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Infant B Cell Memory Differentiation and Early Gut Bacterial Colonization

Anna-Carin Lundell,* Viktor Björnsson,* Annika Ljung,† Margareta Ceder,‡ Susanne Johansen,§ Gunhild Lindehagen,§ Carl-Johan Törnhage,† Ingegerd Adlerberth,† Agnes E. Wold,† and Anna Rudin*

Germ-free animal models have demonstrated that commensal bacterial colonization of the intestine induces B cell differentiation and activation. Whether colonization with particular bacterial species or groups is associated with B cell development during early childhood is not known. In a prospective newborn/infant cohort including 65 Swedish children, we examined the numbers and proportions of CD20+ B cells, CD5+, and CD27+ B cells in blood samples obtained at several time points during the first 3 y of life using flow cytometry. Fecal samples were collected and cultured quantitatively for major facultative and anaerobic bacteria at 1, 2, 4, and 8 wk of life. We found that the numbers of CD20+ B cells and CD5+CD20+B cells reached their highest levels at 4 mo, whereas CD20+B cells expressing the memory marker CD27 were most numerous at 18 and 36 mo of age. Using multivariate analysis, we show that early colonization with Escherichia coli and bifidobacteria were associated with higher numbers of CD20+B cells that expressed the memory marker CD27 at 4 and 18 mo of age. In contrast, we were unable to demonstrate any relation between bacterial colonization pattern and numbers of CD20+B or CD5+CD20+B cells. These results suggest that the intestinal bacterial colonization pattern may affect the B cell maturation also in humans, and that an early gut microbiota including E. coli and bifidobacteria might promote this maturation. 


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Abbreviations used in this article: OPLS, orthogonal partial least squares; PLS, partial least squares; Treg, regulatory T cell; VIP, variable influence on projection.
the B cell population, in a prospectively followed cohort of 65 Swedish infants, using multivariate factor analysis. We demonstrate that children who are colonized early with *Escherichia coli* and/or bifidobacteria have higher numbers of CD27+ B cells at 4 and 18 mo of life than infants who lack these species in the early microbiota. Thus, our results suggest that the bacterial colonization pattern early in infancy may affect B cell maturation later in childhood.

**Materials and Methods**

Subjects and collection of blood samples

Cord blood samples from newborn children (*n* = 41) and peripheral blood samples from children from 3–5 d (*n* = 52), 1 mo (*n* = 53), 4 mo (*n* = 56), 18 mo (*n* = 60), and 36 mo of age (*n* = 55) were obtained from a prospectively newborn infant cohort. The study included 65 healthy Swedish infants (33 boys and 32 girls), born at term (≥38 gestational weeks) in rural areas in southwest Sweden, who were followed to investigate the relation between intestinal bacterial colonization pattern and maturation of the immune system. Ninety-seven percent (63/65) of the children were exclusively or partially breast-fed during the first 6 mo of life. Twelve percent (8/65) of the children were treated with antibiotics in the first 6 mo of life. Peripheral blood was also obtained from 15 healthy adult volunteers, 23–58 y old, who were divided into two groups according to age, that is, individuals younger (*n* = 8) or older (*n* = 7) 35 y of age. All blood samples were collected in preservative-free heparinized tubes. Informed consent was obtained from the parents and volunteers, and the study was approved by the Human Research Ethics Committee of the Medical Faculty, University of Gothenburg, Göteborg, Sweden.

Flow cytometry

Phenotypic characterization of the circulating B cells by flow cytometry was performed within 72 h after venipuncture. Initial experiments showed that this storage time did not affect the numbers or proportions of lymphocytes compared with fresh blood samples (data not shown). All analyses were performed with whole blood samples (50 μl/tube), which were incubated with the respective mAbs for 20 min, 4°C in the dark. The following mAbs were used: PerCP-conjugated anti-CD20 (clone L27, 1/45; BD Bioscience, Erembodegem, Belgium), allophycocyanin-conjugated anti-CD5 (clone UCHT2, 1/90; Pharmingen, San Diego, CA), and FITC-conjugated anti-CD27 (clone L128, 1/20; BD Bioscience). Next, RBCs were lysed with FACS lysing solution according to the manufacturer’s instructions. Allophycocyanin- and FITC-conjugated mouse Ig G1 isotype controls (clone ×40, allophycocyanin IgG1 1/90 and FITC IgG1 1/20; BD Bioscience) were used as references when gating on the CD5- and CD27+ populations within the CD20+ lymphocyte population. As previously described (18), the gate for FOXP3 was set by comparing the expression of FOXP3 in the CD25-, CD5-, or CD27− populations within the CD20+ lymphocyte population. As previously described (18), the gate for FOXP3 was set by comparing the expression of FOXP3 in the CD25-, CD5-, or CD27− populations within the CD20+ lymphocyte population. As previously described (18), the gate for FOXP3 was set by comparing the expression of FOXP3 in the CD25-, CD5-, or CD27− populations within the CD20+ lymphocyte population. As previously described (18), the gate for FOXP3 was set by comparing the expression of FOXP3 in the CD25-, CD5-, or CD27− populations within the CD20+ lymphocyte population. As previously described (18), the gate for FOXP3 was set by comparing the expression of FOXP3 in the CD25-, CD5-, or CD27− populations within the CD20+ lymphocyte population. As previously described (18), the gate for FOXP3 was set by comparing the expression of FOXP3 in the CD25-, CD5-, or CD27− populations within the CD20+ lymphocyte population.

**Sampling and culturing of the gut microbiota**

Fecal samples, which were obtained at 1, 2, 4, and 8 wk of age, were cultured quantitatively for major groups of aerobic and anaerobic bacteria, as previously described (18). In brief, staphylococci were isolated on *Staphylococcus aureus* agar and identified as *Staphylococcus aureus* or coagulase-negative staphylococci with the coagulase test. *E. coli* and other enterobacteria (non-*E. coli* enterobacteria) were isolated on Drigalski agar (Media Department, Clinical Bacteriology, Sahlgrenska University Hospital, Göteborg, Sweden) and identified with the API20E biotyping system (bioMérieux Industry, Marcy l’Etoile, France), whereas enterococci were isolated on Enterococcus agar, in which they hydrolyze esculin (BD Diagnostics, Stockholm, Sweden). Among the anaerobes, *Bacteroides* were isolated on *Bacteroides* Bile Esculin agar (BD Diagnostics), and clostridia were isolated from alcohol-treated samples and identified with the RAPID ID 32A system (bioMérieux Industry). Bifidobacteria were isolated on Biener’s agar (Media Department) and identified by a genus-specific PCR. Putative lactobacilli were identified as Gram-positive unbranched rods growing on Rogosa agar (BD Diagnostics).

**Statistical analysis**

The relations between early intestinal bacterial colonization at 1, 2, 4, and 8 wk (*x*;variables; including *Bacteroides*, bifidobacteria, clostridia, lactobacilli, *E. coli*, other enterobacteria [non-*E. coli*], enterococci, coagulase-negative staphylococci and *S. aureus*) and the numbers of B cells that express CD20, CD5, or CD27 in blood samples collected at 4, 18, and 36 mo of age (*y*-variables) were investigated with multivariate data analysis (SIMCA-P+ version 12; Umetrics, Umeå, Sweden). Projection to latent structures by means of partial least squares (PLS) and orthogonal PLS (OPLS) were implemented to correlate the data matrices X and Y to each other in linear multivariate models. The quality of the analyses was assessed based on the parameters R2 (i.e., how well the variation of the variables is explained by the model) and Q2 (i.e., how well a variable can be predicted by the model). In the OPLS, the importance of each X variable to Y is represented by column bars. Jackknifing was used to calculate SEs displayed as an error bar on each column (representing the 95% confidence interval). Univariate analyses were performed with Kruskal–Wallis test followed by Dunn’s multiple-comparison test, two-tailed Mann–Whitney U test, and Spearman’s rank correlation test (GraphPad Prism; GraphPad Software, La Jolla, CA) as described in the figure legends. A p value ≤0.05 was regarded as being statistically significant (*p* < 0.05, **p* < 0.01, ***p* < 0.001).

**Results**

**Development of numbers and proportions of CD5+, CD20+, and CD27+ B cells during the first 36 mo of life and in adults**

The surface expression of CD5 and CD27 was examined within the CD20+ lymphocyte population (i.e., B cells) based on the gating strategy demonstrated in Fig. 1A. We analyzed these cells both in terms of absolute counts (Fig. 1B–D) and proportions (Fig. 1E–G), in cord blood and peripheral blood obtained from children at the ages 3–5 d, 1 mo, 4 mo, 18 mo, and 36 mo, and from adults. As shown in Fig. 1B and 1E, the numbers and proportions of CD20+ B cells in the circulation did not change during the first days of life. Therefore, this cell population increased considerably, peaked at 4 mo of age, and subsequently decreased in an age-dependent manner. The counts of CD5+ B cells displayed a similar pattern as that observed for the total B cell population (Fig. 1C). The proportion of B cells that expressed CD5 was rather high already at birth (40%) and significantly increased up to 1 mo of age, and at this time point the majority (69%) of the B cells were CD5+. Thereafter, this population clearly decreased with age (Fig. 1F). The numbers of B cells expressing the memory marker CD27 increased more slowly than the counts of CD20+ and CD5+ B cells, and were found to reach a plateau between 18 and 36 mo of age (Fig. 1D). In adults, the numbers of CD27+ B cells were comparable with that observed in children at 4 mo of age. However, as shown in Fig. 1G, the percentage of B cells expressing CD27 was low during the first months of life but increased significantly between 4 and 18 mo, and continued to increase in an age-dependent fashion. We found no differences either in the numbers or in the proportions of the CD20+, CD5+CD20+, or CD27+CD20− B cells between chil-
Children treated or not treated with antibiotics in the first 6 mo of life (data not shown). Moreover, no sex differences in the numbers or in the proportions of the CD20+, CD5+CD20+, or CD27+CD20+ B cells were seen, except for the proportion of CD5+CD20+ at birth when boys had a significantly higher fraction of these cells compared with girls (data not shown).

Early gut colonization by E. coli and bifidobacteria is positively associated with high CD27+ B cell counts later in infancy

It is clear that the gut microbiota has beneficial effects on B cell functions in mice (4, 19), but it is not known whether the intestinal bacterial colonization pattern has an impact on B cell maturation in humans. Thus, in this study, we investigated whether any particular bacterial species or group colonizing the intestine during the first 8 wk of life was associated with the numbers of total CD20+, CD5+, and CD27+ B cells in the circulation at 4, 18, and 36 mo of age.

We investigated the relation between the early intestinal bacterial colonization patterns at 1, 2, 4, and 8 wk of age (x-variables) and total CD20+, CD5+, and CD27+ B cell counts at 4, 18, and 36 mo of age (y-variables), using multivariate factor analysis. Projection to latent structures by means of PLS was performed to determine the influence of each x-variable on the overall model, that is,
variable influence on projection (VIP). VIP values can be used to discriminate between important and unimportant predictors. $x$-variables with VIP values $\geq 1.0$ were included in the final PLS loading scatter plot (Fig. 2A). The scatter plot projected E. coli and bifidobacteria at all time points and CD27$^+$ B cells at 4 mo of age on the right side of the y-axis, indicating a positive association between colonization with these bacteria and high numbers of CD27$^+$ B cells at 4 mo of age. S. aureus colonization at 2, 4, and 8 wk of age, in contrast, were projected on the opposite side, indicating an inverse relation to high CD27$^+$ B cell counts. These association patterns are further depicted in the OPLS loading column plot (Fig. 2B). A similar bacterial association pattern was observed for the proportion of CD27$^+$ B cells at 4 mo of age (data not shown). Clostridia at 1, 2, and 4 wk, enterobacteria (non-E. coli) at 4 wk, and lactobacilli at 4 wk were also projected close to CD27$^+$ B cells at 4 mo (Fig. 2A), but these variables contributed...
less to the model because the smaller the bar and the larger the error bar in the OPLS loading column plot, the smaller and less certain is the contribution (Fig. 2B).

The univariate analyses showed that children colonized with *E. coli* or bifidobacteria at 4 and 8 wk displayed significantly higher numbers of CD27+ B cells at 4 mo of age compared with noncolonized children (Fig. 2C, 2D). In contrast, the numbers of CD27+ B cells tended to be less numerous in children colonized with *S. aureus* at 8 wk compared with children not colonized with such bacteria (Fig. 2E). Regarding CD20+ and CD5+ B cells at 4 mo of age, none of the bacterial species or groups examined was found to be associated to the circulating numbers of these B cell populations, neither in multivariate (Fig. 3A, 3B) nor in univariate analyses (data not shown). Taken together, these results suggest that children who establish an early intestinal flora characterized by *E. coli* and/or bifidobacteria have higher numbers, whereas children colonized with *S. aureus* tended to have lower numbers of CD27+ B cells at 4 mo of age relative to noncolonized children.

Next, we examined the association between bacterial colonization patterns and the counts of different B cell populations at 18 and 36 mo of age. As shown in Fig. 4A, both *E. coli* and bifidobacteria colonization were found to be positively associated to high numbers of CD27+ B cells at 18 mo in multivariate factor analysis. However, the contribution of the x-variables in the OPLS model at 18 mo was less predictive than that obtained at 4 mo of age (Q2 = 0.003 at 18 mo; Q2 = 0.10 at 4 mo). In univariate analyses, children colonized with *E. coli* (at 4 and 8 wk) and bifidobacteria (at 8 wk) displayed significantly higher numbers of circulating CD27+ B cells than children not colonized by these bacteria (Fig. 4B, 4C). Moreover, at 18 mo of age, different association patterns between bacterial colonization and the numbers of CD20+ and CD5+ B cells were observed compared with those obtained at the age of 4 mo (Fig. 5A, 5B and Fig. 3A, 3B, respectively). The bacterial colonization pattern associated to high counts of CD20+ B cells at 18 mo of age was similar to that related to high numbers of CD27+ B cells at the same age, that is, positively associated to *E. coli* and bifidobacteria (Fig. 5A and 4A, respectively). High CD5+ B cell counts in 18-mo-old children were positively associated to early colonization with lactobacilli and bifidobacteria during the first 2 wk of life (Fig. 5B).

Univariate analysis showed that children who were colonized with *E. coli* at 4 wk or lactobacilli at 2 wk had significantly higher numbers of CD20+ or CD5+ B cells, respectively, in the blood at 18 mo of age relative to children not colonized by these bacteria at the same time points (p ≤ 0.05, data not shown). Finally, at 36 mo of age, no associations were found between early intestinal bacterial colonization patterns and the numbers of B cells expressing CD20, CD5, or CD27 (CD20: R2 = 0.29 and Q2 = −0.02; CD5: R2 = 0.27 and Q2 = 0.03; CD27: R2 = 0.13 and Q2 = −0.25; data not shown). In conclusion, children colonized with *E. coli* and/or bifidobacteria during the first 8 wk of life exhibit significantly higher numbers of B cells expressing the memory marker CD27 compared with noncolonized children both at 4 and at 18 mo of age. Early colonization with *S. aureus*, in contrast, was associated to low numbers of CD27+ B cells at 4 mo of age.

Some *S. aureus* strains produce enterotoxins that act as T cell superantigens, and experimental studies demonstrate that *S. aureus* superantigens induce Tregs (20, 21), which have been shown to suppress B cell proliferation and Ig responses (22, 23). In this study, however, no relation between the proportion of FOXP3+ Tregs within the CD4+CD25+ T cell population, and the counts (data not shown) or proportion of CD27+ B cells at either 4 or 18 mo of life was found (Fig. 6A, 6B).

**Discussion**

In this study, we investigated whether early intestinal bacterial colonization patterns were associated to the numbers of CD20+,

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**FIGURE 3.** Relation between early gut bacterial colonization pattern and numbers of CD20+ and CD5+ B cells at 4 mo of age. Orthogonal projections to latent structures by means of PLS plots depicting the association between x-variables (intestinal colonization with bacterial species or groups at 1, 2, 4, and 8 wk of life) and y [the numbers of circulating (A) CD20+ or (B) CD5+ B cells at 4 mo of age]. x-parameters that lie in the same direction as CD20+ or CD5+ B cell counts at 4 mo are positively associated, whereas parameters in the opposite direction are inversely related to high numbers of these cells. Each column displays an uncertainty bar with a 95% confidence interval.
CD5+, and CD27+ B cells later in infancy in a prospectively followed newborn/infant cohort. For the first time, to our knowledge, we demonstrate that gut bacterial colonization pattern may affect B cell activation and maturation also in humans because early colonization with *E. coli* or bifidobacteria was associated to higher numbers of B cells that expressed the memory marker CD27 at 4 mo of age.

**FIGURE 4.** Relation between early gut bacterial colonization pattern and numbers of CD27+ B cells at 18 mo of age. (A) Orthogonal projections to latent structures by means of PLS plot depicting the association between *x*-variables (bacterial species or groups at 1, 2, 4, and 8 wk of life) and *y* (counts of CD27+ B cells at 18 mo of age). *x*-parameters that lie in the same direction as CD27+ B cell counts are positively associated, whereas parameters in the opposite direction are inversely related to high numbers of these cells. Each column displays an uncertainty bar with a 95% confidence interval. (B and C) The blood counts of CD27+ B cells are compared at 18 mo of age for children colonized with (B) *E. coli* or (C) bifidobacteria at 1, 2, 4, or 8 wk of age with children who were not colonized with these bacteria at the same time points. Horizontal bars indicate the median. *p ≤ 0.05 (two-tailed Mann–Whitney U test).

**FIGURE 5.** Relation between early gut bacterial colonization pattern and numbers of CD20+ and CD5+ B cells at 18 mo of age. Orthogonal projections to latent structures by means of PLS plot depicting the association between *x*-variables (bacterial species or groups at 1, 2, 4, and 8 wk of life) and *y* (the numbers of circulating (A) CD20+ or (B) CD5+ B cells at 18 mo of age). *x*-parameters that lie in the same direction as CD20+ or CD5+ B cell counts at 4 mo are positively associated, whereas parameters in the opposite direction are inversely related to high numbers of these cells. Each column displays an uncertainty bar with a 95% confidence interval.
and 18 mo of life. Colonization with S. aureus in the first weeks of life, in contrast, was related to lower numbers of CD27+ B cells at 4 mo of life.

The gut microbiota starts to establish directly after birth and provides a strong stimulus for the development of the immune system (4, 19). Bacteria and bacterial products may cross the intestinal epithelium through several pathways. In mouse models, dendritic cells have the capacity to open up tight junctions and capture Ags using their dendrites (24, 25). In both human and mouse, the small intestine contains lymphoid aggregates that are overlaid by M cells, a unique epithelial cell type specialized for transepithelial transport of macromolecules, particles, and microorganisms (26). Moreover, bacteria that reach high populations in the gut also cross the immature gut barrier and stimulate immune cells via a process termed translocation (27, 28).

In accordance with this, our results show that the presence of E. coli, bifidobacteria, and other cultivable bacteria predicted the variation in numbers of CD27+ B cells to a higher degree at 4 relative to 18 mo of life, and the early bacterial colonization pattern was not at all related to the variation in numbers of memory B cells at 36 mo of age.

B cells are activated by bacterial Ags that bind to the BCR, and B cells also respond to bacterial signals in an innate fashion as they express multiple pathogen recognition receptors, including TLRs. Still, the expression of different pathogen recognition receptors on B cells needs to be further examined in humans, but the expression of TLRs appears to be closely linked to the differentiation stage of the B cell (29–31). Microbial Ags that trigger these receptors can thus directly stimulate the B cells to produce Abs and cytokines. LPS, a cell wall component of E. coli and all other Gram-negative bacteria, activates immune cells that express the LPS receptor TLR4. However, in contrast with mouse B cells, human naive B cells do not express TLR4 (29, 30, 32). In this study, we demonstrate that children who were colonized with E. coli during the first weeks of life had higher numbers of B cells that expressed the memory marker CD27 relative to children not colonized by this bacterium. Because of lack of TLR4 expression on human B cells, it is unlikely that E. coli LPS per se is the factor that expands the number of memory B cells. Moreover, Gram-negative bacteria including Bacteroides and Enterobacteriaceae, other than E. coli, were found to be unrelated to high number of CD27+ B cells. Finally, early colonization with bifidobacteria, a Gram-positive bacterium that colonizes the majority of the children during the first weeks of life, was also associated with increased numbers of CD27+ B cells in the children at 4 and 18 mo of life. Instead, early colonization by E. coli and bifidobacteria, which may reflect successful acquisition of maternal gut bacteria during delivery, could be the trigger of B cell activation. Indeed, the classical infantile bacterial colonization pattern includes E. coli and bifidobacteria among the first bacteria that colonize the large bowel (33, 34).

In parallel with improved sanitary conditions and general cleanliness, the bacterial exposure of the Western infants has been reduced, and apparently this has led to changes in the composition of the gut microbiota since the 1970s (35, 36). As a result, S. aureus, which is primarily considered as a skin bacterium, has emerged as one of the first colonizers of the infantile gut of Swedish infants (37, 38) and colonize ∼70% of the children during the first 8 wk of life (39). The staphylococcal strains can persist for several months in the intestine, but the population counts decrease with age (39), reflecting their poor ability to replicate in a more mature and complex gut flora.

In this study, colonization with S. aureus was associated to lower numbers of circulating CD27+ B cells at 4 mo of age compared with children not colonized with such bacteria. Because presence of intestinal S. aureus might reflect a gut microbiota of low complexity unable to suppress staphylococcal growth, the low numbers of memory B cells in these children could be because of lack of other bacterial species or groups that are normally present in a highly complex microbiota. Moreover, S. aureus enterotoxins have been found to induce Treg in both human and mice (20, 21), and these cells can suppress B cell activation and Ig responses (22, 23). However, we found no relation between the proportion of Treg and proportion of CD27+ B cells in the periphery at either 4 or 18 mo of life. Still, the S. aureus strains isolated in this study need to be further investigated for presence or absence of genes encoding various enterotoxins because these could be differently related to the B cell memory differentiation in the infants.

In accordance with others, we show in this article that the numbers of CD5+ B cells increased during the first 4 mo of life and thereafter decreased in an age-dependent manner (10). In contrast with the counts of CD27+ B cells, we found no relation between the numbers of CD5+ B cells and the early bacterial colonization patterns at 4 mo of age. This indicates that the bacterial colonization patterns may be specifically associated to B cell memory differentiation in early infancy.

In conclusion, children who acquired E. coli and bifidobacteria early in life had higher numbers of circulating CD27+ memory B cells in the circulation later in childhood relative to children not colonized by these bacteria. In contrast, children who harbored

![Graph](https://example.com/graph.png)
S. aureus in the gut microbiota displayed lower circulating numbers of memory B cells. Thus, our results suggest that the early gut bacterial colonization pattern may indeed affect B cell maturation also in humans.

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Disclosures
The authors have no financial conflicts of interest.

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