ALLERGY

Farm exposure in infancy is associated with elevated systemic IgG_4, mucosal IgA responses, and lower incidence of food allergy

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The role of antibodies in protection against food allergy is debated. Here, we designed a longitudinal birth cohort study recruiting participants from an Old Order Mennonite (OOM) community, a traditional farm lifestyle considered to be at low risk for allergic diseases, and participants from urban and suburban Rochester, NY, USA, that were at higher risk for developing allergic diseases. We collected cord and peripheral blood, stool, saliva, and maternal milk samples longitudinally during pregnancy and during the first year of the infant's life to evaluate B cell and antibody responses. Farm exposure led to an accrual of memory and immunoglobulin G–positive (IgG^+) B cells, higher titers of plasma IgG and IgA and salivary and fecal IgA in infants, and higher IgA titers in maternal milk. Moreover, OOM infants and their mothers had higher titers of plasma IgG_4 and IgA antibodies to egg ovalbumin in maternal milk, respectively, a feature that we found to be associated with lower incidence of egg allergy. Infant systemic IgG_4 and IgA were associated with early introduction or more frequent ingestion of lightly cooked egg, respectively, but there was also an effect independent of egg exposure. Several food antigens were detected in cord blood, with in utero exposure potentially explaining the unexpected presence of antigen-specific IgA at birth. These analyses suggest earlier maturation of B cell immunity in farming lifestyle communities.

INTRODUCTION

The prevalence of allergic diseases and asthma has been a growing global issue, placing a substantial burden on individuals with allergies and their families (*1*). The rapid rise cannot be explained by genetics alone; however, changes in the surrounding environment and lifestyle over time could be major contributors (*2*, *3*). Studies in Europe (*4*, *5*) and the United States (*6*, *7*) have suggested that the farm lifestyle is associated with a decreased prevalence of asthma and allergies. Consumption of unpasteurized cow's milk and contact with farm stables and animals have been suggested as contributors to this protection (*5*, *8*). Differential innate immune markers, such as differences in cytokine production [increased interferon-γ (IFN-γ) and tumor necrosis factor–α (TNF-α)] and expression of Toll-like receptor 2 (TLR2) and TLR4 (*8*, *9*) and an increased frequency and function of regulatory T cells (*10*, *11*), have been observed in infants living on farms. This combination of responses may favor immunoregulation and protection against allergic sensitization. Whereas innate and T cell immunity have been a focus (*7*, *12*), less attention has been given to B cells in farming lifestyle studies.

In the Swedish FARMFLORA birth cohort, enhanced B cell maturation at birth was associated with decreased allergic outcomes later in infancy (*13*). Moreover, infants who lived on a farm had elevated concentrations of B cell activating factor (BAFF) at birth, which was found to be associated with increased memory $CD27^+$ B cells at 4 months (*14*). Although immunoglobulin E (IgE) is the main isotype associated with allergy, systemic IgG_4 is thought to be antagonistic to allergic responses by blocking IgE-mediated basophil activation (*15*–*17*). Only one study so far has reported patterns of antibody expression in farm lifestyle children, with coexpression of IgG_1, IgG_4, and IgE responses (as opposed to IgG_1 and IgE alone) being associated with a higher risk of allergic disease (*18*), but only aeroallergen antibody responses and no IgA were reported in these older children. Furthermore, elevated mucosal IgA concentrations in saliva, maternal milk, or feces have been associated with a lower risk of allergic outcomes (*19*–*22*). IgA at mucosal surfaces can trap and limit the translocation of antigens and microbes across the mucosal lumen, protecting against detrimental responses. Mechanistically, IgA has also been implicated in the development of oral tolerance and protection against food allergy and anaphylaxis in animal studies and human immunotherapy trials (*23*–*26*). However, the role of IgA in natural tolerance to food allergens was recently called into question in a study of infants at high risk for food allergy (*27*). Further, little is known about antibody profiles in farming individuals who are naturally protected against allergic diseases, including food allergy.

In 2017, we launched a longitudinal birth cohort study to assess the B cell phenotypes and antibody responses before and after development of atopic disease in two populations: an agrarian farming Old Order Mennonite (OOM) community (*28*, *29*) and urban/suburban infants at higher risk for allergy. OOM infants in the cohort were found to have lower rates of food allergy and atopic dermatitis (AD) in the first year of life (*30*). We hypothesized that OOM infants would have more robust IgA production in early life compared with urban infants. We analyzed B cell and antibody responses to common food and environmental allergens with the goal of understanding how

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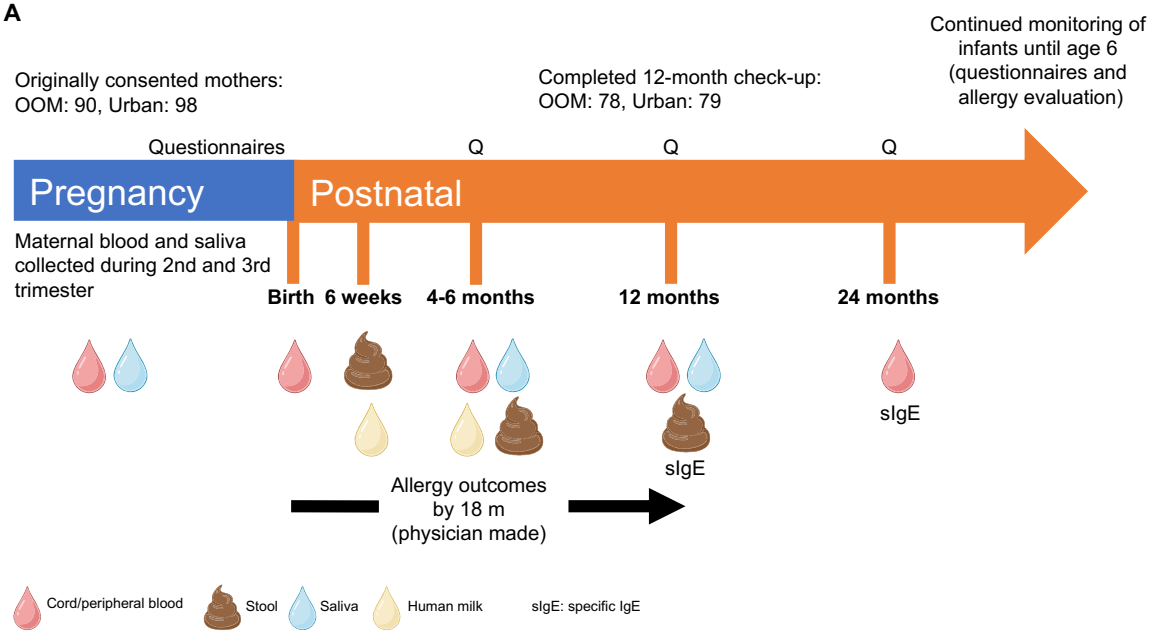
humoral immunity may promote or protect against food allergy. Overall, infants born to farm lifestyle populations demonstrate boosting of both systemic and mucosal antibodies in utero and postnatally, which is associated with natural tolerance to foods.

RESULTS

Allergic outcomes in the infant cohort

We recruited infants from the OOM ($n = 78$) community located in Penn Yan, NY, USA, which is 65 miles southeast of Rochester, NY, USA, who are at low risk for allergic diseases (*28*, *29*), whereas high-risk infants (with a first-degree blood relative with allergy) came from the urban and suburban Rochester community (urban, $n = 79$; Fig. 1). The cohort details and clinical outcomes by 12 months of age have been published (*30*). By 24 months of age, 28 urban infants (35%) were diagnosed with AD, and 15 urban infants (19%) were diagnosed with food allergy; 7 urban infants were sensitized to egg (hen's egg white IgE > 0.35 kU/liter at 24 months of age), and 10 of them had IgE-mediated egg allergy. In the OOM cohort, two infants (3%) were diagnosed with AD, and five were egg-sensitized, but no infant was clinically allergic to egg. Two-year allergic outcomes are reported in table S1.

A



B

Assay	Prenatally	Birth	6 weeks	4-6 months	12 months
Spectral flow cytometry		OOM: 20 Urban: 18		OOM: 20; Urban: 18	OOM: 35 Urban: 34
ELISA (total IgG, IgA)	OOM: 58, 57* Urban: 68, 65*	OOM: 53 Urban: 39		OOM: 46, 74*; Urban: 38, 65*	OOM: 43, 74*; Urban: 31, 59*
ELISA (food antigens)		OOM: 54-55 Urban: 42	OOM: 66-67#^; Urban: 65-66#^		
ELISA (IgA_1, IgA_2)			OOM: 67+, 63#^; Urban: 51+, 63-64#^	OOM: 75+, 67#^; Urban: 63+, 58#^	OOM: 64+; Urban: 65+
Luminex (specific IgG, IgG_4, IgA)	OOM: 58 Urban: 68	OOM: 53-55 Urban: 41-42	OOM: 66-67+, 68#^ Urban: 50-51+, 65#^	OOM: 46-48, 75+, 69#^ Urban: 38-39, 64+, 58#^	OOM: 49-53, 63-64+ Urban: 37-39, 67-68+
B. infantis qPCR			OOM: 68+; Urban: 63+	OOM: 30+; Urban: 36+	

*saliva samples, +stool samples, #human milk, ^maternal samples

Fig. 1. Study timeline. (**A**) Overview of study. Pregnant OOM women carrying low-risk fetuses and Rochester, NY, USA, urban/suburban women with high-risk fetuses were enrolled prenatally. Both maternal and infant sample collection time points are shown. Questionnaires were filled out at the time points displayed. Antigen (hen's egg white, cow's milk, peanut)–specific IgE was measured in infants at 12 and 24 months. Allergy (AD, food allergy) evaluations were done by a physician throughout the study. (**B**) Summary of assays and number of samples used. This figure was created with images from BioRender.com.

The proportions of memory and IgG$^+$ B cells were more pronounced in the farming lifestyle infants

Cord and peripheral blood samples collected from the OOM and urban infants were used to explore B cell populations in cord blood and at 6 and 12 months (Fig. 2 and figs. S1 and S2, A to E). In general, naïve B cells predominated at all time points, and switched and unswitched memory B cells appeared in later infancy (Fig. 2A). We identified no difference in the proportions of total B cells, but we observed higher proportions of total memory B cells ($P = 0.002$), unswitched ($P = 0.004$) and switched ($P = 0.007$) memory B cells, and double-negative ($P = 0.001$) B cells and a lower proportion of naïve B cells ($P < 0.0001$) in OOM samples at 12 months compared with urban samples (Fig. 2, B and C). The absolute numbers of total B cells, as well as several of these subpopulations (fig. S2, F and G), and proportions of transitional and immature B cells and plasmablast populations were comparable between the groups (Fig. 2D). Moreover, there were few differences in B cell subpopulations between urban infants who did and did not develop allergy (AD or food allergy) (fig. S3).

Whereas most circulating B cells expressed IgM, IgG- and IgA-expressing B cells were present over the first year of life (Fig. 2E). At 12 months, OOM infants had a significantly lower proportion of IgM$^+$ B cells ($P = 0.02$) but a higher proportion of IgG$^+$ B cells ($P = 0.004$), and IgA$^+$ B cells were similar (Fig. 2E). IgM$^+$ B cells expressed less Ki67, a marker of proliferative capacity, whereas both IgG$^+$ and IgA$^+$ Ki67 expression increased with age [IgG$^+$Ki67$^+$: cord blood versus 6-month blood ($P < 0.0001$) and cord blood versus 12-month blood ($P < 0.0001$); IgA$^+$Ki67$^+$: cord blood versus 6-month blood ($P < 0.0001$) and cord blood versus 12-month blood ($P < 0.0001$); fig. S4A]. IgM$^+$ B cells expressed more of integrin pair α4β7, an intestinal homing marker, whereas both IgG$^+$ and IgA$^+$ B cells also gradually increased in their expression of α4β7 [IgG$^+$α4β7$^+$: cord blood versus 6-month blood ($P = 0.004$) and cord blood versus 12-month blood ($P = 0.0001$); IgA$^+$α4β7$^+$: cord blood versus 6-month blood ($P < 0.0001$) and cord blood versus 12-month blood ($P < 0.0001$)]; mucosal chemokine receptor (CCR9 and CCR10) expression was low over time (fig. S4, B to D). No differences in these markers were identified between the OOM and urban infants (fig. S5). Together, these data suggest that substantial changes in several B cell populations occur during infancy and that farm exposure leads to a more mature B cell profile.

A farming lifestyle was associated with higher systemic total IgG and IgA in infancy

Because of the differences in IgG$^+$ and IgA$^+$ B cells, we next assessed circulating total immunoglobulin concentrations throughout infancy. Maternal and cord blood IgG concentrations were comparable between urban and OOM infants. Total IgG was present in cord blood because of transplacental transfer (Fig. 3A). By 12 months of age, OOM infants had a significantly ($P = 0.002$) higher concentration of systemic IgG compared with urban infants (Fig. 3A).

Whereas cord blood IgG antibodies are maternal and transplacentally transferred, IgA antibodies reflect intrauterine production by the fetus. Total IgA was low but detected in cord blood (Fig. 3B). Similar to IgG, IgA concentrations in maternal blood and cord blood were comparable between the groups (Fig. 3B); however, OOM infants had significantly elevated systemic IgA compared with urban infants at 6 ($P = 0.002$) and 12 months ($P = 0.004$). In urban infants who did or did not develop allergy, plasma IgG and IgA concentrations were comparable (fig. S6).

Farming and urban infants differed in their systemic antigen-specific antibody concentrations in the first year of life

With the finding that IgM$^+$ B cells have a high capacity to home to the gut, we next evaluated infant plasma IgA, IgG, and IgG$_4$ concentrations to several foods but also animal epithelia and common aeroantigens (fig. S7). IgA to food and environmental antigens significantly increased [ovalbumin $P = 0.005$, β-lactoglobulin $P < 0.0001$, casein $P < 0.0001$, Ara h 2 $P < 0.0001$, crude peanut extract (CPE) $P < 0.0001$, Der f 1 $P < 0.0001$, Der p 1 $P < 0.0001$, Fel d 1 $P < 0.0001$, Phl p 5a $P < 0.0001$, Equ c 1 $P < 0.0001$] after birth (fig. S7A, C to K). IgG to food antigens decreased significantly (ovalbumin $P < 0.0001$, ovomucoid $P < 0.0001$, β-lactoglobulin $P = 0.008$, casein $P = 0.0002$, Ara h 2 $P = 0.002$) after birth, consistent with disappearance of maternal antibodies, reaching a nadir by 6 months (fig. S7, A to E). There was a similar nadir in IgG$_4$ antibody at 6 months for food antigens (ovalbumin $P < 0.0001$, ovomucoid $P < 0.0001$, β-lactoglobulin $P < 0.0001$, casein $P < 0.0001$, Ara h 2 $P < 0.0001$, CPE $P < 0.0001$; fig. S7, A to F). By 12 months, IgA and IgG antibody levels typically reached those found at birth, except for allergen-specific IgG$_4$. This implies that IgG$_4$ antibody responses are slower to develop in the first year.

Comparison of antibody responses between lifestyle groups showed some noteworthy differences. Regarding IgG, OOM infants at birth had significantly higher IgG against house dust mite (Der f 1 $P < 0.0001$, Der p 1 $P = 0.005$) and horse (Equ c 1 $P = 0.029$) (Fig. 4, A to C), whereas urban infants were born with higher IgG against peanut (Ara h 2 $P = 0.003$, CPE $P = 0.006$) and cat (Fel d 1 $P < 0.0001$) (Fig. 4, D to F). These differences in cord blood reflected maternal concentrations (Fig. 4, A, C, D, and F). In infancy, IgG to dust mite (Der f 1 $P = 0.027$) and horse (6 months: $P = 0.004$, 12 months: $P = 0.017$) continued to be higher in OOM infants, whereas IgG to peanut (Ara h 2 $P = 0.0005$) and cat ($P = 0.043$) remained higher in urban infants (Fig. 4, A, C, D, and F). IgG$_4$ at birth mirrored the findings of IgG, with dust mite (Der f 1 $P = 0.0002$) and horse ($P = 0.004$) IgG$_4$ antibodies higher in OOM infants and peanut (CPE $P = 0.04$, Ara h 2 $P = 0.0001$) and cat ($P = 0.012$) antibodies higher in urban infants (Fig. 4, A and C to F). There were elevated maternal IgG$_4$ antibodies to horse ($P = 0.023$, OOM) and cat ($P < 0.0001$, urban) (Fig. 4, C and F). IgG$_4$ antibodies to dust mite (Der p 1 $P = 0.008$), horse ($P < 0.0001$), and timothy grass (Phl p 5a $P = 0.026$) were higher by 12 months in OOM infants (Fig. 4, B, C, and G). Because we had detected total IgA in cord blood, allergen-specific IgA antibodies were of particular interest. Although generally low, urban infants were born with lower IgA to dust mite (Der f 1 $P < 0.0001$) and with higher IgA against peanut (Ara h 2 $P < 0.0001$) and β-lactoglobulin ($P = 0.0002$) (Fig. 4, A, D, and H). Urban mothers had higher IgA against cat ($P = 0.004$) and β-lactoglobulin ($P = 0.018$) (Fig. 4, F and H). In infancy, OOM infants had significantly higher IgA against house dust mite (Der f 1 6 months $P = 0.004$, 12 months $P = 0.046$; Der p 1 $P = 0.005$), horse (6 months $P = 0.001$, 12 months $P < 0.0001$), cat ($P = 0.020$), grass ($P = 0.001$), and casein (6 months $P = 0.0002$, 12 months $P = 0.047$) antigens at 6 and 12 months (Fig. 4, A to C, F, G, and I). Collectively, these findings indicate that whereas cord blood IgG reflects maternal IgG, IgA responses are likely that of infant origin and differential between lifestyles.

We also examined the impact of maternal atopy [no allergy; allergic rhinitis (AR) only; or combination of food allergy, AD, asthma, and AR (combo)] on infant allergen-specific responses. Urban

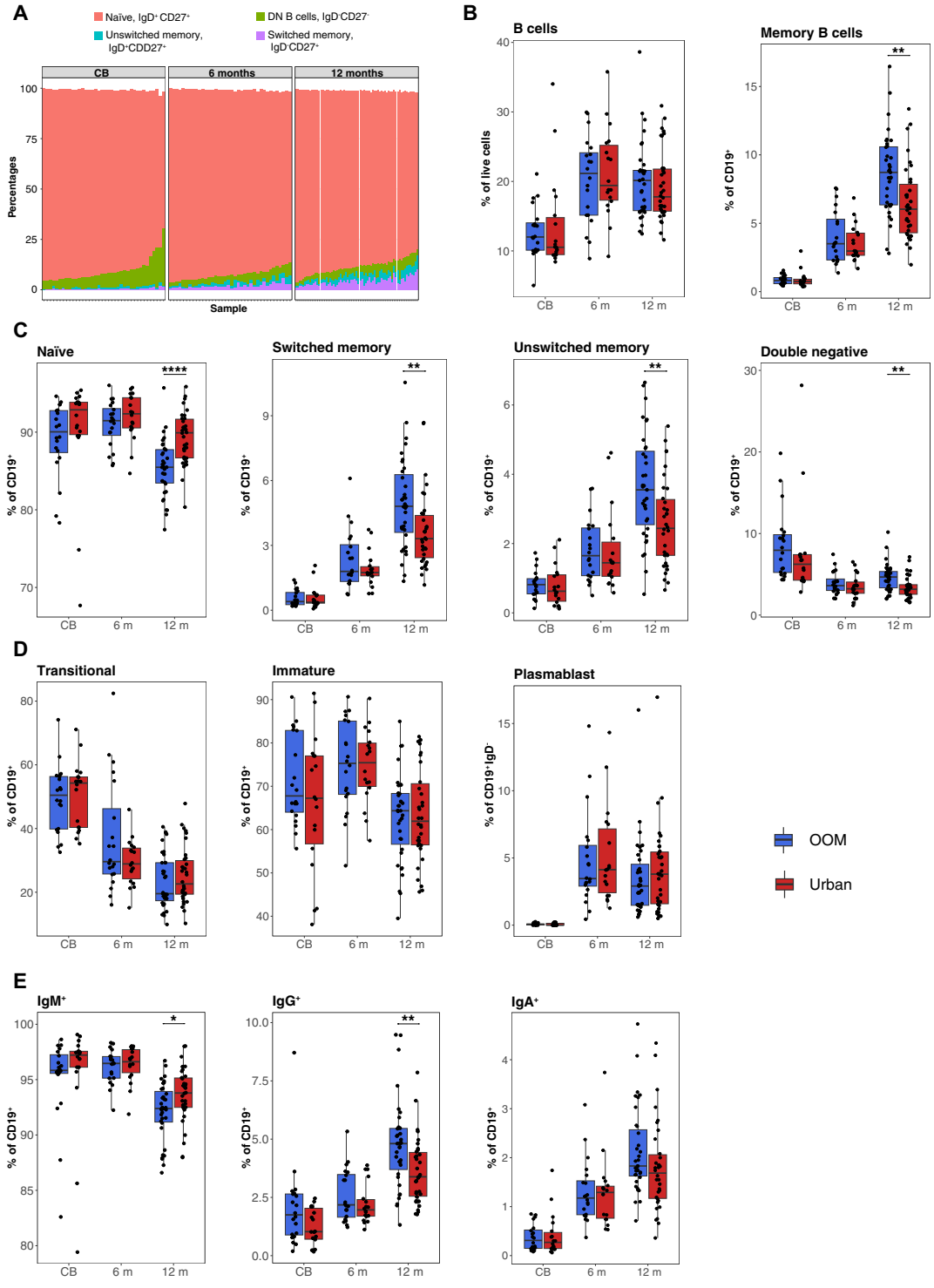


Fig. 2. OOM and urban infant B cell populations. (**A**) Stacked bar chart of percentages of naïve ($CD19^+IgD^+CD27^-$), double-negative (DN) ($CD19^+IgD^-CD27^-$), unswitched ($CD19^+IgD^+CD27^+$), and switched memory ($CD19^+IgD^-CD27^+$) B cells in urban and OOM cord blood (CB) and peripheral blood collected at 6 and 12 months (m) of age (A) in infants (OOM and urban combined). (**B** to **E**) Percentages of total B cells ($CD19^+$) and memory B cells ($CD19^+CD27^+$) (B); naïve B cells, switched and unswitched memory B cells, and DN B cells (C); transitional B cells ($CD19^+CD24^{hi}CD38^{hi}$), immature B cells ($CD19^+CD5^+$), and plasmablasts ($CD19^+IgD^-CD27^+CD38^+$) (D); and IgM-, IgG-, and IgA-expressing B cells (E). Wilcoxon test was used to compare OOM and urban cell populations at each age; $^*P < 0.05$, $^{**}P < 0.01$, $^{****}P < 0.0001$. CB: urban $n = 18$, OOM $n = 20$; 6 months: urban $n = 18$, OOM $n = 20$; 12 months: urban $n = 34$, OOM $n = 35$. Box-and-whisker plots indicate the medians and interquartile ranges, and data points represent individual samples.

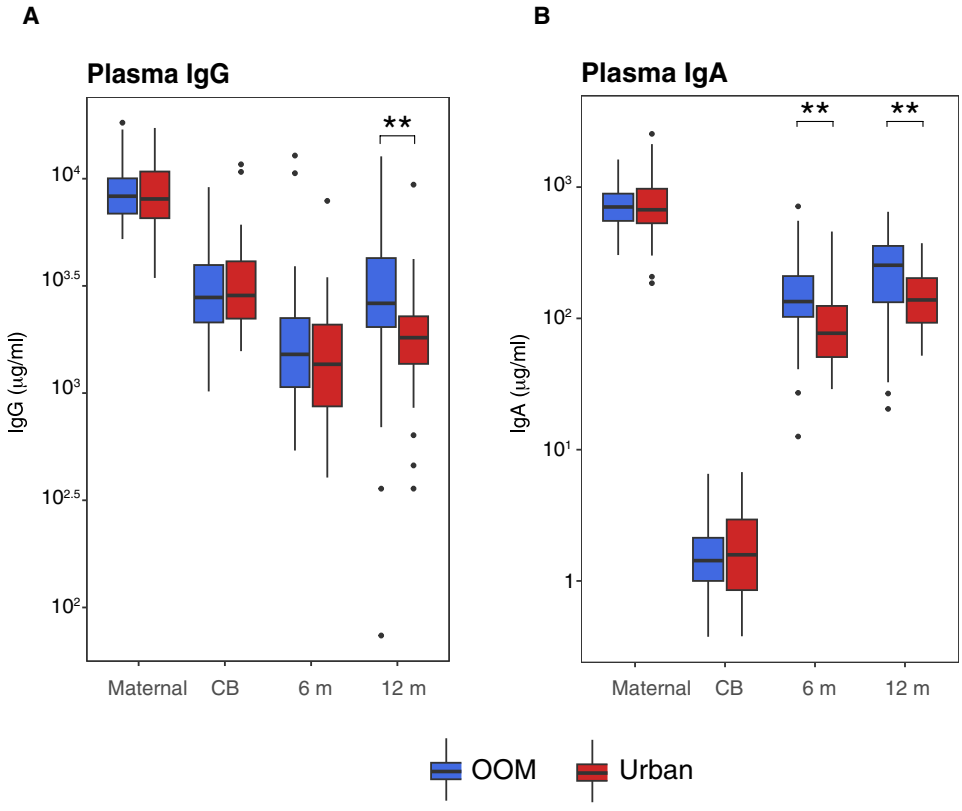


Fig. 3. Total immunoglobulin responses in maternal and infant plasma. (**A** and **B**) Concentrations of infant and maternal plasma IgG (A) and IgA (B). Wilcoxon test was used to compare OOM and urban antibody concentrations at each time point; $^{**}P < 0.01$. CB: OOM $n = 53$, urban $n = 39$; 6 months: OOM $n = 46$, urban $n = 38$; 12 months: OOM $n = 43$, urban $n = 31$; maternal: OOM $n = 58$, urban $n = 68$. Maternal plasma was collected during pregnancy. Box-and-whisker plots indicate the medians and interquartile ranges, and data points represent individual samples.

infants born to nonatopic mothers had lower peanut-specific IgG (Ara h 2 no allergy versus AR only $P = 0.012$, no allergy versus combo $P = 0.034$; CPE no allergy versus AR only $P = 0.004$, no allergy versus combo $P = 0.024$), IgG_4 (Ara h 2 no allergy versus AR only $P = 0.012$; CPE no allergy versus AR only $P = 0.023$, no allergy versus combo $P = 0.023$), and IgA (Ara h 2 no allergy versus AR only $P = 0.019$, no allergy versus combo $P = 0.019$; CPE $P = 0.012$) in their cord blood (fig. S8, A to F). For urban infants, those of nonatopic mothers had lower IgG_4 against dust mite at 6 months (no allergy versus AR only $P = 0.011$) but elevated casein-specific IgG_4 at 12 months (no allergy versus combo $P = 0.034$) (fig. S8, G and H). OOM infants of nonatopic mothers had higher grass-specific IgA in cord blood (no allergy versus combo $P = 0.038$) (fig. S8I).

Egg allergy was associated with lower systemic egg-specific IgG_4

Because egg allergy is the most common food allergy in urban infants by 12 (*30*) and 24 months of age, we had an opportunity to assess whether egg-specific antibodies were associated with protection against egg allergy by comparing the responses with those found in OOM infants, given that no OOM infants developed food allergy. In OOM infants, IgG to ovalbumin was higher at 6 months ($P = 0.002$) and IgG_4 and IgA were higher at 6 (IgG_4 $P = 0.009$, IgA $P < 0.0001$) and 12 months (IgG_4 $P < 0.0001$, IgA $P = 0.017$) relative to those in urban infants (Fig. 4J). Ovomucoid-specific IgG (cord blood $P = 0.003$), IgG_4 (cord blood $P = 0.046$, 6-month blood $P = 0.027$, 12-month blood $P = 0.008$), and IgA (cord blood $P = 0.015$, 6-month blood $P = 0.002$) were similarly elevated in OOM infants (fig. S9A). Whereas urban egg-allergic infants had similar IgG and IgA to ovalbumin at 12 months, their IgG_4 was the lowest (versus OOM $P = 0.002$) (Fig. 5A). OOM infants had significantly higher ovalbumin-specific IgG at 6 months ($P = 0.003$) and IgG_4 and IgA at 6 (IgG_4 $P = 0.034$, IgA $P < 0.0001$) and 12 (IgG_4 $P = 0.002$, IgA $P = 0.016$) months than urban non–egg-allergic infants, and the ratio of ovalbumin-specific IgG_4 to IgG was the lowest in urban egg-allergic infants at 12 months (versus OOM infants $P = 0.005$) (Fig. 5A). Differences in ovomucoid-specific antibody responses were less pronounced, but OOM infants had elevated IgG at birth (versus urban non–egg-allergic infants $P = 0.030$; versus urban egg-allergic infants $P = 0.047$), IgG_4 at 12 months (versus urban egg-allergic infants $P = 0.045$), and IgA at 6 months (versus urban non–egg-allergic infants $P = 0.003$) (fig. S9B). There was a similar low ovomucoid-specific IgG_4 to IgG ratio in urban egg-allergic infants at 12 months (versus OOM infants $P = 0.036$).

Although no OOM infants developed food allergy, several were found to be sensitized to egg (hen's egg white–specific IgE above baseline 0.3 kU/liter at either 12 or 24 months), allowing us to investigate differences in egg-specific responses based on egg sensitization status. Analysis was performed to determine the association between lifestyle and sensitization on egg-specific antibody. Although egg-specific IgG and IgA were elevated by 12 months in egg-sensitized infants (OOM and urban), the lowest IgG_4 amounts were in urban egg-sensitized infants as follows: Sensitization was associated with elevated IgG and IgA at 6 (IgG ovalbumin $P = 0.02$, ovomucoid $P = 0.04$; IgA ovalbumin $P = 0.004$, ovomucoid $P = 0.047$) and 12 months (IgG ovalbumin $P = 0.0002$, ovomucoid $P = 0.003$; IgA ovalbumin $P = 0.0004$, ovomucoid $P = 0.03$); OOM lifestyle had an effect at 6 months only (IgG ovalbumin $P = 0.008$; IgA ovalbumin $P < 0.0001$, ovomucoid $P = 0.02$) (Fig. 5B and fig. S9C). The effect of sensitization on ovalbumin-specific IgG_4 responses at 12 months

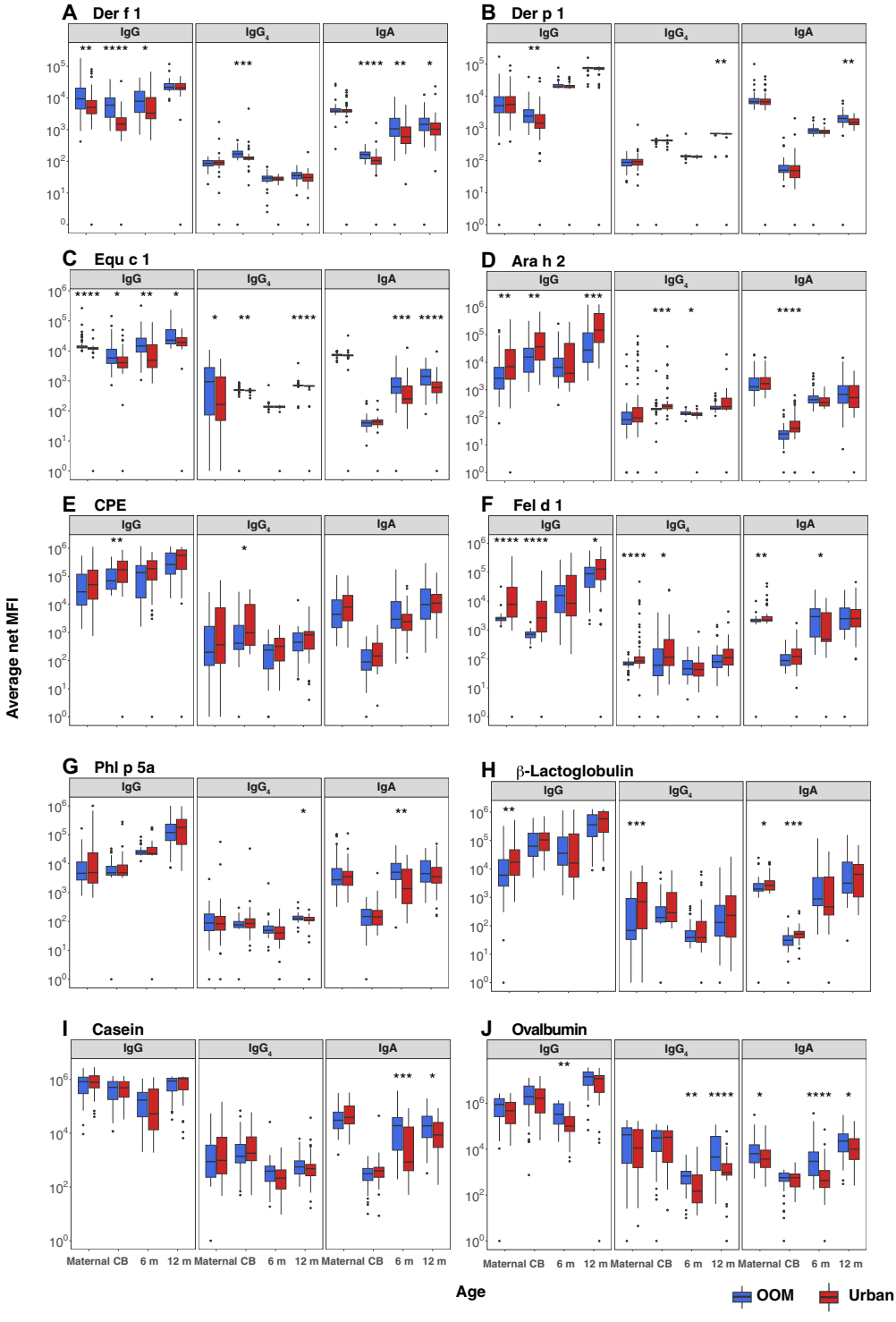


Fig. 4. Antigen-specific immunoglobulin responses in maternal and infant plasma. (**A** to **J**) Infant and maternal plasma IgG, IgG_4, and IgA allergen-specific antibodies against house dust mite [Der f 1 (A), Der p 1 (B)], horse [Equ c 1 (C)], peanut [Ara h 2 (D), CPE (E)], cat [Fel d 1 (F)], timothy grass [Phl p 5a (G)], cow's milk [β-lactoglobulin (H), casein (I)], and ovalbumin (J) antigens. Antigen-specific IgG, IgG_4, and IgA were measured by Luminex; values are reported as average net mean fluorescence intensity (MFI). Wilcoxon test was used to compare OOM and urban antibody concentrations at each time point; $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$. CB: OOM $n = 53$ to 55, urban $n = 41$ or 42; 6 months: OOM $n = 46$ to 48, urban $n = 38$ or 39; 12 months: OOM $n = 49$ to 53, urban $n = 37$ to 39; maternal OOM $n = 58$, urban $n = 68$. Maternal plasma was collected during pregnancy. Box-and-whisker plots indicate the medians and interquartile ranges, and data points represent individual samples.

was opposite in different lifestyle groups; i.e., sensitization was associated with high IgG_4 in OOM infants but low IgG_4 in urban infants (interaction of sensitization and lifestyle ovalbumin $P = 0.0005$, ovomucoid $P < 0.0001$) (Fig. 5B and fig. S9C). In summary, despite elevated systemic egg-specific IgG and IgA, low egg-specific IgG_4 concentrations were associated with egg sensitization and allergy in urban infants.

Lifestyle and age of introduction and frequency of consumption of egg were associated with systemic IgG_4 and IgA to egg

An early age of introduction of egg has been shown to positively associate with median egg-specific IgG_4 concentrations (*31*). Using parental survey data on infant feeding practices (*30*), we determined that the average age of introduction of egg (baked or lightly cooked

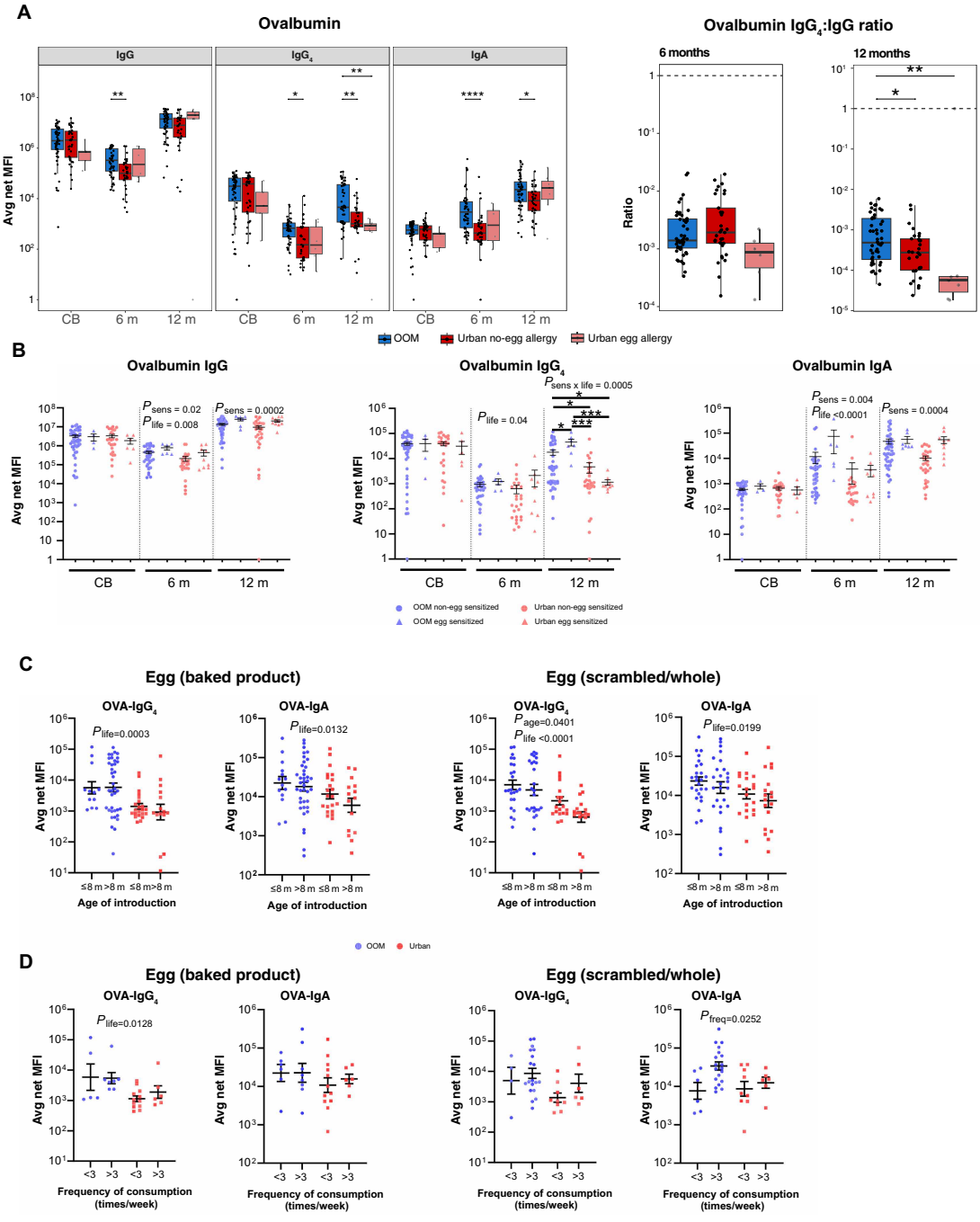


Fig. 5. Infant plasma ovalbumin immunoglobulin responses. (**A**) OOM and urban infant plasma ovalbumin (OVA)–specific IgG, IgG_4, and IgA (left) and OVA-specific IgG_4:IgG ratios (right) were quantified on the basis of their egg allergy status. Dashed line represents a ratio of 1. (**B**) Infant plasma OVA-specific IgG, IgG_4, and IgA based on their egg sensitization status. (**C** and **D**) Twelve-month plasma OVA-specific IgG_4 and IgA based on either the age of introduction [≤8 or >8 months of age (C)] or frequency of egg consumption <3 or >3 times/week [among infants introduced ≤8 months of age (D)] of baked (left) or scrambled/whole egg (right). OVA-specific IgG, IgG_4, and IgA were measured by Luminex; values are reported as average net MFI. Dunn's test with Benjamini-Hochberg correction was used to compare antibody responses in (A). Two-way ANOVA on original [(B) CB IgA] or transformed values [log- (B): 6-month IgG, IgG_4, and IgA and 12-month IgA; (C) and (D): baked IgA, scrambled/whole IgG_4 and IgA; square root– (B): 12-month IgG and CB IgG_4; sin- (B): CB IgG; inverse- (D): baked IgG_4] was used to determine the impact of lifestyle and sensitization (P_{sens}) or lifestyle (P_{life}) and age of egg introduction (P_{age}) or frequency of egg consumption (P_{freq}). Aligned rank transform test was used to analyze the impact of lifestyle and sensitization (P_{sens}) on nontransformed 12-month IgG_4 data (B); $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$. OOM and urban non–egg sensitized $n = 25$ to 45; OOM egg sensitized $n = 4$ to 7; urban egg sensitized $n = 6$ to 10. OOM and urban non–egg allergic $n = 30$ to 55; urban egg allergic $n = 5$ to 7. Baked egg ≤8 or >8 months of age: OOM $n = 12$ to 39, urban $n = 15$ to 22; <3 or >3 times/week OOM $n = 5$ to 8, urban $n = 6$ to 12; scrambled/whole egg ≤8 or >8 months of age OOM $n = 24$ to 27, urban $n = 18$ or 19; <3 or >3 times/week OOM $n = 4$ to 19, urban $n = 6$ to 9. Box-and-whisker and column scatterplots indicate the medians and interquartile ranges and means with SEM, respectively. Data points represent individual samples.

egg) was 8 months of age across infants in our cohort. This was also the age when introduction of lightly cooked egg was performed in an Australian early introduction trial (*31*). We assessed the effect of introduction of baked or lightly cooked egg before or after 8 months and lifestyle on egg-specific antibody concentrations in infants at 12 months. There was a significant lifestyle effect on ovalbumin-specific IgG_4 (baked $P = 0.0003$, lightly cooked $P < 0.0001$) and IgA (baked $P = 0.0132$, lightly cooked $P = 0.0199$), with more found in OOM infants than in urban infants (Fig. 5C). There was a minimal effect of early scrambled/whole egg introduction on IgG_4 (lightly cooked $P = 0.0401$) (Fig. 5C). Additionally, we assessed the effect of frequency of egg ingestion, using three times a week as a cutoff, among those who were introduced to egg before 8 months of age (*31*). There was no association between the frequency of consumption of baked egg and either ovalbumin-specific IgG_4 or IgA. However, a higher frequency of ingestion of scrambled/whole egg (more than three times per week) was associated with higher ovalbumin-specific IgA in both populations (lightly cooked $P = 0.0252$) (Fig. 5D). Overall, these data highlight the association of the farming lifestyle with elevated egg-specific IgG_4 and IgA. Moreover, there appears to be a beneficial effect of ingesting lightly cooked egg, given that early consumption boosted egg-specific IgG_4 and frequent consumption boosted egg-specific IgA regardless of lifestyle.

A farming lifestyle was associated with enhanced mucosal IgA responses

We were next interested in the development of mucosal, as opposed to systemic, immunoglobulin concentrations given the capacity of B cells to home to the gut in infancy. Salivary IgA was significantly higher in OOM mothers ($P < 0.0001$) and 6-month-old infants ($P = 0.003$) but similar at 12 months (fig. S10A). OOM mothers also had elevated salivary IgG ($P < 0.0001$) (fig. S10B). In lieu of direct access to the gut, we developed assays to measure total and allergen-specific IgA in infant stool samples, keeping in mind that only IgA_2 is resistant to protease digestion because of its glycosylation patterns (*32*). Fecal IgA_1 concentrations were found to be significantly higher in OOM infants compared with urban infants without atopic disease (AD or food allergy) at 6 weeks ($P < 0.0001$), 6 months ($P = 0.008$), and 12 months ($P = 0.024$), whereas IgA_2 was higher at 6 weeks ($P = 0.013$) and 6 months ($P = 0.0002$) (Fig. 6A). A similar profile was seen for OOM infants when compared with urban infants with allergies at 6 weeks (IgA_1 $P < 0.0001$, IgA_2 $P = 0.0004$) and 6 months (IgA_1 $P = 0.003$, IgA_2 $P = 0.011$). Fecal IgA_1 and IgA_2 were similar between urban infants with and without allergy. OOM infants who were not sensitized to food (hen's egg white, cow's milk, peanut) had elevated fecal IgA_1 and IgA_2 at 6 weeks (IgA_1 $P < 0.0001$, IgA_2 $P = 0.013$) and 6 months (IgA_1 $P = 0.009$, IgA_2 $P = 0.0002$) compared with urban infants without food sensitization (fig. S11). Compared with urban infants with food sensitization, OOM infants without sensitization had elevated IgA at 6 weeks (IgA_1 $P < 0.0001$, IgA_2 $P = 0.013$) and 6 months (IgA_1 $P = 0.032$). Moreover, fecal IgA_1 ($P = 0.014$) and IgA_2 ($P = 0.039$) were higher at 6 weeks in food-sensitized OOM infants compared with urban infants with food sensitization (fig. S11). This implies that farm exposure regardless of future sensitization induces elevated fecal IgA in early infancy.

Given that maternal milk is a major reservoir of IgA that can be delivered to infants during lactation, we probed IgA concentrations in maternal milk from OOM and urban women. At 6 weeks, OOM mothers had elevated IgA_1 compared with urban mothers who did ($P = 0.021$) or did not ($P = 0.021$) develop allergy (Fig. 6B). Urban mothers of infants who did or did not develop allergy had similar amounts of IgA_1 and IgA_2. Additionally, when we consider infants who did not receive milk, considering their exposure to maternal milk as "0," the results were similar (fig. S12). To assess to what extent maternal milk IgA contributes to the pool of IgA found in the infant gut in early life, we next evaluated the correlation between infant fecal and maternal milk IgA. There were positive correlations between the OOM samples only for IgA_2 at 6 weeks ($P = 0.023$) and 6 months ($P = 0.0071$; Fig. 6, C and D). These data provide evidence of a relatively weak association of maternal milk and infant fecal IgA_1. Another approach to assess the contribution of maternal milk to infant fecal IgA is to compare fecal IgA concentrations between breastfed and non-breastfed infants. We observed an effect of infant feeding on infant fecal IgA_1 but not IgA_2, with infants who were either exclusively breastfed ($P = 0.032$) or consumed any maternal milk ($P = 0.009$) having elevated fecal IgA_1 (fig. S13). In conclusion, breastfeeding was associated with elevated infant fecal IgA.

We then assessed egg-specific IgA responses in infant fecal samples. Similar to total IgA, higher ovalbumin-specific IgA was detected in OOM infants at 6 ($P = 0.003$) and 12 months ($P = 0.046$) compared with urban infants without egg allergy (Fig. 7A). The amount of egg-specific IgA in egg-allergic infants was comparable to that in infants with no egg allergy (Fig. 7A and fig. S14). Regarding maternal milk, ovalbumin-specific IgA at 6 weeks was significantly lower in the milk of mothers of urban infants with egg allergy compared with those of mothers of OOM infants ($P = 0.009$) or urban non–egg-allergic infants ($P = 0.028$; Fig. 7B). After imputing mother's milk IgA as "0" to represent the infants who did not receive milk, at 6 weeks, again, urban egg-allergic infants had the lowest ovalbumin- (versus OOM infants $P = 0.0005$, versus urban infants without egg allergy $P = 0.005$) and ovomucoid-specific (versus OOM infants $P = 0.012$) IgA (fig. S15, A and B). Moreover, there was elevated maternal milk ovomucoid-specific IgA at 6 weeks ($P = 0.008$) in OOM mothers who regularly used egg in the home compared with urban mothers who did not (fig. S15, C and D). Comparisons between fecal and maternal milk egg-specific IgA showed positive correlations for ovalbumin in OOM ($P = 3.1 \times 10^{-12}$) and urban ($P = 0.0013$) samples at 6 weeks and in OOM samples ($P = 0.045$) at 6 months (Fig. 7, C and D). For ovomucoid, a positive correlation was observed in OOM samples ($P = 0.00029$) at 6 weeks. In summary, farm exposure induces a mucosal IgA response in both mothers and their infants, with maternal milk IgA antibodies associated with more frequent exposure and protection against egg allergy before development of egg allergy. The early fecal IgA in OOM infants may be partially due to a contribution by maternal IgA.

Early colonization with *B. infantis* was associated with increased fecal IgA responses

The reason for elevated fecal IgA immunity in OOM infants was of great interest to us. We previously identified increased abundance of total *Bifidobacterium* and specifically *Bifidobacterium longum* subspecies *infantis* (*B. infantis*) in the stools of OOM infants compared with urban infants at an average age of 2 months (*33*). *Bifidobacterium* is of interest because it has several, potentially protective, immunoregulatory properties that may affect maturation of the immune system (*34*, *35*) but is decreased in the gut microbiome of westernized infants in parallel with the rise in allergic diseases (*36*). Moreover, Lundell *et al.* reported a positive

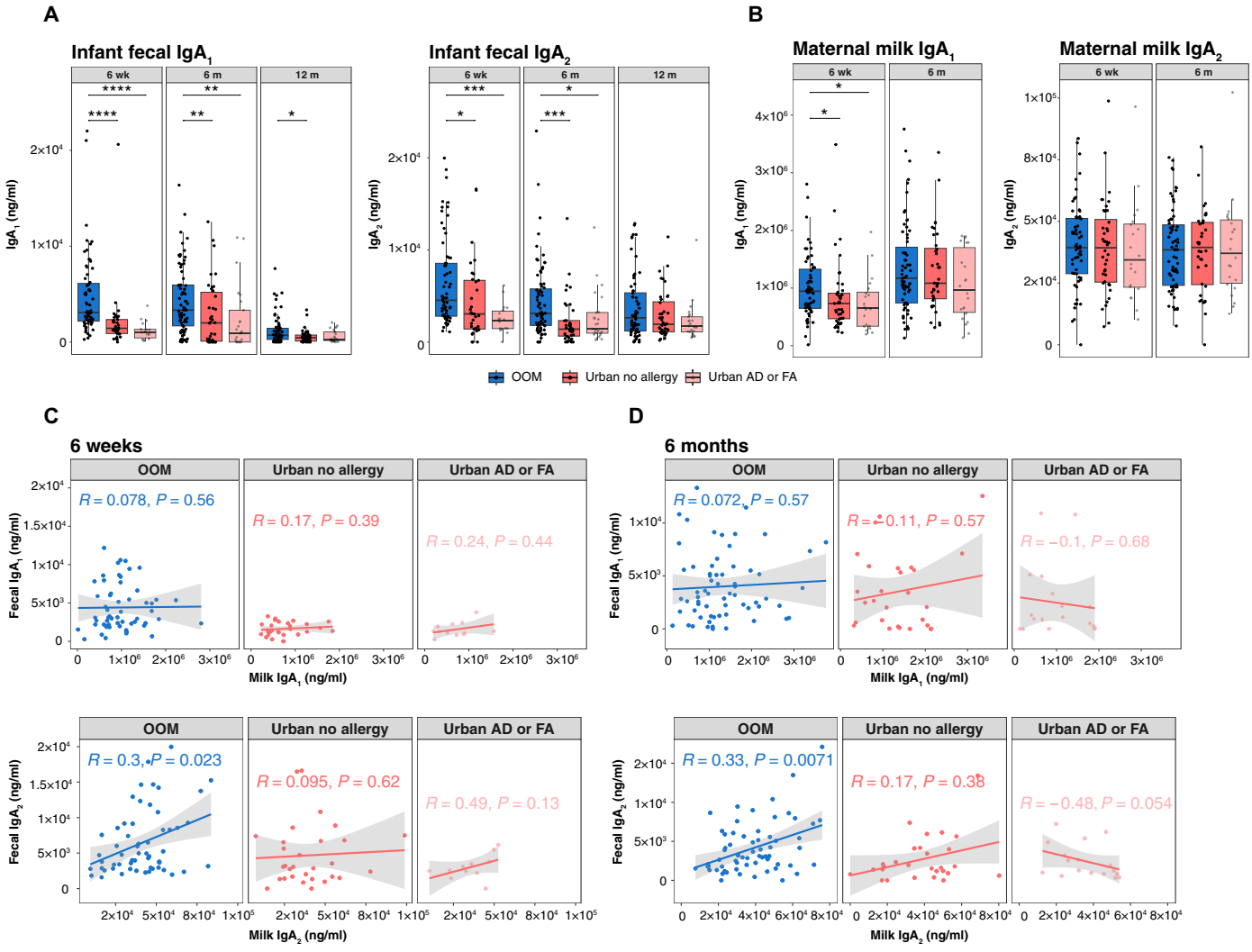


Fig. 6. Infant fecal and maternal milk IgA_1 and IgA_2 based on infant allergy status. (**A** and **B**) IgA_1 and IgA_2 concentrations were measured in OOM and urban infant fecal (A) and their maternal milk (B) samples based on infant allergy [AD or food allergy (FA)] status. (**C** and **D**) Correlation plots of infant fecal and maternal milk IgA_1 (top) and IgA_2 (bottom) at 6 weeks (C) and 6 months (D). Dunn's test with Benjamini-Hochberg correction was used to compare infant fecal and maternal milk IgA_1/IgA_2 concentrations at each age, and Spearman correlation was used to determine the correlations between infant fecal and maternal milk IgA_1/IgA_2; $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$. Fecal: 6 weeks: OOM $n = 67$, urban nonallergic $n = 33$, urban allergic $n = 18$; 6 months: OOM $n = 75$, urban nonallergic $n = 38$, urban allergic $n = 25$; 12 months: OOM $n = 64$, urban nonallergic $n = 40$, urban allergic $n = 25$; maternal milk: 6 weeks: OOM $n = 63$, urban nonallergic $n = 43$ or 44, urban allergic $n = 20$; 6 months: OOM $n = 67$, urban nonallergic $n = 36$, urban allergic $n = 22$. Correlations: 6 weeks: OOM $n = 56$ or 57, urban nonallergic $n = 28$ to 30, urban allergic $n = 12$; 6 months: OOM $n = 65$, urban nonallergic $n = 28$, urban allergic $n = 18$. Box-and-whisker plots indicate the medians and interquartile ranges, and data points represent individual samples.

association between early colonization (at 4 and 8 weeks) with *Escherichia coli* and *Bifidobacterium* and memory B cells at 4 and 18 months of age (*37*). We investigated the presence of *B. infantis* [measured by quantitative polymerase chain reaction (qPCR)] in our current cohort and its association with several B cell responses. Colonization of *B. infantis* at 6 weeks was associated with increased fecal IgA_1 ($P = 0.044$) and IgA_2 ($P = 0.047$) as well as fecal ovalbumin-specific IgA ($P = 0.013$) at 6 months (fig. S16, A and B). However, *B. infantis* colonization at 6 weeks was not associated with systemic total immunoglobulin or memory B cells at 6 months (fig. S16, C and D). Whereas infants colonized with *B. infantis* at 6 months had increased fecal IgA_1 (but not IgA_2) at 12 months ($P = 0.014$; fig. S17A), colonization at 6 weeks or 6 months was not associated with fecal egg-specific IgA, systemic total immunoglobulin, or memory B cells (fig. S17, B to F). Subsequent analysis assessed the interaction of *B. infantis* colonization and lifestyle. Although there was no effect of lifestyle or *B. infantis* colonization on infant fecal total IgA_1 and ovalbumin-specific IgA, a lifestyle effect ($P = 0.007$) was found for fecal IgA_2 at 6 months (fig. S18, A and B). There was a modest effect of *B. infantis* ($P = 0.0382$) on elevated fecal IgA_1 at 12 months (fig. S18C). Overall, early *B. infantis* colonization is associated with mucosal, but not systemic, IgA.

Food antigens were detectable in cord blood and maternal milk

We were surprised about the IgA responses to foods that were already detected at birth, prompting a query into fetal exposure to food antigens. Because the concentrations of infant total IgA were

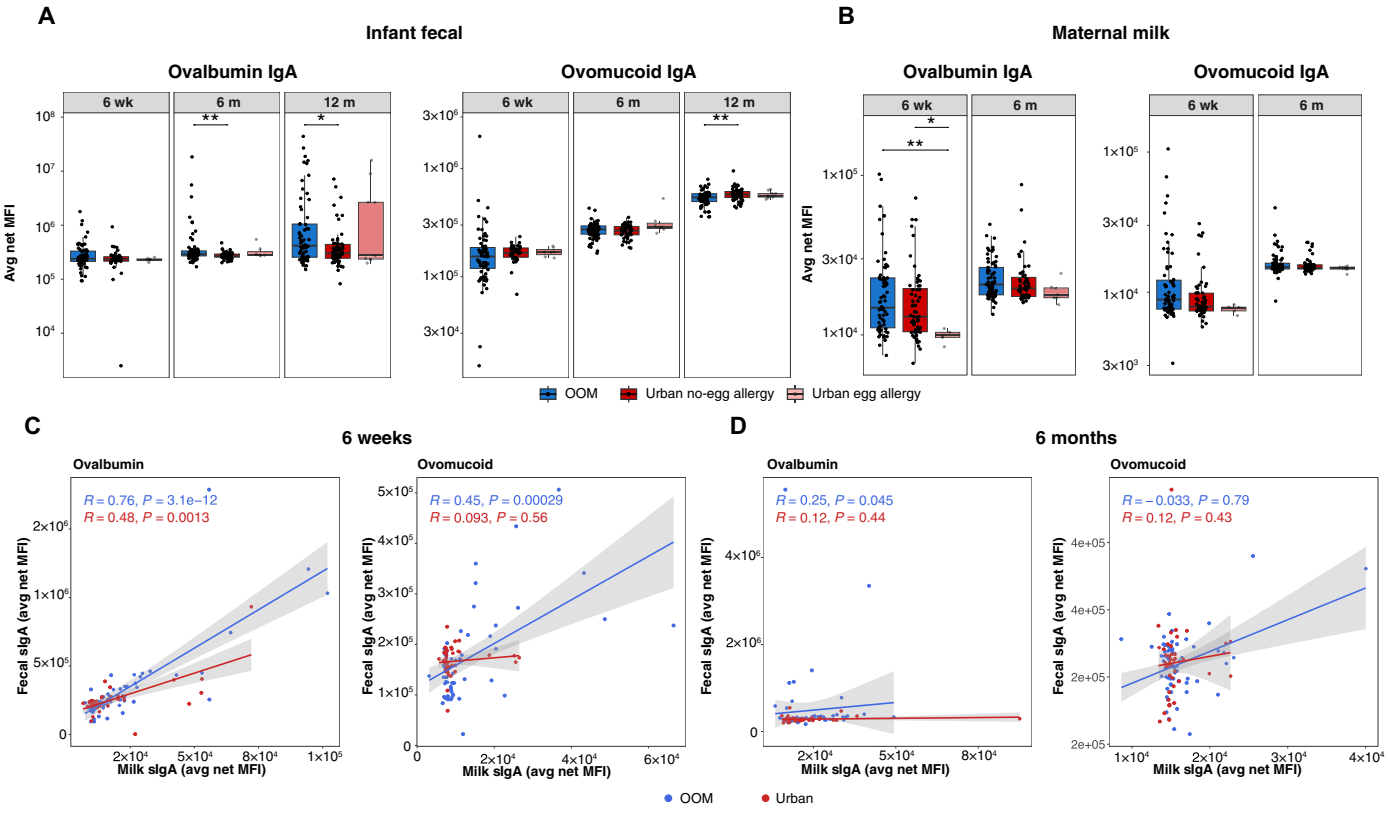


Fig. 7. Egg-specific IgA in infant fecal and maternal milk based on infant egg allergy status. (**A** and **B**) OVA and ovomucoid (OVM)–specific IgA (sIgA) were measured in OOM and urban infant fecal (A) and their maternal milk (B) samples based on infant egg allergy status. (**C** and **D**) Correlation plots of infant fecal and maternal milk OVA- and OVM-sIgA at 6 weeks [6 wk (C)] and 6 months [6 m (D)], colored by lifestyle. Egg-specific IgA was measured by Luminex; values are reported as average net MFI. Dunn's test with Benjamini-Hochberg correction was used to compare fecal and maternal milk sIgA at each time point, and Spearman correlation was used to determine correlations between infant fecal and maternal milk sIgA; $^{*}P < 0.05$, $^{**}P < 0.01$. Fecal: 6 weeks: OOM $n = 66$ or 67, urban non–egg allergic $n = 43$ or 44, urban egg allergic $n = 7$; 6 months: OOM $n = 74$, urban non–egg allergic $n = 57$, urban egg allergic $n = 7$; 12 months: OOM $n = 63$ or 64, urban non–egg allergic $n = 58$ or 59, urban egg allergic $n = 9$; maternal milk: 6 weeks: OOM $n = 68$, urban non–egg allergic $n = 60$, urban egg allergic $n = 5$; 6 months: OOM $n = 69$, urban non–egg allergic $n = 51$, urban egg allergic $n = 7$. Correlations: 6 weeks: OOM $n = 60$, urban $n = 41$ or 42; 6 months: OOM $n = 66$ or 67, urban $n = 45$. Box-and-whisker plots indicate the medians and interquartile ranges, and data points represent individual samples.

very low (<10 μg/ml), we concluded that maternal contamination with blood was unlikely (*38*). We postulated that food antigens may be present in utero, which may be responsible for the induction of food-reactive IgA antibodies. In lieu of amniotic fluid, we measured the presence of food antigens in cord blood as well as 6-week maternal milk samples. β-Lactoglobulin, casein, Ara h 2, and ovalbumin were detected; however, neither the concentrations nor the proportions of infants with detectable antigens were different between OOM and urban cord blood and maternal milk samples (fig. S19, A to D). However, among the infants who had detectable antigen, there were significant positive correlations between cord blood antigens Ara h 2 and casein ($P = 0.0028$), Ara h 2 and β-lactoglobulin ($P = 0.016$), and casein and β-lactoglobulin ($P = 0.018$) (fig. S19E). In maternal milk, there was only a positive correlation between β-lactoglobulin and Ara h 2 ($P = 0.0031$; fig. S19F). These data suggest the potential for antigen exposure both in utero and in maternal milk.

Infant B and T cell populations were associated with systemic antigen-specific immunoglobulin responses

Finally, we applied multivariate regression analysis to understand the association between systemic antibodies and B or T cell populations that we recently reported (*39*). $IgG^{+}\alpha4\beta7^{+}$ B cells significantly positively associated with ovomucoid-specific IgG ($P = 0.041$) and IgA ($P = 0.027$) (Fig. 8). The association of gut-homing B cells and egg-specific antibodies is likely reflective of these B cells producing antibodies in response to oral ingestion of egg during infancy. Among the type 2 helper T-B (T_H2B) clusters, which we recently found to be enriched in infants who went on to develop allergy (*39*), we observed a significant negative association between cluster 58 with ovomucoid-specific IgA ($P = 0.032$) (Fig. 8).

DISCUSSION

We aimed to better understand the impact that farm exposure has on infant B cell development and antibody responses both before and after development of allergic disease. Data from our longitudinal birth cohort demonstrated that farm-exposed infants had higher amounts of circulating IgG^{+} and IgA^{+} cells and respective immunoglobulins in infancy. Notably, we found that elevated systemic IgG_4 is associated with protection against egg allergy and frequency of ingestion of lightly cooked egg. OOM participants also had increased mucosal IgA as demonstrated by elevated fecal IgA throughout

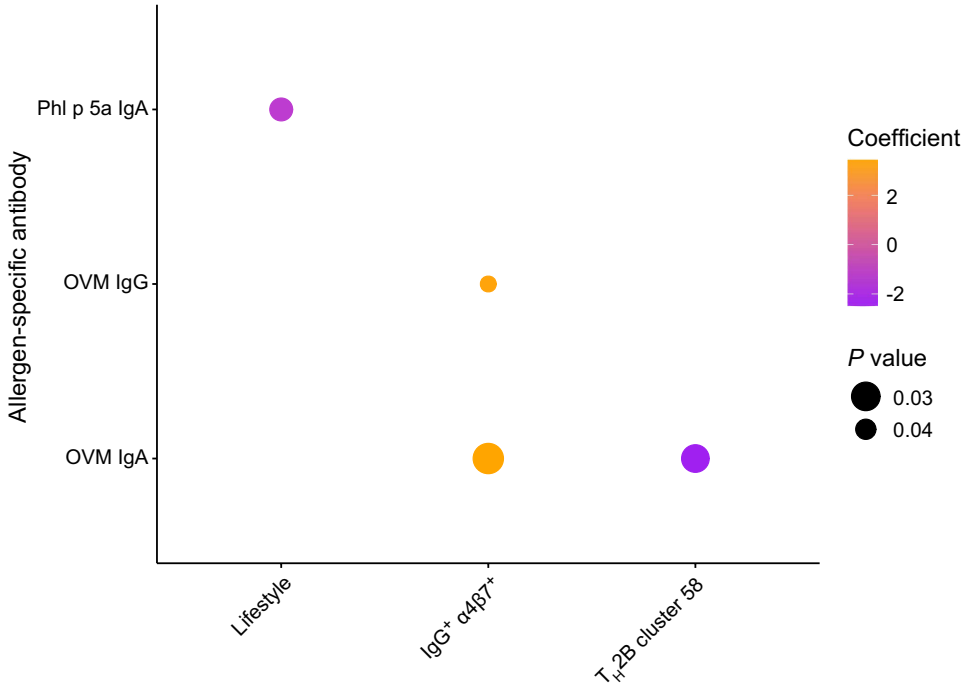


Fig. 8. Infant T cell and B cell populations associated with systemic antigen-specific antibody. Association between systemic allergen-specific antibody concentrations (*y* axis) with lifestyle and B and T cell frequencies (*x* axis) obtained from infants (OOM and urban) at 12 months of age. Dot size corresponds to *P* value, and dot color corresponds to regression coefficient. Quantile regression was used to analyze data; responses with a significant *P* value ($\alpha = 0.05$) were plotted. Urban $n = 22$, OOM $n = 26$. Allergens tested included Phl p 5a (timothy grass) and OVM.

infancy and in maternal milk. Egg-specific IgA in maternal milk was associated with a lack of egg allergy in early life before the development of egg allergy. Although maternal IgA was certainly provided by their milk postnatally, IgA was already in cord blood, indicating an effect of in utero exposure on IgA immunity. Our data support the protective effect of farm exposure against the development of atopic diseases being at least partially mediated through a differential B cell immune response, the origins of which may start prenatally.

Our findings of the accrual of memory and class-switched IgG^+ and IgA^+ B cells by 12 months complements previous reports of the gradual maturation of the infant B cell compartment, likely reflective of antigen experience or cell activation that occurs as exposures to the infants increase after birth (*40*, *41*). Additionally, a proportion of IgG^+ and IgA^+ B cells at 6 and 12 months expressed Ki67, which may reflect their recent generation. These class-switched cells were also found to have the capacity for homing to mucosal surfaces, such as the intestine, as previously described (*42*), possibly facilitating interaction with the developing microbiome or a site of origin for B cell development (*43*). The increased proportion of memory B cells in OOM infants complements a previous report of increased $CD27^+$ B cells in farm-raised infants at 4 months of age from a Swedish birth cohort (*14*).

Enhanced maturation could be driven by differential microbial colonization during early life. We initially focused on *B. infantis*, because we previously found that the microbe is differentially colonized in urban and OOM infants (*33*). Among the different infant B cell responses, we only found a modest association with increased fecal IgA_1. A previous study found that farm exposure led to a more mature infant microbiome at 12 months of age, which was characterized by increased abundances of short-chain fatty acid (SCFA)–producing bacteria like *Roseburia* and *Coprococcus* and was associated with protection against asthma at school age (*44*). SCFAs are one of the most abundant microbially derived metabolites and are known to modulate both innate and adaptive immune cells. Through metabolism of human milk oligosaccharides, *Bifidobacterium* has the capacity to generate SCFAs, specifically acetate and lactate (*45*–*47*). It remains to be seen whether other gut microbiota or metabolites in OOM infants are responsible for their IgA production and are active areas of interest in our laboratory.

OOM infants were found to have elevated systemic concentrations of total IgG and IgA. Additionally, OOM and urban infants differed in their allergen-specific responses. We hypothesized that these differences in antibodies are reflective of the infants' divergent exposures during the first year of life (*30*). Of particular interest were egg antibodies, given the different rates of egg allergy between the groups. Higher IgG, IgG_4, and IgA against egg ovalbumin were already seen at 6 months in OOM infants compared with urban infants. Because egg introduction was rare before this age, even in OOM infants, exposure through maternal milk boosting egg-specific antibodies is one of the few plausible explanations. By 12 months of age, we found that the farming lifestyle was associated with the elevated IgG_4 and IgA responses to egg, although the earlier age of introduction to lightly cooked egg was also positively associated with egg-specific IgG_4, and more frequent ingestion was associated with egg-specific IgA. Besides the amount and route of exposure (oral, skin, lung, or through breast milk) (*48*, *49*), methods of food preparation such as pasteurization can enhance allergenicity, as is the case with cow's milk allergens (*50*). Pasteurization's impact on the T_H2 response to eggs has not been assessed, nor has the effect of consumption of fresh farm eggs, prevalent among the OOM participants, which may require further exploration. We found that gut-homing $\alpha4\beta7^+$ IgG^+ B cells were positively associated with egg-specific IgA and IgG at 12 months of age, which suggests that the generation of the egg-specific antibodies in the gut after egg ingestion may prevent generation of effector memory $\alpha4^+$ T_H2-poised T_H2B cells, which we recently found associated with allergic diseases in infancy (*39*).

The high rate of egg allergy exclusively seen in our urban cohort provided a unique opportunity to examine antibody responses in affected infants. Egg-sensitized infants (OOM and urban) had increased systemic IgG and IgA to egg at 6 and 12 months compared with infants without egg sensitization. This mirrors an "inflammatory," polyisotypic response previously described in cow's milk allergy (*51*), which suggests that, after sensitization, an increased IgA response is seen to the food of interest because of sequential class-switch recombination events generating IgA along with IgE. Therefore, detection of IgA responses in circulation at the time of or after sensitization or allergy is likely associated with sensitization, given that most of the egg-allergic urban infants were diagnosed between 6 and 12 months of age (*30*). However, elevated IgG_4 to both ovalbumin and ovomucoid was detected in the OOM egg-sensitized infants who did not develop clinical allergy compared with their urban peers, suggesting that elevated systemic egg-specific IgG_4 may associate with protection. IgG_4 is suggested to be an antagonist to IgE, functioning as a blocking antibody (*15*). Although the T_H2 cytokine interleukin-4 (IL-4) is required for IgE and IgG_4 induction (*52*, *53*), additional cytokines like IL-10 favor the generation of IgG_4 (*54*); therefore, IgG_4 induction instead of IgE may reflect a modified T_H2 response that is protective against allergy (*55*, *56*). The most compelling antibody response that associated with protection against egg allergy was ovalbumin-specific IgA detected in maternal milk, but not in fecal samples, before development of egg allergy. The lack of an association in fecal samples is not surprising because fecal IgA is a poor proxy for IgA in the intestine, given that it may be susceptible to bacterial proteases as it travels the intestinal tract. IgA in maternal milk potentially provides a better estimation for what will be encountered in the small intestine because of its relative resistance to digestion (*57*–*59*), especially that of IgA_2 (*32*). This finding may suggest protective properties of maternal milk antibodies against food allergy, given that IgA was elevated before development of egg allergy, which is consistent with data from our previous study (*22*). Our clinical trial is about to start to assess the effect of maternal ingestion of egg and peanut on their milk antibody concentrations and protection against infant food allergy.

Low but detectable amounts of IgA antibodies were observed in cord blood. Fecal IgA was also present early on but likely stemmed in part from maternal milk, given that infant fecal and maternal milk IgA_2 was correlated in the OOM group, which had more exclusively breastfeed infants (*30*). Moreover, we found that breastfeeding infants (either exclusively or any consumption) had elevated fecal IgA_1 compared with non-breastfed infants, further supporting the contribution of mothers. The source of cord blood IgA has been debated, suggesting a contribution of maternal IgA by contamination of maternal blood, and its role at birth remains obscure (*60*, *61*). Because systemic total IgA was very low (<10 μg/ml), we concluded that maternal contamination with blood was unlikely (*38*). The detection of IgA and IgA^+ B cells in cord blood and fetal tissues (*62*–*65*) has been previously reported, indicating the potential capacity to mount an IgA response already in utero. The production has been suggested to be due to the presence of food antigens in amniotic fluid (*66*, *67*), which we were not able to assess in this cohort. Alternatively, antigens from maternal circulation could reach the fetal systemic compartment, as suggested from ex vivo placental perfusion studies (*68*, *69*). We observed the presence of several food antigens in cord blood plasma in about one-fifth of infants. To our knowledge, the presence of casein and peanut has not been previously reported, but there are two older reports showing the presence of ovalbumin and β-lactoglobulin in cord blood (*68*, *70*). The paucity in food antigens detected among the infants may be due to the lack of maternal ingestion of specific foods before delivery (*71*–*73*). Assessment of maternal food consumption and circulating concentrations of food antigens matched with ingestion of specific foods may shed light on the kinetics of maternal-fetal food antigen transfer and serve as an estimate of antigen exposure by the fetus throughout the pregnancy. Data have suggested an increase in intestinal permeability during pregnancy (*74*), which may affect passage of antigens that reach the fetal compartment among mothers. In addition, food antigens may be found in complex with immunoglobulin, with IgG immune complexes having the potential to be transferred through the placenta into fetal circulation (*71*, *75*). The role of transplacental antigen transfer, whether free or in a complex, and its impact on infant tolerance development have been long debated and continue to be explored.

The mechanism responsible for boosted IgA immunity in OOM participants is unknown and may be multifactorial. The farming lifestyle effect on infant B cell responses likely initiates with innate and T cells. Among school-aged children, compared with Hutterites, the Amish who are protected against allergic asthma exhibit accrual of a hyporeactive monocyte population that positively associates with activated regulatory T cells (*7*, *12*). Similarly, we recently reported that OOM infants have more gut-homing memory regulatory T cells and memory regulatory T cells that express markers suggestive of enhanced suppressive function (*39*). The OOM, like the Amish, practice single-family farming compared with industrialized communal farming practiced by the Hutterites (*7*, *28*). Further characterization of innate cells is currently ongoing in the laboratory.

Our study is not without limitations. One of the main limitations is the small number of individuals with food allergy in the cohort. Another limitation is that, although we began to explore mucosal responses in infant saliva and stool, we lacked access to explore the cellular and humoral responses directly generated in the infant intestine. Last, we could have included more frequent sample collection time points during the study.

In conclusion, differences in B cell phenotypes during infancy were associated with farm exposure. Infants exposed to a farming lifestyle in early infancy exhibited enhanced B cell maturation and a differential antibody profile. This study provides additional insight into the vast influence of farm exposure on infant immunity (*76*, *77*) and suggests that the farming lifestyle is an important factor enhancing IgG_4 and IgA responses. Elevated systemic IgG_4 and maternal milk IgA responses were associated with protection against egg allergy. Future work should determine additional B cell–intrinsic or B cell–extrinsic differences that may be affected by farm exposure.

MATERIALS AND METHODS

Study design

This study investigated B cell responses in the first year of life in our birth cohort of infants from either a traditional farming or an urban community who are at different risks of allergy development. In particular, we focused on B cell phenotypes (flow cytometry) and immunoglobulin (total and antigen-specific) concentrations [enzyme-linked immunosorbent assay (ELISA), Luminex]. We initially hypothesized that farm-exposed infants will have a more mature B cell

profile. Besides the impact of lifestyle, we also examined responses on the basis of infant sensitization or allergy status. Immunoglobulin concentrations (ELISA, Luminex) in maternal samples were also measured to examine potential relationship(s) between the IgA response in the mothers and their infants. The presence of the bacterium *B. infantis* was measured in infant stool (qPCR) to associate its absence or presence with infant immunoglobulin. Food antigens were measured in cord blood plasma and maternal milk (ELISA) to examine the potential of fetal and infant antigen exposure.

Samples were drawn from the previously described longitudinal Zooming in to Old Order Mennonites (ZOOM) birth cohort, which was recruited to assess the development of the infant immune system in populations at differing risk for allergic diseases (*30*); see overview of the study in Fig. 1A. In the cohort, three OOM mothers were recruited twice, and each had two infants enrolled. The cohort details and clinical outcomes by 12 months of age have been published (*30*); this current study is not applicable to reporting guidelines. Briefly, we recruited infants from the OOM ($n = 78$) community located in Penn Yan, NY, USA, 65 miles southeast of Rochester, NY, USA, who are at low risk for allergic diseases (*28*, *29*), whereas high-risk infants (with a first-degree blood relative with allergy) came from the urban and suburban Rochester, NY, USA, community (urban, $n = 79$; Fig. 1A). Infants were born full term (>36 weeks of gestation), with birth weight >2000 g, and were generally healthy, and their mothers were free from chronic infections, inflammatory diseases, or known immunodeficiencies. Our protocol (RSRB52971) was approved by the institutional review board of the University of Rochester Medical Center, and all parents provided written consent before enrollment to the study.

Unlike for flow cytometry, where we subsampled infants at random, ELISA, Luminex, and qPCR sample sizes were not predetermined but based on sample availability; see number of samples used for assays performed shown in Fig. 1B. Samples were not blinded. Sample sizes and statistical tests used to analyze the data are indicated in the figure legends. Except for flow cytometry, where samples were assayed once, data obtained from ELISA, Luminex, and qPCR were the result of samples assayed in duplicate in a single experiment. Excluded samples were based on sample quality; flow cytometry samples were excluded from analysis if fewer than 20,000 live cells were detected. An average of 223,292 (range: 37,051 to 341,176) live cells were acquired for flow analysis. Outliers were removed in Fig. 6 (C and D).

Diagnosis of sensitization and allergy

Sensitization and allergy outcomes at 12 months among the infants in the cohort have been previously described (*30*). Briefly, plasma IgE concentrations to a panel of allergens were measured by ImmunoCAP (Thermo Fisher Scientific) at 12 and 24 months. We defined egg-sensitized infants as any infant with IgE specific to hen's egg white above baseline (0.3 kU/liter) at either 12 or 24 months of age or a positive skin prick test (wheal >3 mm) performed shortly after an immediate reaction. Food sensitization was defined as any infant with IgE specific to hen's egg white, cow's milk, or peanut above baseline (0.3 kU/liter) at either 12 or 24 months of age. Physician diagnosis of food allergy was based on guidelines from the National Institute of Allergy and Infectious Diseases (NIAID)–sponsored expert panel report (*78*). Specifically, IgE-mediated egg-allergic infants had a history of immediate-onset reaction after isolated ingestion of egg between 5 and 15 months of age and either elevated egg white–specific IgE, a positive skin prick test, or a positive oral food challenge. In addition to food allergy, some urban infants were diagnosed with AD or allergic proctocolitis by a physician. AD was diagnosed based on recurring or chronic red, pruritic rash, whereas allergic proctocolitis was diagnosed by a medical history of visible specks or streaks of blood in stool in a generally healthy infant whose symptoms resolved when the causative food was eliminated and recurred after food challenge. The urban infants defined as being allergic (food allergy or AD) included in this study were diagnosed by 2 years of age.

Integration of immune cell phenotyping data with plasma immunoglobulin

B and T cell frequencies were obtained by flow cytometry applying manual gating strategies (B and T cells) as well as the SWIFT clustering algorithm (T cells) (*79*, *80*). Five of the SWIFT-identified T cell clusters were previously characterized and classified as T_H2B-like cells (*39*). In total, frequencies were obtained for 49 T cell and 19 B cell populations. These frequencies, in addition to demographic variables collected from questionnaires (*30*), were independent variables used in the quantile regression model. The dependent variables, allergen-specific immunoglobulin isotypes (IgA, IgG, and IgG_4), were measured by Luminex. Variable selection of T cell and B cell populations was performed using hierarchical clustering to avoid collinearity. The variable selection was performed separately on T and B cell phenotyping data. The most abundant populations among the correlated clusters were selected, resulting in five T cell and four B cell populations. Additionally, five demographic variables available for 80% of participants were obtained from the survey data. T cell, B cell, and immunoglobulin response variables were log transformed, and the Shapiro-Wilk test was performed, which was significant for five of the nine T cell and B cell variables and 22 of the 33 antibody response variables. Hence, a quantile regression model was fit using the package quantreg (version 5.98) in R (version 4.2.1). The tau value was set to 0.5 to predict the median immunoglobulin response. Significant ($\alpha = 0.05$) responses were plotted using package ggplot2 (version 3.5.1).

Statistical analyses

Individual-level data are presented in data file S1. R (version 4.2.2), RStudio (2022.12.0 Posit Software), and GraphPad Prism 8 (GraphPad) were used to graph and analyze data for statistical significance. Statistical differences between two groups were analyzed using Wilcoxon test, and differences between more than two groups were analyzed by Dunn's test with Benjamini-Hochberg correction. Differences in the antigen-specific response over time were analyzed by paired Wilcoxon test with Benjamini-Hochberg correction. Transformed data (log, square root, sin, inverse) were checked for normality using Kolmogorov-Smirnov or D'Agostino and Pearson test. Two-way analysis of variance (ANOVA) using transformed data determined the effect of lifestyle, sensitization, age of egg introduction, and frequency of egg consumption on egg-specific antibody. It was also applied to determine effect of lifestyle and infant feeding on fecal IgA as well as *B. infantis* colonization and infant B cell responses. If transformed data were not significant, aligned rank transform test was used to analyze the impact of lifestyle and sensitization on nontransformed data. Participants were divided into upper and lower halves according to the average age of introduction (8 months) in our cohort. Eight months was also the age when lightly cooked egg

introduction was performed in an Australian early introduction trial (*31*). Ingestion was considered frequent if given more than three times a week (*31*). The age and the frequency of introduction of allergenic foods, as well as infant breastfeeding were pulled from visit surveys performed at 4 to 6, 12, and 24 months, and regularity of egg use in the home was assessed from visit surveys at 1 and 4 to 6 months (*30*). Fisher's exact test was used to analyze the proportion of infants with a detectable amount of food antigens and ovalbumin-specific IgA_1/IgA_2. Spearman's correlation was used to analyze correlations between food antigens and infant fecal-maternal milk IgA. Statistically significant *P* values were plotted ($\alpha = 0.05$).

Supplementary Materials

The PDF file includes:
Materials and Methods
Figs. S1 to S19
Table S1
Reference (*81*)

Other Supplementary Material for this manuscript includes the following:
Data file S1
MDAR Reproducibility Checklist

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