Colitis development during the suckling-weaning transition in mucin Muc2-deficient mice

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Burger-van Paassen N, van der Sluis M, Bouma J, Korteland-van Male AM, Lu P, Van Seuningen I, Boehm G, van Goudoever JB, Renes IB. Colitis development during the suckling-weaning transition in mucin Muc2-deficient mice. Am J Physiol Gastrointest Liver Physiol 301: G667–G678, 2011. First published June 24, 2011; doi:10.1152/ajpgi.00199.2010.—The mucin Muc2 is the structural component of the colonic mucus layer. Adult Muc2 knockout (Muc2−/−) mice suffer from severe colitis. We hypothesized that Muc2 deficiency induces inflammation before weaning of mother’s milk [postnatal day (P) 14] with aggravation of colitis after weaning (P28). Muc2−/− and wild-type mice were killed at embryonic day 18.5 and P1.5, P7.5, P14, P21, and P28. Colon morphology, influx of T cells, and goblet cell-specific protein expression was investigated by (immuno)histochemistry. Cytokine and Toll-like receptor (TLR) profiles in the colon were analyzed by quantitative RT-PCR. Muc2−/− mice showed an increased and persistent influx of Cd3ε-positive T cells in the colon mucosa as of P1.5. This was accompanied by mucosal damage at P28 in the distal colon but not in the proximal colon. At P14, the proinflammatory immune response [i.e., increased interleukin (IL)-12 p35, IL-12 p40, and tumor necrosis factor-α, expression] in the distal colon of Muc2−/− mice presented with an immune suppressive response [i.e., increased Foxp3, transforming growth factor (TGF)-β1, IL-10, and Ebi3 expression]. In contrast, at P28, a proinflammatory response remained in the distal colon, whereas the immune suppressive response (i.e., Foxp3 and TGF-β1 expression) declined. The proximal colon of Muc2−/− mice did not show morphological damage and was dominated by an immune suppressive response (i.e., Foxp3 and TGF-β1 expression). Importantly, changes in expression of TLRs and TLR-related molecules were observed in the distal colon at P14 and P28 and in the proximal colon only at P28. Colitis in Muc2−/− mice is limited before weaning by immune suppressive responses and exacerbates in the distal colon after weaning because of the decline in the immune suppressive response.

THE EXACT ETIOLOGY OF INFLAMMATORY bowel diseases (IBD), such as ulcerative colitis (UC) and Crohn’s disease (CD), but also necrotizing enterocolitis (NEC), is still partly unknown. Genetic susceptibility and environmental agents are known to be involved in causing IBD (5, 10). In NEC, prematurity as well as dysmaturity and formula feeding are the only consistent risk factors associated with the development of this disease (17, 23, 27). However, the intestinal microbiota seems to play a key role in the onset and perpetuation of inflammation in all these disease entities that share the feature of (entero)colitis. This is supported by the fact that several models for IBD and NEC only develop disease when colonized with bacteria, but not under germ-free conditions (38, 40).

Detection of bacteria in the intestine is mediated by Toll-like receptors (TLRs), which are a family of pattern recognition receptors. Following bacterial recognition, TLRs recruit and activate a variety of signal-transducing adaptor proteins, which trigger a cascade of signaling pathways and ultimately lead to the activation of transcription factors such as NF-κB and interferon regulatory factors (IRFs). Finally, this results in the transcription of proinflammatory cytokines, type I interferons, and chemokines. Intestinal epithelial cells acquire tolerance following colonization, presumably to permit colonization without chronic inflammation (28). However, aberrant expression levels of TLRs have been related to decreased tolerance and the onset and perpetuation of disease. For example, TLR4 is known to be upregulated in CD and UC, and the expression of TLR4 and TLR2 is upregulated in lamina propria macrophages as well as intestinal epithelial cells in IBD patients (7, 13, 15). Additionally, TLR4, which is thought to play an important role in the development of NEC, appears to be upregulated in several rodent NEC models (14, 21, 24–25).

Contact between bacteria and TLRs is limited because of the presence of a physical barrier that is formed by the intestinal mucus layer, of which the mucin MUC2 is the structural component. Johansson et al. (22) elegantly showed that the mucus layer exists of two separate layers. The inner colonic mucus layer, which is in contact with the epithelial cells, is devoid of bacteria. Accordingly, the absence of a protective mucus layer, as in Muc2 knockout (Muc2−/−) mice, allows bacteria to come in direct contact with the epithelial cells, causing intestinal inflammation. Importantly, decreased numbers of goblet cells, the producers of MUC2, and a thinner mucus layer have been related to IBD (20, 35, 37, 46) and NEC (39). Absence of, or breaches in, the mucus layer increase and intensify bacterial-TLR interactions, which in turn might lead to alterations in TLR expression levels.

We previously demonstrated that Muc2−/− mice display clinical and histological signs of colitis from the age of five weeks on (48). It is unknown whether absence of Muc2 already causes development of colitis in the early postnatal period, more specifically, before weaning of mother’s milk. In the present study, we investigated the consequences of Muc2 deficiency during different phases, e.g., during embryonic development, immediately after birth, and during the suckling-weaning transition. We hypothesized that lack of Muc2 in-
duces the development of colitis early after birth, which aggravates during the suckling-weaning transition. We therefore studied morphological changes, influx of immune cells, and expression levels of proinflammatory and immune suppressive cytokines as parameters for colitis development. Furthermore, we analyzed the expression profiles of TLRs and the TLR adaptor protein myeloid differentiation factor 88 (Myd88) during the pre- and postweaning period in wild-type (WT) and Muc2−/− mice.

MATERIALS AND METHODS

Animals. Muc2−/− mice were bred as previously described (48). All mice were generated from Muc2+/− breedings. Mice were housed in the same specific pathogen-free environment with free access to standard rodent pellets (Special Diets Services, Witham, Essex, UK) and acidified tap water in a 12:12-h light-dark cycle. Animal experiments were reviewed and performed with the approval of the Erasmus Medical Center Animal Ethics Committee, Rotterdam, The Netherlands. WT and Muc2−/− mice were tested negative for Helicobacter hepaticus and norovirus infection.

Experimental setup. WT and Muc2−/− littermates were housed together with their birth mother until weaning at the age of 21 days. WT and Muc2−/− mice were killed at the embryonic (E) age of 18.5 days and postnatal (P) ages of 1.5, 7.5, 14, 21, and 28 days. Colonic tissue was excised immediately and either fixed in 4% (wt/vol) paraformaldehyde in PBS, stored in RNA later (Qiagen, Venlo, The Netherlands) at −20°C, or frozen in liquid nitrogen and stored at −80°C.

Histology and histologic grading. Tissue fixed in 4% (wt/vol) paraformaldehyde in PBS was prepared for light microscopy, and 4-μm-thick sections were stained with hematoxylin and eosin (H&E) to study histological changes. To detect differences in mucosal and epithelial thickness in the colon, 10 well-oriented crypts were chosen per intestinal segment and measured using calibrated Leica Application Suite software, version 3.2.0 (Leica Microsystems BV, Rijswijk, The Netherlands). Values are expressed in micrometer (μm) and depicted as box-and-whisker diagrams (maximum value, upper quartile, median, lower quartile, and minimal value).

Immunohistochemistry. Four-micrometer-thick sections were cut and prepared for immunohistochemistry as described previously (50) using the Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA) and 3,3′-diaminobenzidine as staining reagent. Antigen unmasking was carried out by heating the sections for 20 min in 0.01 M sodium citrate (pH 6.0; Merck, Darmstadt, Germany) at 100°C. CD3-positive cells were detected using a mouse monoclonal anti-human CD3ε antibody, which also cross-reacts with mouse CD3ε (1:800 diluted in 1% BSA, 0.1% Triton X-100 in PBS; DAKO, Heverlee, Belgium). To detect trefoil factor 3 (Tff3), rabbit-anti-rat Tff3 [1:3,000 in PBS; a generous gift from D. K. Podolsky (30)] was used. Muc4 was stained using an anti-human-MUC4 rabbit-polyclonal antibody (hHA-1) that recognizes the AGYRPPRPAWTFGD amino acid sequence of the COOH-terminal peptidic region of MUC4ε subunit, which is homologous in humans and mice. The antibody was diluted 1:6,000 in 1% BSA, 0.1% Triton X-100 in PBS. IRF3 was stained using anti-mouse IRF3 (Abcam, Cambridge, UK), diluted 1:500 in 1% BSA, 0.1% Triton X-100 in PBS.

Quantitative real-time RT-PCR (TaqMan technology). Total RNA was prepared using the RNeasy mini-kit from Qiagen, and a total of 1.5 μg was used to prepare cDNA. The mRNA expression levels of all studied genes were quantified using quantitative real-time PCR (qRT-PCR) analysis (TAQman chemistry) based on the intercalation of SYBR Green on an ABI prism 7900 HT Fast Real-Time PCR system (PE Applied Biosystems) as previously described (31). All primer combinations were designed using OLGEO 6.22 software (Molecular Biology Insights) and purchased from Invitrogen. An overview of all primer sequences is given in Table 1. Statistical analysis. Statistical significance was assessed using the Mann-Whitney U-test (Prism, version 5.00; GraphPad software, San Diego, CA). The data were considered statistically significant at P < 0.05.

RESULTS

Significant growth retardation in Muc2−/− mice occurs after weaning. Before birth, at P1.5 days and before weaning (at P14 and P21), there were no significant differences in body weight between Muc2−/− mice and WT mice. However, after weaning, at P28, a significant difference in body weight was observed, as Muc2−/− mice were lighter than WT mice (Fig. 1). No additional clinical symptoms of colitis (i.e., diarrhea, occult blood loss, rectal prolapse) were seen before the age of 28 days.

Characteristic changes in colon morphology in Muc2−/− mice after weaning. H&E staining was used for morphological analysis. No morphological differences were present in the proximal or distal colon between Muc2−/− and WT mice

Table 1. Primer sequences for qRT-PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
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<tr>
<td>TLR2</td>
<td>AAG ATG CGG TCC TGG AAT TGG</td>
<td>TCC AGG GTC TGA GGA ATG C</td>
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<tr>
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<td>ATG CAG CCA CTG AAG TTT T</td>
</tr>
<tr>
<td>TLR9</td>
<td>TTC CCC CAC ATG TCT TCT</td>
<td>AAG GGG GTA CAG ACT TGA</td>
</tr>
<tr>
<td>Myd88</td>
<td>CCT GGG GTC CAT CAC TAT</td>
<td>GCC TCC GCA TGA GTC T</td>
</tr>
<tr>
<td>Cd3</td>
<td>CCA ACC TCA AAT AAT</td>
<td>AAC A</td>
</tr>
<tr>
<td>Cd45</td>
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<td>TGG AGC ACA TGA CTT ATT AG</td>
</tr>
<tr>
<td>Fox3</td>
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<td>GGC AGT GCT TGA GAA AC</td>
</tr>
<tr>
<td>IL-12α</td>
<td>GCC TTC GTA GCA TCT ATG AG</td>
<td>TGG GCA TTG TGA TGA CAG GA</td>
</tr>
<tr>
<td>Ebi3</td>
<td>CCC GGA CAT TCT TCT</td>
<td>GAC GGT CCA GTC ACT TG</td>
</tr>
<tr>
<td>TGF-β1</td>
<td>AAC GAA AGA CAT CTC ACA CA</td>
<td>GCC AGG AAT TGG TGT TAT</td>
</tr>
<tr>
<td>TNF-α</td>
<td>TGG CCT CCC TCT CAT C</td>
<td>GCC TGG CAC CAC TAC TT</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>CCG CAC AGG GTA TGA AA</td>
<td>TCC GAC GTC CTA CTA TTG</td>
</tr>
<tr>
<td>IL-10</td>
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<td>CAT GGC CTT GTA GAC ACC</td>
</tr>
<tr>
<td>Tff3</td>
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<td>AGC TGC CAG GAC TTA C</td>
</tr>
<tr>
<td>Muc4</td>
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<td>TGG CCT CCA TTG TGA G</td>
</tr>
<tr>
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<td>GCT CTT CGG GAT AGA ACT</td>
</tr>
<tr>
<td>β-Actin</td>
<td>GGC ACC TGA GGG ACT AC</td>
<td>TGC CAG AGG ATT GCA TAG</td>
</tr>
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TLR, Toll-like receptor; Myd88, myeloid differentiation factor 88; IL, interleukin; TGF, transforming growth factor; TNF, tumor necrosis factor; IFN, interferon; Tff, trefoil factor; Muc, mucin.
before birth, except for the absence of the characteristic bell-shaped morphology of the goblet cells (Fig. 2). There were no evident signs of inflammation or epithelial damage at E18.5, P1.5, and P14 in the proximal or distal colon. A change in morphology was seen after weaning, with the first signs of colitis, such as epithelial flattening and superficial erosion in the distal colon at P28 (Fig. 2). In contrast, in the proximal colon, morphological damage was still not observed at this day.

Distal colonic crypt length increased during aging and was significantly different between WT and Muc2−/− mice at P21 and P28 (Fig. 3). Increased influx of immune cells in the distal colon of Muc2−/− mice. Because morphological signs of colitis were only observed in the distal colon of Muc2−/− mice, we first focused our attention on the distal colon. Cd3ε was used as a marker for T cell infiltration and was analyzed by immunohistochemistry (Fig. 4, showing representative stained sections of the distal colon of WT and Muc2−/− mice). At P1.5, there was

Fig. 1. Body weight of wild-type (WT) and Muc2 knockout (Muc2−/−) mice during postnatal age (P) 1.5 to P28. Body weight of WT (open bars) and Muc2−/− (hatched bars) mice in time. No significant differences were observed between WT and Muc2−/− mice, except at the age of 28 days. Values are depicted as means ± SE.

Fig. 2. Colonic morphology of WT and Muc2−/− mice. Morphology of the distal colon in WT (top) and Muc2−/− (bottom) mice by hematoxylin and eosin staining. Representative sections of the distal colon are depicted at embryonic day (E) 18.5, P1.5, P14, P21, and P28. The tissue samples are representative of all mice in the studied groups. Note, no evident signs of inflammation or epithelial damage were seen at E18.5, P1.5, and P14. After weaning, at P28, the first signs of colitis (i.e., epithelial flattening and superficial erosion) were seen.

Fig. 3. Crypt length is increased significantly in Muc2−/− mice at P21 and P28. Crypt length in the distal colon of WT (solid boxes) and Muc2−/− (hatched boxes) mice at the age of 14 days (white), 21 days (light gray), and 28 days (dark gray). Crypt length was significantly different between WT and Muc2−/− at the age of 21 and 28 days.
Altered cytokine gene expression profiles in the distal colon of Muc2−/− mice. To assess the development of colitis in the distal colon in further detail, gene expression levels of a subset of cytokines, as mentioned in Table 1, were analyzed. We first focussed on interleukin (IL)-35, a heterodimer of IL-12 p35 and Ebi3, which is produced by Treg cells and inhibits T cell proliferation and thereby limits inflammation. Both IL-12 p35 (Fig. 5D) and Ebi3 (Fig. 5E) were significantly higher in Muc2−/− mice compared with WT mice at P14. Interestingly, IL-12 p35, which is expressed at much lower levels than Ebi3 and therefore determines IL-35 levels (8), decreased significantly from P14 until P28 in Muc2−/− mice, but not in WT mice. Gene expression levels of transforming growth factor (TGF)-β1, another peptide with immune suppressive capacities, also decreased significantly from P14 to P28 in Muc2−/− mice compared with WT mice. Gene expression levels of TGF-β1 significantly increased from P14 until P28 (Fig. 5F). Furthermore, at P28, expression of TGF-β1 was significantly lower in Muc2−/− mice compared with WT mice.

Tumor necrosis factor (TNF)-α, a proinflammatory cytokine produced by macrophages, dendritic cells, and subsets of T cells known as Th1 and Th17 cells, is significantly higher in Muc2−/− mice at P14 as well as P28 (Fig. 5G). Yet, in Muc2−/− mice, TNF-α gene expression levels decreased from P14 until P28, whereas, in WT mice, TNF-α expression remained stable. Interferon (IFN)-γ, which is also a proinflammatory cytokine and generally related to a Th1-type immune response, was not significantly different between WT and Muc2−/− mice at P14 and P28, neither were there significant differences from P14 to P28 in WT nor in Muc2−/− mice (Fig. 5H). IL-10, an immune suppressive cytokine that inhibits the expression of proinflammatory cytokines like IFN-γ, IL-2, and TNF-α, was significantly higher in Muc2−/− mice compared with WT mice at P14 (Fig. 5I). Interestingly, in Muc2−/− mice, IL-10 gene expression levels remained high, since gene expression levels were similar between P14 and P28. Finally, in WT mice, IL-10 expression levels showed a trend toward

Fig. 4. The amount of Cd3-positive T cells is increased in Muc2−/− mice before and after weaning. Infiltration of T cells determined by Cd3 immunohistochemistry on distal colon sections at different ages (P1.5, P14, and P28). Insets show higher magnification to point out individual Cd3-positive T cells. The tissue samples are representative of all mice in the studied groups. Note, the influx of Cd3-positive T cells was increased in WT mice before weaning. Weaning from mother’s milk resulted in exacerbation of colitis in Muc2−/− mice but not WT mice.

already a marked increase in the amount of Cd3ε-positive T cells in Muc2−/− mice, whereas in WT mice there were hardly any Cd3ε-positive T cells visible. Interestingly, the abundance of Cd3ε-positive T cells slightly increased in WT mice before weaning, whereas a subsequent decrease was observed after weaning. In contrast, in Muc2−/− mice, a marked increase in the amount of Cd3ε-positive T cells during aging was seen regardless of weaning. Moreover, the number of infiltrating Cd3ε-positive cells was higher in Muc2−/− mice compared with WT mice at each time point investigated.

In addition to the immunohistochemical analysis, we also determined the presence of immune cells in the distal colon by qRT-PCR (Fig. 5, A–C). Cd45 was used as a marker for all differentiated hematopoietic cells except erythrocytes and plasma/B cells, Cd3ε as a marker for T cells, and Foxp3 as a marker for regulatory T cells (Treg cells). At P14 and P28, Muc2−/− mice showed a significant increase in Cd45 levels and a trend toward increased expression of Cd3ε, respectively, indicating increased numbers of immune cells/T cells in Muc2−/− mice compared with WT mice. Gene expression levels of Foxp3, the marker for Treg cells, which have immune suppressive functions, were increased significantly in Muc2−/− mice compared with WT mice at P14 but not at P28. Moreover, in Muc2−/− mice, expression levels of Foxp3 were decreased significantly at P28 compared with P14.

Altered cytokine gene expression profiles in the distal colon of Muc2−/− mice. To assess the development of colitis in the distal colon in further detail, gene expression levels of a subset of cytokines, as mentioned in Table 1, were analyzed. We first focussed on interleukin (IL)-35, a heterodimer of IL-12 p35 and Ebi3, which is produced by Treg cells and inhibits T cell...
increased expression from P14 to P28. Analysis of the Th2 cytokines IL-4 and IL-13 and the Th17 signature cytokines IL-17A and IL-23 did not reveal differences between Muc2/H11002/H11002 and WT mice at P14 or P28 (Supplemental Fig. 2).

Altered mRNA expression of TLRs and TLR signaling molecules in the distal colon of Muc2/H11002/H11002 mice. Because bacterial colonization plays a pivotal role in the development of colitis, we studied the expression of several TLRs and TLR-signaling molecules in the distal colon during the suckling-weaning transition, i.e., P14 vs. P28, in WT and Muc2/H11002/H11002 mice. Gene expression levels of TLR2, a TLR that recognizes several bacterial ligands such as glycolipids, lipopeptides, and lipoproteins, were not significantly different between WT and Muc2/H11002/H11002 mice at the age of 14 days. Nevertheless, at P28, there was a significantly lower level of TLR2 mRNA in Muc2/H11002/H11002 mice compared with WT mice (Fig. 6A). The mRNA expression of TLR4, a receptor for lipopolysaccharide, was increased significantly in Muc2/H11002/H11002 mice at P14 compared with WT mice (Fig. 6B). Although not significant, there was still a trend toward increased TLR4 mRNA expression in Muc2/H11002/H11002 mice at P28. TLR9, the TLR for unmethylated CpG DNA (i.e., bacterial DNA), showed increased mRNA expression levels in Muc2/H11002/H11002 mice compared with WT mice at P14 (P = 0.0317) and P28 (nonsignificant) (Fig. 6C). Finally, Myd88, an intracellular signaling adaptor protein that is involved in TLR signal transduction, was studied in the distal colon. There were no

![Graphs showing mRNA expression levels of immune response genes](http://ajpgi.physiology.org/)

Fig. 5. Altered expression of immune response genes in the distal colon of Muc2/H11002/H11002 mice before and after weaning. Relative gene expression levels of immune response genes in the distal colon of WT and Muc2/H11002/H11002 mice at P14 and P28. Gene expression levels were normalized to β-actin and depicted as medians. Groups are depicted as follows: WT, P14 (●); Muc2/H11002/H11002, P14 (○); WT, P28 (●); and Muc2/H11002/H11002, P28 (○). Note, a strong immune response was seen before weaning (at P14) with increased expression of Cd45, Cd3ε, Foxp3, interleukin (IL)-12 p35, Ebi3, transforming growth factor (TGF)-β1, tumor necrosis factor (TNF)-α, interferon (IFN)-γ, and IL-10 gene expression levels in the distal colon of Muc2/H11002/H11002 mice. After weaning, at P28, Foxp3, IL-12 p35, TGF-β1, and TNF-α gene expression levels are significantly downregulated in Muc2/H11002/H11002 mice (compared with P14), whereas IL-10 gene expression levels are maintained.
Fig. 6. Altered expression of Toll-like receptors (TLRs) and myeloid differentiation factor 88 (MyD88) in the distal colon of Muc2−/− mice before and after weaning. Relative mRNA expression levels of TLRs and TLR signaling molecules in the distal colon of WT and Muc2−/− mice at P14 and P28. TLR2 mRNA was significantly different between WT and Muc2−/− mice at P28. In Muc2−/− mice, expression significantly decreased during aging (A). Expression of TLR4 was significantly higher in Muc2−/− mice compared with WT mice at P14 (B). TLR9 expression was significantly higher in Muc2−/− mice at P14 compared with WT mice (C). MyD88 significantly decreased during aging in Muc2−/− mice (D). Gene expression levels were normalized to β-actin and depicted as medians. Groups are depicted as follows: WT, P14 (●); Muc2−/−, P14 (○); WT, P28 (●); and Muc2−/−, P28 (○).

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significant differences in MyD88 mRNA expression levels between WT and Muc2−/− mice at P14 or P28. However, in Muc2−/− mice, expression of MyD88 mRNA was decreased significantly at P28 compared with P14, whereas, in WT mice, expression levels remained unaltered (Fig. 6D).

Goblet cell differentiation is altered in the distal colon of Muc2−/− mice. The mucin Muc4 and Tff3 were used as goblet cell markers. In both types of mice, Tff3-positive and Muc4-positive goblet cells were localized from the lower crypt to the surface epithelium at E18.5 and P1.5 (Fig. 7A). A similar localization of Tff3-positive goblet cells was seen at P14 and P28 (Fig. 7A, top). Interestingly, because of crypt lengthening in Muc2−/− mice, the total number of Tff3-positive goblet cells seemed to increase at P28 compared with WT mice at P28 and with Muc2−/− mice at P14. Focusing on Muc4 expression, we observed that Muc2−/− mice hardly showed Muc4-positive goblet cells at P14 and P28 compared with WT mice (Fig. 7A, bottom).

Next to the Tff3 and Muc4 protein localization studies, qRT-PCR was performed to determine Tff3 and Muc4 gene expression levels normalized to villin, which in the colon is mainly expressed by enterocytes (Fig. 7B). In WT mice, Tff3 gene expression decreased from P14 to P28, whereas Muc4 gene expression increased. In Muc2−/− mice both Tff3 and Muc4 gene expression levels did not show any increase or decrease and were thus comparable between P14 and P28.

Remarkably, at P14, Tff3 mRNA levels were significantly lower in Muc2−/− mice than in WT mice. Additionally, analysis of another goblet cell marker, Spdef, showed that Spdef gene expression levels did not differ between WT and Muc2−/− mice at P14 and P28 (Supplemental Fig. S3). However, in both WT mice and Muc2−/− mice, Spdef gene expression levels significantly decreased at P28 compared with P14.

Increased influx of immune cells in the proximal colon of Muc2−/− mice. Although there were no major morphological changes in the proximal colon of Muc2−/− mice before and after weaning as described above (Supplemental Fig. 1), we still decided to determine the presence of immune cells in this part of the colon by qRT-PCR (Fig. 8, A–C). These studies demonstrated significantly increased Cd45 expression levels in Muc2−/− mice at P14 and P28 (Fig. 8B). Cd3ε and Foxp3 expression levels increased from P14 to P28 in Muc2−/− mice and WT mice (Fig. 8, A and C). There were no differences in CD3ε and Foxp3 expression levels between Muc2−/− mice and WT mice at P14; however, at P28, expression levels of these genes trended higher in Muc2−/− mice.

Altered cytokine gene expression profiles in the proximal colon of Muc2−/− mice. To assess the inflammatory response in the proximal colon in further detail, gene expression levels of cytokines, mentioned in Table 1, were analyzed (Fig. 8, D–I). IL-12 p35 and Ebi3 expression showed a similar expression pattern (Fig. 8, D and E). Specifically, in Muc2−/− mice
Fig. 7. Embryonic and early postnatal expression of intestinal goblet cell markers in the distal colon of WT and Muc2−/− mice. A: immunohistochemical staining for trefoil factor 3 (Tff3, top) and Muc4 (bottom) in distal colon of WT and Muc2−/− mice at E18.5 and P1.5, P14, and P28. For each type of staining, panels on top represent WT mice, and panels on bottom represent Muc2−/− mice. The tissue samples are representative of all mice in the studied groups. B: Tff3 (left) and Muc4 (right) gene expression levels normalized to villin gene expression and depicted as medians. Groups are depicted as follows: WT, P14 (●); Muc2−/−, P14 (○); WT, P28 (●); and Muc2−/−, P28 (○). Note, Muc2−/− mice hardly show Muc4-positive goblet cells at P14 and P28, but Muc4 gene expression levels in Muc2−/− mice are comparable with WT mice. An increase in Tff3-positive goblet cell numbers is seen in Muc2−/− mice at P28 compared with WT at P28 and Muc2−/− mice at P14. Tff3 gene expression levels normalized to villin are comparable between Muc2−/− mice at P28, WT at P28, and Muc2−/− mice at P14.
and WT mice, IL-12 p35 and Ebi3 levels significantly increased from P14 to P28. At P14, no differences were found in expression levels of these genes between Muc2−/− and WT mice. Yet, IL-12 p35 and Ebi3 expression levels were significantly higher in Muc2−/− mice at P28. Expression levels of the immune suppressive cytokines TGF-β1 and IL-10 increased significantly from P14 to P28 in both Muc2−/− and WT mice (Fig. 8, G and I). Furthermore, expression levels of TGF-β1 were similar between Muc2−/− and WT mice at P14 and P28. Interestingly, IL-10 expression was significantly higher in Muc2−/− at P14 but not at P28. Finally, IFN-γ gene expression levels were extremely low in the proximal colon and did not differ significantly between Muc2−/− and WT mice at P14 and P28 (Fig. 8H).

Altered mRNA expression of TLRs and MyD88 in the proximal colon of Muc2−/− mice. Because we found major changes in cytokine expression patterns in the proximal colon, we analyzed the gene expression profiles of TLRs and the TLR adaptor protein MyD88 in the proximal colon (Fig. 9). There were no differences in TLR2 expression levels in Muc2−/− mice compared to WT mice at P14 and P28.
mice compared with WT mice at P14 and P28 (Fig. 9A). In contrast, TLR4 expression significantly increased in Muc2−/− mice from P14 to P28, whereas, in WT mice, expression levels remained unaltered (Fig. 9B). TLR9 expression was significantly higher in Muc2−/− mice after weaning. The influx of TLR9-positive T cells in the colonic mucosa shortly after birth is the first indicator of colitis development in these mice. These data also demonstrate that the inflammatory process in Muc2−/− mice is triggered before morphological changes are seen and that Muc2−/− mice are predestined to develop colitis. Interestingly, in WT mice, an increased influx of T cells in the colonic mucosa occurred shortly before weaning (P14), which decreased again after weaning (P28). This influx of T cells might be considered as a physiological phenomenon, since T cells and T cell activity is nec-
The increase in Cd45, Cd3ε, and Foxp3 gene expression levels in combination with increased IL-12 p35, Ebi3, TGF-β1, TNF-α, and IL-10 gene expression levels in the distal colon of Muc2−/− mice before weaning (P14) imply that, in this part of the colon, a vigorous immune response is already developing before weaning. This immune response involves Cd45- and Cd3-positive T cells and the proinflammatory cytokine TNF-α and likely IL-12 (i.e., a heterodimer of IL-12 p35 and IL-12 p40), and is counterbalanced by Foxp3-positive Treg cells and the immune suppressive cytokines TGF-β1, IL-10, and most likely IL-35 (i.e., the heterodimer of IL-12 p35 and Ebi3). After weaning, at P28, Foxp3, IL-12 p35, TGF-β1, and TNF-α gene expression levels are significantly downregulated in the distal colon of Muc2−/− mice (compared with P14), whereas expression levels of the immune suppressive cytokine IL-10 are maintained.

The immune response in the proximal colon of Muc2−/− mice is characterized by increased Cd45 and IL-10 gene expression before weaning (P14) and increased Cd3, Foxp3, IL-12 p35, and Ebi3 levels after weaning (P28) (all compared with WT mice). These data suggest that, in the proximal colon of Muc2−/− mice, the immune response is dominated by an immune suppressive response with the appearance of Treg cells only after weaning. Interestingly, not only Muc2−/− mice but also WT mice showed increased Cd3, Foxp3, IL-12 p35, Ebi3, TGF-β1, and IL-10 levels in the proximal colon from P14 to P28, suggesting suppression of the immune response as reaction upon the changes in diet and microbiota.

Next, we studied the expression of different TLRs and TLR signaling molecules that are involved in recognizing bacterial products in the intestinal lumen. We focused our attention on TLR2, TLR4, and TLR9, since these TLRs enable us to differentiate between responses that are induced by gram-positive bacteria, which mainly induce a TLR2 response; gram-negative bacteria, which signal predominantly through TLR4; and bacterial CpG-DNA motifs, which signal via TLR9 (42). Decreased expression of TLR2 was seen after weaning (P28) in the distal colon of Muc2−/− mice, correlating with the occurrence of severe signs of colitis. Loss of TLR2 has been related to exacerbation of intestinal inflammation (36). Additionally, it has also been demonstrated that TLR2 deficiency predisposes to stress-induced injury of tight junction modulated barrier function, leading to perpetuation of mucosal inflammation and apoptosis (6). Thus, besides loss of barrier function because of the absence of a protective mucus layer, barrier function in Muc2−/− mice might be impaired further when these mice are exposed to stressors, since Muc2−/− mice suffer from partial TLR2 deficiency.

In Muc2−/− mice, increased TLR4 and TLR9 gene expression was observed in the proximal colon at P28 and in the distal colon at P14, although only trending at P28. Together with the fact that morphological damage was only seen in the distal colon of Muc2−/− mice at P28, these data suggest that TLR4 and TLR9 play an important role in limiting colitis and also in aggravation of colitis. In line with this, in the intestinal mucosa of IBD patients, overexpression of TLR4 is seen (7). Furthermore, in dextran sulfate sodium (DSS)-induced colitis in mice, TLR4 appeared to be involved in the induction of colitis and the recovery phase of colitis (47). Finally, Obermeier et al. (33) showed that CpG-DNA from endogenous bacteria and thus TLR9 contributes to the perpetuation of chronic intestinal inflammation, which also corroborates with our data. Expression levels of MyD88 mRNA, which forms a signaling complex with the activated TLRs, were significantly lower in the distal colon of Muc2−/− mice at P28 compared with P14. MyD88 is crucial for the activation of the innate immune defense against microorganisms (1, 26, 34). Moreover, it was shown that MyD88−/− mice exhibited increased susceptibility to DSS-induced colitis and that the MyD88-dependent pathway may directly promote the proliferation and survival of colitogenic CD4-positive T cells to sustain chronic colitis, implying a role for MyD88 in colitis (3, 45). One could speculate that MyD88 expression is downregulated in the distal colon of 28-day-old Muc2−/− mice to protect the intestinal epithelium from aberrant TLR signaling due to deviant bacterial colonization; however, more studies are necessary to confirm this. On the other hand, MyD88 gene expression levels were significantly higher in the proximal colon of Muc2−/− mice at P28, the location and moment were mucosal damage is not observed. This implies that MyD88 might play a role in the induction of an immune suppressive response, thereby preventing/limiting mucosal damage in the proximal colon. In addition to the molecules involved in the inflammatory response, we studied the function and differentiation of goblet cells in the distal colon during colitis development. Immunohistochemical analysis revealed an increase in Tff3-positive goblet cell numbers in Muc2−/− mice at P28 compared with WT at P28 and Muc2−/− mice at P14. It is highly likely that this increase in goblet cell numbers is the result of the observed crypt elongation in Muc2−/− mice, which leads to an increase in epithelial cell numbers in general and thereby also to more goblet cells. The increase in goblet cells is most likely not caused by a predominant differentiation to goblet cells at the expense of other epithelial cell types, since Tff3 gene expression levels normalized to villin (i.e., a gene, which in the colon is mainly expressed by enterocytes) are comparable between Muc2−/− mice and WT at P28 and between Muc2−/− mice at P28 and Muc2−/− mice at P14. Similarly, gene expression of Spdef, another marker of goblet cells, is comparable between Muc2−/− mice and WT mice at P14 and P28. Nevertheless, because Tff3 is known to be an important factor that contributes to healing after mucosal injury throughout the gastrointestinal tract (16, 41, 43–44), the increased Tff3-positive goblet cell numbers in Muc2−/− mice at P28 might be a compensatory mechanism stimulating mucosal regeneration.

Finally, Muc2−/− mice hardly show Muc4-positive goblet cells at P14 and P28. Yet, Muc4 gene expression levels in Muc2−/− mice are comparable with WT mice. In conjunction, these results imply that Muc4 protein secretion is increased in Muc2−/− mice compared with WT mice. This might be caused by a deviant bacterial colonization in Muc2−/− mice compared with WT mice and/or could be caused by inflammatory cytokines that are highly expressed in Muc2−/− mice. Indeed, several in vitro and in vivo studies confirm that bacterial products and proinflammatory cytokines are able to influence mucus synthesis (2, 4, 12, 19, 32).

In conclusion, we demonstrated that diminished epithelial protection due to absence of Muc2 plays a major role in the induction and perpetuation of inflammation in the colon. Muc2−/− mice showed an influx of Cd3ε-positive T cells in the


