership, as well as higher maternal and...
maternal occupational social contact, was associated with higher aTreg at 12 months with evidence of dose–response (Figure 2C).

### 3.3 | Assessment of nTreg measures over multiple timepoints

We examined the longitudinal patterns of both nTreg and aTreg by trajectory analysis (Figure 3 and Figure S1) and demonstrate some differences by whether an early life factor was present or not.

We then examined how the key prenatal factors influenced overall longitudinal Treg proportions across all three timepoints in the first postnatal year. Infants from larger households had higher nTreg ($p < .001$) and aTreg ($p = .007$) proportions across the first postnatal year. Infants with a pet dog also had higher nTreg proportions throughout the first year ($p = .01$; Table 3). Infants who were breastfed had higher longitudinal nTreg and aTreg proportions (Table 2; Table S2). Infants who attended formal childcare in the first postnatal year had higher longitudinal nTreg proportions ($p = .02$; Table 2) but not aTreg proportions ($p = .41$; Table S2).

### 3.4 | T regulatory cell profile and atopic disease at 1 year of age

Having previously reported higher birth nTreg proportions were associated with reduced food allergy at 1 year of age,$^{14}$ we conducted an ROC analysis to evaluate the predictive performance of higher birth nTreg proportions and resultant average Youden Index was a cutoff of 3% (Figure S2). Overall, infants with 3% or more nTreg at birth were markedly less likely to have positive allergic outcomes (Table 4).

### 3.5 | Additional analyses

With multiple environmental microbial exposures per time period and four allergic outcomes, it was not feasible to undertake detailed confounder detection and evaluation analyses.$^{25}$ Therefore, we applied the E-value approach.$^{30,31}$ For unmeasured confounding to have explained the association between 3% or more nTreg and reduced food allergy (Table 5), the unmeasured confounder would
need to have a ninefold positive risk ratio for this nTreg profile and be associated with a ninefold lower risk of food allergy. To account for the association between larger household size and a twofold increase in the likelihood of this nTreg profile, a potential confounder would need to positively increase the likelihood of the nTreg profile 2 or more-fold and also be associated with a 2- or more-fold increase of a larger household. Table 5 shows that none of these potential confounders listed have these profiles, and thus, it is unlikely that these findings are due to unmeasured confounding.

Inverse probability weighting to rebalance the samples to better reflect women invited to enter the cohort at baseline did not alter the key findings above. Further, exclusion of cord blood samples with potential maternal contamination did not materially alter key findings. Lastly, we investigated how infant allergic outcomes predicted child allergic disease. Polysensitization at age 1 year was strongly associated with any allergic disease (food allergy, atopic eczema, and/or atopic asthma) at ages 4 years with an AOR of 9.26 (95% CI 2.46, 34.85), respectively. We have previously reported that food allergy at 1 year predicted subsequent hay fever and atopic wheeze.23

4 | DISCUSSION

Here, multiple environmental microbial factors were associated with Treg development at birth and during the first year of life. For the first time, to our knowledge, larger household size was associated with higher nTreg proportions at birth and in longitudinal analyses, it was associated with higher proportions of nTreg and aTreg over the first postnatal year. Higher nTreg proportions at birth but not 6 or 12 months predicted allergic outcomes by 12 months of age. Taken together, these findings indicate that inadequate prenatal immune priming is important in allergic disease development.

The major strength of this study is the availability of comprehensive environmental microbial and serial Treg measures over the first year of life, as well as objective infant allergic outcomes for a large population-based cohort. While the longitudinal Treg measurements at all timepoints were limited to 191 infants, this sample is larger than most previous studies, particularly a recent study with serial Treg measures.32 The availability of comprehensive data allowed an assessment of potential confounding and selection bias33 that did not reveal these to be important explanations. In particular, the key associations were of high magnitude, reducing the risk of a contribution due to unmeasured confounding.31 Limitations include the identification of additional subpopulations of Treg or alternate staining methods and the lack of functional suppressive assays. These aspects will be part of future studies; however, the nTreg and aTreg populations measured here have both previously been shown to be suppressive12 which provides some functional relevance to the link between lower suppressive nTreg and allergic outcomes described here. In our past work on lower cord blood Treg proportions and subsequent food allergy, we also found in parallel that gene demethylation at the FOXP3 and TIGIT loci (markers of suppressive Treg function) was lower in the sorted CD4+ cells of food allergic infants.16 Additionally, we confirmed good correlations between our birth nTreg proportions and FoxP3 demethylation site (TSDR) \( r = 0.50, p = .004, n = 31 \) and TIGIT demethylation \( r = 0.43, p = .017, n = 31 \), indicating that the FoxP3 gene in the nTreg population that we measured is functionally stable.

The nTreg index of 3% or more requires replication in independent studies. We did not examine long-term allergic outcomes, but infant polysensitization at 12 months is important in its own right and a key determinant of child allergic disease here and in other studies.9,10

Our findings are consistent with past work, indicating that a lower Treg profile in the prenatal period24 and at birth is associated with higher allergic disease risk.18 Children born in a farming environment have higher Treg at birth,35 Bacterial strain inoculation into pregnant rodents leads to FoxP3 Treg induction, but this must occur before the neonatal period.35 Prenatal factors such as dog ownership and farm milk exposure have previously been related to upregulated nTreg proportions in cord blood.18

The finding of a relative nTreg deficit at birth being associated with higher allergy risk is consistent with the concept of delayed maturation of T cell function in early life underlying the allergic phenotype.34,37 Genetic risk of atopy is associated with delayed maturation of T cell competence.36 Consistent with past work,18,38,39 a positive parental allergy history was associated with lower cord blood nTreg and we describe, for the first time, a steeper trajectory of Treg maturation over the first six months. Compared with no

### TABLE 3 Association between microbial-related factors and longitudinal naïve and activated T regulatory cells levels in the first year of life in the Treg cohort (n = 191)

| Prenatal Factors                          | Naïve T regulatory cells* | Activated T regulatory cells* |
|------------------------------------------|----------------------------|-------------------------------|
|                                          | Mean difference (95% CI)   | p value                       |
| Parental allergy history                 | -0.22 (-0.85, 0.41)       | .49                           |
| Residents (3 or more)                    | 0.67 (0.30, 1.04)         | <.001                         |
| Pet dog                                  | 0.46 (0.10, 0.82)         | .01                           |
| Occupational social contact (mother)     | 0.16 (-0.21, 0.52)        | .40                           |
| Occupational social contact (father)     | 0.13 (-0.24, 0.50)        | .49                           |
|                                          | 0.03 (-0.11, 0.16)        | .67                           |

Note: * T regulatory cells as a % of CD4+ cells. Bold value indicate p<0.05 threshold.
| Naive Treg cells (as a % of CD4+ cells) | Polysensitization | Food allergy | Atopic eczema | Atopic wheeze |
|--------------------------------------|-------------------|-------------|--------------|--------------|
|                                      | Number            | AOR (95% CI) | p value      | AOR (95% CI) | p value      | AOR (95% CI) | p value      |
| All available measures               |                   |             |              |              |              |              |              |
| Birth*++                             | 463/1074          | 0.85 (0.62, 1.17) | .32          | 0.65 (0.45, 0.93) | 0.02          | 0.68 (0.48, 0.96) | .03          | 0.80 (0.57, 1.12) | .19          |
| 6 months¹                           | 600/1074          | 0.95 (0.72, 1.26) | .74          | 0.88 (0.69, 1.11) | 0.28          | 0.76 (0.58, 0.99) | .04          | 0.95 (0.72, 1.24) | .70          |
| 12 months²                          | 675/1074          | 1.01 (0.81, 1.25) | .95          | 0.89 (0.73, 1.09) | 0.27          | 0.89 (0.71, 1.10) | .27          | 0.97 (0.78, 1.20) | .77          |
| 3% or more at birth*++              | 463/1074          | 0.42 (0.16, 1.07) | .07          | 0.21 (0.09, 0.50) | <.001         | 0.26 (0.10, 0.64) | .004         | 0.46 (0.16, 1.34) | .15          |
| Treg cohort                         |                   |             |              |              |              |              |              |
| Birth*++                            | 191               | 0.68 (0.40, 1.13) | .14          | 0.55 (0.32, 0.95) | 0.03          | 0.47 (0.24, 0.89) | .02          | 0.49 (0.26, 0.92) | .03          |
| 6 months¹                           | 191               | 1.23 (0.86, 1.76) | .25          | 0.93 (0.63, 1.37) | 0.73          | 0.81 (0.50, 1.30) | .38          | 0.96 (0.63, 1.46) | .85          |
| 12 months²                          | 191               | 1.19 (0.91, 1.56) | .20          | 0.92 (0.67, 1.26) | 0.60          | 0.96 (0.68, 1.35) | .81          | 0.90 (0.62, 1.29) | .56          |
| 3% or more at birth*++              | 191               | 0.22 (0.07, 0.71) | .01          | 0.17 (0.06, 0.50) | 0.001         | 0.23 (0.07, 0.82) | .02          | 0.25 (0.07, 0.88) | .03          |

Note: Model * was assessed to fit the data better than model ++: model * had an pseudo r-squared = 0.06 and a log likelihood test p = .01 and model ++ had a pseudo r-squared = 0.03 and a log likelihood test p = .11. Bold value indicate p<0.05 threshold.

¹Adjusted for gestational age and sex, in all with birth measure n = 463.
²Adjusted for age at 6 month blood draw and sex, in all with 6 month measure n = 600.
³Adjusted for age at 12 month skin prick test and sex, in all with 12 month measure n = 675.
TABLE 5  Association between early life factors, a birth naïve T regulatory cell profile of 3% or more (as a proportion of CD4⁺ cells), household size, infant polysensitization, and food allergy at 12 months in all participants with nTreg at birth measured (n = 463)

| Characteristic                        | Naïve Treg 3% or more at birth OR* (95% CI) | Residents (3 or more) OR* (95% CI) | Polysensitization OR* (95% CI) | Food allergy OR* (95% CI) | Atopic Eczema OR* (95% CI) | Atopic wheeze OR* (95% CI) |
|---------------------------------------|---------------------------------------------|-------------------------------------|---------------------------------|--------------------------|---------------------------|---------------------------|
| Prenatal factors                      |                                             |                                     |                                 |                          |                           |                           |
| Parental allergy history (yes vs. no) | 0.97 (0.39, 2.44)                           | 0.88 (0.46, 1.70)                   | 1.19 (0.34, 4.14)               | 1.88 (0.43, 8.25)        | 1.11 (0.32, 3.88)         | 1.68 (0.38, 7.46)         |
| Number of residents (≥3 vs. <3)       | **                                          | **                                  | 0.55 (0.25, 1.21)               | 0.52 (0.23, 1.16)        | 0.79 (0.34, 1.81)         | 1.07 (0.44, 2.58)         |
| Number of Siblings (≥1 vs. none)      | 1.85 (1.02, 3.38)                           | **                                  | 0.74 (0.34, 1.60)               | 0.70 (0.32, 1.51)        | 1.04 (0.46, 2.36)         | 1.41 (0.59, 3.36)         |
| Pet dog (yes vs. no)                  | 1.55 (0.84, 2.84)                           | 1.25 (0.81, 1.93)                   | 0.35 (0.15, 0.81)               | 0.18 (0.06, 0.48)        | 0.44 (0.19, 1.01)         | 0.42 (0.17, 0.99)         |
| Maternal social contact               | 1.01 (0.72, 1.41)                           | 0.66 (0.51, 0.86)                   | 0.77 (0.50, 1.17)               | 0.84 (0.55, 1.30)        | 0.99 (0.64, 1.56)         | 0.70 (0.44, 1.09)         |
| Paternal social contact               | 1.10 (0.74, 1.63)                           | 1.14 (0.86, 1.53)                   | 0.67 (0.40, 1.12)               | 0.70 (0.42, 1.17)        | 0.85 (0.50, 1.45)         | 0.66 (0.38, 1.15)         |
| Number of adults (more than 2 vs. rest) | 1.10 (0.71, 1.70)                           | -                                   | 0.89 (0.47, 1.69)               | Omitted in Stata         | 0.90 (0.47, 1.71)         | 0.95 (0.50, 1.81)         |
| SEIFA disadvantage (per % increase)   | 0.92 (0.64, 1.32)                           | **                                  | 1.41 (1.08, 1.82)               | 1.30 (0.80, 2.13)        | 1.11 (0.68, 1.81)         | 0.82 (0.50, 1.34)         |
| Maternal history of smoking (yes vs. no) | 1.40 (0.53, 3.75)                           | **                                  | 0.37 (0.20, 0.68)               | 1.13 (0.37, 3.45)        | 0.82 (0.24, 2.85)         | 1.66 (0.59, 4.66)         |
| Perinatal factors                     |                                             |                                     |                                 |                          |                           |                           |
| Gestational age (weeks)               | 0.58 (0.44, 0.77)                           | 1.01 (0.86, 1.18)                   | 1.02 (0.77, 1.35)               | 1.25 (0.92, 1.71)        | 1.13 (0.83, 1.53)         | 0.96 (0.72, 1.28)         |
| Sex (Male vs. Female)                 | 1.33 (0.73, 2.42)                           | 1.17 (0.76, 1.79)                   | 1.82 (0.82, 4.02)               | 1.05 (0.48, 2.27)        | 1.36 (0.61, 3.02)         | 2.70 (1.11, 6.60)         |
| Caesarean section (Any vs. none)      | 1.57 (0.77, 3.20)                           | 1.08 (0.67, 1.75)                   | 0.67 (0.28, 1.63)               | 1.10 (0.48, 2.53)        | 0.85 (0.40, 2.26)         | 0.76 (0.31, 1.88)         |
| Labor time (hour)                     | 0.92 (0.88, 0.96)                           | 0.78 (0.73, 0.83)                   | 1.00 (0.94, 1.07)               | 1.03 (0.98, 1.09)        | 1.02 (0.96, 1.08)         | 0.98 (0.92, 1.06)         |
| Z scores birth                        | 1.12 (0.80, 1.57)                           | 1.64 (1.27, 2.13)                   | 0.77 (0.49, 1.22)               | 0.77 (0.49, 1.21)        | 0.80 (0.51, 1.27)         | 0.76 (0.47, 1.23)         |
| Birth nTreg 3% or more                | -                                           | 2.19 (0.20, 3.99)                   | 0.43 (0.17, 1.07)               | 0.19 (0.08, 0.44)        | 0.30 (0.12, 0.74)         | 0.58 (0.20, 1.63)         |
| Postnatal factors                     |                                             |                                     |                                 |                          |                           |                           |
| Breast feeding duration               | 1.07 (0.78, 1.48)                           | 1.26 (0.99, 1.59)                   | 1.27 (0.83, 1.94)               | 1.09 (0.72, 1.65)        | 0.76 (0.50, 1.16)         | 1.11 (0.72, 1.72)         |
breastfeeding, exclusive or any breastfeeding was associated with higher nTreg proportions at 6 months as well as higher longitudinal nTreg and aTreg proportions throughout the first year. A recent study (n = 38) reported that compared with non-breastfed infants, exclusively breastfed infants had increased naïve Treg proportions 3 weeks after birth and a Treg-dependent reduction in proliferative T cell antigenic responses.40 We did not have Treg measures available during the first six months, when breastfeeding is most common, and lacked in vitro suppressive data, but our findings indicate more intensive studies are required for this issue. Childcare attendance was associated with higher longitudinal nTreg proportions in the first year.

Regarding aTreg postnatal development, parental occupational social contact was associated with higher aTreg at 12 months of age. This is a previously identified possible determinant of reduced type 1 diabetes21 and higher child leukemia risk,41 possibly through increased early infection,21,41 reflecting that activated Treg cells develop in response to postnatal antigenic stimuli.42 Several features indicating possible causality were present.25 For example, high-magnitude prospective associations were reported for larger family size and birth nTreg proportions and also the birth nTreg profile and reduced infant allergic outcomes with dose–response trends. These findings were consistent with past laboratory work.43,44 The mechanism underlying the association between larger family size and increased nTreg in cord blood requires further elucidation. Relevant factors to a larger household include the presence of additional children and increased exposure to shared microbes,45 a greater number of viral/bacterial infections,46 reduced cleanliness, and the presence of pet/s.47 Several protective immune factors have previously been related to birth order and protection from atopy. These include reduced maternal and cord IgE with subsequent pregnancies,48 as well as dampened cord serum Th1 responses.49 Additional research should, therefore, include evaluating the immune priming role of previous pregnancies in the mother50 and the impacts of labor duration.14 To elucidate the influence of factors on the development of Treg, further work on prenatal programming of Treg cells should include epigenetic studies. Aspects of the gut microbiota have also been shown to influence Treg development.31,52 We have reported in this birth cohort that larger household size is associated with a higher abundance of maternal prenatal Prevotella copri, which is, in turn, associated with a markedly reduced risk of allergic disease at 12 months.7 The influence of the maternal, as well as infant, gut microbiome on the infant immune profile requires further evaluation.53

### 5 | CONCLUSION

A range of prenatal and postnatal environmental microbial factors may work in concert to influence early Treg cell development. In this study, the most consistent of these was larger household size, which was associated with an increased proportion of nTreg in cord

| Characteristic | Naïve Treg 3% or more at birth OR* (95% CI) | Avoid egg at 6m (yes vs. no) | Avoid peanut at 12m (yes vs. no) | Polysensitization OR* (95% CI) | Polyvalent OR* (95% CI) |
|---------------|------------------------------------------|-----------------------------|---------------------------------|------------------------------|------------------------|
| Residents (3 or more) | 1.22 (0.46, 2.73) | 0.97 (0.31, 3.03) | 2.48 (1.15, 5.38) | 0.97 (0.40, 2.35) |
| Polysensitization | 1.21 (0.64, 2.28) | 2.32 (1.45, 7.12) | 1.43 (0.62, 3.29) | 0.86 (0.33, 2.20) |
| Polyvalent | 1.62 (1.01, 2.60) | 1.87 (0.85, 4.13) | 1.43 (0.62, 3.29) | 0.92 (0.36, 2.38) |
| Polyvalent | 1.23 (0.60, 2.51) | 1.41 (0.62, 3.19) | 0.92 (0.36, 2.38) | 0.92 (0.36, 2.38) |
blood at birth, as well as with higher proportions of nTreg and αTreg throughout the first postnatal year. Higher cord blood nTreg proportions were associated with a reduced risk of allergic polysensitization, food allergy, atopic eczema, and atopic wheeze by one year of age. In utero programming of nTreg development may partly underlie the well-replicated association between larger household size and reduced allergic disease. Our findings are consistent with recent calls for studies and trials targeting fetal immune development for the prevention of allergic disease.54

AUTHOR CONTRIBUTIONS
Anne-Louise Ponsonby: Conceptualization (lead); Data curation (lead); Formal analysis (lead); Funding acquisition (lead); Investigation (lead); Methodology (lead); Project administration (lead); Resources (lead); Supervision (lead); Visualization (equal); Writing – original draft (lead); Writing – review & editing (lead). Fiona Collier: Conceptualization (lead); Data curation (equal); Formal analysis (equal); Funding acquisition (equal); Investigation (lead); Methodology (lead); Project administration (equal); Resources (equal); Supervision (equal); Visualization (equal); Writing – original draft (equal); Writing – review & editing (equal). Martin O’Hely: Conceptualization (equal); Data curation (equal); Formal analysis (lead); Investigation (equal); Methodology (lead); Project administration (equal); Resources (equal); Supervision (equal); Visualization (lead); Writing – original draft (equal); Writing – review & editing (equal). Lawrence EK Gray: Conceptualization (lead); Data curation (equal); Funding acquisition (equal); Investigation (equal); Methodology (equal); Writing – original draft (equal); Writing – review & editing (equal). Sarah Ranganathan: Conceptualization (equal); Data curation (equal); Funding acquisition (equal); Investigation (equal); Supervision (equal); Writing – original draft (equal); Writing – review & editing (equal). David Burgener: Data curation (equal); Funding acquisition (equal); Investigation (equal); Methodology (equal); Project administration (equal); Supervision (equal); Writing – original draft (equal); Writing – review & editing (equal). Richard Saffery: Conceptualization (equal); Data curation (equal); Funding acquisition (equal); Investigation (equal); Methodology (equal); Project administration (equal); Supervision (equal); Writing – original draft (equal); Writing – review & editing (equal). Leonard Harrison: Conceptualization (equal); Investigation (equal); Methodology (equal); Supervision (equal); Writing – original draft (equal); Writing – review & editing (equal). Peter Vuillermin: Conceptualization (lead); Data curation (equal); Formal analysis (equal); Funding acquisition (equal); Investigation (lead); Methodology (lead); Project administration (equal); Resources (equal); Supervision (equal); Writing – original draft (equal); Writing – review & editing (equal).

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**SUPPORTING INFORMATION**

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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