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ORIGINAL ARTICLE

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Breastfeeding promotes early neonatal regulatory T-cell expansion and immune tolerance of non-inherited maternal antigens

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ABSTRACT

Background: Breastfeeding is associated with long-term health benefits, such as a lower incidence of childhood infections, asthma, obesity and autoimmune disorders. However, little is known regarding how the maternal and neonatal immune systems interact after parturition when the neonate receives nutrition from maternal breast milk.

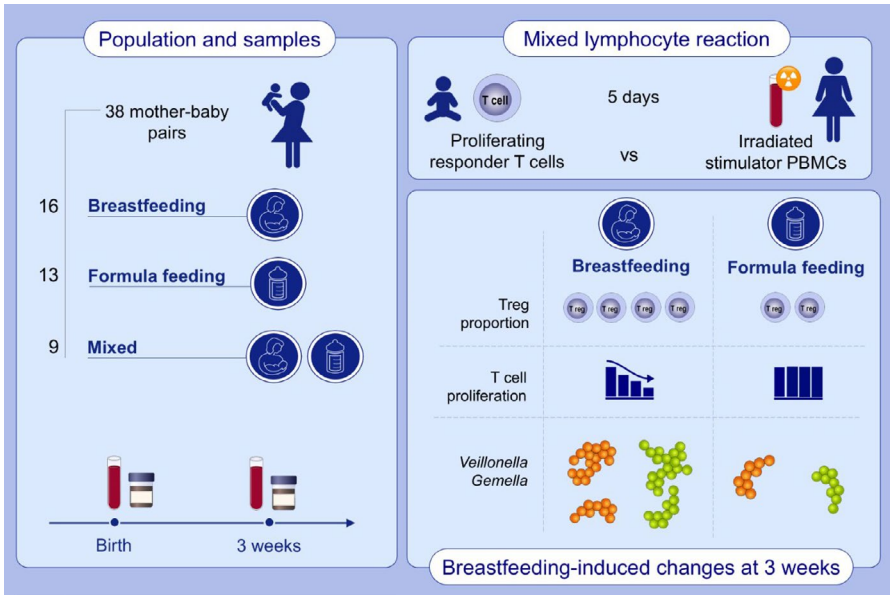
Methods: We undertook a comparative analysis of immune repertoire and function at birth and 3 weeks of age in a cohort of 38 term neonates born by caesarean section grouped according to feeding method (breast milk versus formula). We used flow cytometry to study the immune phenotype in neonatal and maternal blood samples and mixed lymphocyte reactions to establish the proliferation response of neonatal versus maternal lymphocytes and vice versa. The microbiome of neonatal stool samples was also investigated using 16S rRNA sequencing.

Results: We show that the proportion of regulatory T cells (Tregs) increases in this period and is nearly twofold higher in exclusively breastfed neonates compared with those who received formula milk only. Moreover, breastfed neonates show a specific and Treg-dependent reduction in proliferative T-cell responses to non-inherited maternal antigens (NIMA), associated with a reduction in inflammatory cytokine production. We also observed the enrichment of short chain fatty acid producing taxa (*Veillonella* and *Gemella*) in stool samples of exclusively breastfed neonates.

Conclusions: These data indicate that exposure of the neonate to maternal cells through breastfeeding acts to drive the maturation of Tregs and 'tolerizes' the neonate towards NIMA.

KEYWORDS
breastfeeding, microbiome, neonate, non-inherited maternal antigen, regulatory T cell, Th17

Hannah Wood and Animesh Acharjee contributed equally.



GRAPHICAL ABSTRACT

- The proportion of Tregs is nearly twofold higher in exclusively breastfed neonates compared with those who received formula milk only.
- Breastfed neonates show a specific and Treg-dependent reduction in proliferative T-cell responses to non-inherited maternal antigens.
- Short chain fatty acid producing taxa (Veillonella and Gemella) are enriched in stool samples of exclusively breastfed neonates.

Abbreviations: PBMC, peripheral blood mononuclear cell; Treg, regulatory T cell.

1 | INTRODUCTION

Exposure and adaptation to extra-uterine life represents a substantial homeostatic challenge for the cardiovascular and respiratory as well as the immune systems of neonates. Paramount amongst these are modifications within immune function which must facilitate acquisition of a symbiotic microbiome whilst protecting against pathogen challenge. Infection is a significant cause of morbidity and mortality in neonates[1-3] but current understanding of the functional capacity of the neonatal immune system in the first few weeks of life remains limited. Substantial differences are observed between neonatal and adult immune function.[4,5] For example, CXCL8 (IL-8) is the major effector chemokine of neonatal T cells whilst production of IFN-γ is markedly suppressed and a reduction in natural killer (NK) cell numbers is observed.[4,6]

Elegant multi-dimensional analyses have revealed a significant increase or decrease in various immune cell subsets as well as plasma protein levels within the first week of life, initiating a stereotypic immune differentiation pathway. This profile is seen in both preterm and term infants and as such appears to represent a response to multiple environmental cues, predominantly microbial, that are received after birth.[7] Dynamic alterations in the interferon and complement pathways, as well as neutrophil-associated signalling, are particularly prominent.[8] However, less is known regarding neonatal immune function and the potential impact of nutritional intake on immune homeostasis.

An important consideration in relation to neonatal immune function is the threshold of immune response versus tolerance in the early postnatal period. A range of factors act to limit alloreactive immune responses during pregnancy and avoid immunological rejection of the foetus. This includes an increase in both maternal and foetal-derived regulatory T cells (Tregs)[5,9] together with preferential differentiation of foetal CD4+ cells towards Treg phenotype[10] mediated through increased 'tolerogenic' dendritic cell activity.[11] It is likely that this balance towards relative immune suppression continues into neonatal life but the profile of this, and its relative dependence on the postnatal environment, as well as the presence of non-inherited maternal antigens (NIMA) remain unclear.

The perinatal establishment of the gut microbiome is likely to be a dominant regulator of neonatal immune development. Indeed, colonization with specific commensal bacteria can enhance the development of Treg responses,[12] whilst dysbiosis disturbs stereotypic immune development and promotes T-cell activation.[7] Vertical transmission of maternal microbiota is the initial, and potentially most important, determinant of the neonatal microbiome. In this regard, it is notable that the mode of delivery is a critical factor and its influence on childhood microbiome extends for at least 7 years.[13] The influence of microbiome composition on long-term health outcomes is an area of considerable interest and atypical colonization has been associated with a range of conditions including impaired immune function and increased risk of allergy.[14]

The importance of nutrition as a determinant of the profile of neonatal immunity has been poorly investigated.[15,16] The natural nutrition for neonates is from breast milk which contains a range of complex nutrients as well as antimicrobial proteins. Breast milk also contains bacteria and maternal cells, and as such, it is not surprising that exposure to either breast milk or formula milk significantly influences the composition of the gut microbiome.[17]

Differential microbiome composition is likely to act as an indirect influence on how nutrition can modify the neonatal immune profile but there may also be a direct effect from exposure to maternal cells and antigenic proteins within milk. We analysed how immune phenotype and function evolve between birth and 3 weeks of age in a cohort of healthy neonates born by caesarean section. This timeframe was chosen to allow for changes related to the type of milk received to occur but to avoid potential confounding effects of immunization and acquired infections. We designed mixed lymphocyte reactions that allowed us to study the interaction of the maternal and neonatal immune systems. Our aim was to determine prospective changes in the neonatal adaptive immune response in relation to source of nutrition and neonatal gut microbiome with a specific focus on regulatory T cells.

2 | METHODS

A total of 38 healthy pregnant women who were planned to deliver electively at gestational term by caesarean section were sampled in our study at Birmingham Women's Hospital, UK. Peripheral blood samples were collected prior to the caesarean section and all women were not in labour and had intact membranes. Sixteen out of 38 babies (42%) were exclusively breastfed for the duration of the study, whilst 9 babies received mixed feeding and 13 babies were exclusively formula-fed. Further characteristics of the neonatal population are male/female: 17/21, gestational age: 39 [39–39] weeks and birth weight: 3530 [3298–3733] g.

Cord blood samples were taken immediately after delivery. A peripheral blood sample (up to 2 mL) was taken from the neonate at 3 weeks of age. In addition to the blood samples, a neonatal stool sample was collected at birth and at 3 weeks of age from 29 of the recruited neonates. Exclusion criteria included multiple pregnancy, sepsis risk factors (especially maternal fever or chorioamnionitis), Group B Streptococcus positivity in the current pregnancy, genetic conditions of the foetus or the mother, maternal HIV, maternal tuberculosis, maternal new-onset viral infection, maternal hypertensive disorder, maternal endocrine condition or diabetes, maternal asthma and maternal autoimmune conditions, as well as maternal medication other than pregnancy supplements. Peripheral blood samples were also collected from 13 healthy, non-pregnant adults (male/female: 6/7) who were age-matched to the pregnant women.

Informed written consent was obtained from all pregnant women and healthy volunteers. The study was reviewed and approved by the East Midlands—Nottingham 2 NHS Research Ethics Committee (reference 16/EM/0379).

2.1 | Mononuclear cell isolation from blood samples

Blood samples were collected into EDTA anticoagulated tubes. Peripheral blood mononuclear cells (PBMCs) were isolated by density centrifugation using Lymphoprep (Stemcell Technologies, Seattle, WA, USA). PBMCs were washed with RPMI-1640 medium (Sigma-Aldrich, St. Louis, MO, USA) and used either directly or cryopreserved in foetal bovine serum containing 10% dimethyl sulphoxide (DMSO) (Sigma-Aldrich). After thawing, cells were washed with RPMI and re-suspended in enriched media (RPMI-1640 + foetal bovine serum 10% + 2 mM glutamine). Viability of thawed cells was consistently >90% as assessed by methylene blue exclusion. For immunophenotyping, a small portion of cells was frozen from all blood samples and samples were processed in batches after thawing. For mixed lymphocyte reactions, cord blood and neonatal blood samples at 3 weeks of age were used directly. Pregnant and non-pregnant adult samples were split at the time of sampling and one part of the samples was used directly with cord blood, whilst the other part was frozen and retained to be used 3 weeks later with the neonatal blood.

2.2 | Immunophenotyping

Mononuclear cells were prepared as described above for two immunophenotyping panels. Panel 1 was designed to study intracellular cytokine production following stimulation, using unstimulated cells as controls. Cells were stained with FITC-conjugated CD107a (Biolegend, San Diego, CA, USA) at the time of stimulation, as this marker is only detectable on the cell surface upon degranulation in conjunction with stimulation.[18] Stimulation was performed with phorbol myristate acetate (PMA, 50 ng/mL, Sigma-Aldrich) and ionomycin (1 μg/mL, Sigma-Aldrich) for a total of 3.5 hours at 37 degrees centigrade. After 30 minutes, 1.25 μg/mL of monensin (Sigma-Aldrich) was added to stimulated cells for the remaining 3 hours. Cells were then washed with phosphate-buffered saline (PBS, Sigma-Aldrich) and re-suspended in MACS buffer. Cell surface staining was performed for 30 minutes on ice in the dark as follows: BV510-conjugated CD4, PerCP-Cy5.5-conjugated CD8, ECD-conjugated CD14/CD19/CD56, APC-Cy7-conjugated CD3 (all from Biolegend) and Live/Dead (red, 488 nm, Invitrogen, Carlsbad, CA, USA). After washing, cells were fixed with fixation/permeabilization solution (eBioscience, San Diego, CA, USA) for 30 minutes at room temperature in the dark. Cells were washed with permeabilization buffer (eBioscience). Following centrifugation and re-suspension in MACS buffer, the following intracellular dyes were added for 30 minutes at room temperature in the dark: PE-Cy7-conjugated IL-6, AF700-conjugated IFN-γ, AF647-conjugated IL-4, BV421-conjugated IL-17A and PE-conjugated IL-8 (all from Biolegend). Cells were then washed and run on an LSRII flow cytometer equipped with blue, red and violet lasers (BD Biosciences, San Jose, CA, USA). The staining procedure was identical for unstimulated cells. At least 50,000 cells were recorded per sample. Unstained and single-stained samples were used as compensation controls in flow cytometry experiments. All antibodies and dyes were used in concentrations recommended by the relevant manufacturer.

Panel 2 assessed the immunophenotype of cells without mitogenic stimulation. Cells were surface stained in MACS buffer

with PE-Cy7-conjugated HLA-DR, BV510-conjugated CD4, PerCP-conjugated CD69, ECD-conjugated CD14/CD19/CD56, AF700-conjugated CD45RA, APC-Cy7-conjugated CD3, BV421-conjugated CD25, BV605-conjugated CD31 (all from Biolegend) and Live/Dead (red, 488 nm, Invitrogen). After washing, cells were fixed with fixation/permeabilization solution (eBioscience) for 30 minutes at room temperature in the dark. Cells were washed with permeabilization buffer (eBioscience). Following centrifugation and re-suspension in MACS buffer, AF647-conjugated FoxP3 (Biolegend) antibodies were added for 30 minutes in the dark. Cells were then washed and run on an LSRII flow cytometer equipped with blue, red and violet lasers (BD Biosciences). At least 50,000 cells were recorded per sample. Unstained and single-stained samples were used as compensation controls in flow cytometry experiments. All antibodies and dyes were used in concentrations recommended by the relevant manufacturer.

In both panels during the gating process, doublets were first excluded based on FSC-A and FSC-H characteristics. Lymphocytes were then identified based on FSC-A and SSC-A characteristics. Dead, as well as CD14+, CD19+ and CD56+ cells were excluded based on positivity in the ECD channel. Further gating was performed within CD3+ cells (Supplementary Figure 1). Flow cytometry data were analysed using the FlowJo software package.

2.3 | T cell proliferation using mixed lymphocyte reaction assay

Mononuclear cells were prepared as described above. Stimulator cells were extracted and re-suspended in enriched media for irradiation. Cells were irradiated at 3000 rad. For responder cells, T-cell enrichment was performed using the Easy Sep T cell enrichment antibody mix (1 μL per 10^6 cells), magnetic beads and Easy Sep Purple Magnet separation (Stemcell Technologies) as described earlier.[19] Mixed lymphocyte reactions (MLRs) were applied in the following combinations: maternal T-cell responders vs irradiated cord blood cells, cord blood T-cell responders vs irradiated maternal cells, maternal T-cell responders vs irradiated neonatal cells and neonatal T-cell responders vs irradiated maternal cells. For the set-up of neonatal MLRs, all corresponding maternal cells had undergone prior cryopreservation and were thawed as described above. In selected maternal, cord blood and neonatal samples (n = 6 each), the T cell-enriched suspensions were split between non-CD25-depleted and CD25-depleted samples. Suspensions that were retained for CD25 depletion were depleted using CD25 MicroBeads with magnetic MACS microcolumn separation (Miltenyi Biotec, Bergisch Gladbach, Germany).

To trace proliferation of responder T cells in the assay, 1 μL of CellTrace Violet dye (Invitrogen) was added per 10^6 cells and incubated at 37 degrees for 20 minutes. 2 x 10^5 irradiated stimulator mononuclear cells were added at a 2:1 ratio to each responder sample of 1 x 10^5 in a 96-well round-bottom plate in enriched media. Each sample was run in duplicate. Positive control samples were established with the addition of 5 μL of CD3/CD28 activator Dynabeads (Thermo Fisher Scientific, Waltham, MA, USA) instead of stimulator cells. Negative controls were established in enriched media only. To each sample and positive control, 10 U of IL-2 cytokine was added. Samples were incubated for 5 days. At day 3, 150 μL of media per well was replaced with fresh media. Each sample was harvested and washed with phosphate-buffered saline (PBS) (Sigma-Aldrich). Surface staining was performed in MACS buffer using APC-Cy7-conjugated CD3, BV510-conjugated CD4 and PerCP-Cy5.5-conjugated CD8 (all from Biolegend). Samples were washed and then re-suspended. 1 μL of propidium iodide (Biolegend) was added for live/dead discrimination to each sample immediately before flow cytometry was performed on an LSRII flow cytometer equipped with blue, red and violet lasers (BD Biosciences). At least 20,000 cells were recorded per sample. Unstained and single-stained samples were used as compensation controls in flow cytometry experiments. All antibodies and dyes were used in concentrations recommended by the relevant manufacturer.

During the gating process, doublets were first excluded based on FSC-A and FSC-H characteristics. Lymphocytes were then identified based on FSC-A and SSC-A characteristics. Dead cells were excluded based on positivity in the ECD channel. Further gating was performed within CD3+ cells (Supplementary Figure 1). Flow cytometry data were analysed using the FlowJo software package.

2.4 | Cytokine production

MLRs were applied in the combinations as described above. On day 5, supernatants from each well (100 μL) of selected samples (n = 13) were collected and frozen. The concentration of cytokines in each supernatant sample was analysed in batches after thawing using a custom designed Luminex plate as per the manufacturer's instructions (Bio-Techne, Minneapolis, MI, USA). The concentration of IFN-γ, IL-4, IL-6, IL-8, IL-10, IL-17 and TNF-α were analysed using a Bio-Plex 200 plate reader and the Bio-Plex Manager 6.1 software (Bio-Rad, Hercules, CA, USA).

2.5 | Stool DNA extraction, amplification and sequencing

2.5.1 | Neonatal stool collection

Whole nappies were removed by the parents and placed into a transport bag. Samples were then taken from the nappies by a gloved study personnel using a sterile scoop and then placed into sterile glass containers. Stool samples were frozen immediately and stored at −20 degrees until DNA extraction.

2.5.2 | Extraction of neonatal stool DNA

DNA was extracted from thawed stool samples using the QIAamp Fast DNA Stool Mini Kit (Qiagen, Hilden, Germany). For all extractions, 290–310 mg of stool was transferred into Lysing Matrix E

2 mL tubes (MP Biomedicals, Illkirch-Graffenstaden, France). Tubes without stool were used as negative controls for each batch of samples. Samples underwent 4 cycles of bead beating for 30 seconds using the FastPrep-24 5G Instrument (MP Biomedicals). The suspensions were heated to 95 degrees for 5 minutes and then centrifuged (2 minutes, 12000 rpm). The remaining extraction steps were performed according to the manufacturer's instructions. DNA was eluted using 100 μL of elution buffer and samples were centrifuged for 2 minutes to elute the DNA. DNA yield was assessed using the Qubit dsDNA HS Assay kit with a Qubit 2.0 fluorometer (Invitrogen). Extracted DNA was stored at −80 degrees until amplified.

2.5.3 | Amplification of bacterial DNA

16S rRNA genes were amplified with primers targeting the V4 region using the standardized Earth Microbiome 16S Illumina Amplicon protocol.[20] Samples were processed in batches with appropriate negative controls to ensure there were no contaminants arising from the DNA extraction kits as described earlier.[21] Following clean-up, the amplicon fragment lengths were assessed for quality using Tapestation (Agilent, Santa Clara, CA, USA). DNA was quantified for each amplicon using the Qubit dsDNA HS Assay kit with a Qubit 4.0 fluorometer (Invitrogen). Each DNA library was normalized to a DNA concentration of 4 nM and then pooled to contain 5 μL DNA from each sample. The quality of the pooled DNA sample was assessed on Tapestation and demonstrated an average base pair length of 401 bp. Using the Qubit method described above, the pooled DNA was quantified to an average 1.61 ng/μL.

2.5.4 | Sequencing and Identification of bacterial DNA

The pool of stool DNA was sequenced in one sequencing run. Sequences were obtained using an Illumina MiSeq paired-end 250-bp protocol for 500 cycles. The PCR was performed in one batch with appropriate negative controls following which paired-end sequencing (2 × 250 bp) was performed on the Illumina MiSeq platform (Illumina, San Diego, CA, US) and processed using the Quantitative Insights Into Microbial Ecology 2 (QIIME2) pipeline.[22] Samples were rarefied prior to alpha and beta diversity analysis. Taxonomy assignment was done against the Silva-132–99% OTUs database and differences in relative abundance of taxa between cohorts were analysed using linear discriminant analysis (LDA) effect size (LEfSe). Taxa with LDA >2 at a p value <0.05 were considered significant.

2.6 | Univariate statistical modelling

2.6.1 | Statistical analysis

Comparisons were made using the Kruskal-Wallis test or the Mann-Whitney U test as the distribution of data appeared to be non-normal according to the Shapiro-Wilk test. p values <0.05 were considered significant. Data are presented as median [interquartile range]. Statistics were calculated using the GraphPad Prism 5 and 8 software.

2.6.2 | Random Forest machine learning method

We used Random Forest (RF) for data integration and individual data set analysis. RF is a machine learning ensemble method in conjunction with multiple learning algorithms to obtain better predictive performance.[23] RF can be used for both classification and regression. In our analysis, we used RF for classification using the feeding method (exclusively breastfed vs exclusively formula-fed) as outcome variable and treating each of the data sets separately. We used ntree = 500 and mtry = square root of variables in our models. We used two packages for RF analysis (randomForest and varSelRF) in R (v3.6.1).

2.6.3 | The Backward elimination method

To select features automatically, we iteratively fitted random forests, at each iteration building a new forest after discarding 20% of the features with the smallest variable importance. The selected set of features was used as a predictor to fit the model to check the 'out of bag' (OOB) error rate. We examined the OOB error rates from all fitted random forests. We chose the solution with the smallest number of variables whose error rate was within one standard error. This procedure was performed iteratively using the varSelRF package in R (v3.6.1).

2.6.4 | Network analysis

We used the qgraph package in R (v3.6.1) to perform network analysis. A network is a set of nodes and a set of edges, where each node represents either an immune parameter or an operational taxonomic unit (OTU) from microbiome analysis whereas the edges represent associations amongst them. Pearson correlation coefficients were used to quantify the strength of associations between combinations of immune parameters or OTUs.

3 | RESULTS

3.1 | Neonates develop both protective and tolerogenic adaptive immune responses in the first three weeks of life

The proportion of CD3+ cells was lower in cord blood compared with maternal blood, whilst that of CD4+ cells was nearly twofold higher. CD8+ cell percentages both at birth and at 3 weeks of age were nearly half of those seen in maternal and non-pregnant adult samples (Figure 1a).

The proportion of CD4+ FoxP3+ CD25hi Tregs increased from 6.4% in cord blood to 8.0% within the first 3 weeks of life, comparable to the level in non-pregnant adults but remained lower than third trimester maternal samples (Figure 1b). Interestingly, within the different feeding groups at 3 weeks of age, the frequency of Tregs was nearly twofold higher (9.3% vs 4.9%) in exclusively breastfed compared with exclusively formula-fed neonates (Figure 1c&d). We also examined the expression of selected cell surface markers on Treg cells at birth and at 3 weeks of age. HLA-DR expression increased during the first 3 weeks of life, potentially reflecting recent activation; however, no difference was detected in the expression of CD45RA, CD31 or CD69 between the two time points (Figure 1e). Of note, no difference was observed in the expression of these markers between the different feeding groups.

We then went on to determine the functional activity of T cells through analysis of intracellular cytokine and surface CD107a expression, a marker of degranulation in response to mitogenic stimulation. The proportion of IL-8+ CD4+ cells was lower, whereas that of IFN-γ+ CD4+ cells was higher in maternal compared with neonatal samples (Supplementary Figure 2). No differences were observed in the profile of IFN-γ, IL-4, IL-6 or IL-8 expression by CD4+ and CD8+ cells between birth and 3 weeks of age. However, the number of IL-17+ CD8+ cells, as well as the mean fluorescence intensity of IL-17 in CD4+ and CD8+ cells, increased during the first three weeks (Figure 2a and Supplementary Figure 3). Interestingly, at 3 weeks of age the mean fluorescence intensity of IFN-γ in CD4+ and CD8+ cells was nearly three times higher in exclusively formula-fed neonates compared with those receiving breast milk (Figure 2b). No further differences were observed between feeding groups.

A higher proportion of CD8+ cells expressed CD107a, a marker of cytotoxic degranulation, in non-pregnant adult samples compared with neonatal samples at birth and 3 weeks of age. The mean fluorescence intensity of CD107a expression on CD8+ cells

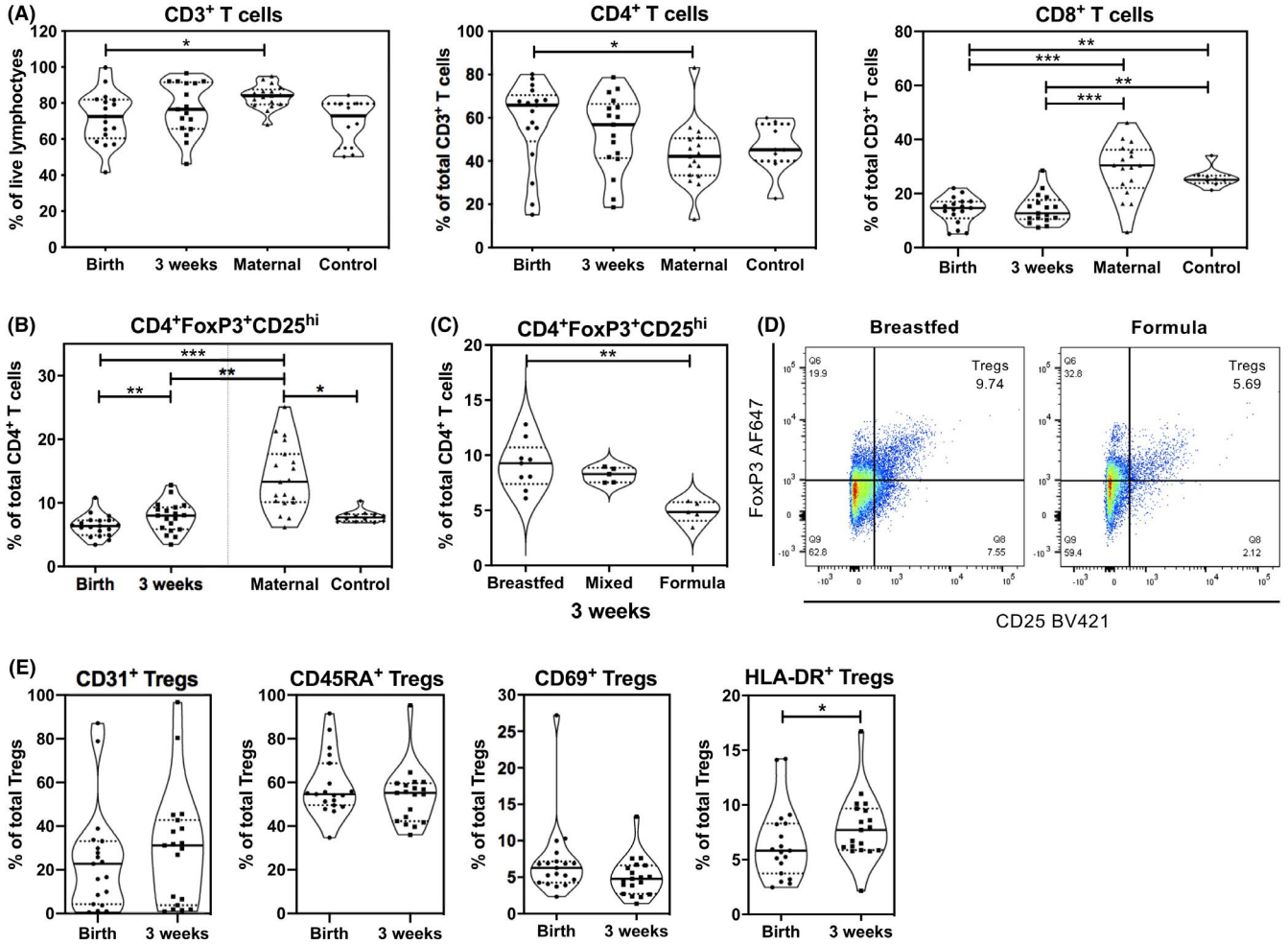


FIGURE 1 Alterations of T-cell subsets and the regulatory T-cell (Treg) phenotype in neonates between birth and 3 weeks of age. **A,** The frequency of CD3+, CD4+ and CD8+ cells in neonatal blood samples (n = 17) at birth and at 3 weeks of age, as well as in maternal blood (n = 17) and healthy controls (n = 8). **B,** The frequency of CD4+ FoxP3+ CD25hi cells in neonatal blood samples (n = 19) at birth and at 3 weeks of age, as well as in maternal blood (n = 19) and healthy controls (n = 13). **C,** The frequency of CD4+ FoxP3+ CD25hi cells in neonatal blood samples at 3 weeks of age grouped according to the feeding method: exclusively breastfed (n = 9), mixed feeding (n = 5) and formula-fed (n = 5) neonates. **D,** Representative dot plots of CD4+ FoxP3+ CD25hi cells in a breastfed and a formula-fed neonate at 3 weeks of age, gated within CD4+ cells. **E,** The expression of selected cell surface markers on Tregs in neonatal blood samples at birth and at 3 weeks of age (n = 19). Horizontal lines represent medians and interquartile ranges. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

was higher in adult compared with neonatal samples (Figure 2c and Supplementary Figure 4). No differences were observed between feeding groups.

3.2 | T cells of exclusively breastfed neonates show reduced proliferation in response to stimulation by maternal cells

We performed MLRs on blood samples of 37 mother-and-baby dyads. Initially, maternal cells were stimulated with irradiated cord or neonatal cells and here we observed increased proliferation of CD3+ and CD4+ cells in response to neonatal PBMCs at 3 weeks of age compared with birth (Supplementary Figure 5a). A similar increase (CD3+: 5.4 vs 15.5% median proliferating cells, CD4+: 5.3 vs 18% median proliferating cells) was observed when responder T cells of a non-pregnant healthy adult were used in combination with neonatal stimulator cells (Supplementary Figure 5b).

We next utilized cord and neonatal T cells in MLRs against irradiated maternal PBMCs (Figure 3a). The rate of proliferation on Figure 3a is represented by the proportion of cells with a lower concentration of the CellTrace Violet dye, signifying the dilution of the dye in proliferating cells. Interestingly, here we observed decreased proliferation in neonatal CD3+ and CD8+, but not CD4+ cells in response to maternal stimulator cells at 3 weeks of age compared with birth (Figure 3b).

We further examined the influence of neonatal nutrition on these proliferative responses at 3 weeks of age. Sixteen neonates had been exclusively breastfed whereas 13 had received only formula and 8

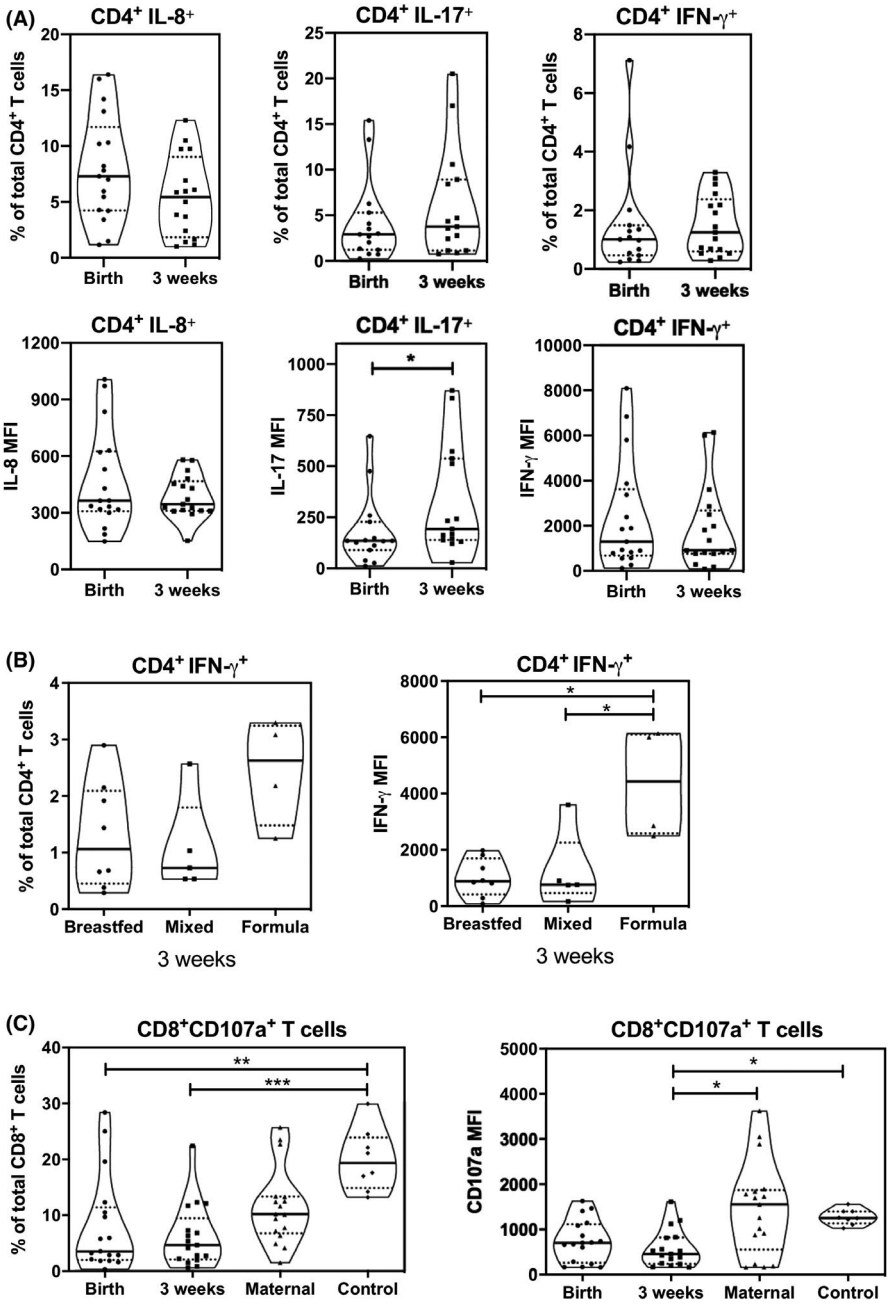


FIGURE 2 Alterations of the pro-inflammatory and cytotoxic immunophenotype in neonates between birth and 3 weeks of age. **A,** The intracellular frequency and mean fluorescence intensity (MFI) of selected pro-inflammatory cytokines in CD4+ cells in neonatal blood samples at birth and at 3 weeks of age (n = 17). **B,** The intracellular frequency and MFI of interferon gamma (IFN-γ) in CD4+ cells in neonatal blood samples at 3 weeks of age grouped according to the feeding method: exclusively breastfed (n = 8), mixed feeding (n = 5) and formula-fed (n = 4) neonates. **C,** The frequency of CD8+ and CD8+ CD107a+ cells and MFI of CD107a in CD8+ cells in neonatal blood samples (n = 17) at birth and at 3 weeks of age, as well as in maternal blood samples (n = 17) and healthy controls (n = 8). Horizontal lines represent medians and interquartile ranges. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

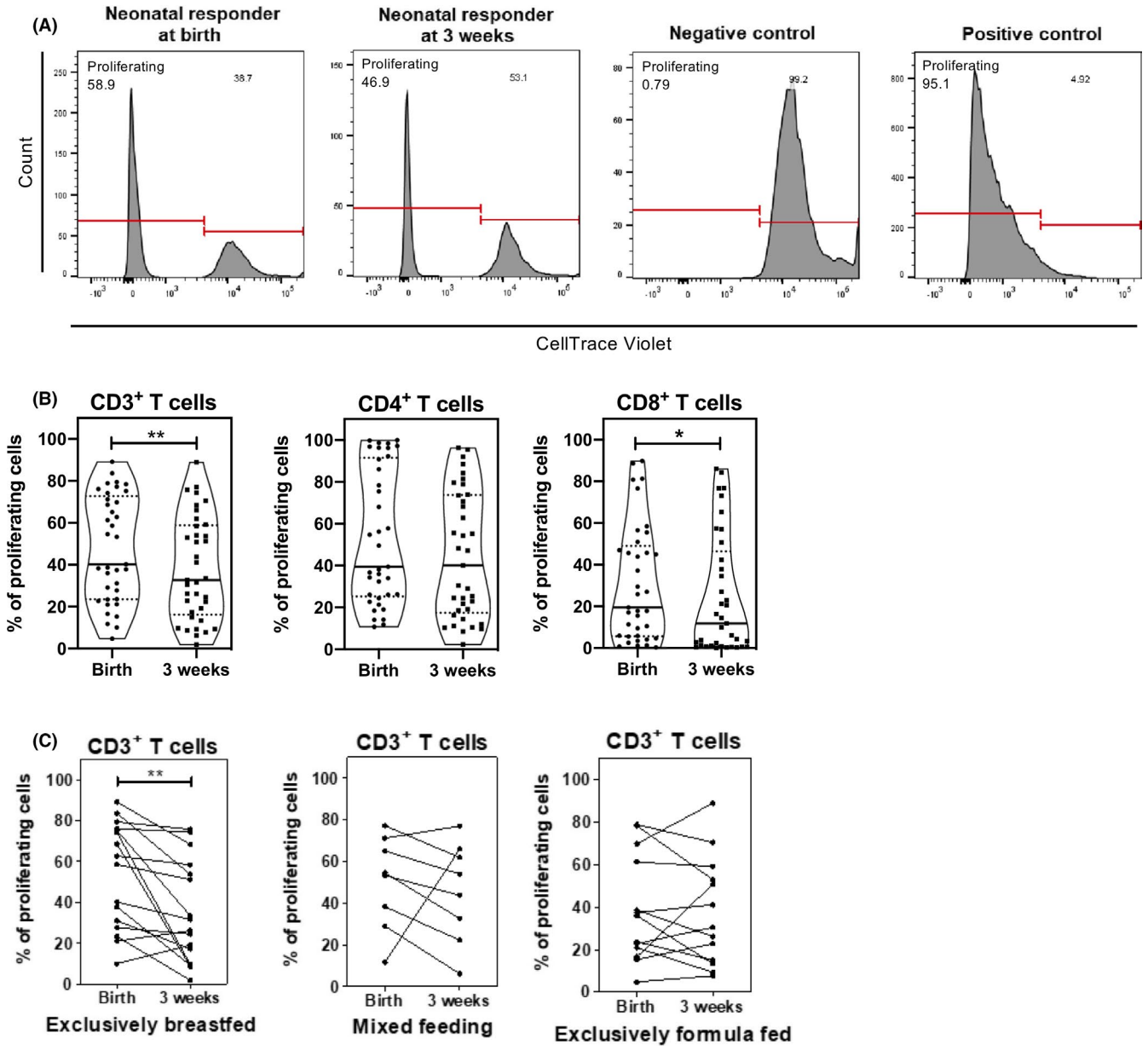


FIGURE 3 Neonatal T-cell response upon maternal antigen stimulation. **A,** Representative sample of a mixed lymphocyte reaction (MLR) at birth and at 3 weeks of age with negative and positive controls. Positive controls were established with the addition of CD3/CD28 activator beads instead of stimulator cells. Negative controls were established in enriched media only. Samples were incubated for 5 days. **B,** Percentage of neonatal proliferating T cells (n = 37) at birth and at 3 weeks of age in response to maternal irradiated cells in the CD3+, CD4+ and CD8+ cell subsets. **C,** Percentage of neonatal proliferating CD3+ cells at birth and at 3 weeks of age in response to maternal irradiated cells grouped according to the feeding method: exclusively breastfed (n = 16), mixed feeding (n = 8) and formula-fed (n = 13) neonates. Horizontal lines represent medians and interquartile ranges. * $p < 0.05$, ** $p < 0.01$.

had undergone a mixed milk intake. The decrease in the proliferation rate of CD3+ cells was still evident in exclusively breastfed neonates (60.7 vs 28.9% median proliferating cells) but was not present in the mixed feeding and exclusively formula-fed groups (Figure 3c). The same pattern was observed in CD4+ and CD8+ cells. Interestingly, the proliferation rate of CD3+, CD4+ and CD8+ cells of exclusively breastfed neonates was comparable at birth and at 3 weeks of age when PBMCs of a non-pregnant healthy adult were used as stimulators (n = 6), reflecting that the neonatal tolerance is specific to maternal antigens.

3.3 | Neonatal immune tolerance promoted by breastfeeding is mediated by regulatory T cells and is associated with a reduction in release of inflammatory cytokines

Having observed that breastfeeding promotes the expansion of Tregs and suppresses proliferative responses against maternal antigen, we next investigated whether this immune tolerance was dependent on the presence of Tregs. To this end, we repeated the MLRs on a set of 6 mother-and-baby dyads where babies were exclusively breastfed

following the depletion of CD25+ cells (Figure 4a). Pregnancy is associated with peripheral accumulation of Tregs and the proliferation of maternal CD3+ cells in response to neonatal antigens increased both at birth (20.4 vs 59.7% median proliferating cells) and at 3 weeks of age (34.1 vs 57.1% median proliferating cells) following depletion of CD25+ cells (Figure 4b). The same pattern was observed for CD4+ but not in CD8+ cells. Of note, the proliferation of neonatal CD3+ cells in response to maternal antigens increased after depletion of CD25+ cells in samples taken at 3 weeks of age (71.4 vs 85.1% median proliferating cells) but this was not seen with the use of cord blood cells (Figure 4c). The same pattern was observed for CD4+ but not in CD8+ cells.

We also measured the concentration of cytokines in MLR supernatants (n = 11) produced by neonatal T cells of exclusively breastfed neonates in response to maternal antigens. The concentration of IFN-γ and TNF-α was found to be lower at 3 weeks of age compared with birth, whereas no difference was observed in case of the other cytokines tested (IL-4, IL-6, IL-8, IL-10, IL-17). The maximal IFN-γ and TNF-α producing capacity of neonatal T cells, tested by culturing with CD3/CD28 activator beads, was higher both at birth and at 3 weeks of age compared with the level seen in response to maternal antigens at 3 weeks (Figure 4d). As such, breastfeeding is seen to suppress the inflammatory Th1 cytokine response of neonatal T cells in response to maternal antigen stimulation.

3.4 | Breastfeeding has modest impact on the gut microbiome in neonates born by caesarean section within the first 3 weeks of life

To evaluate the impact of breastfeeding on the neonatal gut microbiome, we analysed stool samples from exclusively breastfed and exclusively formula-fed neonates collected at 3 weeks of age. Meconium samples had also been collected at birth but the amount of DNA extracted from these samples was consistently <0.15 ng/μL. PCR amplification using 16S primers on these DNA samples yielded undetectable product for further analysis, reflecting minimal or no microbial colonization immediately after birth.

In the stool samples at 3 weeks of age, following microbial 16S rRNA gene amplification, a median frequency of 18,354 amplicon

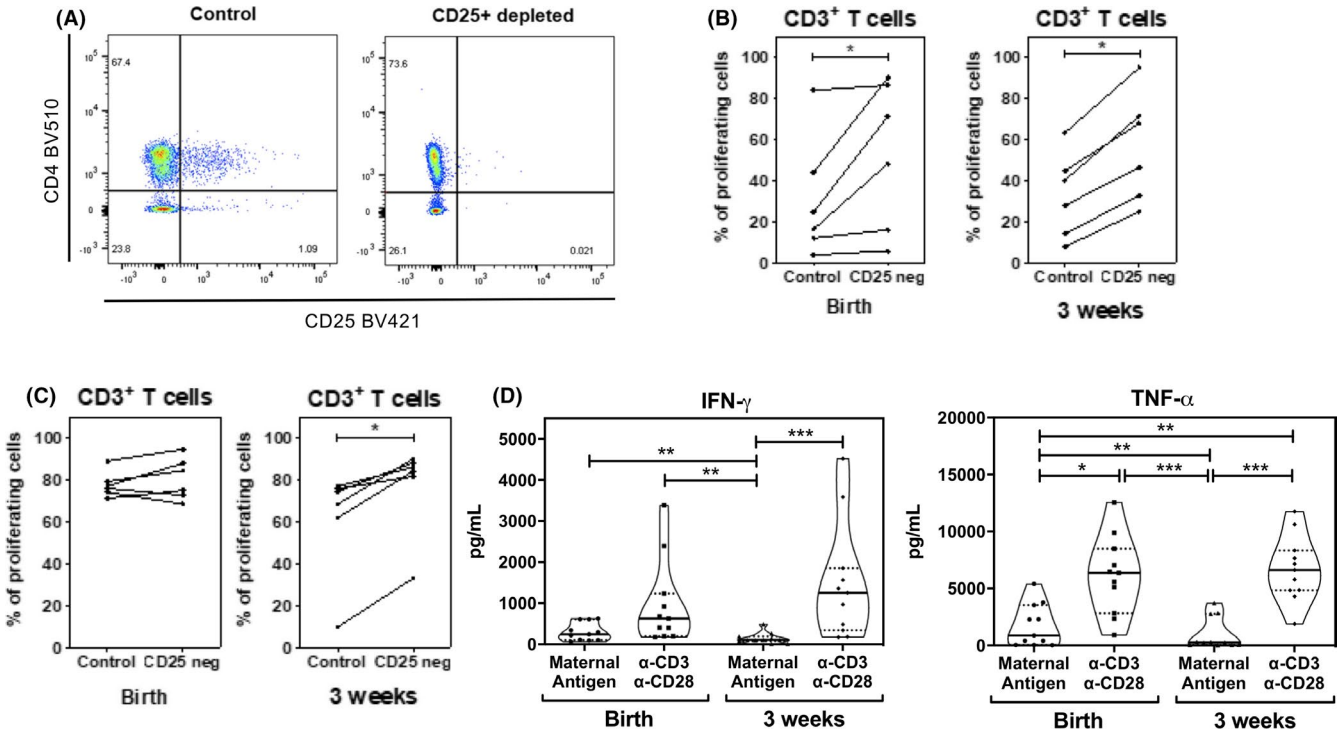


FIGURE 4 Maternal and neonatal T-cell response in mixed lymphocyte reactions (MLR) following the depletion of CD25+ cells and pro-inflammatory cytokine production by neonatal T cells in exclusively breastfed neonates in MLR upon maternal antigen stimulation at birth and at 3 weeks of age. **A,** Representative dot plots with and without the depletion of CD25+ cells in a neonatal sample at 3 weeks of age, gated within CD3+ cells. CD25+ cells were depleted using magnetic microbead separation. **B,** Percentage of maternal proliferating T cells (n = 6) in response to neonatal stimulator cells of exclusively breastfed neonates from birth and 3 weeks of age in the CD3+ subset. **C,** Percentage of neonatal proliferating T cells (n = 6) in response to maternal stimulator cells at birth and at 3 weeks of age in the CD3+ subset of exclusively breastfed neonates. **D,** The concentration of IFN-γ and TNF-α was found to be lower at 3 weeks of age compared with birth (n = 11). The maximal IFN-γ and TNF-α producing capacity of neonatal T cells was also assessed at birth and at 3 weeks of age by culturing them with CD3/CD28 activator beads. The production of IFN-γ and TNF-α was higher both at birth and at 3 weeks of age compared with the level seen in response to maternal antigens at 3 weeks. Horizontal lines represent medians and interquartile ranges. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

sequence variants (ASV) per sample were retained after trimming and filtering. The composition of gut microbiota from exclusively breastfed and exclusively formula-fed neonates was broadly similar, and no differences in any alpha or beta diversity metrics were seen (Figure 5a). Principal component analysis (PCA) of gut microbiota composition of exclusively breastfed and exclusively formula-fed neonates demonstrated that individuals in these groups cluster closely together, and breastfeeding is associated with the presence of *Gemella* (Figure 5b). Linear discriminant analysis (LDA) effect size (LEfSe) revealed enrichment of the *Veillonella* and *Gemella* taxa in exclusively breastfed neonates (Figure 5c). Random forest (RF) analysis identified the presence of *Staphylococcus*, followed by that of *Gemella* to be the most significant parameters distinguishing the two groups of exclusively breastfed and exclusively formula-fed neonates (Figure 5d).

3.5 | Network modelling links Veillonella to regulatory T-cell expansion whilst skin-associated bacteria enhance T-cell proliferation in breastfed neonates

Finally, we undertook an integrative analysis of the combined data from flow cytometry, MLR and microbiome sequencing of neonates at 3 weeks of age. Network modelling revealed a range of positive and negative correlations between these parameters. In breastfed neonates, CD4+ and CD8+ proliferative responses against maternal antigen were strongly correlated with *Gemella* and skin-associated taxa (Figure 6a). The presence of *Veillonella* within the microbiome correlated with the prevalence of Tregs at 3 weeks, and this effect was independent of nutrition history (Figure 6b). In breastfed neonates, *Veillonella* was also associated with HLA-DR expression on CD4+ cells and IFN-γ production in CD8+ cells. These findings suggest that *Veillonella* may act to enhance regulatory responses in the early period of life whereas skin-associated bacteria, potentially acquired through breastfeeding, may act to promote proliferative responses.

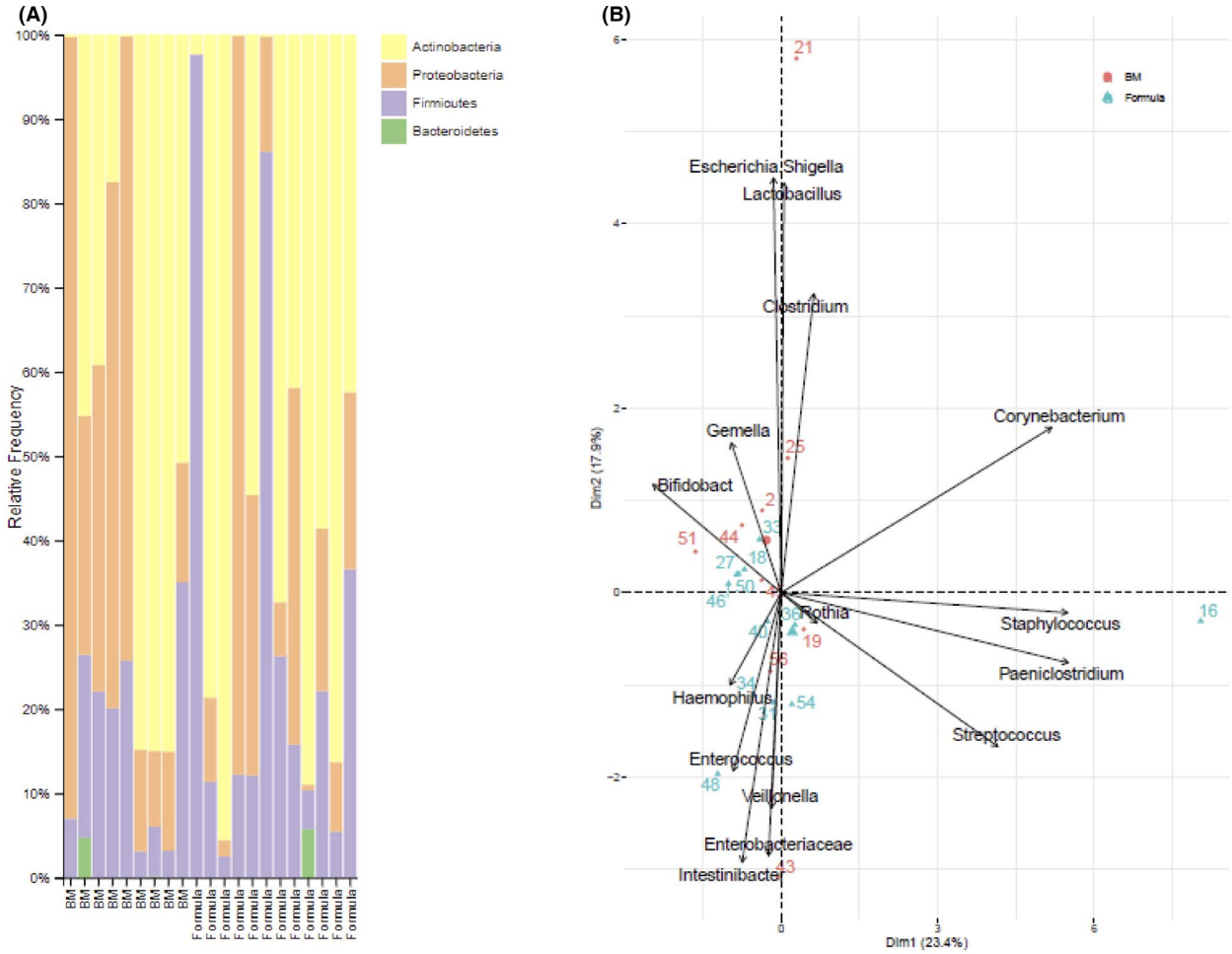
4 | DISCUSSION

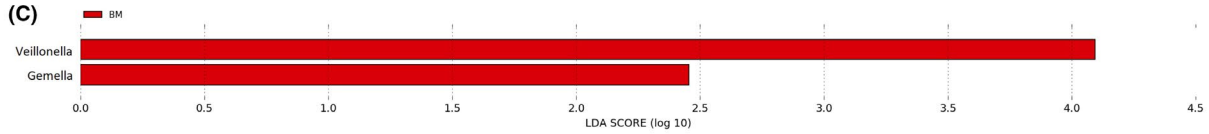
The influence of early life nutrition on the development of the immune response has not previously been studied in the first few weeks of life. This is an important question as epidemiological data suggest that breastfeeding is associated with long-term health benefits, such as a lower incidence of childhood infections, asthma, obesity and autoimmune disorders,[24-26] although it may not reduce the risk of food allergy.[27] This mechanism may be imprinted early after birth when the immune system faces the dual challenge of establishing inflammatory capacity against pathogens whilst developing tolerance towards harmless antigens.

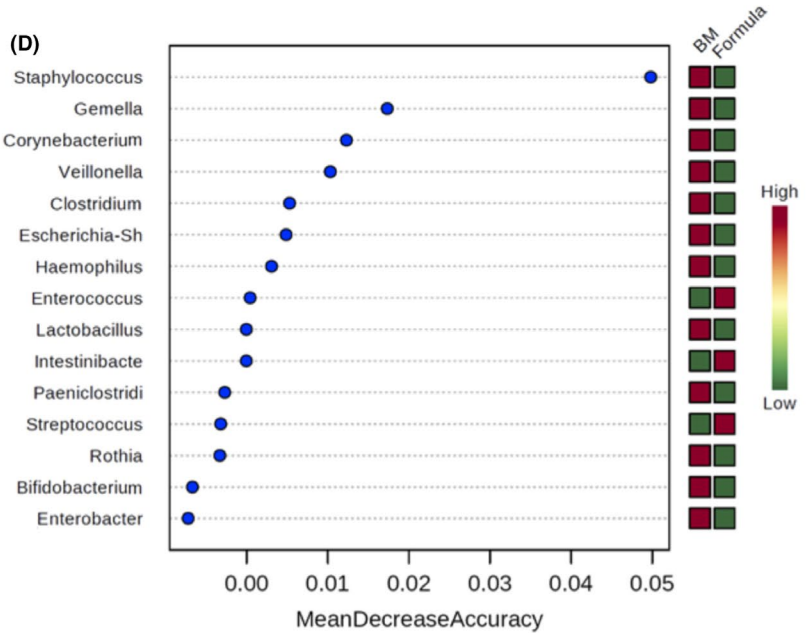
A striking finding was that Tregs expand substantially in the first 3 weeks of life and this expansion was more profound in breastfed babies in contrast to those receiving formula feed. Tregs of breastfed neonates also display an activated phenotype with increased expression of HLA-DR, a marker of increased suppressive activity.[28] A recent mouse study on the role of maternal milk in setting the frequency of Tregs in the offspring reported the importance of a double-negative feedback loop, vertically transmitted via the enteromammary axis that governs a set point for Tregs in the gut.[29] On the other hand, changes in the profile of pro-inflammatory cytokine production were also observed at this early stage of life. IL-8 production, a major phenotypic attribute within cord blood, was largely maintained but a striking feature was the increase in intensity of IL-17 production by both CD4+ and CD8+ cells by 3 weeks of age. This is likely to reflect recognition of bacterial antigen during establishment of the microbiome and may be balanced by the coordinated Treg expansion during this period. An increase in serum IL-17 has been shown at 4 weeks and is likely explained by these observations.[7] Most of these features were independent of nutrition although IFN-γ production by T cells was lower in exclusively breastfed neonates. However, in the whole study population, we did not observe any difference in the proportion of Th1 and Th2 cells between birth and 3 weeks of age. Earlier studies indicated that Th1 development may be guided by environmental exposure via epigenetic changes and would therefore be expected to occur at later stages (first months to years) of development. Low microbial exposure during early life was reported to increase the risk of allergic disease by reducing demethylation induced activation of the IFN-γ gene of naive T cells.[30] Our observation that CD8+ cells are less abundant and have reduced cytotoxic capacity in neonates compared with adults confirms previous results.[31,32]

One of the most interesting findings was that T cells from breastfed neonates display reduced proliferative responses and produce substantially lower levels of Th1 cytokines when challenged with maternal cells. This was specific to maternal antigens and was not present against unrelated PBMC and did not result from an intrinsic reduction of cytokine producing capacity. As such, this reflects the development of immunological tolerance against non-inherited maternal antigens (NIMA) in exclusively breastfed neonates.

FIGURE 5 Microbiome analysis of neonatal stool samples at 3 weeks of age in exclusively breastfed (n = 9) and exclusively formula-fed (n = 12) neonates. **A,** Relative frequency of bacterial phyla in the two cohorts. **B,** Principal component analysis (PCA) of gut microbiota composition of exclusively breastfed (red) and exclusively formula-fed (blue) neonates at 3 weeks of age determined by bacterial 16S rRNA amplification. Numbers represent the individual study number of each participant. Pooled variables of milk received are represented by the large red circle for breast milk and the large blue triangle for formula, respectively. **C,** Association of specific microbial taxa with the feeding method by linear discriminant analysis (LDA) effect size (LEfSe). Red indicates taxa enriched in exclusively breastfed neonates. **D,** Ranking of gut microbial strains using the random forest (RF) method in exclusively breastfed (BM) and exclusively formula-fed neonates at 3 weeks of age.


(A)
Actinobacteria
Proteobacteria
Firmicutes
Bacteroidetes
Relative Frequency
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
BM
Formula
(B)
BM
Formula
Escherichia-Shigella
Lactobacillus
Clostridium
Corynebacterium
Gemella
Bifidobact
Rothia
Staphylococcus
Paeniclostridium
Haemophilus
Streptococcus
Enterococcus
Veillonella
Enterobacteriaceae
Intestinibacter
Dim1 (23.4%)
Dim2 (17.9%)


(C)
BM
Veillonella
Gemella
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5
LDA SCORE (log 10)


(D)
Staphylococcus
Gemella
Corynebacterium
Veillonella
Clostridium
Escherichia-Sh
Haemophilus
Enterococcus
Lactobacillus
Intestinibacte
Paeniclostridi
Streptococcus
Rothia
Bifidobacterium
Enterobacter
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0.01
0.02
0.03
0.04
0.05
MeanDecreaseAccuracy
BM
Formula
High
Low

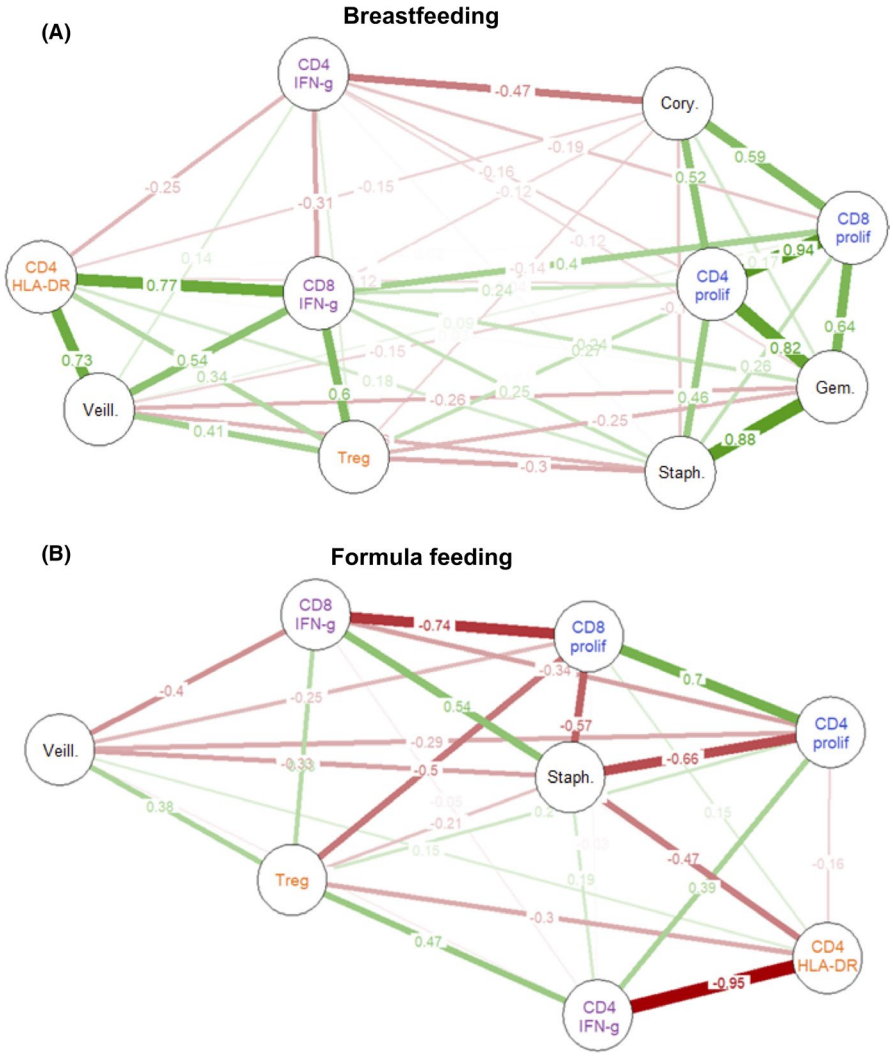

FIGURE 6 Integrated analysis of mixed lymphocyte reaction (MLR), flow cytometry and microbiome data of **A,** exclusively breastfed (n = 9) and **B,** exclusively formula-fed (n = 5) neonates at 3 weeks of age. Various positive and negative correlations between the studied parameters were revealed. Green represents a positive correlation, whereas red represents a negative correlation. Thicker lines represent stronger correlations. Nodes in blue represent MLR data, nodes in black represent microbiome data, and nodes in purple and orange represent flow cytometry data from the panels with and without mitogenic stimulation, respectively.

Importantly, we were also able to show that NIMA-specific tolerance was mediated by Tregs and is therefore linked to the expansion of this population in breastfed neonates.

An additional observation was that neonatal PBMC at 3 weeks of age triggered stronger immune responses from maternal PBMC compared with cord blood cells. This may reflect maturation of antigen presentation function by 3 weeks of age, although this was not assessed in this study. This also indicates that the foetal immune system may contribute to suppression of maternal immune recognition during pregnancy by maintaining a tolerogenic phenotype prior to parturition.

It is interesting to speculate on potential mechanisms by which breastfeeding can promote NIMA-specific tolerance in neonates. Our observations likely reflect immune tolerance to gastrointestinal presentation of maternal cells within breast milk.[33] Transplacental passage of cells during pregnancy leads to reciprocal microchimerism that can persist for many years. Furthermore, this 'microchiome' of maternal cells supports fertility in female offspring by promoting immune tolerance to NIMA during next-generation pregnancies.[34] Beneficial effects are also seen when NIMA are shared between donors and recipients of allogeneic renal or hemopoietic stem cell transplantation. Importantly, the establishment of NIMA-specific tolerance has been shown to be dependent on breastfeeding and nutritional history is also a determinant of NIMA-associated transplant outcome.[35,36] Our findings show that breastfeeding promotes the development of Tregs that suppress recognition of NIMA, thus potentially supporting maternal microchimerism and conferring lifelong benefits in relation to fertility and immune protection against infectious agents and cancer.[34,37]

Additionally, recent studies highlighted the possible immune modulating effects of microplastics released from feeding bottles, which may be of relevance in the context of our study.[38,39] However, the possible contribution of the above mechanism to the differences observed between our study groups was not assessed.

A further recent observation is that breastfeeding, through the transfer of human milk oligosaccharides, exerts important prebiotic and immunomodulatory effects including the development of tolerogenic dendritic cells which prime Tregs.[40,41]

We were also interested to assess how nutrition could impact on the formation of the early microbiome and how this might correlate with immune function. Microbial composition was broadly comparable in breastfed and formula-fed neonates, and this is

likely to reflect the fact that all babies in our cohort were delivered by caesarean section. Dysbiosis of the microbiota has been found to occur following delivery by caesarean section and in infants who are not breastfed.[42,43] Nevertheless, although it may take several months for nutrition to markedly influence microbiome composition[44] subtle differences in microbial diversity were already apparent at 3 weeks of life. In line with previously published results,[45] we observed that the gut microbiome of breastfed neonates is more abundant in short chain fatty acid (SCFA) producing bacterial genera, such as *Gemella* and *Veillonella*. SCFAs, in particular propionate and butyrate, play an important role in promoting Treg differentiation and proliferation via the inhibition of histone deacetylases.[12] This notion is supported by a link between the presence of *Veillonella* and the proportion of Tregs at 3 weeks of age in our network modelling analysis. Furthermore, a recent study demonstrated paucity of Veillonella and other anaerobic taxa in the microbiome of extremely preterm infants with postnatal growth failure compared with appropriate postnatal growth, indicating its role in early metabolic programming.[46] The relative abundance of Veillonella further increases by 2 months of age.[47]

We selected elective caesarean deliveries for our study as labour is known to promote pro-inflammatory changes,[48,49] which could have introduced unwanted variation in the study immune parameters in our population depending on the length and characteristics of labour and parturition. Further studies will therefore be needed to establish the relative contribution of mode of delivery and nutritional history to the development of NIMA-specific tolerance in the neonate. The applied method of collecting stool samples from nappies is limited by possible contamination with environmental and skin bacteria, which was taken into account during the analysis of microbiome data. A further limitation of this work is the relatively low number of neonates in the examined feeding groups. Nevertheless, we established a unique and homogenous cohort of healthy neonates who were sampled at 3 weeks of age exclusively for the purposes of this study.

In summary, we demonstrate that the neonatal immune system undergoes substantial maturation in the first 3 weeks of life with an increase in IL-17 production in T cells and a simultaneous increase in the Treg population. Moreover, breastfed neonates show a specific and Treg-dependent reduction in proliferative T-cell responses to NIMA, associated with a reduction in inflammatory cytokine production. These findings add to our understanding of mechanisms by which early life nutrition can determine long-term health outcomes.[50]

CONFLICT OF INTEREST STATEMENT

The authors declare no competing interests.

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DATA AVAILABILITY STATEMENT

The data sets generated and analysed during the current study are available from the corresponding author on request. Microbiome sequencing data were deposited in the SRA database (accession number PRJNA629085).

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SUPPORTING INFORMATION

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

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