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Intestinal steroid profiles and microbiota composition in colitic mice

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Keywords: inflammatory bowel disease, colorectal cancer, intestinal microbiota, neutral sterols, bile acids, cholesterol, coprostanol

Abbreviations: BA, bile acid; CA, cholic acid; DCA, deoxycholic acid; FISH, fluorescence in situ hybridization; IBD, inflammatory bowel diseases; IL-10, interleukin 10; α-MCA, alpha-muricholic acid; ω-MCA, omega-muricholic acid

Reduced gut microbiota diversity in conjunction with a bloom of a few bacterial species is a common feature in inflammatory bowel disease (IBD) patients. However, the environmental changes caused by inflammation and their possible impact on the microbiota are largely unknown. Since IBD is associated with an impaired intestinal steroid metabolism, we hypothesized that changes in intestinal steroid and particularly bile acid (BA) concentrations affect microbial communities. We used Interleukin-10 deficient (IL-10$^{-/-}$) mice as a model for chronic gut inflammation. Healthy wild-type mice served as controls. In these animals, intestinal steroid concentrations and gut microbial diversity were analyzed at 24 weeks of age. The IL-10$^{-/-}$ mice developed moderate inflammation in cecum and colon and colorectal tumor formation was observed in 55% of the animals. Compared to the healthy conditions, gut inflammation was associated with higher intestinal cholesterol and cholic acid concentrations and a reduced microbial diversity. The latter was accompanied by a proliferation of *Robinsoniella peoriensis*, *Clostridium innocuum*, *Escherichia coli* and *Enterococcus gallinarum*. All these species proved to be highly bile acid resistant. We concluded that chronic colitis in IL-10$^{-/-}$ mice is associated with changes in intestinal steroid profiles. These changes may be due to alterations in gut microbiota composition or vice versa. Whether the bacterial sterol and bile acid metabolism is implicated in colitis and colorectal carcinoma etiology remains to be clarified.

Introduction

The etiology of inflammatory bowel diseases (IBD) has not fully been elucidated. It is generally accepted that IBD results from an excessive immune response towards the commensal intestinal microbiota in a genetically susceptible host.[1] Animal IBD models and IBD patients show a significantly reduced intestinal microbiota diversity, often associated with an increased abundance of some bacterial species.[2-5] However, it has remained unclear whether an altered gut microbiota is the cause or a consequence of the disease.

To better understand the interactions between commensal gut bacteria and the host under chronic gut inflammation, we previously analyzed the intestinal microbiota of Interleukin-10 knockout (IL-10$^{-/-}$) mice, a widely used IBD model. The microbiota in the inflamed gut of these mice is less diverse than in the healthy wild-type controls and dominated by only a few bacterial species including *Escherichia coli* and *Blautia producta*. We speculated that the chronic inflammatory state changes the environmental conditions in the gut, which in turn may affect growth of some gut bacteria. In this way, bacteria capable of adapting to these conditions could benefit from the disease.[6]

One factor possibly affecting the gut microbiota in the inflamed intestine is an impaired metabolism of neutral sterols and bile acids (BAs), which is a common clinical feature of IBD.[7,8] BAs exert antimicrobial effects by damaging the cytoplasmic membrane or the DNA, or by inducing oxidative stress[9-11] and preferentially inhibit the growth of strict anaerobes.[12,13] However, the ability to transform BAs is widespread among commensal gut bacteria.[14]

We therefore hypothesized that the steroid metabolism is impaired under chronic gut inflammation and that the resulting higher BA concentrations influence gut microbiota composition by stimulating BA-resistant and/or inhibiting BA-susceptible bacterial species. To find experimental support for this hypothesis, we measured intestinal steroid concentrations in 24-week-old IL-10$^{-/-}$ mice with chronic gut inflammation and in healthy wild-type animals. In addition, the intestinal microbiota composition was analyzed with 16S rRNA gene sequencing. Selected bacterial groups were quantified by fluorescence in situ hybridization

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Table 1. Intestinal steroid concentrations in the intestine of 24-week-old IL-10$^{-/-}$ and wild-type mice (n = 4 per group)

	Small intestine		Cecum		Colon		Feces	
	WT	IL-10$^{-/-}$	WT	IL-10$^{-/-}$	WT	IL-10$^{-/-}$	WT	IL-10$^{-/-}$
Steroids								
Cholesterol	2.09 ± 1.20	2.16 ± 1.08	0.69 ± 0.29	1.63 ± 0.63*	0.64 ± 0.13	1.27 ± 0.30**	0.76 ± 0.07	1.15 ± 0.27*
Coprostanol	0.01 ± 0.01	0.00 ± 0.00	0.07 ± 0.02	0.10 ± 0.09	0.09 ± 0.04	0.08 ± 0.10	0.11 ± 0.05	0.09 ± 0.11
CA	132.71 ± 32.88	90.06 ± 15.25	0.09 ± 0.01	0.20 ± 0.10	0.10 ± 0.03	0.31 ± 0.25	0.05 ± 0.01	0.10 ± 0.02***
DCA	0.24 ± 0.05	0.09 ± 0.02	0.27 ± 0.11	0.04 ± 0.03*	0.22 ± 0.07	0.06 ± 0.04*	0.11 ± 0.04	0.01 ± 0.01**
12-keto DCA	0.04 ± 0.00	0.03 ± 0.00	0.08 ± 0.02	0.03 ± 0.01*	0.08 ± 0.03	0.06 ± 0.03	0.11 ± 0.03	0.04 ± 0.01**
α-MCA	21.44 ± 4.34	19.29 ± 6.50	0.07 ± 0.01	0.07 ± 0.02	0.10 ± 0.04	0.10 ± 0.05	0.04 ± 0.01	0.05 ± 0.01
ω-MCA	20.18 ± 3.47	23.50 ± 16.33	0.37 ± 0.07	0.27 ± 0.05	0.35 ± 0.12	0.33 ± 0.15	0.21 ± 0.05	0.13 ± 0.06

Results are presented in mg/g dry weight of intestinal contents (mean ± SD). Primary BAs are cholic acid (CA) and α-muricholic acid (α-MCA). Secondary BAs are deoxycholic acid (DCA), 12-keto-deoxycholic acid (12-keto DCA), and ω-muricholic acid (ω-MCA). Significance of differences between both groups in a given gut compartment is indicated (*p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001).

(FISH). BA-resistant gut bacteria were enumerated and isolated using classical culturing techniques. The isolates were characterized and tested for their BA resistance.

Results

Animal health status. Histopathological scoring revealed moderate gut inflammation in the cecum (2.97 ± 1.88) and the colon (2.69 ± 1.45) of the 24-week old IL-10$^{-/-}$ mice. The wild-type control animals did not show any signs of inflammation in the cecum and colon (0.61 ± 0.22 and 0.56 ± 0.17, respectively). We observed colorectal carcinoma formation in 55% of the IL-10$^{-/-}$ animals but no tumors in the wild-type mice.

Dry matter and pH of intestinal contents. Chronic gut inflammation might be associated with diarrhea or loose stool. Since differences in intestinal water content can influence the interpretation of the results from steroid measurements, we determined the intestinal and fecal dry weight. No differences in the dry weight of small intestinal contents were observed between IL-10$^{-/-}$ and control animals (~21%). Dry matter of cecal and colonic material was slightly higher in wild-type than in IL-10$^{-/-}$ mice (25.7 ± 1.8% vs. 23.5 ± 1.5% and 26.5 ± 3.2% vs. 22.6 ± 2.6%, respectively). Fecal dry matter was significantly higher in wild-type than in IL-10$^{-/-}$ animals (42.2 ± 0.7% vs. 33.0 ± 4.5%, p ≤ 0.01). Fecal pH did not differ significantly between IL-10$^{-/-}$ (6.18 ± 0.62) and wild-type mice (6.38 ± 0.47).

Intestinal neutral sterol concentrations. We hypothesized that an altered gut microbiota composition influences the intestinal steroid metabolism under chronic gut inflammation or vice versa and measured intestinal and fecal concentrations of neutral sterols.

Cholesterol made up the majority of neutral sterols measured in the intestine of the mice. Coprostanol, whose formation is brought about by bacterial cholesterol modification, was either not detected or present at only very low concentrations (**Table 1**). In both animal groups, the sterol concentrations were higher in the small than in the large intestine and in the feces. These differences were more pronounced in the wild-type animals. However, the large intestinal and fecal sterol concentrations were significantly higher in the IL-10$^{-/-}$ mice (**Fig. 1**).

We observed high inter-individual differences in the proportion of cholesterol to coprostanol. However, proportions were always lower in the wild-type mice (data not shown). Since the fecal dry matter between IL-10$^{-/-}$ and wild-type mice differed significantly, neutral sterol concentrations were also calculated based on fecal wet weight. This re-calculation did not change the principal findings.

Intestinal bile acid concentrations. Concentrations of the primary BAs cholic acid (CA) and alpha-muricholic acid (α-MCA) and of the secondary BAs deoxycholic acid (DCA), 12keto-DCA and omega-muricholic acid (ω-MCA) were measured in intestinal material from the experimental animals. A fecal wet weight-based re-calculation of BAs did not change the principal outcome.

CA was the major primary BA whereas the secondary BAs were mainly represented by DCA and ω-MCA in all animals (**Table 1**). High inter-individual differences in intestinal and fecal BA concentrations were observed. However, concentrations of total BAs and total primary BAs were significantly lower in the small intestine of IL-10$^{-/-}$ mice. No differences were observed for total small intestinal secondary BAs. This was also true for total BAs and total primary and secondary BAs in the colon. In cecal contents and feces, concentrations of total BAs and total secondary BAs were significantly lower in the IL-10$^{-/-}$ than in the wild-type mice (**Fig. 2A–C**).

A closer look at the single primary and secondary BAs showed that the animals did not significantly differ in any BA measured in the small intestine. The cecal DCA and 12-keto DCA concentrations were significantly lower in the IL-10$^{-/-}$ mice. This was also observed for colonic DCA concentrations. Fecal CA was higher while fecal DCA and 12-keto DCA were lower in the colitic animals (**Table 1**).

Given that the relative concentrations of primary and secondary BAs indicate the bacterial BA conversion, our data suggest a lower transformation capacity in the IL-10$^{-/-}$ mice (**Fig. 2D**).

Intestinal microbiota composition. Analysis of bacterial 16S rRNA gene libraries indicated a greater microbial diversity

in the colon of the wild-type animals compared to the IL-10$^{-/-}$ mice (**Table 2**). The Firmicutes in the wild-type mice were represented by 27 species affiliated within 8 different families. In contrast, this phylum was exclusively represented by *Blautia producta, Enterococcus gallinarum, Clostridium innocuum, Robinsoniella peoriensis* and *Lactobacillus murinus* in the IL-10$^{-/-}$ mice. The relative proportion of Bacteroidetes sequences was 25% higher in the wild-type mice and both Porphyromonadaceae and Bacteroidaceae were detected. The latter were absent from the IL-10$^{-/-}$ mice. The only Proteobacteria detected were the Enterobacteriaceae which were exclusively represented by *Escherichia coli* contributing a high proportion of total sequences in the IL-10$^{-/-}$ but not in the wild-type mice. Verrucomicrobia was a minor phylum in wild-type mice and not detected in IL-10$^{-/-}$ mice.

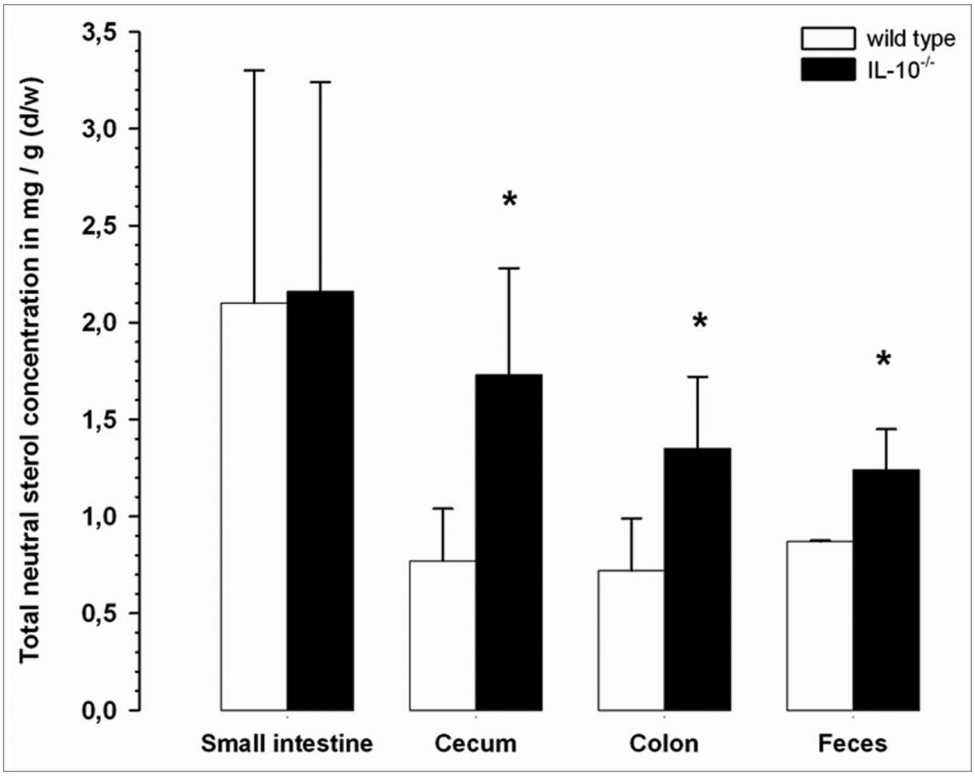

Figure 1. Total neutral sterol concentrations (cholesterol + coprostanol) in the intestine of 24-week-old IL-10$^{-/-}$ and wild-type mice (n = 4 per group). Significance of differences between the experimental groups is indicated (*p ≤ 0.05).

Members of selected bacterial groups were quantified in cecal contents with FISH. Total bacterial numbers were slightly but significantly higher in wild-type than in IL-10$^{-/-}$ mice (**Fig. 3**). The same was true for the members of Bacteroides-Prevotella group. In contrast, members of the *Eubacterium rectale-Clostridium coccoides* cluster were more abundant in IL-10$^{-/-}$ mice. The strongest difference between both groups was observed for the Enterobacteriaceae. Lactic acid bacteria, including lactobacilli and enterococci, did not differ between both groups.

Quantification and isolation of bile acid resistant bacteria. BA-resistant bacteria in fecal contents were enumerated on bile-esculin agar. Under anaerobic conditions, the counts for both groups were 10^9 bacteria/g. Under aerobic conditions, 10^8 bacteria/g were detected for the IL-10$^{-/-}$ mice and 10^7 bacteria/g for the wild-type mice. These differences were not statistically significant. Independent of the mouse genotype, bacteria isolated from agar plates incubated under aerobic conditions were identified as *E. gallinarum* and *E. coli*. However not statistically significant, higher numbers of *E. gallinarum* (10^8 vs. 10^6 bacteria/g fecal material, p = 0.051) and *E. coli* (10^7 vs. 10^6 bacteria/g fecal material, p = 0.166) were observed in IL-10$^{-/-}$ mice. When the agar plates were incubated under anaerobic conditions, *E. gallinarum* and *Lactobacillus murinus* were the most frequently isolated bacteria from wild-type mice (35% and 25% of the isolates, respectively). In addition, different Clostridium, Eubacterium and Bacteroides species were isolated at low frequencies (5 to 10% of the isolates) indicating a high diversity of BA-resistant bacteria in the wild-type mice. In contrast, bacteria from IL-10$^{-/-}$ mice cultured under these conditions mainly belonged to *E. gallinarum*, *L. murinus*, *E. coli*, *R. peoriensis* and *C. innocuum* (42, 17, 13, 13 and 8% of the isolates, respectively). According to their growth on media containing 2% bile and to the results from the Vitek system (40% bile), all isolates were considered highly BA-resistant.

Discussion

Low intestinal microbiota diversity and a bloom of few bacterial groups are common features in IBD patients and in animal models for gut inflammation. However, it is not clear whether these changes are crucial for the onset and perpetuation of IBD or rather a consequence of altered environmental conditions in the intestine caused by the inflammation. To better characterize the micro-environmental situation in the inflamed gut, we used IL-10$^{-/-}$ mice as a model for human IBD. In line with previous observations, these animals developed inflammation in the colon and cecum associated with a high colorectal carcinoma incidence.[15,16] As expected, gut inflammation in the IL-10$^{-/-}$ mice was associated with a reduced microbiota diversity in conjunction with an increased proportion of few bacterial species. These results confirm our previous findings in this mouse model.[6] It has to be taken into consideration that this observation is based on a low number of sequences analyzed in our study and that these sequences were obtained from pooled sample material. Since it can be deduced from recent human studies that at least

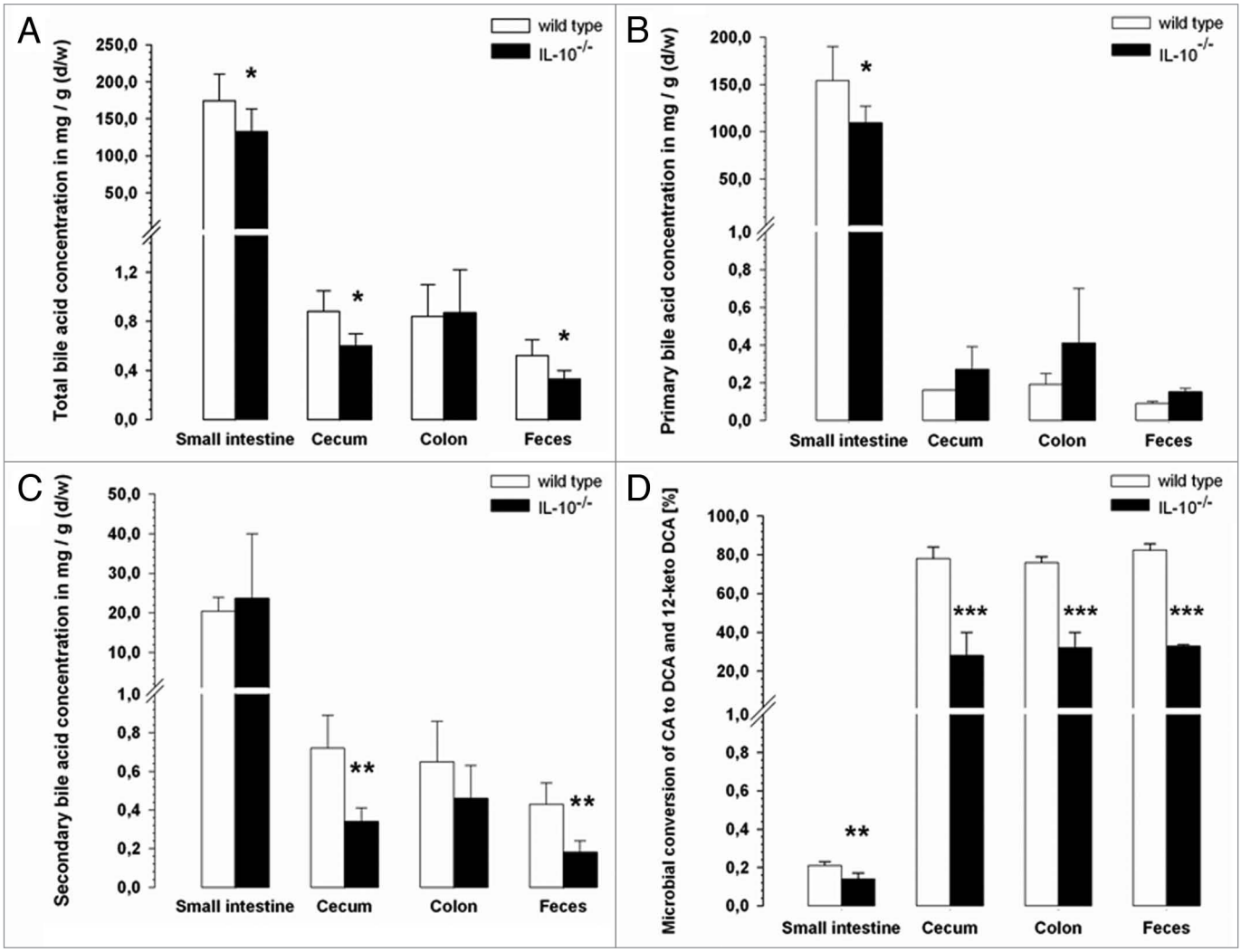

Figure 2. Bile acid concentrations in the intestine of 24-week-old IL-10$^{-/-}$ and wild-type mice (n = 4 per group). (A) Total BA concentrations are the sum of cholic acid (CA), α-muricholic acid (α-MCA), deoxycholic acid (DCA), 12-keto-deoxycholic acid (12-keto DCA) and ω-muricholic acid (ω-MCA). (B) Primary BA concentrations are the sum of CA and α-MCA. (C) Secondary BA concentrations are the sum of DCA, 12-keto DCA and ω-MCA. (D) Microbial conversion of CA to DCA and 12-keto DCA (in %) in the intestine of 24-week-old IL-10$^{-/-}$ and wild-type mice. Significance of differences between the experimental groups is indicated (*p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001).

several hundred clones per individual study subject are needed to describe total microbiota diversity in the inflamed gut,[17] we were not able with our approach to address the full complexity of the intestinal ecosystem in IL-10$^{-/-}$ and wild-type mice. However, together with the results from the culture-based and FISH analyses, we assume that changes in the dominant bacterial populations in the intestine of our experimental mice were monitored. Interestingly, some of the observed differences between colitic and healthy mice in major bacterial groups are in good agreement with findings in IL-10$^{-/-}$ mice kept under different housing conditions. In these animals, a lower intestinal abundance of phylotypes belonging to the Clostridiaceae and Lachnospiraceae was strongly associated with a decreased disease activity.[18]

We hypothesized that these changes in gut microbiota composition might be associated with an impaired intestinal steroid metabolism since a high fecal excretion of neutral sterols and low sterol plasma levels have been reported in IBD patients.[19,20] In addition, fecal primary BAs are higher while secondary BAs are lower in IBD patients than in healthy controls.[21,22] Thus, we measured neutral sterol and bile acid concentrations in intestinal contents and feces from IL-10$^{-/-}$ and healthy control mice.

We did not observe differences in small intestinal neutral sterol concentrations between IL-10$^{-/-}$ and wild-type mice suggesting that biliary cholesterol secretion was not affected in IL-10$^{-/-}$ mice. In contrast, neutral sterol concentrations were significantly higher in cecal, colonic and fecal material of IL-10$^{-/-}$ mice and, thus, an impaired sterol uptake under chronic gut inflammation may be deduced. We observed higher cholesterol but lower coprostanol concentrations in samples from IL-10$^{-/-}$ mice. Since coprostanol is a product of bacterial cholesterol conversion, these finding may be indicative for a depletion of cholesterol-metabolizing bacteria in IL-10$^{-/-}$ mice.

If the higher neutral sterol concentrations in the intestine of the colitic animals were the cause or a consequence of an altered

microbiota composition remains elusive. It has been shown that adding 0.5% cholesterol to the diet increases the abundance of intestinal Lactobaciliales and Clostridiales in mice.[23] On the other hand, selected gut bacteria are capable of influencing small intestinal cholesterol absorption.[24] In addition, fecal excretion of neutral sterol depends on intestinal microbiota composition and gut bacterial cholesterol-to-coprostanol-conversion capacity.[25]

The observed differences in the BA profiles between colitic and healthy animals might also be explained by a reduced bacterial transforming capacity since intestinal bacteria convert primary to secondary BAs.[14] The decreased microbiota diversity in the IL-10$^{-/-}$ mice was associated with a reduced BA transformation: higher concentrations of CA and lower DCA and 12-keto DCA levels were observed. Such an effect of an intestinal microbiota modified by antibiotic treatment has been shown previously in rats. The partial elimination of gut bacteria in these animals resulted in lower fecal concentrations of total and secondary BAs and an increase of primary BAs. In agreement with our findings, the conversion of cholesterol to coprostanol was also negatively affected in this study.[26]

A modified intestinal steroid profile might not only result from changes in microbiota composition but, conversely, may be the reason for these changes in the intestinal bacterial community. It has been demonstrated that BAs, particularly secondary BAs, inhibit bacterial growth and that anaerobic bacteria are more susceptible than aerobic bacteria.[12,13] In our study, numbers of total BA-resistant bacteria did not differ significantly between the animal groups. However, 16S rRNA gene sequencing indicated that BA-resistant *B. producta*, *E. gallinarum*, *E. coli*, *L. murinus*, *C. innocuum* and *R. peoriensis* are dominant species in the inflamed gut. This finding was partly supported by FISH analysis indicating higher numbers of *B. producta* (EREC482 probe) and *E. coli* (Enter1432 probe) in the IL-10$^{-/-}$ mice. Thus, it might be concluded that higher intestinal CA levels provide a colonization advantage to BA-resistant intestinal bacteria in conjunction with an inhibition of BA-susceptible bacteria.

We did not explore whether the 55% tumor incidence and gut inflammation in the IL-10$^{-/-}$ mice was directly affected by high intestinal primary BA concentrations. However, high intestinal neutral sterol and BA concentrations have been demonstrated in patients with large adenomas and colorectal carcinomas.[27,28] Especially secondary BAs have been implicated in cancer development and gut inflammation, but their exact role has not been fully elucidated.[29,30] Additional studies are needed for clarification.

In conclusion, chronic gut inflammation in IL-10$^{-/-}$ mice is associated with differences in intestinal neutral sterol and BA profiles. These alterations might affect and/or might be affected by changes in intestinal microbiota composition. Whether bacterial sterol and BA transformation directly influences the onset and perpetuation of colitis and colorectal cancer development remains to be clarified.

Materials and Methods

Animal experiment. Interleukin-10 knock-out (IL-10$^{-/-}$) mice (129(B6)-Il10tm1Cgn/J, n = 10) and the respective wild-type animals (129S6/SvEvTac, n = 10) were housed in individually ventilated cages (IVC) at constant room temperature (22°C ± 2°C), air humidity (55% ± 5%) and a light/dark cycle of 12 hours (lights on at 6:00). Water and feed (Altromin 1310) were given ad libitum. At the age of 24 weeks, animals were killed. One IL-10$^{-/-}$ and one wild-type mouse each were excluded from sampling because of tumor formation in testis and liver, respectively. Samples from the cecum and the colon were taken for histopathology analyses. Intestinal contents from 4 animals per group were collected for the measurement of neutral sterols and BAs, intestinal dry matter and fecal pH. Intestinal material from the remaining 5 animals per group was used for molecular and culture-dependent microbiological analyses.

Table 2. Relative proportions of bacterial 16S rRNA gene sequences in colonic contents of 24-week-old IL-10$^{-/-}$ and wild-type mice (150 sequences per group)

	Relative proportion of sequences			
Bacteria	**Wild-type mice**		**IL-10$^{-/-}$ mice**	
Firmicutes	**80**	**(53.3)**	**89**	**(59.3)**
Blautia	1	(0.7)	53	(35.3)***
Clostridiaceae	29	(19.3)	0	(0.0)***
Enterococcaceae	0	(0.0)	23	(15.3)***
Erysipelotrichacea	0	(0.0)	5	(3.3)
Eubacteriaceae	6	(4.0)	0	(0.0)*
Lachnospiraceae	30	(20.0)	7	(4.7)*
Lactobacillaceae	1	(0.7)	1	(0.7)
Ruminococcaceae	9	(6.0)	0	(0.0)**
Veillonellaceae	2	(1.3)	0	(0.0)
Oscillospiraceae	2	(1.3)	0	(0.0)
Bacteroidetes	**68**	**(45.3)**	**30**	**(20.0)*** **
Bacteroidaceae	13	(8.7)	0	(0.0)***
Porphyromonadaceae	55	(36.7)	30	(20.0)**
Proteobacteria	**1**	**(0.7)**	**31**	**(20.7)*** **
Enterobacteriaceae	1	(0.7)	31	(20.7)***
Verrucomicrobia	**1**	**(0.7)**	**0**	**(0.0)**
Verrucomicrobiaceae	1	(0.7)	0	(0.0)
Total number of OTUs (%)	**150**	**(100.0)**	**150**	**(100.0)**

Significance of differences between both groups are indicated (*p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001).

Histopathology scoring. Tissue material was fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at 4 μm and stained with hematoxilin and eosin (H&E) for light microscopic examination. Blinded scoring was conducted using a scale of 0 (no inflammation) to 6 (severe inflammation) as described elsewhere in reference 31.

Steroid analysis from intestinal contents. The method for neutral sterol and BA analysis in intestinal contents was adapted from Keller and Jahreis.[32] An aliquot of 50–200 mg of lyophilized intestinal contents containing 5α-cholestane (internal standard) was hydrolyzed with ethanolic sodium hydroxide and the sterols were extracted with cyclohexane. The extract was concentrated and re-dissolved in decane. Sterols were determined with a

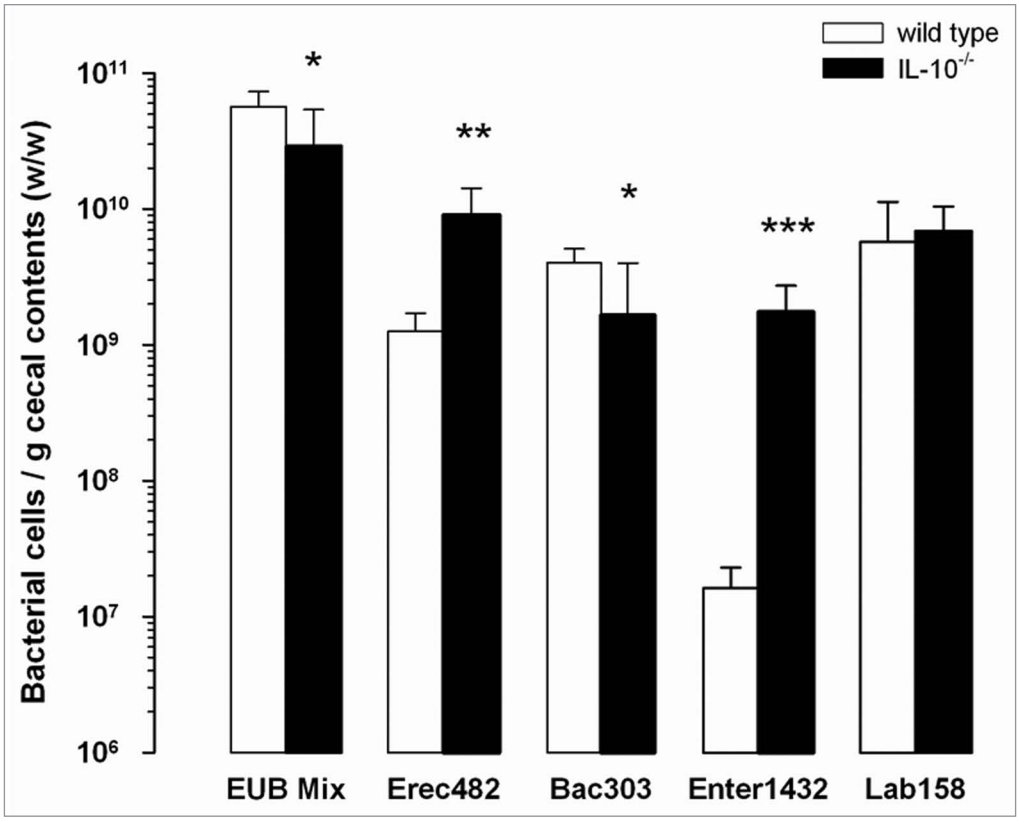

Figure 3. Quantification of dominant bacterial groups determined by FISH in cecal contents of 24-week-old IL-10$^{-/-}$ and wild-type mice (n = 5 per group). Statistical significance of differences between the groups is indicated (*p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001).

GC-FID 17A instrument (Shimadzu), equipped with a capillary column (Optima δ3; 30 m length; 0.25 mm inner diameter; 0.25 μm film thickness). The sum of the concentrations of coprostanol and cholesterol represent the total neutral sterol concentration.

The remaining aqueous phase was saponified and subsequently acidified to pH = 1 for BA analysis. After extraction of BAs and addition of the internal standard (23nor-cholic acid, 23nor-CA) the dried residue was methylated and silylated. Two microliters of the dried and re-dissolved BA methyl ester silyl ethers were injected into a GC17-QP5000 gas chromatograph (Shimadzu), equipped with a capillary column (ZB5; 30 m; 0.25 mm; 0.25 μm).

The mass spectrometric detection was performed in multi ion current (23nor-CA: m/z = 253.20 u; DCA: m/z = 255.30 u; α-MCA: m/z = 403.00 u, CA: m/z = 343.15 u; 12keto-DCA: m/z = 231.25 u; ω-MCA: m/z = 195.05 u). According to Eyssen et al. CA and α-MCA were summarized as primary BAs. DCA, 12keto-DCA and ω-MCA represent the secondary BAs in mice.

The standard substances of cholesterol (C8667), coprostanol (C7578), cholic acid (C1129) and deoxycholic acid (D2510) were purchased from Sigma-Aldrich. The standards of 12keto-deoxycholic acid (C1650), 23nor-cholic acid (N2450) and alpha-α-muricholic acid (C1890-000) and omega-muricholic acid (C1888-000) were purchased from Steraloids.

Molecular analysis of the colonic microbiota. Bacterial DNA from colon contents was extracted with the Fast DNA SpinKit (Qbiogene, 6540-400). DNA from the 5 mice per group was pooled. 16S rRNA genes were amplified by PCR using primers 27-f and 1492-r[34] and the following PCR program: 4 min at 94°C; 35 cycles of 1 min at 94°C, 1 min at 50°C and 2 min at 72°C; and finally 10 min at 72°C. PCR products were checked on 1% agarose gels and subsequently purified using the innuPREP PCRpure kit (Analytic Jena, cat # 845-KS-5010010). Amplicons were cloned into the PGEM T-Easy vector and transformed into *Escherichia coli* JM109 competent cells (Promega, cat # A1380). Inserts were checked by PCR with vector specific T7 (5'-TAA TAC GAC TCA CTA TAG GG-3') and SP6 (5'-GAT TTA GGT GAC ACT ATA G-3') primers. One-hundred-fifty clones per group were sequenced using vector specific primers and the cycle sequencing technology (MWG Biotech). The sequences were used for genome database (GenBank, www.ncbi.nlm.nih.gov) searches by using the BLAST function.[35] Sequences with a sequence identity of ≥98% to a known bacterial species were affiliated to this species. Sequences with a sequence identity under 98% to a known bacterial species were designated to the next known bacterial family. Results were additionally confirmed by using the seqmatch function of the Ribosomal Database project (http://rdp.cme.msu.edu/).[36]

Fluorescence in situ hybridization (FISH). Bacteria in cecal sample material were enumerated after hybridization with 5'-Cy3 labeled 16S rRNA-targeted oligonucleotide probes. For the enumeration of total bacteria an equimolar mixture of five

bacteria specific probes (Eub338, Eub785, Eub1055, Eub1088, Eub927) was used.[37] Enterobacteria were quantified with Enter1432,[38] members of the *Eubacterium rectale-Clostridium coccoides* group with Erec482,[39] Bacteroides-Prevotella with Bac303,[40] and lactic acid bacteria with Lab158.[41] Sample preparation, hybridization and enumeration of bacteria by epifluorescence microscopy were performed as described elsewhere in reference 42. Briefly, the cecal samples were diluted tenfold and homogenized by vortexing. Debris was removed by centrifugation (1 min at 300x g, 4°C) and bacterial cells were fixed with paraformaldehyde. Hybridization was performed overnight at 46°C for EUB-mix, Bac303 and Lab158, at 41°C for Enter1432 and at 50°C for Erec482. This was followed by a washing step at 2°C above the specific hybridization temperature for 20 min. Samples were always counter stained with 4,6-diamidino-2-phenylindole (DAPI; Carl Roth, 6335.1). Bacteria were counted using an Axioplan epifluorescence microscope (Carl Zeiss) and bacterial numbers are expressed as $\log_{10}$/g wet weight of cecal material.

Isolation, identification and culture of bacteria isolated from mouse intestine. A tenfold dilution [w/v] of fresh fecal contents was prepared in reduced PBS (37 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.47 mM KH_2PO_4, 0.1 gl^{-1} peptone, 0.3 gl^{-1} L-cysteine x HCl and 1 mgl^{-1} resazurine, pH 7.4, under a gas atmosphere of 80% N_2 and 20% CO_2). Six sterile 3 mm glass beads were added and samples were homogenized for 5 min by vigorous shaking. The fecal suspensions were centrifuged (300x g, 1 min) to sediment debris. The supernatant with the bacterial cells was used for tenfold dilution series in reduced PBS (10^{-2} to 10^{-8}). Aliquots of 100 µl per dilution were plated on bile-esculin agar (Oxoid, CM0888) and bacteria were cultured at 37°C under aerobic and anaerobic conditions. After 24 h of aerobic and 48 h of anaerobic incubation, colonies on triplicate plates were counted. Bacterial numbers are expressed as $\log_{10}$/g wet weight of intestinal contents. For isolation and identification of cultured bacteria, colonies from one representative animal per group were randomly picked with a sterile toothpick, cultured overnight at 37°C in Brain Heart Infusion medium (Oxoid, CM1136) and re-streaked on Columbia sheep blood agar (Biomerieux, 43049). Biochemical characterization of isolated bacteria was done with the Vitek system (Biomerieux) according to the instructions of the manufacturer. All isolated bacteria were subjected to 16S rRNA gene sequence analysis. Briefly, genomic DNA was isolated using the RTP Bacteria DNA Mini Kit (Invitek, 1033200200) and the 16S rRNA genes were amplified with the primers 27-f and 1492-r using the PCR program described above. PCR products were purified and sequenced (MWG Biotech). Sequences were used for genome database (GenBank, www.ncbi.nlm.nih.gov) searches to identify the most closely related bacterial species.

16S rRNA gene sequences of the isolated bacteria were submitted to the NCBI nucleotide database. GenBank accession numbers are indicated in parentheses: *E. gallinarum* (GU811873), *E. coli* (GU811877), *L. murinus* (HQ668465), *C. innocuum* (GU811875) and *R. peoriensis* (GU811874).

Statistical analysis. Statistical analysis was performed with SPSS for Windows version 14.0 using students t-test or one-way analysis of variance with Scheffé and Student-Newman-Keul test for post hoc comparison. The Gaussian distribution of the samples was determined by visual inspection of normal probability plots and tested with the Kolmogorov-Smirnov-Test. Logarithmic transformations were used for the variables that did not show a normal distribution. The z-test was used for comparison of proportions of the 16S rRNA gene sequences from colonic contents and of proportions of fecal bacterial isolates between the two different groups. Differences were considered significant at $^*p \leq 0.05$, highly significant at $^{**}p \leq 0.01$ and very highly significant at $^{***}p \leq 0.001$. All data is presented as means ± SD.

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