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ORIGINAL RESEARCH

Effect of nonpathogenic *Escherichia coli* monoassociation on small intestinal brush-border glycoconjugate moieties and cytokine production after colonization in ex-germ-free rats and pigs

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Abstract: We evaluated the contribution of nonpathogenic *Escherichia coli* O83 after colonization of germ-free (GF) rat pups and piglets on development of terminal α2,6- and α2,3-sialylated and broad range of terminal α1,2-, core α1,6-, and α1,3-, α1,4-fucosylated glycoconjugates in the suckling period relative to noncolonized GF and conventional (CV) counterparts. The ELISA-lectin approach was used to specify and quantify sialylated and fucosylated glycans in brush-border membrane vesicles (BBMV). Levels of pro- and anti-inflammatory cytokines in sera and splenocyte secretions were demonstrated using ELISA. Rat and pig intestinal responses to *E. coli* O83 monoassociation were different from those of GF and more similar to the CV animals in the decline of sialylated glycans, which is in agreement with the shorter life span of enterocytes in *E. coli* O83 monoassociated rats and pigs. No significant effect of *E. coli* O83 colonization on labeling fucosylated glycans was observed for immature fucosylation at the late suckling period. We demonstrate spontaneous secretion of pro-inflammatory cytokine IL-18 and anti-inflammatory IL-10 by GF rat splenocytes, and its suppression of IL-18 in *E. coli* O83 associated rat pups, and suggest that these cytokines serve as an immunomodulatory pool during the suckling period indicating the balance between T helper (Th) 1 and Th2 phenotypes.

Keywords: nonpathogenic *E. coli*, brush-border vesicles, glycoconjugates, plant lectins, cytokines

The brush-border membrane of epithelial cells of the small intestine represents a good model of cell differentiation. During the 72–96 hours during proliferation and migration cycle the enterocytes become differentiated and acquire typical highly specialized lining, along with the brush border with its membrane-bound digestive enzymes, transport systems, and cell surface receptors. These systems are organized in lipid rafts with a high content of glycosphigolipids.[1]

In rodents dramatic changes of the brush-border structure and function occur during transition from suckling to weaning in the third and fourth week after birth, presumably due to changes in hormonal and nutritional factors and binding of microflora to receptors.[2] A crucial role in morphological and functional changes of intestinal mucosa is played by developmental changes of glycosylation processes, including the biosynthesis of surface glycan chains of brush-border glycoproteins and glycolipids. Oligosaccharide chains are attached to a protein by N- or O-glycosidic bond. The known brush-border digestive enzymes are glycoproteins with the protein part penetrating the lipid bilayer and the oligosaccharide component protruding into the intestinal lumen. The structure

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of glycan chains plays a role in digestive enzyme integration into the apical membrane. It was shown that O-linked glycan and the associated action of N- and O-linked glycans mediate apical sorting of sucrase-isomaltase and dipeptidyl peptidase IV through association with lipid rafts, respectively.[3] Developmental studies suggest that during the transition from suckling to weaning of rats and mice the highly sialylated glycoforms of brush-border enzymes, with sialic acid in the terminal position of oligosaccharide chains, are converted to asialylated, adult forms.[4,5] This strikingly diminished sialylation of glycoproteins is accompanied by increased fucosylation, so the molar ratio between sialic acid and fucose is reversed on weaning.[6] Studies by eg, Büller et al[7] have shown that terminal sugars of both N-and O-linked oligosaccharide chains of lactase-phlorizin hydrolase shift from sialic acid in suckling rats to fucose in adults. The results strongly agree with the developmental pattern of sialyltransferase and fucosyltransferase activities in the small intestine.[8–10] The highly expressed sialyltransferase of suckling rat intestine has been attributed to α2,6-sialyltransferase, a major enzyme of suckling rat small intestine.[11–13] The production of fucosylated glycoconjugates is associated with accumulation of α1,2-fucosyltransferase mRNA.[14,2]

Both sialylated and fucosylated surface glycans are implicated in the interaction between microbes, both commensals and pathogens, and intestinal host cells. In germ-free mice the changes in sialyl- and fucosyl-transferases maintain the immature pattern. Conventionalization of germ-free mice by introduction of mouse microflora leads to maturation of glycosyltransferases.[15] It has been postulated that binding of segmented filamentous bacteria to small intestinal epithelial cells is critical for epithelial surface glycoproteins and glycolipids and exhibits strict specificity for the host animal. Thus, segmented filamentous bacteria induce fucosylasialo GM1 glycolipid[16] and α1,2-fucosylated glycans are regulated by *Bacteroides thetaiotaomicron*[14] on the intestinal epithelial cells of mice. Concerning pathogenic types of *Escherichia coli*, α1,2 fucosyltransferase determines the susceptibility of pig small intestinal epithelium to *E. coli* F18 adhesion.[17] Complex sialoglycoprotein of rabbit microvillus membranes acts as receptor for enteropathogenic *E. coli* pilus, which induces cytoskeleton disruption.[18] Similarly, a sialoglycolipid of piglet enterocyte membrane serves as a receptor for *E. coli* K99 fimbriae.[19,20]

In order to gain more insight into microbial-host interaction to control glycosylation, we studied germ-free rat and pig baseline glycosylation of brush-border membrane from various compartments of the small intestine. We studied the effect of nonpathogenic *E. coli* strains in monoassociated rats and pigs on the expression of sialylated and fucosylated glycoconjugates by comparing them with germ-free and conventional animals. We used ELISA-lectin approach to specify sialyl and fucosyl glycans and compared changes in glycosylation of germ-free rats, born from germ-free mother, with germ-free piglets delivered by hysterectomy, reared without the mother in sterile isolaters, and hand-fed with milk formula. As mother's colostrum and milk constituents, such as EGF, are required for normal growth and protection of the intestine from the injury,[21] core 1,6-fucosylation that regulates affinity binding of EGF to EGF receptor[22] was also studied here. We also considered the difference in cytokine production between these two animal models.

Materials and methods

E. coli strain used for colonization of GF pregnant rats and GF piglets

E. coli O83 (strain A034/86 of serotype O83:K24:H31) was initially isolated from porcine feces, characterized,[23] and used in Czech pediatric clinics as Colinfant Newborn (Dyntec, Czech Republic). It is efficient in prophylaxis and treatment of nosocomial infections and diarrhea of preterm and newborn infants.[24]

Animals

Rats

Two pregnant GF rats of Wistar AVN strain were transferred each from a sterile Trexler type of isolaters into another sterile isolater of the same type and fed sterile pellet diet (ST1, Bergman, Kocanda, Czech Republic, 59 kGy γ-irradiated for 30 minutes) and sterile water *ad libitum*.[25] CV pregnant rats were reared under standard conditions and fed the same but nonsterile diet. All animals were kept in a room with a 12 hour light-dark cycle. One of the pregnant rats was monocolonized *per os* through a silicone tube 0.2 mm in diameter (10^8 colony-forming units (CFU)/1 ml of PBS, phosphate buffered saline) with *E. coli* O83. Newborn pups were monocolonized naturally from the mother and until day 25 they could also reach the solid food, giving them a free choice between intake of milk or of solid food. Fecal samples of GF and monocolonized rats were evaluated weekly for the presence of aerobic and anaerobic bacteria, moulds, and yeast using standard microbiological methodology.[26] On day 25 the pups of monocolonized GF and CV groups were anesthetized and sacrificed. Their small intestines were divided into three segments – duodenum, jejunum, and ileum, and prepared for isolation of brush-border membrane vesicles (BBMV). Sera

were prepared for analysis of cytokine levels and spleens were removed under sterile conditions for assessment of splenocyte cytokine production. To study postnatal development of carbohydrate structures in higher age groups, both GF and CV pups were separated from their mothers on day 25.

Pigs

In this study Minnesota miniature pigs derived by repeated crossing with outbred black Vietnamese-Asian and white Malaysian-derived pigs were used as previously described.[27] GF piglets were delivered by hysterectomy under systemic halothane-oxygen anesthesia and housed in special sterile isolators. GF and monocolonized piglets were hand-fed with full-cream condensed cow's milk diluted to 16% dry matter and then autoclaved for 10 minutes at 124°C. Vitamins provided in the diet were: A (1000 IU), D_2 (200 IU), and K_2 (10 mg/1000 ml). Fe-dextran (200 mg Fe^{2+}/kg), was injected intramuscularly soon after birth. The piglets were fed six times daily with a baby flask *ad libitum*, the amount corresponding to age and size. Amounts varied from 50 ml per day for newborns to approximately 840 ml per day for 21-day-old piglets. For each litter delivered by hysterectomy, piglets were randomly assigned to two regimes – GF and *E. coli* O83. Each of the regimes was kept in sterile isolators. On postnatal day 8, GF piglets were given a single dose of nonpathogenic *E. coli* O83 in 1 ml of the milk diet and reared under otherwise sterile conditions for a further 2 weeks. The association of piglets with bacteria was monitored microbiologically. At the end of the experiments (day 22), piglets in the GF and monoassociated groups that weighing between 900 g and 1200 g were chosen for further experimental evaluation. Conventional controls were born normally and reared with their mothers until day 22. On day 22 the animals were bled via *arteria carotis* under halothane/oxygen anesthesia, then their small intestines were removed and divided into duodenum, jejunum, and ileum.

All experiments performed on rats and pigs were approved by the ethical committee of the Institute of Microbiology at the Academy of Sciences, Czech Republic.

Preparation of brush-border membrane vesicles (BBMV)

Brush-border membranes were isolated from duodenal, jejuna, and ileal scrapings prepared independently from each animal, according to the method set out by Kessler et al.[28] Briefly, the mucosal layer was gently scraped off, weighed, frozen in liquid nitrogen, and placed into a deep-freeze until BBMV preparation. BBMV were obtained by calcium precipitation using solid $CaCl_2$ added to the homogenate (1:100 in 50 mmol/l mannitol, 2 mmol/l Tris, pH 7.1) to a final concentration of 10 mmol/l. After standing at 4°C for 20 minutes, the homogenate was centrifuged for 15 minutes at 2,000 × g to spin down precipitated nuclei, mitochondria, and most of the basolateral membranes. The supernatant was centrifuged at 100,000 × g for 60 minutes. The pellet containing vesicles from brush-border membranes was resuspended in 10 mmol/l KCl and stored at –40°C until analysis.

The following lectins were used to characterize carbohydrate epitopes on the isolated brush borders:

SNA (*Sambucus nigra*) – epitope Neu Ac (2,6) GAL, NeuAC (2,6) GAL NAc

MAL II (*Maackia amurensis*) – epitope Neu Ac (2,3) GALβ1,4 Glc NAc

UEA I (*Ulex europaeus* I) – epitope Fucα (1,2) GALβ1,4 Glc NAc

AAL (*Aleuria aurantia*) – epitope core Fucα (1,6) Glc NAc, Fucα (1,2) Gal, Fucα (1,3) Glc NAc, Fucα (1,4) Glc NAc

For the BBMV ELISA lectin labeling was performed in 96 well microtiter plates (Nalge Nunc International, Rochester, NY, USA). BBMV (protein content 10 ng per well or 50 ng per well for MAL II labeling) were fixed onto the plate in 100 µl 0.05 M carbonate buffer, pH 9.25 overnight at 4°C under a slight shaking condition. After successive washings with PBS pH 6.5 and incubation in blocking solution the plates were washed in TTBS (0.1% Tween 20 in Tris buffered saline – 0.05 M Tris-HCl, 0.15 M NaCl, pH 7.5) and incubated in this solution for 1 hour. After liquid removal the BBMV were incubated for 1 hour with SNA-, MAL II-, UEA I-, or AAL-biotin conjugate (10 µg/ml TBS) (Vector Laboratories Inc., Burlingame, CA, USA). After three washes in TTBS, the BBMV were incubated with streptavidin-POD (0.3 U peroxidase/ml) in TTBS. After washings in TBS, peroxidase substrate ABTS solution was added and after 30–60 minutes of absorbance at 405 nm it was measured using the Wallac Victor 1420 Multilabel Counter (East Lyme, CT, USA). The readings represented labeling units for individual lectins. Streptavidin-POD and ABTS were products of Vector Laboratoties Inc., Burlingame, USA.

Spleen cell cultivation

At sacrifice spleens were aseptically removed and single cell suspensions were prepared using RPMI 1640 medium containing 10% fetal calf serum, 2 mM glutamine, 100 U penicillin, and 100 µg/ml streptomycin. Splenocytes were cultured (5×10^6 cells/500 µl) in flat bottom 48 well plates (Corning Incorporated, Acton, MA, USA) with or without 1.5 µg/ml ConA (Sigma-Aldrich, Deisenhofen,

Germany) under 5% CO_2 atmosphere at 37°C. After 48 hours supernatants were collected and stored at −40°C until analysis.

Cytokine assays

Cytokine production was measured in sera and splenic supernatants by commercially available ELISA kits according to manufacturer's instructions: interleukin (IL)-6, IL-10, and tumor necrosis factor (TNF)-α (RD systems, Minneapolis, MN, USA), IL-18 (Demeditec Diagnostics GmbH, Kiel, Germany), and interferon (INF)-γ (Biosource International, CA, USA). For IL-18 and INF-γ measurement supernatants restimulated by Con A were diluted 1:1 and 1:6, respectively; all other samples were assayed undiluted. Results were read using an Infinite M200 (Tecan Group Ltd., Grödig, Austria) and expressed as pg/ml.

Statistics

All values are expressed as mean ± SEM. Statistical significance between means was evaluated by either one-way or two-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test, performed by Graph Pad Prizm version 5.03 software. $P < 0.05$ was considered to be significant.

Results

Postnatal changes in sialylated glycoconjugates in the BBMV in the small intestine of GF and CV rats

Detection of sialic acid residues performed using either SNA that recognizes α2,6-linked sialic acid (Figures 1A and 1B) or MAL II that recognizes α2,3-linked sialic acid (Figures 1C and 1D) in BBMV of CV rats (full line) was highest in suckling rat pups and sharply decreased until weaning. Recognition of α2,6- and α2,3-linked sialic acid revealed higher and delayed labeling of BBMV of GF rats (dotted line), due most probably to less mature GF small intestine having a longer life span of epithelial cells compared with CV ones. The difference in lectin labeling between CV and GF groups of rats was more pronounced in the ileum than in jejunum.

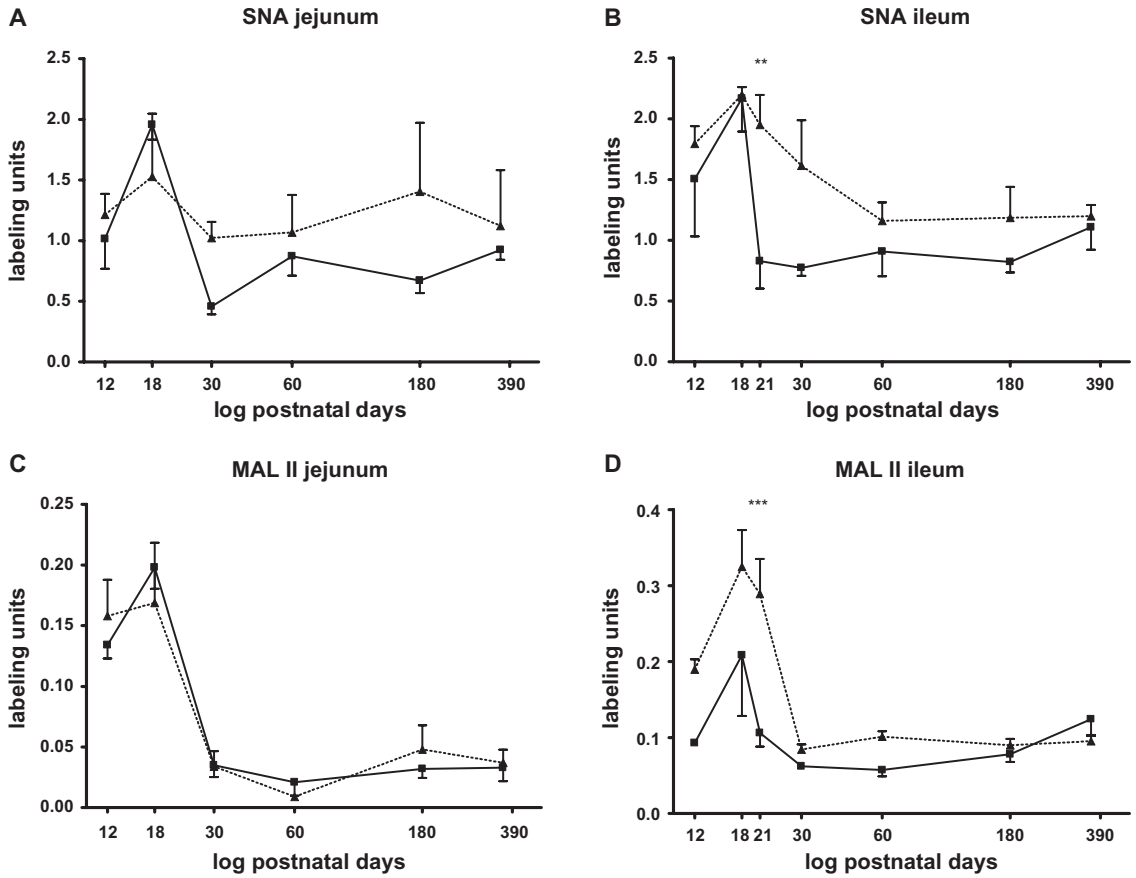

Figure 1 Postnatal changes in sialylated glycoconjugates in BBMV of CV (full line) and GF (dotted line) rats. SNA detection of α2,6 sialylated glycans in **A**) jejunum; in **B**) ileum. MAL II detection of α2,3 sialylated glycans in **C**) jejunum; in **D**) ileum. Values represent the mean ± SEM of 3–5 BBMV preparations. They were submitted to two-way ANOVA analysis. $^{**}P < 0.01$, $^{***}P < 0.001$.

Postnatal changes in fucosylated glycoconjugates in the BBMV of the small intestine of CV and CF rats

Detection of fucose residues was performed using UEA-I lectin specific for α1,2- linked fucose and AAL lectin that recognizes α1,6-, α1,2-, α1,3-, α1,4-linked fucose. In CV rats the UEA-I (Figures 2A and 2B) and AAL (Figures 2C and 2D) labeling started in the suckling period and reached the maximum in the jejunum of weaned rats (on day 30); in the ileum the maximal appearance of labeling with UEA-I and AAL was postponed to 60 days (full line). In the less mature intestines of GF rats the labeling with UEA-I and AAL of the BBMV was reduced compared to that in CV rats (dotted line). The difference in labeling between CV and GF groups of rats was more pronounced in the jejunum than in the ileum. Thus, the result shows that regional variations in fucosylation differ from that of sialylation.

Effect of *E. coli* monoassociation on sialylation of BBMV in rats and pigs

SNA labeling of α2,6-linked sialic acid in GF rat BBMV was significantly higher in both jejunum and duodenum of 25-day-old rats compared with those of time-matched controls (CV group). In the ileum, the region of most abundant sialylation, the difference between GF and CV groups was not significant because of a higher scatter of SNA labeling (Figure 3A). The same figure shows that in *E. coli* O83 monoassociated rats α2,6-sialylation of BBMV is diminished in all three regions, with that in the jejunum being significantly modified compared with GF α2,6-sialylation. Results were similar when they were obtained for piglets monoassociated with *E. coli* O83 strain (Figure 3C). In *E. coli* O83 monoassociated piglets α2,6-sialylation of BBMV in both the jejunum and ileum was significantly diminished compared with that of GF piglets and reached

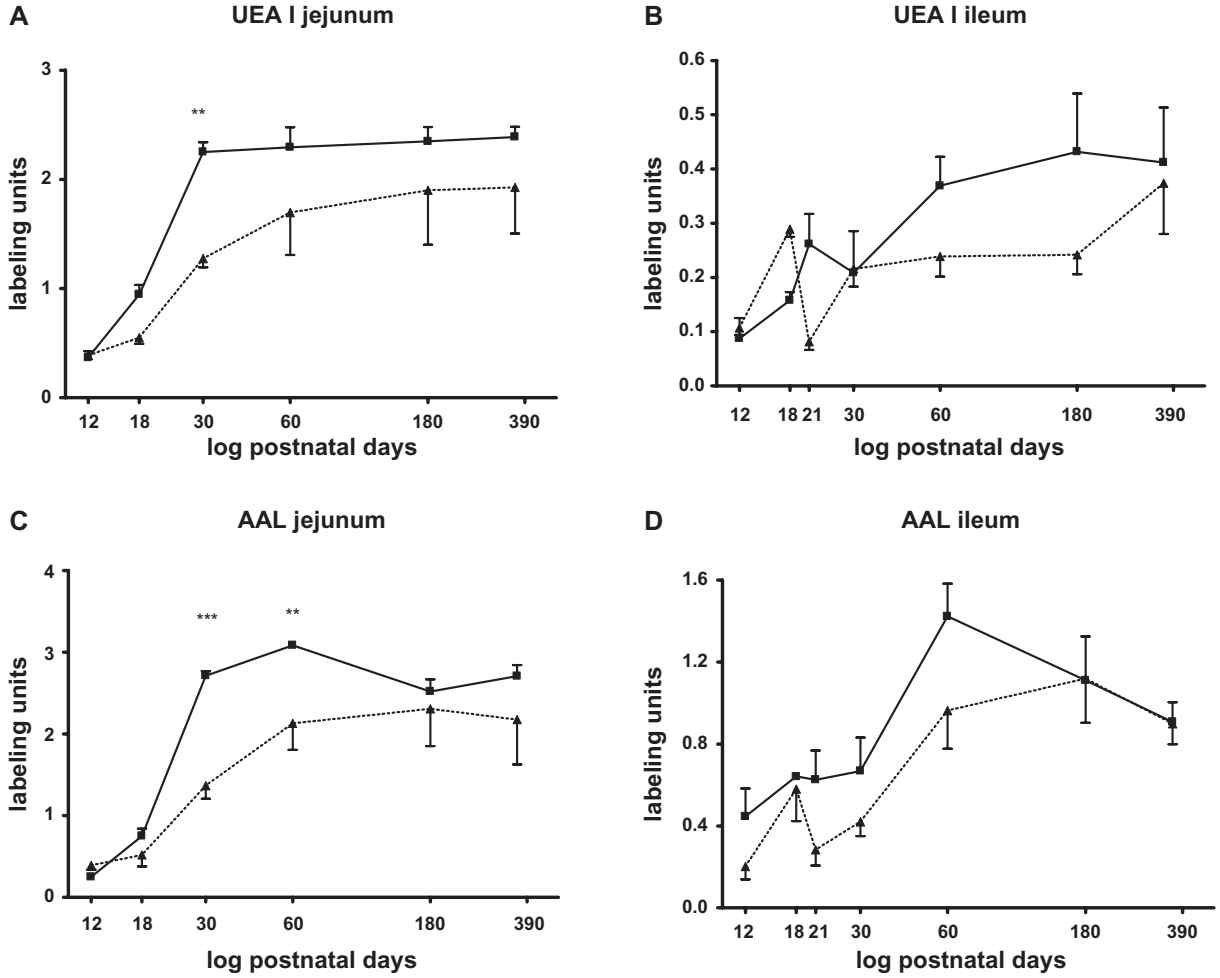

Figure 2 Postnatal changes in fucosylated glycoconjugates in BBMV of CV (full line) and GF (dotted line) rats. UEA I detection of α1,2 fucosylated glycans in **A**) jejunum; **B**) ileum. AAL detection of α1,6-, α1,2-, α1,3-, α1,4-fucosylated glycans in **C**) jejunum; **D**) ileum. Values represent the mean ± SEM of 3–5 BBMV preparations. They were submitted to two-way ANOVA analysis. $^{**}P < 0.01$, $^{***}P < 0.001$.

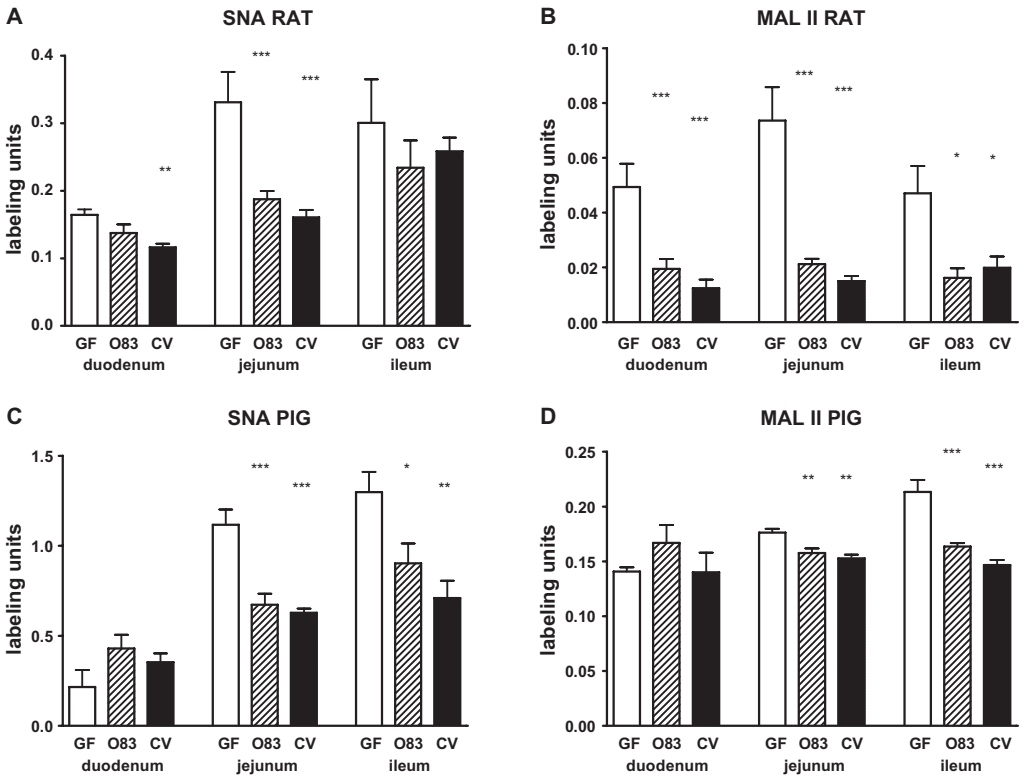

Figure 3 Effect of *E. coli* O83 monoassociation on sialylation of BBMV in duodenum, jejunum, and ileum of rats and pigs. SNA labeling of BBMV in *E. coli* O83 monoassociated rats **A**) and pigs **C**), is diminished versus GF group. MAL II labeling of BBMV in *E. coli* monoassociated rats **B**) and pigs **D**) is diminished versus GF group. Data from experimental groups GF, *E. coli* O83, and CV were analyzed by one-way ANOVA to determine the treatment effect versus GF conditions, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

almost the level found in CV group. No effect of colonization of the intestine with *E. coli* bacteria on SNA labeling was apparent in duodenum.

As seen in Figure 3B MAL II reactive sites (α2,3 sialylation) in CV rats are much less expressed than SNA reactive sites and no progressive reactivity towards MAL II labeling was observed along the length of the small intestine. The difference between higher GF and lower CV amount of Mal II brush-border labeling was highly significant from the duodenum down to the ileum. *E. coli* O83 monoassociation markedly diminished the MAL II labeling almost to the level found in CV rats. In the pig intestine the difference between GF and CV of MAL II labeling, though significant, is smaller in both jejunum and ileum than in case of SNA labeling. Except for duodenum, the *E. coli* strain decreased significantly the MAL II labeling in jejunal and ileal BBMV compared with that in GF piglets (Figure 3D).

Effect of *E. coli* monoassociation on fucosylation of BBMV in rats and pigs

Colonization of the small intestine with single strain, nonpathogenic *E. coli* O83, in rat from birth up to day 25 (Figure 4A) and GF pig from day 8 up until day 22 (Figure 4C) did not significantly modify UEA-I labeling of α1,2-fucose residues compared with those of their GF and CV counterparts. The reason might be that neither in rat nor in pig is fucosylation fully developed. High scatter of labeling enabled us to notice the tendency to increase UEA-I labeling after *E. coli* O83 in more proximal region of the small intestine. AAL labeling of α1,6-, α1,2-, α1,3-, and α1,4-linked fucose in BBMV of *E. coli* associated group was not significantly different from that of CV one and it was evenly distributed along the length of the small intestine (Figure 4B). Otherwise the tendency to increase AAL labeling after *E. coli* colonization above that in GF and CV counterparts is shown in pig duodenum (Figure 4D).

Cytokine expression in rat sera and secretions from splenocytes in GF, *E. coli* monoassociated, and CV rat pups

In this study it was established that sera of rat pups that suckled the milk of GF, *E. coli* O83 monoassociated, and CV mothers exhibit low but detectable levels of TNF-α (Figure 5B) and IFN-γ (Figure 5D). As shown in Figure 5, 5–10 times higher levels of IL-18 (Figure 5A) and IL-10 (Figure 5C) were obtained in sera. Essentially, no significant

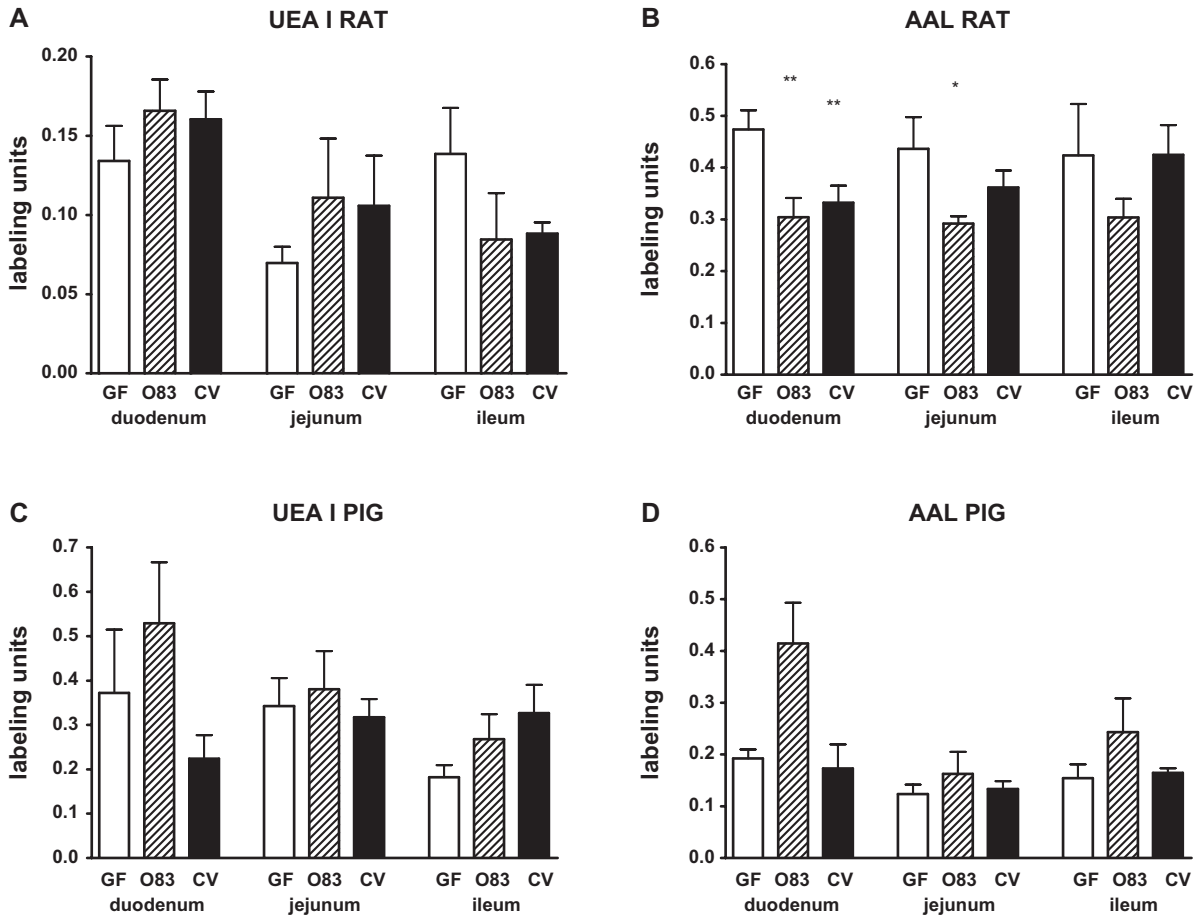

Figure 4 Effect of *E. coli* O83 monoassociation on fucosylation of BBMV in duodenum, jejunum, and ileum of rats and pigs. UEA I labeling of BBMV in *E. coli* O83 monoassociated rats **A**) and pigs **C**) showed nonsignificant tendency to increase versus GF group in proximal regions of the small intestine. AAL labeling in *E. coli* O83 monoassociated rats **B**) showed significant decrease in duodenum and jejunum versus GF group; in pigs **D**) only tendency to increase AAL labeling versus GF group was shown. Data from experimental groups GF, *E. coli* O83, and CV were analyzed by one-way ANOVA to determine the treatment effect. $^{*}P < 0.05$, $^{**}P < 0.01$.

variations between the three groups of rats were found. Appearance of low amounts of TNF-α, IFN-γ, and IL-18 in sera is consistent with reduced gene expression of IL-18, IL-1β, and IFN-γ in the small intestine of naturally suckled rat pups.[29]

To determine whether immune response elicited by *E. coli* O83, accompanied by a nonsignificant increase of IL-18 and IL-10 in sera, is of Th1 or Th2 cytokine type, we followed the secretion of these cytokines from isolated splenocytes (Figure 6). As shown in Figure 6A and Figure 6B pro-inflammatory IL-18 and anti-inflammatory IL-10 are spontaneously secreted by splenocytes under GF condition but *E. coli* monoassociation significantly lowers IL-18 secretion. Precocious nonspecific stimulation of splenocytes with mitogen Con A (concanavalin A) did not change the character of IL-18 secretion from splenocytes of GF, *E. coli* monoassociated, and CV rats. Interestingly, splenocyte stimulation with Con A changes CV involvement in IL-10 secretion from inhibitory to stimulatory effect. TNF-α (Figure 6C), IFN-γ (Figure 6D), and IL-6 (Figure 6E) are secreted in higher proportions only after precocious mitogenic Con A stimulation of splenocytes, with the secretion being partly reduced in *E. coli* monoassociated rats.

Discussion

Bacterial colonization of GF animals plays a crucial role in gene expression of several components of immune[30–32] and intestinal epithelial differentiation[33] systems. Comparison of germ-free and conventionally reared animals revealed that components of microflora modify epithelial cell differentiation including glycoconjugate production.[34] In the present study, using lectin detection of glycoconjugates shows the important role of nonpathogenic *E. coli* in changes of glycoconjugate structures at enterocyte brush-border membrane and in the role of the mother's milk in controlling the production of cytokines during the suckling period. It was important to define the baseline for sialylation and fucosylation of the small intestinal brush-border vesicles of GF rats

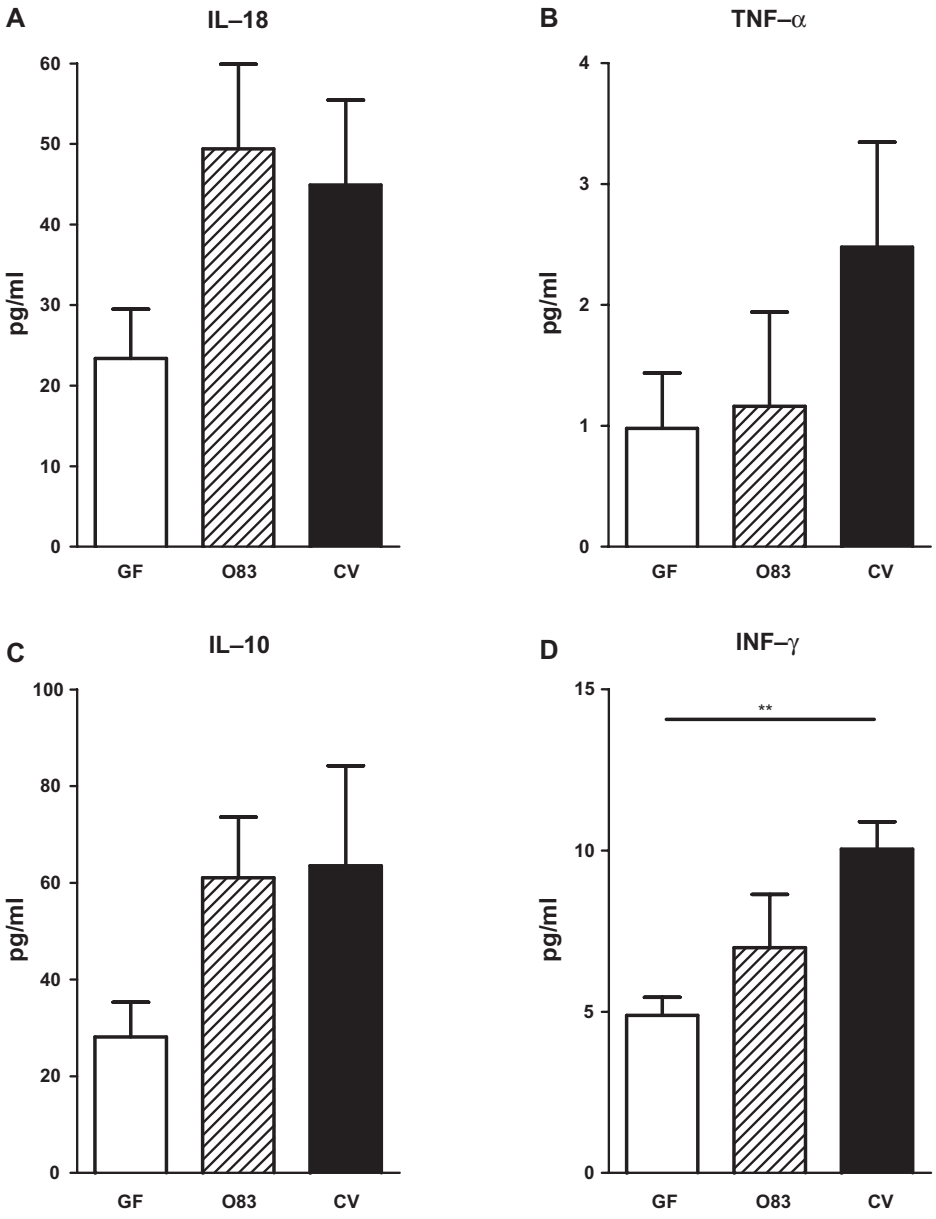

Figure 5 Cytokine levels in rat sera of GF, *E. coli* O83 monoassociated, and CV groups. Sera of rat pups exhibited very low levels of TNF-α, **B**) and IFN-γ, **D**). Some 5–10 times higher levels of IL-18, **A**) and IL-10, **C**) were detected. No significant variations between the three groups were obtained. Data from experimental groups GF, *E. coli* O83, and CV were analyzed by one-way ANOVA to determine the treatment effect versus GF conditions. $^{**}P < 0.01$.

and pigs and to evaluate the contribution of defined bacterial species, monoassociated *E. coli* O83 to the glycosylation pattern, as well as to cytokine production. Because of the known role of nutritional and hormonal – glucocorticoids, thyroxine, insulin – factors[2] on the glycosylation process it was therefore useful to minimize these factors in both GF and monoassociated groups of animals. Variations of these factors were minimized in GF and *E. coli* O83 monoassociated piglets, both were deprived of the trophic effect of colostrum and instead fed milk formula. Similarly concerning rat pups fed with mother's milk of GF and *E. coli* O83 monoassociated mothers, the same nutritional conditions allowed us to compare glycoconjugate expression of these two groups. Besides, the contribution of the constituents of mother's milk in modulation of cytokine production could be evaluated.

Sialylation

Our results show that the programmed developmental decline in the expression of α2,6- and α2,3-sialylated glycoconjugates in rats is completed during the suckling period. In GF rats the slower decline in the expression of these sialylated glycoforms, postponed to the weaning period, compared with time-matched CV rats is in agreement with longer life span

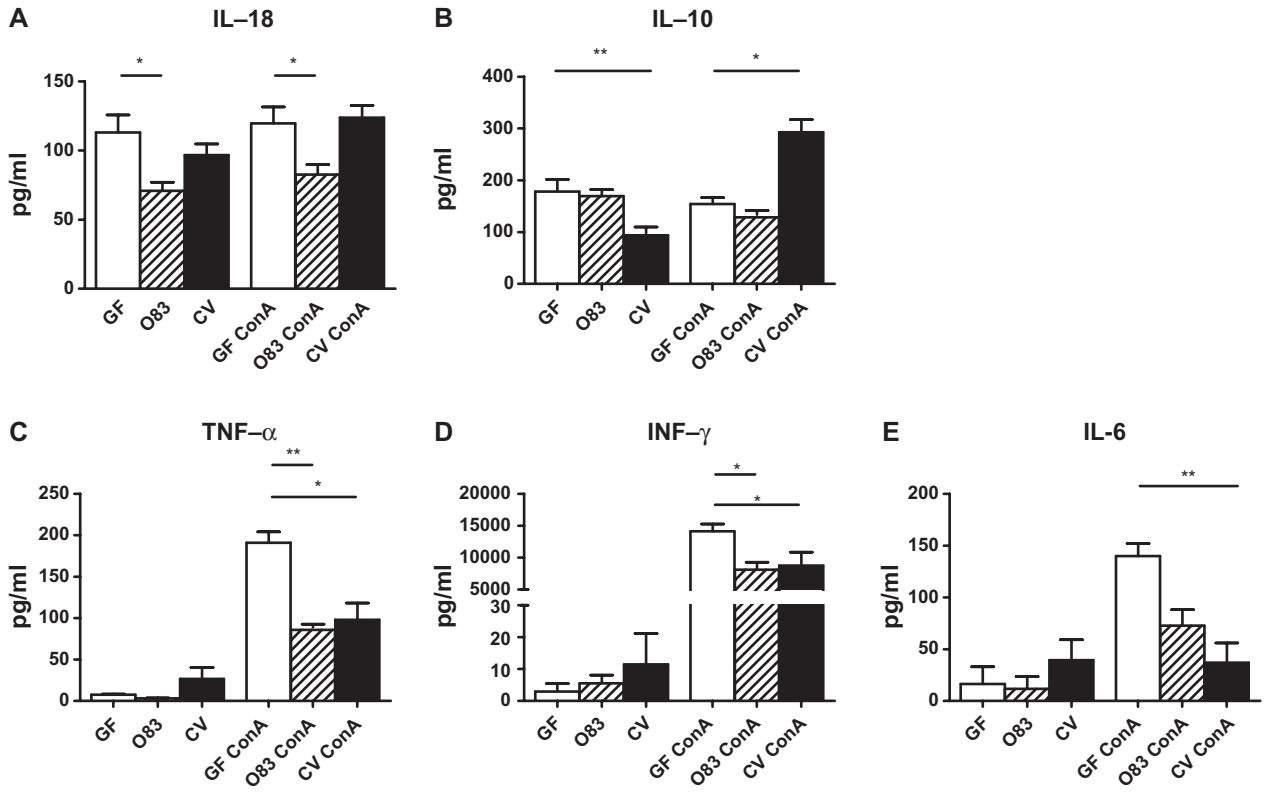

Figure 6 Cytokine secretion from isolated splenocytes of GF, *E. coli* O83 monoassociated, and CV rat groups. As shown here, IL-18 (A) and IL-10 (B) are spontaneously secreted by splenocytes in GF rats but *E. coli* O83 monoassociation significantly lowers the secretion of IL-18 (A). Precocious stimulation of splenocytes with mitogen Con A did not change the character of secretion in **A**) and **B**). TNF-α **C**), IFN-γ **D**) and IL-6 **E**) are secreted in higher proportions only after precocious Con A stimulation of splenocytes, *E. coli* O83 monoassociation decreasing the effect. Data from experimental groups GF, *E. coli* O83, and CV were analyzed by one-way ANOVA to determine the treatment effect versus GF conditions. $^{*}P < 0.05$, $^{**}P < 0.01$.

with altered kinetics of epithelial cell turnover described.[35] For study of the effect of *E. coli* O83 on SNA and MAL II labeling we used 25-day-old rats where the difference in labeling between CV and GF rats for both sialylated and fucosylated glycans was observed. Until the age of 25 days the rat pups were naturally raised with their mothers and could access solid food. *E. coli* monoassociation remarkably substitutes the total microflora of CV rats in suppressing both SNA and MAL II labeling which is in agreement with the shorter life span of enterocytes in *E. coli* monocolonized rats. Similar results we obtained for this remarkable involvement of *E. coli* in regulation of the expression of sialylated glycans in 22-day-old piglets that were fed an artificial diet. In support of our results is recent detailed morphometric analysis of pig intestine after monocolonization by nonpathogenic *E. coli* showing, in contrast with GF pigs, intestinal morphology resembling that of CV pigs[36] with shorter villi and deeper crypts, characterizing shorter life span of enterocytes.

Fucosylation

Fucosylation in the small intestine is a complex process involving coordinated development of fucosyltransferase activities, endogenous protein inhibitor of fucosyltransferase activity displaying an opposite developmental pattern, and availability of the sugar-nucleotide-substrate GDP-fucose (guanidine diphosphate fucose).[37,38] Fucosylation was found to increase during weaning, accompanied by nutritional changes and variations in two fucosyltransferase activities FUT1 and FUT2, both expressed in rat[39] and pig[40] small intestines.

Detection of α1,2-linked fucose by UEA-I lectin and fucoglycoconjugates recognized by AAL lectin in the brush borders indicated appearance of these structures in the suckling period with an increase after weaning up to a maximum between 30–60 days of conventionally raised rats (Figure 2). Our data are consistent with the developmental qualitative and quantitative raise of brush-border fucoglycoproteins[41] showing that the fucosylation is completed in rat intestine between 38–48 days after birth. In the pig intestine fucosylated structures were investigated as antigenic determinants of erythrocyte histoblood group A0 phenotypes. Both FUT1 and FUT2 contribute to formation of α1,2-fucosylated antigenic structures[40] as determinants of pig erythrocyte blood groups, whose full expression was evident at 5–8 weeks of life.[42–44] Binding specificity of AAL with AAL-Sepharose

column[45] and crystal structure of the complex between AAL and fucose[46] showed that this lectin recognizes not only α1,6-core fucosylated N-linked glycoproteins but also α1,2-, α1,3-, and α1,4-fucosylated residues. The broader range of fucose potential binding sites of AAL indicates that also some mucin O-glycans, important for adhesion, cell signaling, and defining various blood group epitopes, react with AAL. It could be expected that the membrane-associated form of Muc3 enriched in brush-border fraction[47] of rat small intestine or the Muc4 membrane mucin[48] of differentiated epithelium of the small intestine, are mucin candidates for AAL binding.

It has been found that intestinal microflora plays a critical role in modifying glycoconjugate production.[33,34] Here we show that the production of UEA-I and AAL fucoglycoconjugates is reduced in the brush-border vesicles of GF rats; in 30-day-old rats this decrease is more pronounced in the jejunum than in the ileum. The decrease in UEA-I fucoglycoconjugate labeling is much less pronounced than that found in the mouse.[34] However, this reduction is not delayed. It starts on day 18 and by the age of 60 days the lectin detection indicated no significant difference between GF and CV rats in both UEA-I and AAL labeling. Of note is the bacterial involvement in regional variation of fucosylation, prevailing in jejunum, that differs from sialylation prevailing in the ileum.

The colonization with a single strain, nonpathogenic *E. coli* O83, of GF rats from birth up to day 25 and GF piglets on day 8 up to day 22, did not significantly modify labeling of α1,2-fucose linked glycoconjugates compared with GF and CV counterparts. The reason is probably the less developed fucosylation at the age mentioned, in both rat pups and piglets, independent of whether they suckled their mother's milk or artificial formula. Only the tendency to increase UEA-I and AAL labeling in the *E. coli* O83 group in the duodenum is masked by high scatter in labeling within the same litter of animals.

Cytokine production in rat pups

In patients with necrotizing enterocolitis[49] and Crohn's disease,[50] chronic lesions exhibit overproduction of pro-inflammatory cytokines, such as IFN-γ, TNF-α, IL-6, and IL-18. However, the regulated expression of inflammatory cytokines during normal physiological processes is essential in intestinal homeostasis and different aspects of development, for which recognition of commensal bacteria by Toll-like receptors (TLR) and replacement of intestinal epithelium plays a crucial role.[51] In rat pups suckling maternal milk of GF, *E. coli* O83 monoassociated, and CV mothers, we observed only negligible levels of TNF-α and IFN-γ but somewhat higher levels of IL-18 and IL-10 in sera, which did not vary significantly between the three groups. Our results are consistent with reduced gene expression of IL-18, IL-1β, and IFN-γ in the small intestine of naturally suckled rat pups.[29] However, they vary from the stimulated gene expression of these cytokines in the ileum of formula fed subjects[29] and increased level of TNF-α in sera of formula fed *E. coli* monoassociated piglets.[27] It means that there exists increased immune response after feeding formula in early postnatal development before immunomodulatory mechanisms, such as oral tolerance, have fully developed. The data suggests maternal milk plays an important role in regulating the immune response mainly by milk derived transforming growth factor (TGF)-β present in high concentrations in milk until weaning.[29] Since the preweaned rats were still on mother's milk, the presence of various immunoregulatory and antimicrobial factors in milk such as EGF, a potent inducer of intestinal migration and wound healing,[52] IL-10 the anti-inflammatory cytokine, IL-1-receptor antagonist, secretory IgA, and spermine,[53] may act to alter inflammatory response.[54] The higher expression of pro-inflammatory IL-18 and anti-inflammatory IL-10 in the sera led us to speculate whether immune response elicited by *E. coli* O83 was of the Th1 or Th2 cytokine type. We therefore examined the production of IL-18 (Th1) and IL-10 (Th2) in the immune organ, the spleen. IL-18 and IL-10 appeared to be the only spontaneously secreted cytokines studied here, the secretion of IL-18 by splenocytes being suppressed in *E. coli* O83 associated rat pups. This might mean that pro-inflammatory IL-18 and anti-inflammatory IL-10, known to be constitutively expressed in many cell types, serve as imunomodulatory pool and that rat pups display both Th1 and Th2 responses. IFN-γ, TNF-α, and IL-6 are secreted in higher proportions only after previous mitogenic stimulation of splenocytes, the secretion being partly reduced in *E. coli* monoassociated rats. In formula fed pigs the nonpathogenic *E. coli* increases the overall enterocyte turnover rate by activation of apoptosis (caspase-3, cystein-activated protease abundance) via TNF-α and FasLigand, and activation of proliferation as well.[55] The correlation between TNF-α and apoptosis is supported by our finding of stimulated gene expression of proapoptotic marker Bax in intestinal epithelial cell line IEC-6,[56] whereas IFN-γ was found to suppress gene expression of antiapoptotic gene Bcl2 in IEC-6, and to inhibit enterocyte migration rate through disruption of gap junction communication between adjacent enterocytes.[57]

While the rat milk model presents information on *E. coli* involvement in immune response toward Th1 and Th2 cytokine type and Th1/Th2 cytokine balance, the *E. coli* monoassociated formula fed pig model has been useful in showing the significant role of nonpathogenic *E. coli* in enterocyte migration rate along the crypt/villus axis by inducing inflammatory responses[55] as previously predicted for TNF-α.[58,27] Both models are suitable for following the changes in the sialylation of brush-border membrane during the suckling period.

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Disclosure

The authors report no conflicts of interest in this work.

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