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Mode of Delivery Shapes Gut Colonization Pattern and Modulates Regulatory Immunity in Mice

Camilla H. F. Hansen,*1 Line S. F. Andersen,*1 Łukasz Krych,† Stine B. Metzdorff,* Jane P. Hasselby,‡ Søren Skov,* Dennis S. Nielsen,† Karsten Buschard,§ Lars H. Hansen,¶‖ and Axel K. Hansen*  

Delivery mode has been associated with long-term changes in gut microbiota composition and more recently also with changes in the immune system. This has further been suggested to link Cesarean section (C-section) with an increased risk for development of immune-mediated diseases such as type 1 diabetes. In this study, we demonstrate that both C-section and cross-fostering with a genetically distinct strain influence the gut microbiota composition and immune key markers in mice. Gut microbiota profiling by denaturing gradient gel electrophoresis and 454/FLX-based 16S rRNA gene amplicon sequencing revealed that mice born by C-section had a distinct bacterial profile at weaning characterized by higher abundance of Bacteroides and Ruminococcaceae, and less Rikenellaceae and Ruminococcus. No clustering according to delivery method as determined by principal component analysis of denaturing gradient gel electrophoresis profiles was evident in adult mice. However, the adult C-section–born mice had lower proportions of Foxp3+ regulatory T cells, tolerogenic CD103+ dendritic cells, and less II10 gene expression in mesenteric lymph nodes and spleens. This demonstrates long-term systemic effect on the regulatory immune system that was also evident in NOD mice, a model of type 1 diabetes, born by C-section. However, no effect of delivery mode was seen on diabetes incidence or insulitis development. In conclusion, the first exposure to microorganisms seems to be crucial for the early life gut microbiota and priming of regulatory immune system in mice, and mode of delivery strongly influences this. The Journal of Immunology, 2014, 193: 000–000.

Environmental microbes start colonization of the sterile gastrointestinal tract of the newborn immediately after birth. Initially, the mucosal surfaces become exposed to commensal bacteria of primarily the mother’s vaginal and intestinal microbiota in both humans and mice (1, 2), but infants born by Cesarean section (C-section) experience a delayed colonization and acquire bacterial members resembling those of the skin (3). Even though variation usually exists in the neonate gut microbiota (4), the altered bacterial diversity in the human microbiota associated with delivery mode has been detected up to 7 y of age (5). Especially a lower frequency of Bacteroides and Bifidobacterium spp. has been reported in several studies on children born by C-section compared with vaginal delivery (6–8). Considering the importance of these species in promoting the development of immune-regulatory mechanisms including TGF-β and IL-10 production (9–11), it is not unlikely that mode of delivery skews immune homeostasis because of differences in microbiota exposure. This is consistent with previous findings of a stronger humoral immune response associated with C-section; these children had more IgA- and IgG-secreting cells in an ELISPOT assay on PBMCs isolated at 3, 6, and 12 mo of age (12). Similarly, a decreased production of various cytokines, including TNF-α and IL-1β, was observed in blood drawn from Cesarean-delivered newborns compared with cases of vaginal delivery (13). Innate immune recognition of the first microbial stimuli during vaginal delivery is associated with a spontaneous activation of intestinal epithelial cells and subsequent acquisition of TLR tolerance (14). However, it only occurred in vaginally born mice, but not after C-section or in TLR4-deficient mice, which demonstrates the importance of initial endotoxin-rich colonizers on the maturation of the immune system. The importance of LPs from Gram-negative bacteria was indicated in TLR4-deficient NOD mice with accelerated diabetes development and reduced suppressive function of regulatory T cells (Tregs) (15). These studies can partially explain why children born by C-section are at increased risk for development of immune disorders such as allergic asthma (16), type 1 diabetes (T1D) (17), celiac disease (18), and obesity (19), in which impaired immune regulation seems to be central. Interestingly, not all autoimmune diseases, but especially those with a certain relation to the gut, are influenced by C-section. Development of multiple sclerosis was, for example, not at excess risk in cases of C-section delivery (20). To a certain extent, T1D has been associated with gut microbiota alteration in a similar manner to children born by C-section (21). Lactobacillus and Bifidobacterium were found in higher abundance in feces from bio-breeding diabetes-resistance rats compared with bio-breeding diabetes-prone rats, which contained more Bacteroides,
Eubacterium, and Ruminococcus spp. at the time of diabetes onset (22). This notion is supported by our recent findings in NOD mice, in which vancomycin treatment before weaning attenuated diabetes development later in life (23). According to the hygiene hypothesis, the increasing incidence of immune-mediated diseases is caused by a deregulated immune response to harmless Ags because of lack of microbial stimuli in early life (24, 25). Consistent with this, defective Tregs have been suggested to explain the imbalance of T lymphocyte subsets found in T1D patients (26–28). Dysfunctional tolerogenic dendritic cells (DCs) and a reduced percentage of Foxp3+ Tregs in the gut of T1D patients support the notion that the mucosal immune system, in parallel with the early gut microbiota, plays an important role in the pathogenesis (29).

Genotype has a profound role in the composition of the gut microbiota as demonstrated in monozygotic twins (30). Also, differences in the gut microbiota between inbred mouse strains show that it is to a great extent defined by genotype (31–33). Even though the gut microbiota is influenced by its surroundings during early life, once it is established, it remains relatively stable (34). However, early-life incidents may overrule this genetic priming of the gut microbiota and alter the pattern of colonization, which in early life is known to strongly influence the development of the immune system (35). We therefore hypothesized that murine offspring delivered by C-section and exposed to a different microbial environment by cross-fostering them with a genetically distinct strain, would not only alter the gut microbiota, but also skew the immune system in a state resembling the foster mouse strain. In our recent study, the switching of early-life environment by cross-fostering mice, in which vancomycin treatment before weaning attenuated diabetes development later in life (23), was considered diabetic and killed when blood glucose levels exceeded 12 mmol/l on 2 consecutive days. At the age of 30 wk, all remaining NOD mice were killed by cervical dislocation.

**Histology**

H&E-stained pancreatic sections from 30-wk-old nondiabetic NOD mice were evaluated for insulitis score in a blinded fashion by two persons. Lymphocytic infiltration was graded as follows: 0, no infiltration; 1, intact islets but with few mononuclear cells surrounding the islets; 2, peri-insulitis; 3, islet infiltration <50%; 4, islet infiltration >50%. Twenty-five islets for each of six nondiabetic mice in each group were scored.

The formalin-fixed, paraffin-embedded tissue was cut into 4-μm-thick sections and deparaffinized and rehydrated according to standard procedures. Pretreatment included heat-induced epitope retrieval for 20 min at 99°C in target retrieval solution buffer at pH 9. Incubation with the primary Ab was performed for 15 min at ambient temperature. The anti-rat/mouse Foxp3 Ab (clone FJK-16s; ebBioScience, San Diego, CA) was applied, using a dilution of 1:40. For detection, the Dako animal research kit (Ark) protocol (Dako North America, Carpinteria, CA) was applied. Finally, the slides were counterstained and coverslipped.

**Gut microbiota analyses**

The samples analyzed included feces samples and ileum content aseptically collected from the B6, BALB/c, and NMRI pups when they were killed at 5 wk of age, and feces samples collected from the NOD offspring at 4 wk of age and when they were killed. Feces and ileum content were also sampled from the mothers when they were killed. The samples were stored at −80°C until DNA extraction and subsequently the V3 region of the 16S rRNA gene was PCR-amplified followed by denaturing gradient gel electrophoresis (DGGE) as described previously (37). The resulting DGGE profiles were analyzed using BioNumerics Version 4.5 (Applied Maths, Sint-Martens-Latem, Belgium).

The composition of the prokaryotic community of feces samples from the NOD mothers and the 4-wk-old NOD pups was determined using tag-encoded 454/FLX Titanium Roche pyrosequencing of the V3 and V4 region of the 16S rRNA gene by the National High Throughput DNA Sequencing Centre, University of Copenhagen, Denmark, as previously described (23). Pyrosequencing data were analyzed as described by Krych et al. (32) using an open source software package, Quantitative Insight Into Microbial Ecology (QIIME version 1.7.0; National Center for Biotechnology Information database accession No. SRP032495). Principal coordinate analysis (PCoA) plots were generated using the Jackknife Beta Diversity workflow based on 10 distance metrics calculated using 10 subsampled operational taxonomic unit (OTU) tables. These were built using 94% relatedness between clusters, which corresponds to genus-level OTUs. The number of sequences to include in each jackknifed subset was set to 2000 reads/sample. Analysis of similarities was used to evaluate group differences weighted and unweighted UniFrac distance metrics that were generated based on the rarefied OTU tables. For testing the significance of group separation along given PCs, a paired Student t test was used, α diversity measures, including observed species number (97% sequence identity threshold), Chao1, Shannon, and Phylogenetic diversity indexes, were calculated for rarefied OTU tables. A nonparametric t test was used to compare α diversities using 1000 Monte Carlo permutations. Testing association of OTUs (generated at 97 and 94% sequence similarity, representing species- and genus-level similarity, respectively) with delivery mode was performed using out_category_significance.py script. The G test of independence (q_test) and ANOVA were determined, respectively: qualitative (presence/absence) and quantitative (relative abundance) association of OTUs with the groups. These were calculated based on 1000 subsampled OTU tables rarefied to an equal number of reads (2000 per sample).

**Cell isolation and flow cytometry**

Mice were killed and the organs immediately placed on ice. DC (CD11c+ CD11b+CD103+) and Treg (CD4+Foxp3+) staining of mesenteric lymph nodes (MLNs), spleens, small intestinal lamina propria (siLP), and colonic lamina propria (clLP) were performed as previously described (23). All Abs were purchased from eBioscience, and analysis was performed using an Accuri C6 flow cytometer (Accuri Cytometers, Ann Arbor, MI).

**Cytokine expression by quantitative PCR**

Immediately after the NOD mice were killed, a 1/2- to 1-cm fragment of spleen and pancreas was placed in RNAlater (Ambion, Austin, TX). Homogenization, RNA isolation with MagMAX-96 RNA Isolation Kit (Ambion), and cDNA synthesis using High-Capacity cDNA Reverse Transcriptase Kit (Applied Biosystems, Foster City, CA) were performed as described previously (38). Tgfβ, Il10, Tnfa, Ibfy, Il1β TaqMan gene ex-

Materials and Methods

The experiments were carried out in accordance with the Council of Europe Convention European Treaty Series 123 on the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes, and the Danish Animal Experimentation Act (LBK 1306 from 23/11/2007). The study was approved by the Animal Experimentation Inspectorate, Ministry of Justice, Denmark (license No. 2007-561-1434 C3), and all efforts were made to minimize suffering and the number of animals used.

**Animals, breeding, and birth delivery**

Seven-week-old male and female outbred barrier bred BomTac-NMRI, inbred male and female barrier-bred C57BL/6Ntac (B6), and inbred male and female barrier-bred BALB/cAnNtac and NOD/MikTac were purchased from Taconic (Germantown, NY) and time-mated separately in our barrier-protected animal facility (Faculty of Health and Medical Sciences, University of Copenhagen, Frederiksberg, Denmark) under standard conditions in open cages without filter lids. C-section and cross-fostered with other NOD mice, to clarify the importance of Cesarean delivery alone; the effect of delivery mode on T1D development was further investigated in the NOD mice.

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pression assays (Applied Biosystems) were used for quantitative PCR analysis, and data were analyzed as described previously (38). In brief, fold changes in gene expression were calculated by the comparative cycle threshold (Ct) method. The expression of target genes was normalized to the reference gene Actinß: \( \Delta \text{Ct} = \text{Ct}_{\text{Sample}} - \text{Ct}_{\text{Reference}} \). Fold change in gene expression was calculated as \( 2^{-\Delta \Delta \text{Ct}} \), where \( \Delta \Delta \text{Ct} = \Delta \text{Ct}_{\text{Sample}} - \Delta \text{Ct}_{\text{Calibrator}} \) and where the mean \( \Delta \text{Ct} \) of samples from the control mice was used as calibrator.

Statistics

GraphPad Prism version 5.02 (GraphPad Software, San Diego, CA) was used for statistical analysis, and \( p \) values < 0.05 were considered significant. Cumulative diabetes incidence was calculated using the Kaplan–Meier estimation, whereas statistical significance was evaluated by the log-rank test. Other differences were estimated by two-tailed t test or one-way ANOVA test with Tukey’s posttest or by Kruskal–Wallis test with Dunn’s posttest on data that did not assume Gaussian distributions. DGGE profile comparison was performed by the Dice similarity coefficient with a band position tolerance and optimization of 1% using the unweighted pair group method with arithmetic averages clustering algorithm and by principal component analysis (PCA).

Results

Both C-section and cross-fostering between distinct strains influence the pattern of early gut colonization

The fecal gut microbiota of NMRI and B6 mothers clustered separately on the PCA plot (Fig. 1A; \( p < 0.05 \) for PC1, explaining 16.2% of the variance), whereas no difference was found between the NMRI and BALB/c mothers (Fig. 1B). The fecal gut microbiota of the C-section–born B6 offspring resembled that of the NMRI mother, which they were fostered by, and was significantly different from the vaginally delivered B6 offspring (Fig. 1A; \( p < 0.001 \) for PC1 and \( p < 0.05 \) for PC3, explaining 10.3% of the variance) and their B6 mothers (\( p < 0.001 \) for PC1). Despite the similar gut microbiota between NMRI and BALB/c mothers, there was also a near-significant tendency for the BALB/c vaginally delivered offspring to cluster separately from the BALB/c C-section offspring (Fig. 1B; \( p = 0.1 \) for PC1, explaining 14.9% of the variance, and \( p = 0.058 \) for PC3, explaining 8.4% of the variance), but it was only the vaginally delivered offspring that clustered significantly different from the mothers (Fig. 1B; \( p < 0.05 \) for PC1).

A similar pattern for the B6 mice was seen in the ileum samples; the C-section offspring clustered separately from both the vaginally delivered B6 offspring (Fig. 1C; \( p < 0.001 \) for PC1, explaining 15% of the variance) and their B6 biological mothers (\( p < 0.05 \) for PC2, explaining 11.7% of the variance). This was consistent with the fact that there was no difference between the B6 C-section–born offspring and their NMRI foster mothers. The NMRI and B6 mothers visually seemed to cluster separately on the PCA plot, but this was not significant (Fig. 1C; \( p = 0.11 \)), most likely because of the limited number of samples.

Even though the NMRI and BALB/c mothers had a similar fecal microbiota, their ileac microbiota was significantly different (Fig. 1D; \( p < 0.05 \) for PC1, explaining 35.8% of the variance, and \( p < 0.05 \) for PC2, explaining 9.9% of the variance). However, the cross-fostering had no effect on ileac microbiota in the BALB/c offspring, and the microbiota of both the vaginally delivered and C-section–delivered BALB/c offspring were similar to the BALB/c biological mothers (Fig. 1D). In all PCA plots, no differences were evident between sexes or between the litters within the individual groups, which is in agreement with a previous study on litters of mothers that were sisters (31).

To differentiate the effect of delivery mode from cross-fostering on the gut microbiota, we collected feces samples from NOD mice born by C-section and fostered by other NOD mice with a similar microbiota and also analyzed them by DGGE. PCA of DGGE profiles revealed clearly distinguishable main clusters separating feces samples from newly weaned mice born by either C-section or vaginally (Fig. 1E; \( p < 0.001 \) for PC1 explaining 17.3% of the variance and \( p < 0.05 \) for PC2 explaining 13% of the variance). No cage–litter effect was seen on the gut microbiota; thus, the difference observed in the pups were associated only to delivery mode. However, later in life, when the mice were diagnosed with diabetes, this difference was no longer evident (Fig. 1F), indicating that genetics is a strong determinant for the permanent gut microbiota composition.

To further characterize the impact of delivery mode on the gut microbiota in mice, we analyzed the feces samples from the NOD pups collected at weaning and from their biological and foster mothers by tag-encoded 16S rRNA gene-based pyrosequencing. The raw number of reads generated from all 38 feces samples scored 642133. Sequences that met all requirements of the quality control (minimum length = 300 bp, quality score \( \geq 25 \)) and free from chimeric reads yielded 554391, providing an average of 4888 sequences per sample (minimum = 2045, maximum = 88321, SD = 17199) with a mean sequence length of 457 bp (minimum = 300, maximum = 470). PCoA based on weighted UniFrac distance metrics separated only along PC3, which described 7.7% of the information (\( p \leq 0.05 \); Fig. 2A), but PCoA plots based on unweighted UniFrac distance metrics showed clear clustering (\( p \leq 0.01 \), analysis of similarities) of the two groups (Fig. 2B). The most abundant OTUs (94% sequence similarity threshold) were located in the middle of the plots, which indicate that it was the low abundant taxa situated in the peripheries that resulted in separate clustering of the two groups. Differences in \( \alpha \) diversity using observed species and chao1 indexes revealed a higher diversity in the C-section group compared with vaginally delivered pups (\( p \leq 0.05 \)). However, testing differences in Shannon and Phylogenetic diversity indexes did not reach significance.

ANOVA analysis conducted on OTUs clustered at 97 (Table I) and 94% (Supplemental Table I) sequence similarity revealed differences in the relative abundance of taxa mainly belonging to two bacterial orders: Bacteroidales and Clostridiales. Three clusters annotated to the Bacteroides genus, one cluster assigned to S24-7 family, and seven clusters from Lachnospiraceae family were more abundant in the C-section offspring. Two clusters characterized as Bacteroides acidifaciens together with unclassified members of Rikenellaceae and Clostridiaceae families, and genera classified as Oscillospira and Ruminococcus were more abundant in the vaginally delivered pups. Lastly, three clusters annotated as Lachnospiraceae family also constituted a higher proportion of the gut microbiota in the vaginally delivered group. A similar group association was shown with G test of independence (Supplemental Table II). Because of the high number of observations (OTUs) and relatively small samples size, the more conservative Bonferroni and false discovery rate corrections of \( p \) values were no longer significant. However, the presented differences are the most likely microbial taxa that accounts for the separate clustering of PCA (DGGE) and PCoA (sequencing) plots. No significant differences in gut microbiota were evident between the biological and foster NOD mothers. Thus, the difference in gut microbiota in the offspring was only due to delivery mode.

Development of tolerogenic immunity is dependent on delivery mode

Next, it was analyzed how cross-fostering influenced the development of Foxp3\(^+\) Tregs. NMRI mothers had significantly lower proportion of Tregs compared with B6 and BALB/c mothers. Interestingly, this seemed to influence the pups by cross-fostering. Tregs were significantly less abundant in both MLN and spleen
FIGURE 1. PCAs of DGGE profiles of fecal and ileal gut microbiota in Cesarean-delivered and cross-fostered mice. (A) PCA plot based on DGGE profiles of 16S rRNA gene PCR-derived amplicons of feces samples collected from 5-wk-old vaginally delivered (VD) B6 mice (yellow) weaned by their own B6 mothers (red), and C-section (CS) B6 mice (blue) cross-fostered by NMRI mothers (green). (B) PCA plot of DGGE profiles of feces samples collected from 5-wk-old VD BALB/c mice (yellow) weaned by their own BALB/c mothers (dark blue) and CS BALB/c mice (light blue) cross-fostered by NMRI mothers (green). (C and D) PCA plots of DGGE profiles of ileum content samples collected from the same mice as in (A) and (B). (E) PCA plot of DGGE profiles of feces samples collected from 4-wk-old VD NOD mice (yellow) and CS NOD mice (blue) cross-fostered by other NOD mice. (F) PCA plot of DGGE profiles of feces samples collected from newly diagnosed diabetic or 30-wk-old nondiabetic VD NOD mice (yellow) and CS NOD mice (blue) cross-fostered by other NOD mice. ANOVA based on the first (X), second (Y), and third (Z) principal component was used to compare the groups.
isolated from offspring delivered by C-section compared with their biological B6 or BALB/c mother, whereas there were no differences when compared with their NMRI foster mother and NMRI control pups (Fig. 3A, 3B). Also, both the B6 and BALB/c C-section pups had significantly lower proportions of Tregs in the spleens than the vaginally delivered control pups of same strains fostered by their biological mothers. This was evident in both the B6 and the BALB/c strain cross-fostered by NMRI mothers, indicating an effect on Tregs by the different gut microbiota vertically transferred from NMRI mothers to the pups. There were, however, no significant differences between the vaginally delivered NMRI, B6, and BALB/c; thus, the low Treg level in the cross-fostered mice might be more because of a separate effect of delivery mode besides cross-fostering. The effect on Treg levels was either way likely mediated by the gut microbiota because there was a tendency for the feces PC1 values of Cesarean-delivered B6 pups to positively correlate with the proportion of Tregs in the spleen ($p = 0.09$).

The effect of delivery mode alone was therefore further investigated in NOD mice delivered by C-section, but fostered by other NOD mice of the same strain. The development of Foxp3$^+$ Tregs and CD103$^+$ DCs were monitored in siLP, cLP, MLN, and spleen by flow cytometry. Interestingly, higher proportions of Tregs (Fig. 3C) and tolerogenic DCs (Fig. 3D) were evident also in these mice in both spleen and MLN of vaginally delivered mice compared with C-section mice, indicating an effect of delivery mode alone on these tolerogenic immune cell compartments. In contrast, no significant differences in the immune system were observed in siLP and cLP. To elucidate how these differences might have influenced the balance of Th1 and Th17 cells, we also monitored the IFN-$\gamma$– and IL-17–producing CD4 T cells by flow cytometry. However, no differences were detected between mice born by C-section or vaginally at any of the investigated sites (Fig. 3E, 3F).

**Systemic IL10 gene expression is low in C-section–delivered pups**

To further support the flow-cytometry results that demonstrated less anti-inflammatory immune cells systemically in C-section mice compared with vaginally delivered mice, we analyzed gene expression of several anti-inflammatory and proinflammatory cytokines in the spleen and pancreas isolated from the NOD mice. No differences were evident in the pancreas, but lower expression level of the anti-inflammatory cytokine IL10 was found in the spleen in C-section offspring compared with vaginally delivered NOD mice ($p < 0.05$, Fig. 4).

**C-section in NOD mice has no effect on diabetes development**

The cumulative diabetes incidence and onset time were found not to be influenced by delivery mode (Fig. 5A). The diabetes incidence at 210 d was 50% ($n = 16$) in the vaginally delivered NOD mice and not significantly different from the 59% ($n = 17$) in the C-section NOD mice. Histological evaluation of insulitis in pancreatic sections from nondiabetic 30-wk-old offspring also did not reveal any significant effect of delivery mode on insulitis score (Fig. 5B, 5C). The number of infiltrating Foxp3$^+$ cells per islet was also not significantly different between vaginally and C-section–delivered NOD mice (Fig. 5D, 5E). The systemic immune changes found because of delivery mode were thus not sufficient to influence the local pancreatic environment.

**Discussion**

It has been suggested that increased risk for chronic inflammatory diseases associated with C-section is a result of the long-term dysbiosis observed in children delivered by C-section (reviewed in Ref. 39). In this study, we demonstrated in a murine model that mode of delivery had a major impact on the early composition of the gut microbiota in mice, as analysis by 16S rRNA gene se-
Table I. Significant quantitative differences in microbial taxa abundance analyzed by partial 16S rRNA gene sequencing of fecal bacteria (97% sequence similarity) in Cesarean- and vaginal-delivered NOD mice at weaning

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<th>Genus</th>
<th>Species</th>
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<th>Bonferroni-corrected p $^b$ for 161 OTUs</th>
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<th>Mean (%) C-section</th>
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$^a$ANOVA analysis verifies whether the relative abundance of given OTU (97% sequence similarity) is different between C-section- and vaginal-delivered NOD mice. The p value was calculated based on 1000 subsampled OTU tables rarefied to the identical number of reads (2000 per sample).

$^b$The p value after correction for multiple tests with Bonferroni correction.

$^c$The p value after correction with false discovery rate (FDR).
sequencing confirmed a separate clustering of the vaginally delivered and C-section pups. A distinct bacterial profile enriched in especially Bacteroides and Lachnospiraceae and with lower abundance of Rikenellaceae and *Ruminococcus* was characteristic for the C-section pups. On the contrary, C-section has been shown to cause decreased colonization rate of *Bacteroides* and increased prevalence of *Clostridia* in humans (40). These differences may, besides genetic differences, be because of the initial colonization pattern being very susceptible to environmental changes. When human babies are not exposed to maternal vaginal and gut microbiota at birth, the main exposure is more likely through contact with the mother’s skin during nursing, which is also reflected in the composition of the gut microbiota of C-section–delivered humans (3). In mice, the early-life events differ from humans, as the contact between the mother’s feces and the newborn pups, independent of delivery mode, is much stronger for murine pups housed on bedding-covered surfaces. The sterile treatment of the newborn mice until they are transferred to this “dirty” environment of the foster mother might also delay colonization of microbes that are crucial to further colonization pattern and subsequent immune development (35, 41).

B6 mice that were cross-fostered by an NMRI mother developed a gut microbiota similar to the foster mother and distinct from the biological B6 mother. Thus, in these mice, it is clear that the early environment to which the newborn pups are introduced governs the gut microbiota composition later in life up to at least 5 wk of age, despite the genetic drive. However, the cross-fostered mice were also born by C-section, which makes it difficult to differentiate between the effects of delivery mode and cross-fostering on the gut microbiota. In the Cesarean-delivered BALB/c mice, their gut microbiota had a strong tendency to cluster differently from the vaginally delivered BALB/c mice, even though their NMRI foster mothers had a fecal microbiota similar to the BALB/c mothers. This effect was therefore most likely caused by C-section, which was subsequently confirmed in Cesarean-delivered NOD mice fostered by other NOD mice with a similar microbiota to the biological NOD mothers. The difference in clustering between the ileal versus fecal microbiota in the BALB/c mice can likely be explained by the much higher diversity of species in feces compared with the ileum.

**FIGURE 3.** Flow cytometric analyses of immune cells in Cesarean-delivered and cross-fostered mice. Flow cytometric analysis of cells isolated from MLN, spleen, siLP, and colon lamina propria (cLP) as indicated. (A and B) Percentages of Foxp3* regulatory CD4* T cells isolated from 5-wk-old vaginally delivered (VD) B6, BALB/c, and NMRI mice fostered by their own mothers (light gray) and C-section (CS) B6 and BALB/c mice cross-fostered by NMRI foster mothers (dark gray). (C) Percentages of Foxp3* regulatory CD4* T cells, (D) CD103* DCs (CD11b* CD11c*) after gating on live cells with 7-aminoactinomycin D, (E) IFN-γ-producing CD4* T cells, and (F) IL-17-producing CD4* T cells isolated from VD (light gray) and CS (dark gray) NOD mice when diagnosed as diabetic or nondiabetic at 30 wk of age. Error bars represent SEM. *p < 0.05, **p < 0.01, ***p < 0.001.

**FIGURE 4.** Quantitative PCR analyses of cytokines in Cesarean-delivered NOD mice. Relative gene expression of the cytokines *Ifnγ* (A and B), *Il1b* (C and D), *Il10* (E and F), *Tgfb* (G and H), and *Tnf* (I and J) in pancreas and spleen as indicated from vaginally delivered (VD) or C-section NOD mice when diagnosed with diabetes or as nondiabetic at 30 wk of age. Data were normalized to *Actb* and then to mean VD group, which was defined to 1. Error bars represent SEM. *p < 0.05.
Figure 5. Effect of delivery mode on insulitis and diabetes incidence. (A) Cumulative diabetes incidence in vaginally delivered (VD, \( n = 16 \)) or C-section (CS, \( n = 17 \)) NOD mice as indicated. All NOD mice were diagnosed as diabetic and killed when blood glucose levels exceeded 12 mmol/l on 2 consecutive days. Comparisons of the two survival curves were tested by log-rank test. (B) Average insulitis score for non-diabetic VD (\( n = 8 \)) and CS (\( n = 8 \)) NOD mice at 30 wk of age. (C) Percent of islets with a given score in non-diabetic 30-wk-old VD (\( n = 8 \)) and CS (\( n = 8 \)) NOD mice. White, no infiltration; light gray, few mononuclear cells infiltrated; gray, peri-insulitis; dark gray, <50% islet infiltration; black, >50% islet infiltration. Error bars represent SEM. (D) Number of Foxp3 positively stained cells per islet of Langerhans in non-diabetic VD (\( n = 8 \)) and CS (\( n = 8 \)) NOD mice. (E) Fluorescence image representing immunohistochemical analysis of pancreatic Foxp3 Tregs in a VD mouse presented in (D). Arrows point to examples of positively stained Foxp3 cells. Original magnification, \( \times 20 \).

Small differences in only few taxa in ileum can therefore result in more pronounced clustering between groups than would be expected in feces, and changes in taxa solely found in feces may vice versa not result in clustering in ileum (42). In either way, it seems that the early-life environment and acquisition of microbes that the mice are first exposed to strongly influences the later composition of the gut microbiota. In this context, it is interesting that we in a previous study found that inoculation of germ-free mice with a microbiota at 3 wk of age, but not at 1 wk of age, made the feces samples from these mice cluster with the inoculum at 9 wk of age in a PCA analysis (35). It seems as if there is a “window” both in a short period after birth and again during the weaning process around 3 wk of age, where the microbial colonization is crucial in shaping the distinctive gut microbiota.

C-section compared with vaginal delivery reduced the tolerogenic immune response in the offspring. This is in accordance with recent epidemiological studies in humans examining the relation between delivery mode and development of the immune system and chronic inflammatory diseases (43). The link is challenging to investigate because of the lack of suitable animal models resembling human birth events. However, in addition to a change in the gut microbiota, we demonstrated that Tregs were less abundant in MLNs and spleens from Cesarean-delivered NOD mice compared with vaginally delivered NOD mice. This was also evident in spleens of cross-fostered B6 and BALB/c mice born by C-section. The B6 and BALB/c mother had more Tregs than the NMRI foster mothers; therefore, the low proportions of Tregs in the C-section mice could also be a result of the acquired microbiota resembling the foster mother. However, because there were no differences in Treg levels between the vaginally delivered BALB/c, B6, and NMRI pups, the difference is more likely a result of the changed microbiota caused by an effect of C-section rather than cross-fostering. These results are in agreement with previous studies demonstrating that the composition of the gut microbiota influences the development of Tregs (11, 44). It is further supported by a tendency for fecal PC1 values of the Cesarean-delivered B6 pups to positively correlate with the proportion of Tregs in the spleen. In addition, low proportion of CD103+ DCs, which have been shown to induce Tregs, and decreased II10 gene expression further point toward a not fully developed regulatory immunity in the Cesarean-delivered NOD mice. Future studies using gnotobiotic mice would be able to answer whether the changes in gut microbiota caused by delivery mode are sufficient and necessary to the immune phenotype present in C-section offspring.

It is not unlikely that the lack of Foxp3+ Tregs, CD103+ tolerogenic DCs, and II10 gene expression is caused by the observed shift in the early gut microbiota. However, other possible causative factors than the gut microbiota have been associated with C-section. It was, for example, shown that increased level of glucocorticoids during stress response to vaginal delivery is linked with maturation of the highly immunoreactive gut (45). Furthermore, it is speculated that altered epigenetic regulation of genes during C-section may modify the immune response (43). In this study, no significant correlations were found between taxon or PC values of the PCoA plots and immunological parameters in NOD mice. This suggests that these features are independent of each other in C-section offspring. Nonetheless, high abundance of Lachnospiraceae was in prediabetic bio-breeding diabetes-prone rats, one of the most prominent differences in the gut microbiota, compared with bio-breeding diabetes-resistance rats (22), and in humans, more abundant Ruminococcaceae and less of several Bacteroides species was evident in diabetic children at the time of \( \beta \) cell autoimmunity (21, 46). Furthermore, in a recent study, a low \( \alpha \) diversity was observed in the hormone-dependent gut microbiota associated with diabetes protection in male mice compared with female mice (47). This was all supportive of our results, so we sought to investigate whether the changes observed in gut microbiota and the reduced regulatory immune cell subsets would increase the incidence of T1D. However, this was not the case in our study. No differences in diabetes incidence or insulitis were observed between C-section and vaginally delivered mice despite the difference observed in gut microbiota and immunity. The changes in systemic Foxp3 Treg levels were also not evident in the islets of Langerhans, further indicating that delivery mode had no effect on the local pancreatic environment.

Although C-section in NOD mice did not increase diabetes incidence, it did result in a different colonization pattern in early life and caused permanent changes in the regulatory immune system; this should be taken into consideration when using first-generation rederived rodent models because it may modify the results of experiments performed on models for immune-mediated diseases. It should also be kept in mind that the NOD mouse is in no way a model that fully resembles T1D in humans (48). In humans,
T1D may be regarded a cluster of different diseases with different cause but same result (49), and therefore it is possible that the changes in gut microbiota observed in children born by C-section also link to defective regulatory immunity, which in humans may be more likely to increase immune disorders such as T1D. In conclusion, the first exposure to microorganisms seems to be crucial for the establishment of a gut microbiota and subsequent priming of regulatory immune system in mice, and mode of delivery strongly influences this, although the impact on the microbiota in some aspects is different from the impact observed in humans.

Disclosures

The authors have no financial conflicts of interest.

References