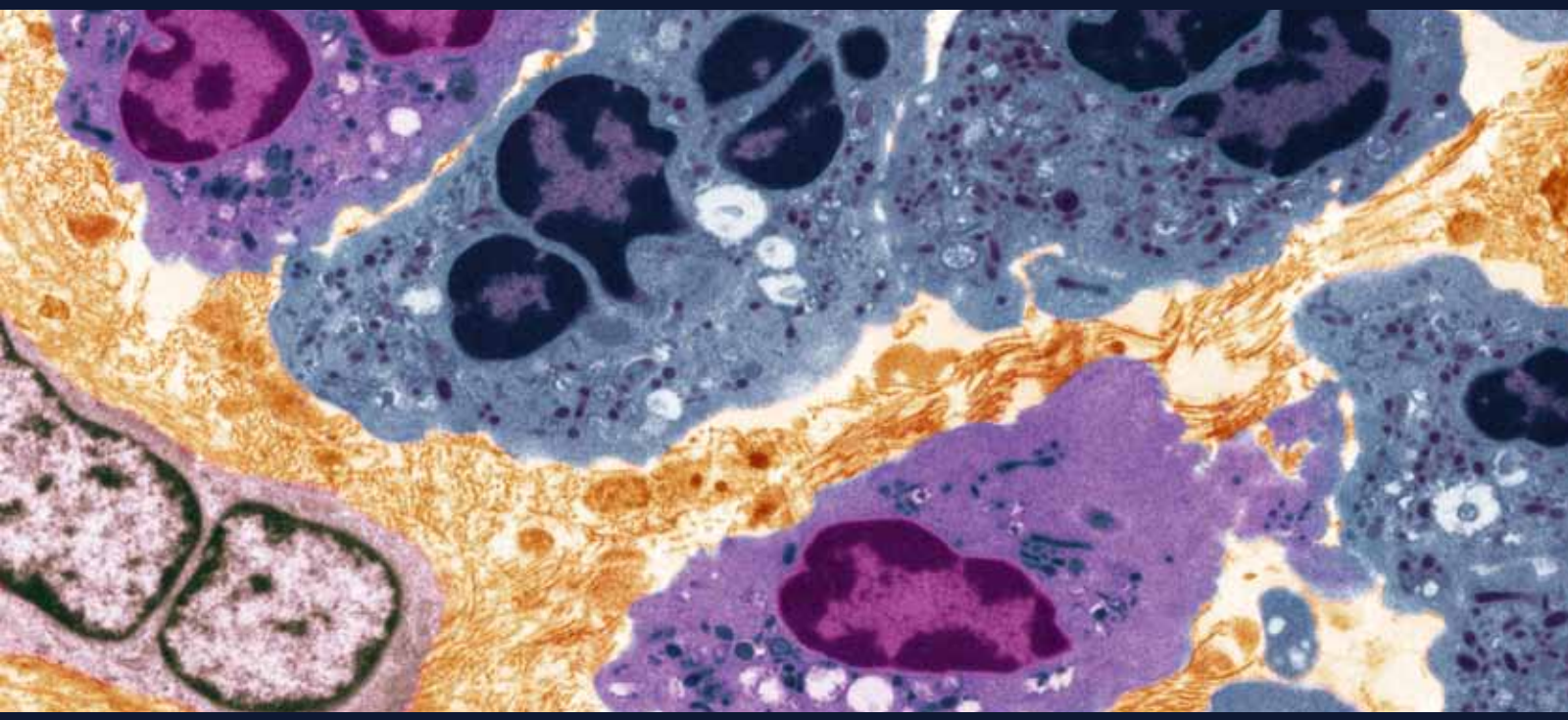
International Journal of Inflammation

Microbiota in Chronic Mucosal Inflammation

Guest Editors: G. Gerhard Rogler, Dirk Haller, and Christian Jobin



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Editorial

Microbiota in Chronic Mucosal Inflammation

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The human gastrointestinal tract is occupied by a complex and abundant microbial community reaching as high as 10^{13}–10^{14} microorganisms in the colon. This microbiota participates in a symbiotic relationship with their eukaryotic host, and this partnership is viewed as essential for maintaining homeostasis. The microbiome contains a wealth of information, encompassing 150-fold as many genes as the human genome and performing essential and nonredundant tasks (e.g., nutrition/energy balance, immune system toning, and pathogen exclusion) for the host. Recent studies have hinted that a core human microbiome exists at the gene level, with a large number of microbial genes and pathways shared among individuals. Deviations from this core microbiome could potentially affect human health and promote disease state. The coexistence of the host with its intestinal microbiota is tightly controlled at various levels and an accumulating body of evidence suggests that the failure of this homeostasis is an important contribution to disease development. Recent discoveries clearly suggest that the gut microbiota of individuals throughout their lifespan is a powerful determinant of chronic diseases and that the mechanisms underlying this link involve the development of inflammatory activity. The microbiota has already been linked to cardiac development, angiogenesis, innate and adaptive immunity, metabolism, nutrient acquisition, and gastrointestinal development and homeostasis. Furthermore, alterations in microbial community composition are associated with multiple diseases, including obesity, fatty liver disease, type 1 and type 2 diabetes, kidney disease, arthritis, and inflammatory bowel diseases (IBD).

On the host side, a series of mechanisms help contain the formidable antigenic power of the microbiota. One of the main mechanisms, which could be regrouped under the wide umbrella of functional mucosal barrier, includes the formation of epithelial tight junction, mucus production, antimicrobial peptide secretion, and immunoglobulin A release. Equally important is the presence of a sophisticated repertoire of innate receptors, each recognizing specific conserved microbial patterns present on various microorganisms. Because the basic structure of bacteria is relatively conserved, eukaryotes have developed throughout evolution sensing systems to detect these bacterial signatures which include cell wall components, locomotion system, and nucleic acids. These microbial sensors are termed pattern recognition receptors (PRR-) and include retinoic acid inducible gene-I-like RNA helicases (RLH), C-type lectin receptors (CLR), nucleotide-binding domain leucine-rich repeat proteins (NLR), also known as Nod-like receptors, and Toll-like receptors (TLR). This arsenal of innate sensors plays a critical role in maintaining intestinal homeostasis through elimination of pathogenic microorganisms and preventing the formation of a dysbiotic microbiota. Interestingly, modulation of the gut microbiota and prevention of a dysbiotic state have become the focus of intense research. Indeed, various strategies including the introduction of prebiotic, probiotic, dietary compounds, and recently fecal transplantation to treat recurrent *C. difficile* infection have received special attention to treat various intestinal inflammatory disorders.

Clearly, one could appreciate the complexity of these host-microbe interaction and the deleterious consequences

of an improper response to both the microbiota and infectious microorganisms. For example, Nod2 polymorphisms causing a loss of bacterial recognition mechanisms have been identified as susceptibility factors for Crohn's disease. Moreover, NOD2 variants are also implicated in the development of gingivitis and Graft-versus-host disease after bone marrow transplantation.

This special issue of the International Journal of Inflammation brings into focus the complexity of bacteria-host interactions and the impact of these interactions on intestinal homeostasis, and conversely in the development of various pathological conditions.

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Research Article

A Strong Impact of Genetic Background on Gut Microflora in Mice

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Genetic background affects susceptibility to ileocolitis in mice deficient in two intracellular glutathione peroxidases, GPx1 and GPx2. The C57BL/6 (B6) GPx1/2 double-knockout (DKO) mice have mild ileocolitis, and 129S1/Sv (129) DKO mice have severe inflammation. We used diet to modulate ileocolitis; a casein-based defined diet with AIN76A micronutrients (AIN) attenuates inflammation compared to conventional LabDiets. Because luminal microbiota induce DKO ileocolitis, we assessed bacterial composition with automated ribosomal intergenic-spacer analysis (ARISA) on cecal DNA. We found that mouse strain had the strongest impact on the composition of microbiota than diet and *GPx* genotypes. In comparing AIN and LabDiet, DKO mice were more resistant to change than the non-DKO or WT mice. However, supplementing yeast and inulin to AIN diet greatly altered microflora profiles in the DKO mice. From 129 DKO strictly, we found overgrowth of *Escherichia coli*. We conclude that genetic background predisposes mice to colonization of potentially pathogenic *E. coli*.

1. Introduction

Gut microbiota play an important role in several diseases including inflammatory bowel disease (IBD), type-1 diabetes, and obesity [1]. Recent metagenomic studies of the gut microbiota have shown that bacteria dominate the gut ecosystem [2]. Between 90 and 98% of bacteria, sampled either from gut-surface-adherent population or feces, belong to four bacterial phyla *Firmicutes*, *Bacteroidetes*, *Proteobacteria,* and *Actinobacteria* [3, 4]. Although there is a large variation in bacterial population in different individuals, the same bacterial phyla predominate in the stomach, small intestine, colon, and feces from the same individual [2, 5]. However, some IBD patient guts have decreased bacterial diversity with depletion of members of *Firmicutes* and *Bacteroidetes* [3, 4]. Since understanding gut microbiota may provide insight for IBD risk, pathogenesis, and treatment strategies, there is surprisingly little information on the microbiota information in mouse models of IBD.

While the metagenomic sequencing study on human fecal microbial genes has expanded the database of bacterial genomes deposited in the GenBank, the findings on the gut microbiota composition also confirm the results from methods based on bacterial 16S ribosomal RNA (rRNA) gene sequences [3–5]. Other noncultured PCR-based methods have been used to appraise gut microbial composition; these include automated ribosomal intergenic spacer analysis (ARISA) and terminal restriction fragment length polymorphisms [6, 7]. ARISA utilizes conserved 16S and 23S rRNA gene sequences coupled with variability in the length of the intergenic spacer to discriminate among bacterial species. The PCR products are separated by an automated capillary electrophoresis system with single-nucleotide resolution and detected by a sensitive laser beam to produce an electropherogram.

ARISA has been used as a crude microbe assay. Metagenomic study has estimated that each individual harbors at least 160 bacterial species and entire cohort harbors between 1,000 and 1,150 prevalent bacterial species [4]. A single ARISA primer set on fecal samples only yields 20–30 consensus products and 100 across all subjects [7–9]. Nevertheless, because ARISA generates a highly reproducible

microbiota profile with conventional instrumentation, we applied this method to assess cecal microbiota in a mouse IBD model.

We have generated a mouse IBD model by disruption of two genes encoding for two intracellular glutathione peroxidases, GPx1 and GPx2 [10, 11]. These GPx1/2-double knockout (DKO) mice (on a mixed C57BL/6 and 129S1/Sv genetic background) have microflora-dependent ileocolitis, since germ-free mice do not have inflammation [11]. Similar to other mouse IBD models, genetic background has a profound effect on disease severity in GPx1/2-DKO mice. B6 DKO mice have mild ileocolitis, the mixed-strain B6; 129 DKO mice have more severe disease [12], and 129 DKO mice have the most severe inflammation (from this study). Since B6 and 129 strains may have different innate immune responses, which can modulate microflora community [2, 13], we hypothesized that these two strains of mice also have different gut microbiota.

In addition to genetics, diet also can modulate IBD. Patients with Crohn's disease (CD) can be managed by prescribed diets, which are almost as efficacious as anti-inflammatory corticosteroids [14–16]. For pediatric CD patients, the enteral nutrition is preferred to corticosteroids to avoid adverse effects in European countries [17]. The major impact of enteral nutrition may rely on changes in gut microbiota [16]. Thus, we also tested whether diet impacts on the ileocolitis and microflora in the DKO mice.

In this manuscript, we analyzed the dietary effect on mouse IBD on both B6 and 129 genetic backgrounds. Based on the current knowledge on gut microbiota in IBD patients, we tested whether mouse genetic background, inflammation (DKO genotype), and diet affected gut microbiota.

2. Materials and Methods

2.1. Mice and Diets. Generation of GPx1/2-DKO mice on the C57BL/6J (B6) × 129S1/SvimJ (129) background (B6;129) was described previously [10]. B6 colony was obtained after backcrossing B6;129 mice to B6 for 8 generations. N5 and N10 129 colonies were from B6;129 mice backcrossing to 129 strain for 5 and 10 generations, respectively. Mice were fed either commercial chows (LabDiet, Richmond, IN) or casein-based defined diets with AIN76A micronutrients (AIN; Harland-Teklad, Madison, WI) (Table 1). As specified in the experiments, some AIN diets were supplemented with brewers' yeast or inulin (Oliggo-Fiber Inulin, a gift from Cargill Inc., Minneapolis, MN).

When on commercial chows, breeders were maintained on a high-fat LabDiet, and pups weaned to a low-fat LabDiet at 22 days of age. When on AIN diets, breeders had 10% corn oil (CO) and pups had 5% CO. Morbidity describes wasting mice, which were likely to die in the next 24–48 hours, or with poor health indicated by low body weight, no weight gain and diarrhea, and unlikely to recover. When describing diet effects on the pups before weaning, the diet refers to the breeder diet. All experiments performed on these mice were approved by City of Hope IUCUC.

2.2. Histology. Distal ileum and the entire colon were processed for histopathology analysis. Tissues were scored for inflammation and pathology in a blinded fashion using a 14-point system described previously [11]. Scoring includes lymphocytes and neutrophils infiltration (0–3 points), ileal Paneth cell or colonic goblet cell degranulation (0–2 points), epithelium reactivity, including crypt distortion (0–3 points), inflammatory foci (0–3 points), and apoptotic figures (0–3 points). The threshold for inflammation corresponds to a score of 6–7 [18].

2.3. Microbiota Census with Noncultured ARISA and Culture Methods. Cecal microflora were characterized in 22-day-old pups or younger (16- to 21-day-old) sick mice when morbidity criteria dictated.

For noncultured ARISA, DNA was isolated from mouse cecal contents in 1 mL TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0), 0.15 mL phenol, and 0.2 g of 1 mm Zircon-ia/Silica beads (BioSpec Products, Inc., Bartlesville, OK) using a minibead-beater (BioSpec Products, Inc.) [19]. Approximately 150–300 μg DNA was extracted from the cecal contents of each mouse. The ribosomal integenic DNA was amplified by PCR using a primer set of ITSF (5′-GTCGTAACAAGGTAGCCGTA-3′) and ITSReub (Hex-5′-GCCAAGGCATCCACC-3′) as described [6]. The Hex-tagged fluorescent reverse primer is used to identify the products on the DNA sequencing instrument. One μL of 25 μL reaction mixture was analyzed with a capillary DNA analyzer (Hitachi AB model 3730) along with Genescan1000-ROX standard (Applied Biosystems; City of Hope Sequencing Core). To identify the DNA amplicons, the rest of PCR products were separated in agarose gels, and major DNA bands were excised and cloned into dT-tailed pCR2.1 (Invitrogen) and sequenced. Cloning total PCR products without gel separation only yielded one new sequence. DNA sequence identified was determined by BLAST (http://www.ncbi.nlm.nih.gov/).

The cecal content was also cultured under aerobic and microaerobic conditions. The cecal contents were collected individually; the volume was measured and diluted with 10 volumes of sterile phosphate-buffered saline (PBS). Each sample was diluted 10,000 times the original sample volume with PBS and then plated with the original volume on two Luria-Bertani (LB) plates. One plate was incubated for 24 hours in aerobic condition, the other for 6-7 days under microaerobic conditions, both at 37°C [20]. For microaerobic culture, plates were placed in a GasPak (Becton Dickerson, Cockeysville, MD) with an activated Anaerocult A insert (EM SCIENCE, Gibbstown, NJ). The assembly was purged with CO_2. After counting the colonies, up to 10 colonies from each plate were collected and DNA was amplified using ARISA primers. The PCR products were resolved in 1.3% agarose gels and DNA fragments were extracted with Qiagen Gel Extraction Kit and sequenced with the ARISA primers.

2.4. Statistics. For comparing time-to-event endpoints, such as survival time on different diets, the results were plotted

Table 1: Diet compositions.

Contents	LabDiet[1] 5061	LabDiet 5062	AIN[1] 5% CO	AIN 10% CO	AIN + inulin 5–10% CO	Brewer's 1% BY	Yeast (BY)[2] 10% BY
Protein	Mixed	Mixed	Casein	Casein	Casein	Mixed	Mixed
(%)[3]	(23.4)	(20.5)	(23.4)	(17.7)	(20–26.5)	(17.8)	(17.7)
Yeast	Brewer's	Brewer's	—[4]	—	—	Brewer's	Brewer's
(% wt)	(1)	(1)				(1)	(10)
DL-Methionine, %	(0.43)	(0.48)	(0.3)	(0.3)	(0.3)	(0.3)	(0.3)
Fat	Mixed	Mixed	Corn oil	Corn oil	Corn oil	Corn oil	Corn oil
(%)	(5.5)	(9.6)	(5.0)	(10.0)	(5–10)	(10.3)	(10.7)
Sucrose, %	(3.7)	(0.4)	(50)	(39)	(5.0–38.4)	(38)	(35)
Other CHO[5]	Mixed	Mixed	Corn[5]	Corn	Corn	Corn	Corn
(%)	(56)	(53)	(5)	(15)	(27.5–15.5)	(15)	(15)
Cellulose (fiber), %	(5.3)	(2.7)	(18.4)	(11.5)	(13.4–6.5)	(11.5)	(11.5)
Kcal/g	3.3	3.8	3.1	3.8	3.1–3.8	3.8	3.8

[1] LabDiets contained crude ingredients and micronutrients similar to AIN-76A (http://www.testdiet.com/). Other diets were defined diets containing AIN-76A mineral and vitamin mix. All defined diets contained ~0.002% ethoxyquin as an antioxidant, and had an adequate amount (0.2%) of choline bitartrate or choline chloride.
[2] Protein, carbohydrate, and fat in Brewer's yeast are not included in other contents.
[3] All % content is by weight.
[4] A dash means not present.
[5] CHO means carbohydrate, and Corn means corn starch.

with Kaplan-Meier curves and analyzed with the log-rank test. Analysis of variance (ANOVA) was used to compare the means ± standard deviations (SDs). To compare pair-wise diets versus a control, Dunnett's correction was made for multiple testing and the Dunnett-corrected P-values were applied. Each ARISA data panel represents results pooled from 6 to 21 mice analyzed individually. The electropherograms were digitized, and the results for each group were averaged using the statistical programming language R [21, 22]. The data were cleaned and passed to the Ribosort package created for R by Scallan et al. [23]. Ribosort detects and classifies peak-generating fragments in ARISA data with a two-pass algorithm [24]. Output from Ribosort contains information on the ribotype (represented by a specific size of PCR product) abundances, ribotype proportions, and sequencer detections. A Euclidean discriminant test was applied to the final step in ARISA data set analysis. Finally, a Czekanowski similarity index was run for pair-wise comparisons from all panels in the ARISA Figures 4, 6, and 8. The quantitative version of the Czekanowski similarity index is defined as $2W/(A + B)$, where A and B are the abundance of species in two given sample conditions and W is the number of species shared in the two samples. A convention for interpreting the Czekanowski similarity index is as follows: index between 0.5 to 0.75 indicates similar abundances; index between 0.25 to 0.5 indicates different abundances; index between 0 to 0.25 indicates very different abundances.

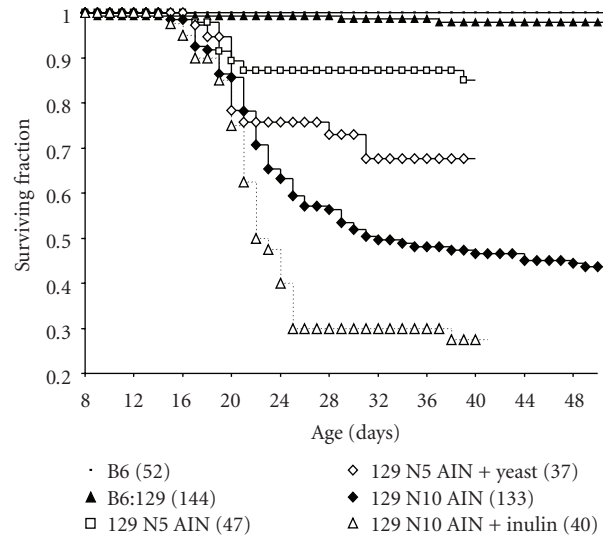

Figure 1: Kaplan-Meier curves showing the effect of genetic background and diet on DKO mouse survival. All mice are on AIN base diets. Monitored from 8 days of age, the surviving fraction excludes dead and culled, moribund mice. The numbers in parenthesis are the number of mice in each group. The data for yeast are pools both 1 and 10% as no difference could be distinguished. There are log rank differences for all interstrain comparisons on AIN diet: $P < .0001$. 129 N5, yeast-supplemented AIN (AIN + yeast) diet versus AIN; $P = .0087$. 129 N10, inulin-supplemented AIN (Ain + inulin) diet versus AIN; $P = .0003$.

3. Results

3.1. Genetic Background and Diet Had a Profound Effect on GPx1/2-DKO Morbidity. Mouse strain background has a big effect on morbidity outcome in GPx1/2-DKO mice (Figure 1). As we noted before the strain difference in survival on the conventional LabDiet, here we report the same genetic effect on mouse survival on the AIN diet. The AIN diet, formulated to mimic LabDiet for calories,

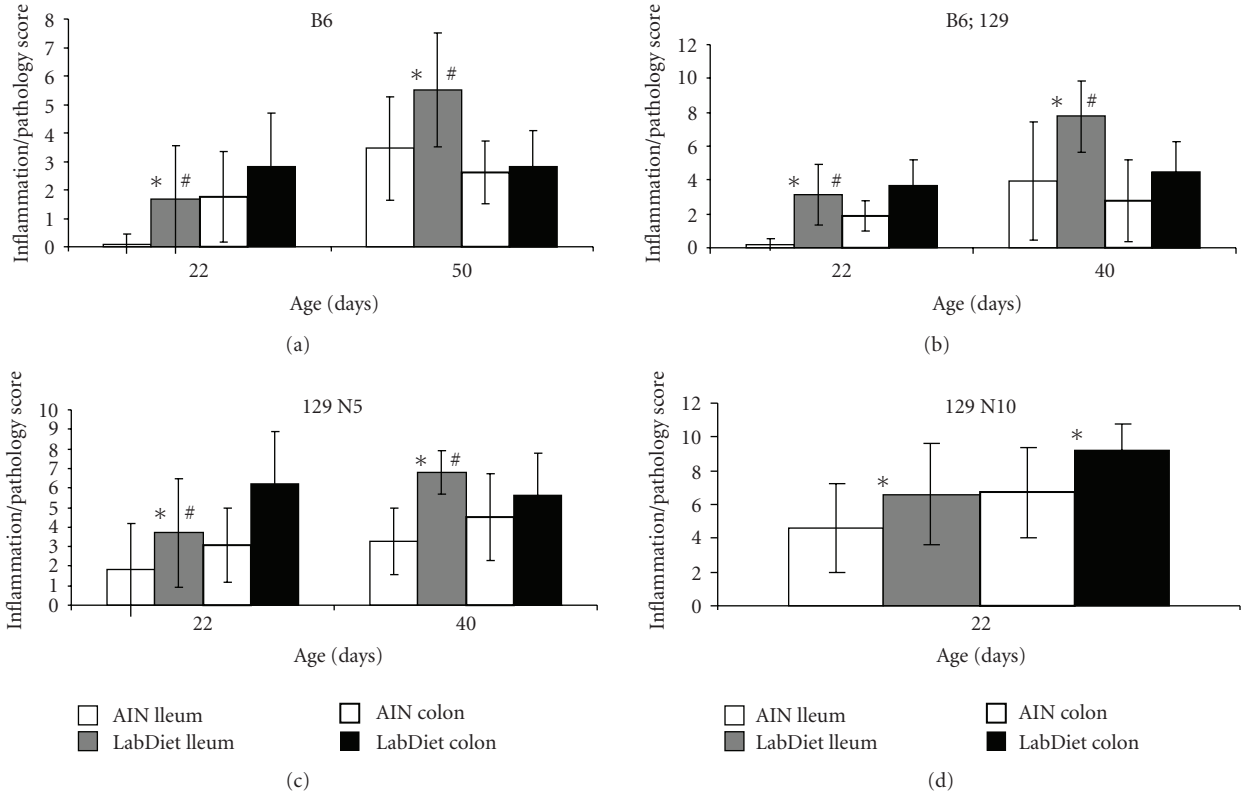

Figure 2: Effect of diet on ileum and colon histology at weaning and peak pathology by strain in GPx1/2-DKO mice. Mice on LabDiet or AIN diet were analyzed at 22 days of age or upon signs of morbidity, and at the peak of ileitis, which is 40 days for B6;129 or 129 N5 and 50 days for B6. 129 N10 mice were only analyzed at 22 days of age due to high morbidity. The AIN diet alleviated ileitis significantly in all strains at both 22 days of age and at peak inflammation (the "$*$" sign indicates $P < .011$). The ileal inflammation/pathology scores increased significantly from 22 days to peak of inflammation in all strains ($^{\#}P < .034$). A diet-associated difference in colitis was only observed in 22-day-old 129 N10 pups ($^{*}P < .001$).

macro- and micronutrients, maintained better health than LabDiets for the DKO mice.

B6 GPx1/2-DKO mice maintained fairly good health on either a LabDiet or AIN diet. The surviving fraction of B6 DKO mice at 45 days on the AIN is 100%, which is virtually the same as mice on LabDiet with 98% survival compared at 45 days of age (Figure 1 and data not shown). The B6;129 mixed-strain DKO mice had marginal health, with 97% survival on AIN diet compared to 85% on LabDiet at 45 days of age. As expected, the 129 DKO mice had poor health. Eighty-five % of 129 N5 DKO on AIN diet survived 40 days when only 65% survived on LabDiet. Only 47% of 129 N10 on AIN diet survived 40 days when merely 5% of 129 N10 on LabDiet made it ($P < .0001$, Log rank; differences for strains between AIN diet and LabDiet, except B6).

For 129 DKO mice, the early morbidity is associated with colitis. The postweaning morbidity in B6 and B6;129 DKO mice is correlated with later developing ileitis and rarely involves diarrhea. Disease in the 129 N5 and N10 DKO starts before weaning based on symptoms of wet tail and runting as early as 11 days of age on AIN diet.

3.2. Morbidity of GPx1/2-DKO Mice Is Correlated with Gut Inflammation. Morbidity at any time appears to be reflection of acute inflammation, reflected in the inflammation/pathology scores of 6 or greater [18]. B6 DKO mice had mean ileal pathology score around 5.5 at 50 days of age, the peak of inflammation in this strain (Figure 2). 129 N10 DKO mice had mean ileal and colonic pathology scores of 6.5 and 9, respectively, at 22 days of age. The pathology scores correlate with morbidity. Typical histopathology in 129 at 22 days of age showed acute inflammation in the cecum and distal colon with frequent skipping or less severe inflammation in the proximal colon (Figure 3). In morbid mice, disease often extended into the proximal colon. By contrast, B6 mice had almost no pathology in the cecum, proximal, and distal colon.

Diet significantly modified ileitis severity in all genetic backgrounds, that is, B6, B6;129, as well as 129 N5 and N10 DKO mice (Figure 2). Only in 129 strains was a dietary effect on colitis observed. Non-DKO mice did not have gut pathology.

3.3. Yeast and Inulin Supplementations to AIN Diet Increased Morbidity on 129 N5 and N10 DKO Mice. Since LabDiet has 1% brewer's yeast, and yeast antigens are associated with some CD, we tested its effect in 129 N5 DKO mice [25]. Feeding 129 N5 DKO mice with AIN diet supplemented

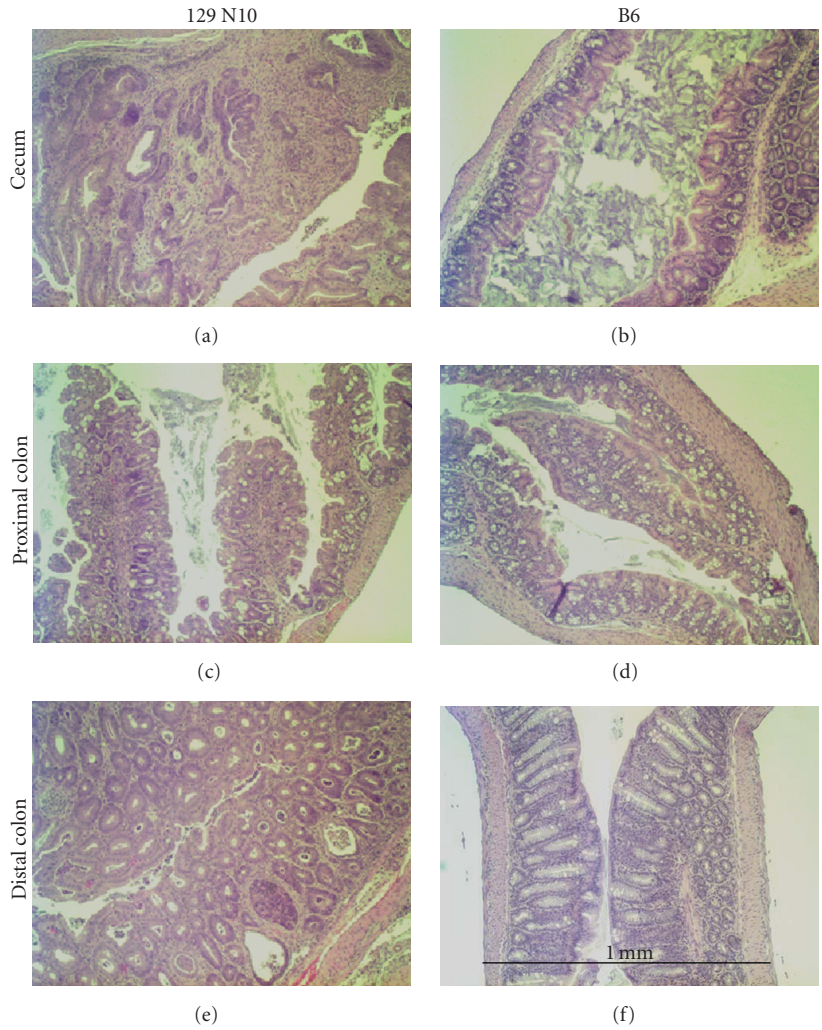

Figure 3: Representative histopathology in the colon of 129 N10 and B6 mice on AIN at 22 days of age. Slides were stained with hematoxylin and eosin. By 22 days of age, 90% of 129 N10 DKO mice have disease signs of diarrhea and many are lethargic. B6 DKO mice are without these signs. This is correlated with histopathology in the cecum and colons of these mice. 129 mice show inflammation and distortion of the cecum and distal colon with frequent crypt abscesses in the distal colon. The inflammation frequently skips the proximal colon (shown here). In severe cases, where lethargy has progressed to wasting, the proximal colon can be involved. The cecum and colon of B6 mice show mucin depletion and mild distortion due to apoptosis and proliferation.

with 1% and 10% brewer's yeast increased their morbidity ($P = .0087$)(Figure 1 shows pooled, no difference 1% versus 10%). The morbidity curve on yeast-containing AIN diet resembles that obtained with LabDiet (data not shown). Although the pups experienced more pronounced diarrhea, the pathology score was not elevated in the N5 DKO mice on the yeast-containing diet. The median colon pathology score was 3 at 22 days of age on both diets ($P = .58$), and 4.5 versus 4 for mice on yeast-containing AIN versus AIN diets at 40 days of age ($P = .9$; Mann-Whitney test). Thus, yeast-supplementation had an adverse effect on the DKO mice by increasing morbidity through intensifying diarrhea without exacerbating colitis.

Inulin, a nondigestible fructooligosaccharide is a food fiber with prebiotic properties, which may have prophylactic or therapeutic potential for IBD [1, 26]. We supplemented 5% inulin in AIN diet to 129 N10 DKO mice on the premise that it may retard the morbidity by fostering the colonization of probiotic microflora. Disappointedly, supplementing 5% inulin to AIN diet increased morbidity in 129 DKO pups (Figure 1; $P = .0003$). However, inulin did not affect colon inflammation; both diets yielded a median disease score of 7, $P = .28$ (Mann-Whitney test). Thus, inulin also had an adverse effect on DKO mice by increasing morbidity without affecting colitis.

3.4. Diet Modified Cecal Microbiota Content in Wild-Type (WT) B6 but Not DKO Mice. Since diet can alter gut microflora [16], and microflora are essential for IBD, we used ARISA to profile microbiota in these mice. We chose cecal content to analyze microbiota for the following reasons. First,

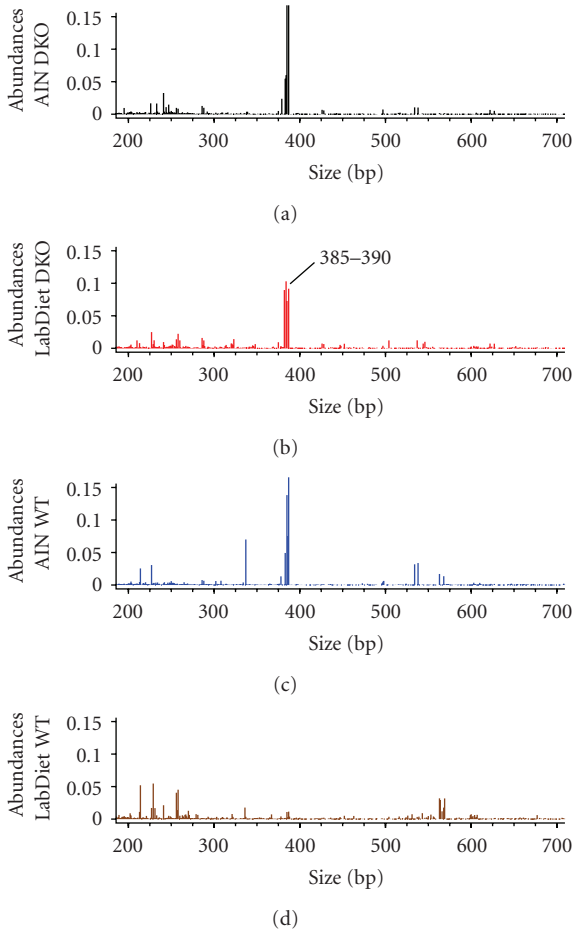

Figure 4: ARISA analysis of DNA from cecal contents of B6 on LabDiet or AIN diet. DNA amplified using primers corresponding to 16S and 23S ribosomal genes and oriented to contain the ribosomal intergenic spacer regions was resolved with a capillary DNA analyzer. The x-axis shows the size of the DNA peak, and y-axis shows the relative intensity of each peak. Each panel represents the average result of 6 mice.

the cecum is a disease site in GPx1/2-DKO mice. Severity of typhlocolitis is correlated with colitis in the 129 strain ($R^2 = 0.63$). Although the cecum has milder pathology than the colon (with the median disease score of 6 in the cecum and 8 in the colon of 129 N10 DKO, pooling AIN and LabDiet scores), the cecum was the major disease site in about 15% of mice. Second, unlike the colon, the cecum is rarely empty even in very sick mice. Third, little variability of microbiota is detected from six major subdivisions of healthy human colon: cecum, ascending colon, transverse colon, descending colon, sigmoid colon, rectum, and feces from the same person [5].

The microbiota profiles on B6 DKO and WT mice were determined individually on either LabDiet or AIN diet, and a combined profile was generated for each group of six mice by the Ribosort package programs (Figure 4). Each electropherogram was checked against the combined panel to confirm that the dominant DNA ribotypes in the panel were a consensus feature of the group. We used Czekanowski similarity index to compare the ribotype profiles between different mouse groups (Figure 5). This index is based on both presence and abundance of ribotypes without the bias of focusing on dominant ribotypes. Similar profiles were found in B6 DKO mice on either AIN or LabDiet and B6 WT mice on the AIN diet. B6 WT on LabDiet had the most distinct ribotype profile with dramatically diminished PCR products of 385–390 bp, which were prominent in the other 3 groups. The 385–390 bp products were sequenced and identified as either *Lactobacillus* species or *Bacteroides sp*. (Table 2). This result supports the notion that DKO mice have diminished diversity and thus more resistant to diet-associated alteration of microbiota.

3.5. Genetic Background and Yeast Supplementation Had a Dramatic Impact on Cecal Microbiota Studied in B6 and 129 N5 Groups. We then compared ARISA profiles in 129 N5 DKO mice and their non-DKO littermates (heterozygous at *Gpx1* and/or *Gpx2*) on LabDiet, AIN diet, or yeast-supplemented AIN diet. Only minor differences were detected between each group of 7–18 mice, except the DKO mice on yeast-supplemented diet (Figure 6). Comparison by Czekanowski similarity index of ribotype profiles between different groups of 129 N5 mice indicated the most distinct profile was produced in the yeast-supplemented DKO mice (Figure 5). However, when comparing between B6 and 129 strains on either the same diet or the same GPx genotype, there is a striking difference in the ribotype profiles.

Noticeably, the prominent 385–390 bp products in B6 DKO were nearly nonexistent in the 129 N5 DKO mice on the LabDiet. The 442 bp ribotype was more prominent in 129 N5 DKO mice than their non-DKO littermates and was not detected in B6 groups. The 129 N5 DKO mice on the yeast-containing AIN diet produced a dominant ribotype at 317 bp and enhanced 385–390 bp ribotypes compared to the ARISA profile of 125 N5 DKO mice on the AIN diet (Figure 6). The 317 and 385–390 bp ribotypes were matched to *Lactobacillus sp*. or *Bacteroides sp*.

3.6. Association of the 442 bp DNA with Inflammation in the N5 129 DKO Mice. Since the microbiota profiles from the N5 129 strain mice were very similar, we stratified the DKO mice into sick versus well groups, regardless of the diet (Figure 7). In this comparison, the "sick" group had fewer ribotypes and a more prominent 442 ribotype.

To identify the ARISA DNA products, we have performed sequence analysis on 9 different sizes of DNA PCR products, which matched to 3 bacterial phylum, *Firmicutes*, *Bacteroides,* and *Proteobacteri* (Table 2). Since multiple ribosomal intergenic spacers exist in each bacterium, some of the PCR products (such as the 442, 517, and 710 bp) belong to the same bacterium (*Escherichia coli, Shigella*, or *Salmonella*) from *γ-Proterobacteri* phylum. As expected, none of the clones were matched to the mouse genome [6, 7].

3.7. Inulin Had an Opposite Effect on Cecal Microbiota in 129 N10 DKO and Non-DKO Control Mice. The ARISA profiles

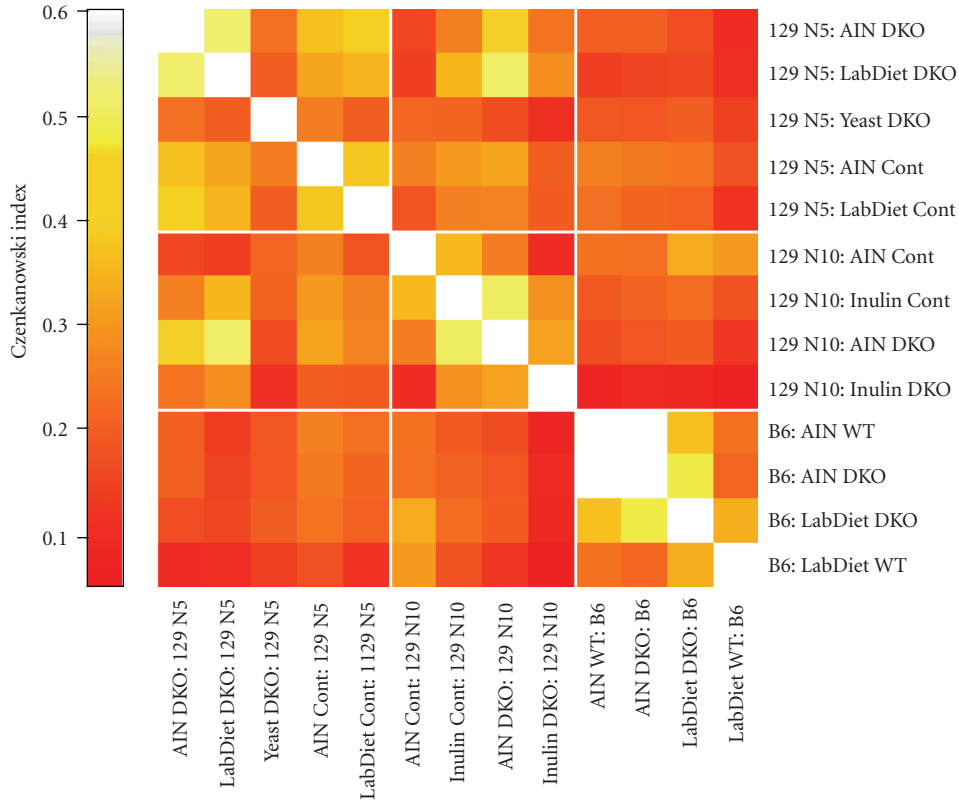

Figure 5: Heat map of Czekanowski similarity indexes of the ARISA profiles from Figures 4, 6, and 8. An index of 0.6 (white) is moderate similarity and an index of 0 (dark orange) is most distinct. "Cont" means the non-DKO sibs control of 129 DKO mice.

of the cecal bacterial content from the inulin-supplemented 129 N10 DKO showed increase of 12 ribotypes (240, 246, 270, 290, 295, 421, 425, 540, 542, 645, 650, 652–655 bp) (Figure 8). However, the prominent putative *E. coli* ribotype, 442 bp, in the AIN-fed DKO mice remained predominant. In the control non-DKO (with at least one WT allele for both *GPx1* and *GPx2* genes) mice, inulin supplementation caused reduction of many ribotypes. Czekanowski similarity index shows that the diversity of microbiota in inulin-supplemented 129 N10 DKO is most different from that in 129 N10 control non-DKO mice on AIN diet (Figure 5). Unexpectedly, the inulin-fed 129 N10 control mice had similar microbiota as 129 N10 DKO mice on the AIN diet.

Comparison of Czekanowski similarity index between 129 N10 groups with 129 N5 and B6 groups shows that the 129 N10 had a more different microbiota profile from B6 groups than 129 N5 groups (Figure 5). Yeast-fed 129 N5 DKO mice had distinct microbiota from other groups. 129 N10 DKO on AIN diet had a similar and simplified ribotype profile as 129 N5 DKO on either LabDiet or AIN diet. The more diversified ribotype profiles present in 129 N10 non-DKO control mice on the AIN diet and 129 N10 DKO on inulin-AIN were also more distinct from those in 129 N5 groups.

3.8. Overgrowth of E. coli in the Cecum of 129 DKO Mice but Not 129 Non-DKO and B6 DKO or WT Mice. We cultured cecal contents under aerobic and microaerobic conditions to verify the identity of ribotypes obtained from the noncultured method and to estimate levels of *E. coli*. The putative *E. coli* colonies were identified by PCR with the ARISA primers, resolving of the dominant 442 and 517 bp products in agarose gels, and BLAST alignment of the sequences obtained from the PCR products. When the PCR was performed side-by-side with DH5α, a bioengineered strain of *E. coli*, the same products were obtained, confirmed by sizing and sequencing (data not shown). These colonies were identified as *E. coli* by the Microbiology Laboratory at City of Hope. Thus, the 442 bp PCR product is most likely indicative of *E. coli*.

The prediction that the 129 N10 DKO mice would show overgrowth of *E. coli* was substantiated by counts of cultures from cecal contents on LB plates (Figure 9). *E. coli* was detected in 28 of 31 mice on AIN diet at an average of $\sim 1 \times 10^9$ CFU/g cecal contents. On LabDiet, 6 of 6 DKO mice had detectable *E. coli* at an average $\sim 1.5 \times 10^9$ CFU/g. WT mice had average *E. coli* levels of $\sim 1 \times 10^6$ CFU/g, detected in 3 of 12 mice on AIN diet, which are at the lower limit of detection.

The 129 N5 cohort was not available for these follow-up studies; so their descendents at N7 were studied. *E. coli* colonies were isolated from 129 N7 DKO on either LabDiet (2 of 2 mice) or AIN diet (3 of 8 mice) cultured under both aerobic and microaerobic conditions at an average

Table 2: Identification of ARISA peak DNA.

Base pair	Top BLAST hits of ARISA peak DNA[1]	Phylum	Matched from bacterial culture[2]
293[1]	*Lactobacillus animalis/murinus*	*Firmicutes*	*Lactobacillus sp.*[3]
300		*Firmicutes*	*Enterococcus gallinarum*
317[1]	*Lactobacillus animalis/murinus*	*Firmicutes*	
350		*Firmicutes*	*Enterococcus faecalis*
380		*Firmicutes*	*Staphylococcus hemolyt./saprophy.*
385–390[1,3]	*Lactobacillus sp.*	*Firmicutes*	*Lactobacillus sp.*[3]
	Uncultured bacteria CD[4]	*Bacteroides sp.*	
400		*Firmicutes*	*Staphylococcus simulins*
421[1]	*Bacteroides sp.*	*Bacteroidetes*	
	Uncultured bacteria CD[4]	*Bacteroides sp.*	
442[1]	*Escherichia coli, Shigella, Salmonella*	*γ-Proteobacteri*	*E. coli*[3]
480		*Firmicutes*	*Lactobacillus sp.*[3]
517[1]	*E. coli, Shigella, Salmonella*	*γ-Proteobacteri*	*E. coli*[3]
	Uncultured bacteria CD[4]	*Bacteroides sp*.	
586[1]	*Dysgonomonas wimpennyi*	*Bacteroidetes*	
710[1]	*E. coli APEC, Shigella, Salmonella*	*γ-Proteobacteria*	
792[1]	*Helicobacter ganmani*	*ε-Proteobacteria*	
800		*Actinobacteria*	*Bifidobacterium sp.*

[1]Eight prominent ARISA peak DNAs plus the 710 bp DNAs were cloned into plasmid and sequenced. The identity of the ARISA fragments was determined using a Basic Local Alignment Search Tool (BLAST; http://www.ncbi.nih.gov/).
[2]The ARISA peak DNA size was matched to the PCR product of cultured bacterial DNA from mouse cecal content using the same primers.
[3]Due to the clustering of DNA products in the 385–390 bp and limitation of the cloning/sequencing vector, the specific size of the clones cannot be determined. Because multiple ribosomal intergenic spacers exist in each bacterium, single *E. coli* colonies produced amplicons at ~442, ~517, and ~710 bp and single colonies of *Lactobacillus* produced amplicons at ~293, 385–390, and ~480 bp.
[4]DNA sequences obtained from at least one clone each of ARISA DNA at 385–390, 421, and 517 bp were matched to an uncultured bacteria CD, LMOACA3ZDO3RM1 clone, submitted by Manichanh et al. obtained from the fecal sample of a Crohn's disease patient [27].

~2×10^8 CFU/g. No putative *E. coli* colonies were obtained from 129 N7 non-DKO mice (0 of 7 mice). These include 2 non-DKO siblings on LabDiet and 5 non-DKO 129 mice on AIN diet under either aerobic or microaerobic conditions.

Several other species of commensal bacteria were also isolated from 129 N7-N10 DKO mice. Under aerobic condition, *Staphylococcus* and *Enterococcus sp.* were cultured from two 129 N7 DKO mice (one well, one sick) on the AIN diet. No *E. coli* was detected in these two 129 DKO mice. The anaerobic plates from 5 of 8 129 samples yielded *Lactobacillus*, which also matched to a rat gut-uncultured bacterium, DR6-87 [28].

No *E. coli* was isolated from B6 DKO ($n = 5$) or WT mice ($n = 7$) on the AIN diet. On the LabDiet, *E. coli* was detected in 3 of 14 B6 DKO mice at an average level of ~2×10^7 CFU/g. Under aerobic conditions, *Staphylococcus* was identified from 2 B6 DKO mice on the LabDiet and *Enterococcus hirae* from 1 mouse on the LabDiet. However, all mice produced *Lactobacillus sp*. (DR6-87) on microaerobic plates. *E. faecalis* and *Bifidobacterium* were isolated from one DKO mouse each on the LabDiet and the AIN diet, respectively.

4. Discussion

The genetic background of GPx1/2-DKO mice is known to have a strong influence on disease course. Here, we show that the genetic background also has a strong impact on cecal microbiota. In fact, the genetic background of the inbred mice has the strongest impact on the gut microbiota compared to diet and GPx genotype. Since the mouse colonies were housed on the same cage rack, it is unlikely that the different microbiota come from physical separation. Since B6 and 129 strains may have different innate immune responses, which can modulate microflora community [2, 13], our result provides an evidence to support the notion that host genetics can affect gut microbiota. A strong genetic effect on microflora has been observed in humans from studies of monozygotic and dizygotic twins versus cohabitating, unrelated individuals [29–31].

Since Paneth cells in the mouse ileum express multiple genes encoding for antimicrobial peptides including α-defensin-related cryptidins and the mouse-specific cryptidin-related sequences (Defcr-rs) [32], it is possible that the quantity and/or types of antimicrobial peptides affect gut microbiota composition (our unpublished observation from Agilent 44K Mouse Expression Array analysis). The Defcr-rs gene subfamily that codes for several cysteine-rich-sequence-4C (CRS4C) peptides is unique to mice and these CRS4C peptides also have bactericidal activity [32]. We have found that 129 strain mice have elevated *Defcr-rs2* (encoding for CRS4C-1), *-rs10* (CRS4C-4) *and -rs12* (CRS3C-5) gene expression in the ileum of both DKO and WT mice, when B6 has low or undetectable expression of these genes. Shanahan et al. reported that *Defcr-rs2 and -rs10* genes were highly expressed in SAMP1/YitFc mice (which had spontaneous

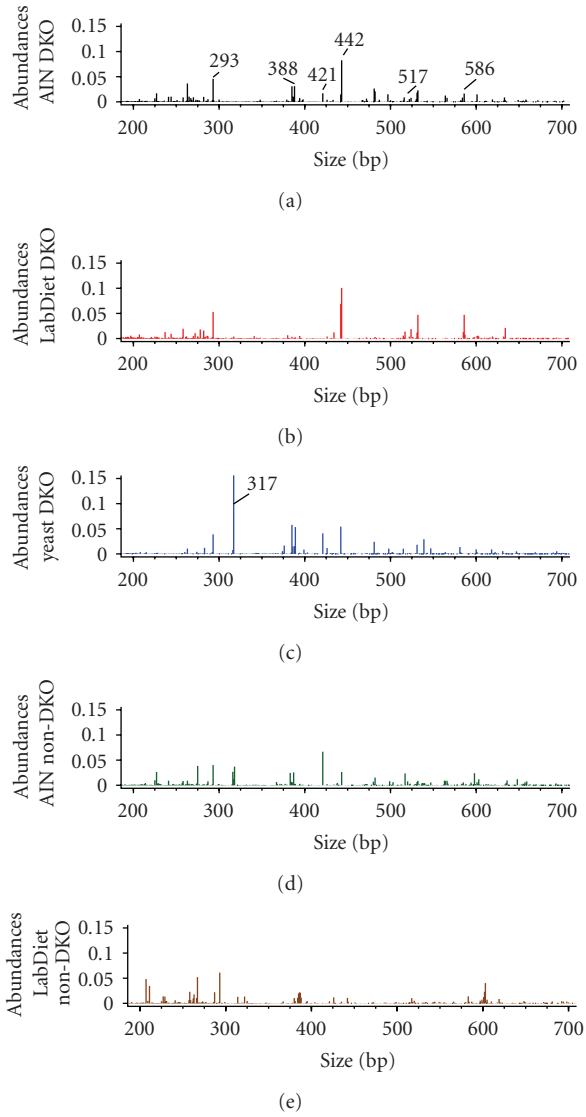

Figure 6: ARISA analysis of DNA from cecal contents of 129 N5. This DKO colony was established by backcrossing the mixed strain B6;129 colony to 129Sv/J for 5 generations. The plots were generated from 15 DKO mice on AIN diet (AINDKO), 18 DKO mice on LabDiet (LabDietDKO), 7 DKO mice on AIN diet supplemented with 1% yeast (Yeast DKO), 13 non-DKO mice on AIN diet (AIN non-DKO), and 13 non-DKO mice on LabDiet (LabDiet non-DKO). Non-DKO mice were the sibs of DKO mice, heterozygous for *Gpx1* and/or *Gpx2* (thus no inflammation). DKO mice on LabDiet did not have the distinctive peaks at 385–390 bp, 421 bp, 481 bp, and 498 bp found in other groups. DKO mice on the yeast-containing diet had a similar profile as AIN-fed mice, except having a prominent 317 bp peak. The 442 bp ribotype found in these 129 N5 DKO mice had lower abundance in the non-DKO sibs on both LabDiet and AIN diets.

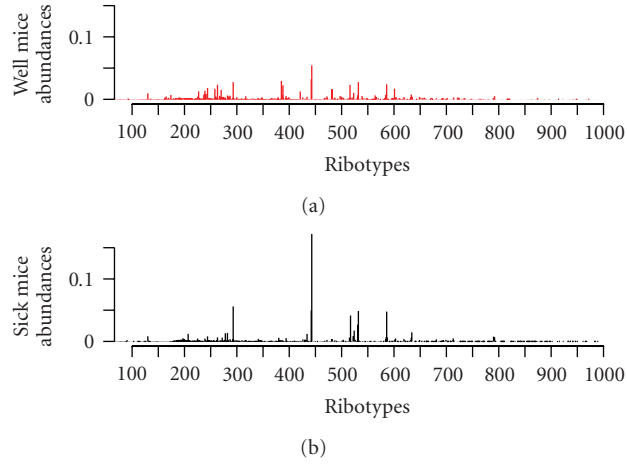

Figure 7: ARISA analysis of DNA from cecal contents of 129 N5 DKO mice stratified by the disease severity. Seven of 18 DKO mice on LabDiet, 13 of 14 DKO mice on AIN diet, and 9 of 16 DKO mice on AIN diet with daily contact of soiled bedding from LabDiet group were combined in the well group (29 mice) based on pathology. The other 19 DKO mice were stratified in the sick group. The median colon pathology score for well mice was 3 (range of 0–5) and 6 for sick mice (range of 6–11).

ileitis) and barely detectable in B6 strain. Since both 129 strain mice and SAMP1/YitFc mice with high expression of some of the *Defcr-rs* genes are prone to inflammation, it was postulated that the cysteine-rich CRS4C peptides may also affect cellular homeostasis such as affecting autophagy, unfolded protein response, and apoptosis. Because of the complex nature of the defensin gene family in mice, we plan to explore this issue further and present it in a comprehensive manner in the future.

Although the ARISA profile has limited resolution, we were able to identify 9 species that belong to 3 bacteria phyla, *Firmicutes*, *Bacteroides*, and *Proteobacteri*, commonly present in human gut. From our small list of bacteria, we have identified *E. coli* as a potential harmful bacteria species, where overgrowth is associated with 129 but not B6 mice. With the ARISA primers, *E. coli* produces a 442 bp PCR product, which is present in the 129 strain and absent in B6 mice. In the 129 strain, this 442 bp ribotype is more abundant in the DKO than that control non-DKO mice, with the only exception of inulin-fed non-DKO mice. Furthermore, this 442 bp PCR product is more prominent in the sick than well 129 N5 DKO mice. Although the 442 bp ribotype is also matched to *Shigella* and *Salmonella* in the *Proteobacteri* bacteria phyla by sequence analysis, our isolation of *E. coli* species from several 129 DKO mice supports the *E. coli* identity of this 442 bp DNA PCR product. The sick 129 DKO mice also have a more prominent 517 bp DNA also produced by our cultured putative *E. coli* clones as well as from a laboratory strain of *E. coli,* DH5α. Detection of the 442 bp but not the 517 bp PCR products may be because the larger product is not as efficiently amplified as the shorter one when *E. coli* DNA is present in a mixture of other bacteria DNA. The results shown in the ARISA panels coupled with counts from diluted cecal contents on LB plates suggest that detection of the 442 bp ribotype peak required 1×10^8-1×10^9 CFU/g. Since most of the well mice, B6 or 129, have counts less than 2×10^7 CFU/g, the counts obtained for sick 129 DKO mice appear to represent an overgrowth of

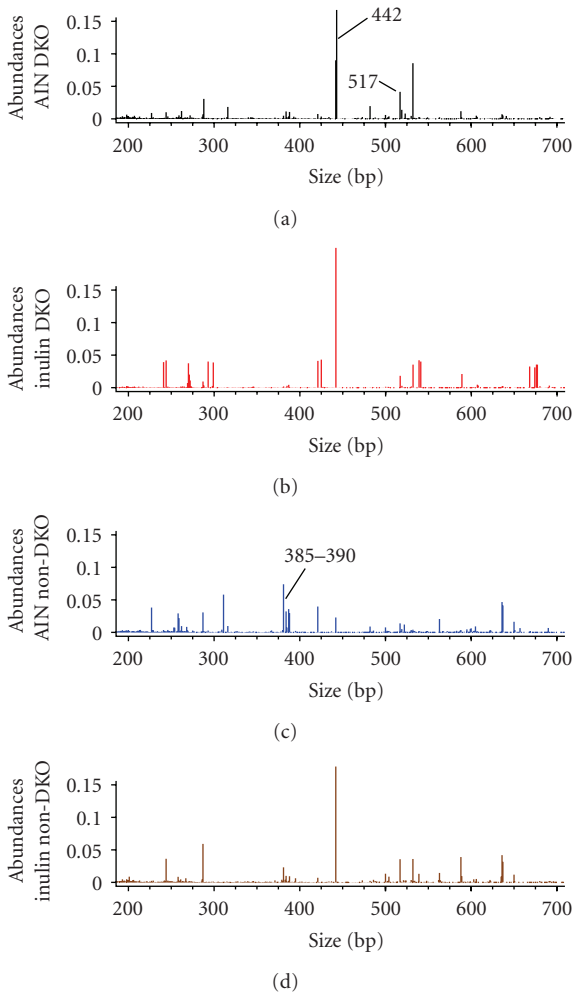

Figure 8: ARISA analysis of DNA from cecal contents of 129 N10. This DKO colony was established by backcrossing the mixed strain B6;129 colony to 129Sv/J for 10 generations. The group sizes are 21 DKO mice on AIN diet, 10 non-DKO mice on inulin-supplemented AIN diet, 7 non-DKO mice on AIN diet, and 15 DKO mice on inulin-supplemented AIN diet. The non-DKO mice are heterozygous DKO with 2 or 3 WT alleles for combined *Gpx1* and *Gpx2*.

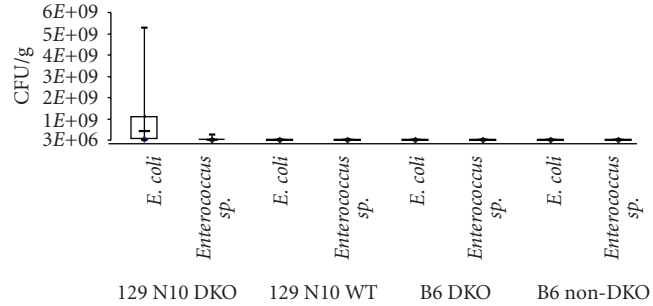

Figure 9: Box and whisker plots of the levels of cecal *E. coli* (large colonies on LB plates under aerobic conditions) and *Enterococcus sp*. (small colonies on LB plates; *E. faecalis, E. hirae,* or *E. gallinarum*) for mice on AIN diet. Bacteria counts are based on colony forming units per gram of cecal contents (CFU/g) at 24 hours, 37°C. Representative colonies were stabbed with a micropipet tip and transferred to ARISA PCR mix. The PCR products were resolved on agarose gels for comparison to laboratory *E. coli* and the PCR products were further isolated and sequenced for final identification of species.

E. coli. Enteropathic *E. coli* has been linked to Crohn's disease and *Salmonella* to enterocolitis by opportunistic invasion of inflamed areas [7, 33–37]. These bacteria may create the opportunity for overcoming colonization resistance by eliciting an inflammatory response and then invading the inflamed region [37].

The DKO mice are more resistant to dietary impact on microbiota profiles than control mice. This result is consistent with the notion that inflammation in DKO mice diminishes bacteria diversification relative to that in the control mice, which was reported in human IBD patients [3, 4]. The limitations on bacterial diversity in DKO mice can explain why diet alters ARISA profiles only in B6 WT but not in B6 DKO mice. Although 129 N5 mice on either AIN or LabDiet have rather similar profiles, DKO mice on these two diets have higher similarity index than non-DKO mice on these two diets. Non-DKO mice were used because they were littermates of DKO mice, thus shared the same bedding but did not have gut pathology.

Supplementation of yeast to the AIN diet changed bacteria profile dramatically in 129 N5 DKO mice. Yeast produced a prominent 317 bp ribotype matched to *Lactobacillus animalis* or *L. murinus* in *Firmicutes* phylum. Although the *Lactobacillus sp.* have been used as probiotics, they can also induce reactive oxygen species (ROS) from macrophages [38]. This pro-oxidant property of these bacteria may blunt their probiotic activity in the DKO mice. This may explain why the yeast-AIN diet increased morbidity and diarrhea, but not colon inflammation, in 129 N5 DKO mice. It is unclear why the LabDiet (which has 1% brewer's yeast) did not produce this 317 bp ribotype in both 129 N5 DKO and non-DKO mice. Perhaps other ingredients in the LabDiet have masked or countered the effect of yeast. IBD patients tend to have high antibody titers against yeast [39], and mannan-containing yeast-wall components are strong activators for macrophages and neutrophils [40]. However, our result does not support the proinflammatory activity of yeast.

Inulin is a prebiotic, which has been used to manage IBD by changing gut microbiota [1, 26]. Dietary supplementation of inulin increases the number of *Bifidobacteria* in humans and animals [41, 42]. Interestingly, supplementing inulin to AIN diet has an opposing effect on microflora between 129 N10 DKO and control mice. Inulin increases bacteria diversity in DKO but decreases diversity in control mice. Consequently, the diverse ARISA profiles are more distinct from each other, when the converged profiles become similar. Most disappointedly, inulin did not eliminate the 442 bp, likely from *E. coli* colonization. This may explain the lack of anti-inflammatory activity of inulin in 129 DKO mice.

The impact of the GPx1/2-DKO construct on microbial diversity is likely due to increases in reactive oxygen species and activation of innate immune response. We have shown that the DKO intestine has higher levels of lipid peroxidation and myeloperoxidase/lactoperoxidase activity compared to non-DKO control mice [10]. This increased oxidative stress may explain the elevated apoptotic cell death in the crypt epithelium accompanied by degranulation of Paneth cells and mucin depletion [11]. The primary function of enteric defensins is thought to be a regulator of intestinal microbiota [43]. Mucin excretion can inhibit bacterial adhesion [44]. Conceivably, overexpression or depletion of these antimicrobial molecules can alter luminal microflora.

5. Conclusions

In summary, using a nonculture ARISA we examined the effect of mouse strain background, diet and DKO genotype on the gut microbiota. We found that B6 versus 129 strain background has the greatest overall impact on the microbiota with similarity indexes from 0.26 to 0.12 when matched for diet and GPx status. GPx status did not affect the strain-based dissimilarity. In comparing AIN and LabDiet, DKO mice were more resistant to change than the non-DKO or WT mice in both B6 and 129 strains. This may be due to inflammation in the DKO gut, which has restricted microflora diversity. However, supplementing yeast and inulin to AIN diet greatly altered microflora profiles in the DKO mice. We have identified a 442 bp DNA from *E. coli*, as a proinflammatory bacteria associated with 129 strain mice especially with the DKO genotype.

Acknowledgments

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Review Article

The Opportunistic Pathogen *Listeria monocytogenes*: Pathogenicity and Interaction with the Mucosal Immune System

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Listeria monocytogenes is an opportunistic foodborne pathogen causing listeriosis, an often fatal infection leading to meningitis, sepsis, or infection of the fetus and abortion in susceptible individuals. It was recently found that the bacterium can also cause acute, self-limiting febrile gastroenteritis in healthy individuals. In the intestinal tract, *L. monocytogenes* penetrates the mucosa directly via enterocytes, or indirectly via invasion of Peyer's patches. Animal models for *L. monocytogenes* infection have provided many insights into the mechanisms of pathogenesis, and the development of new model systems has allowed the investigation of factors that influence adaptation to the gastrointestinal environment as well as adhesion to and invasion of the intestinal mucosa. The mucosal surfaces of the gastrointestinal tract are permanently exposed to an enormous antigenic load derived from the gastrointestinal microbiota present in the human bowel. The integrity of the important epithelial barrier is maintained by the mucosal immune system and its interaction with the commensal flora via pattern recognition receptors (PRRs). Here, we discuss recent advances in our understanding of the interaction of *L. monocytogenes* with the host immune system that triggers the antibacterial immune responses on the mucosal surfaces of the human gastrointestinal tract.

1. Introduction

Listeria monocytogenes is a Gram-positive foodborne pathogen that is ubiquitously found in diverse environments such as soil, water, various food products, animals, and humans [1]. Infection by *Listeria monocytogenes* occurs almost exclusively after ingestion of contaminated food. Because the bacteria are readily inactivated at pasteurization temperature, the main source of infection represents contaminated raw food that is subjected to minimal further processing, such as soft cheeses, frankfurters, pâtés, vegetables and postprocessed contaminated milk products [2]. In individuals with impaired cell-mediated immunity such as neonates, pregnant woman, elderly persons, and immunocompromised patients suffering from transplantation events, the bacterium may cause mother-to-fetus infections, septicemia, or meningoencephalitis. Listeriosis is relatively rare and annual incidence is decreasing; in the United States from 7.7 cases per million population in 1990 to 3.1 cases per million population in 2003. In France, the incidence of listeriosis declined from 4.5 cases per million population in 1999–2000 to approximately 3.4 in 2002–2003 [3]. Although the incidence is low, the high mortality rates (about 30%) associated with listeriosis make *L. monocytogenes* one of the most deadly human food-borne pathogens. In contrast to the severe invasive disease recent outbreaks demonstrated that infection of healthy individuals with *L. monocytogenes* often leads to the development of a febrile gastroenteritis [4].

The organisms are well adapted to the conditions in the gastrointestinal tract and pursue different strategies to counteract changes in acidity, osmolarity, oxygen tension, or the challenging effects of antimicrobial peptides and bile. The finding that the bacteria are able to colonize and persist in the gallbladder [5] suggests the occurrence of long-term and chronic infections and demonstrates the ability of pathogenic *Listeria* to survive within the various microenvironments of the gastrointestinal tract.

Although other animals, such as guinea pigs, seem to be better suited to study the immune response to

L. monocytogenes mice have been proven the most useful model for immunological studies due to availability of knock-out mice deficient in specific genes. Hence, most of our knowledge of how the immune system functions has been learned from experimental infections of mice using *L. monocytogenes* and the subsequent analysis of the innate and adaptive immune responses [6]. The molecules that function as pattern recognition receptors (PRRs) on epithelial cells, macrophages, and dendritic cells (DCs), thereby triggering the innate immune system after contact to bacterial pathogens, comprise Toll-like receptors (TLRs) and nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs). The recognition of pathogen-associated molecular patterns (PAMPs) by the PRRs on mucosal cells drives the activation of subsequent signaling cascades including NF-κB, interferon (IFN) response factors (IRFs), activator protein 1 (AP1), and mitogen-activated protein kinases that promote the induction of proinflammatory cytokines and antimicrobial peptides, as well as the maintenance of epithelial barrier function and epithelial cell proliferation [7]. The vast amount of knowledge that has been gathered through proteomic and transcriptomic approaches makes *L. monocytogenes* one of the best-studied bacterial pathogens for investigations on the interplay of intracellular pathogens and the intestinal immune system. In this paper, we focus on the recent developments in the analysis of the interaction between *L. monocytogenes* and the mucosal immune system of the host gastrointestinal tract.

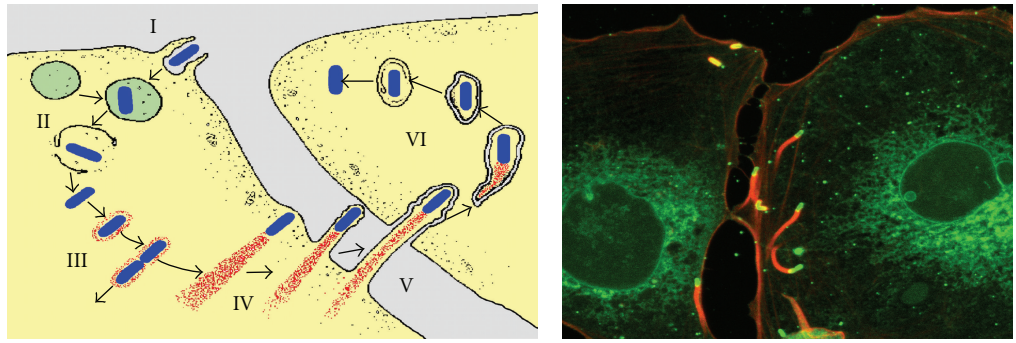
2. Adaptation of *Listeria monocytogenes* to the Conditions of the GI Tract

Along the gastrointestinal tract *L. monocytogenes* cells have to face a hostile environment characterized by altered osmolarity, low oxygen pressure, low pH, and presence of bile. In a recent article Lungu et al. [8] reconsider growth, survival, proliferation and pathogenesis of *L. monocytogenes* under low oxygen or anaerobic conditions. Other studies have shown that *L. monocytogenes* is able to launch acid resistance systems to respond to the low pH conditions in food or in the host [9, 10]. In *L. monocytogenes,* the glutamate decarboxylase (GAD) system confers resistance to acidic environments. Cotter and colleagues demonstrated that the expression of the GAD system correlates directly with acid tolerance of *L. monocytogenes* and is an absolute requirement for survival during the transmission of the stomach [9]. This system has already been associated with acid resistance in many other bacteria that need to transit the stomach before they reach their site of infection. The GAD system leads to intracellular consumption of protons by irreversible decarboxylation of extracellularly sourced glutamate and subsequent export of gamma-aminobutyrate (GABA) via a glutamate:GABA antiporter. Surprisingly, it was shown that acid-adapted *L. monocytogenes* were more successful in entering and proliferating in Caco-2 cells in contrast to cells that were not previously exposed to acid-stress [11].

L. monocytogenes encounters changes in the osmolarity not only in the gastrointestinal tract of its host but also in the food industry, as a preservation method or in their environmental niches. The strategy that *Listeria* and also other bacteria apply to respond to conditions of elevated osmolarity is the cytoplasmic accumulation of compatible solutes or osmolytes (reviewed by Sleator and Hill [13]). The finding that *L. monocytogenes* is able to colonize the gall bladder of infected mice demonstrates the organisms abilities to tolerate high concentrations of bile stored in this compartment [14]. The genes conferring the principal bile-resistance to *L. monocytogenes* are BSH and BilE [5]. This resistance has important consequences as Dussurget et al. [15] reported that bile salt hydrolase activity is essential for *L. monocytogenes* pathogenesis. Furthermore, carnitine uptake by the pathogen is essential for survival in the small intestine and transient colonization of the murine gastrointestinal tract [16, 17].

In the context of adaptation of *L. monocytogenes* to the conditions along the GI tract, the transcription factor Sigma^B (σ^B) was identified as the key factor that triggers the manifold adaptation mechanisms. The crucial role that the σ^B protein plays in acid-tolerance was identified by Wiedmann et al. [18], whereas Becker et al. [19] independently identified σ^B as the relevant factor for adaptation of *L. monocytogenes* to alterations in osmolarity and temperature. Also, the genes for BSH and BilE responsible for bile-tolerance are preceded by σ^B dependent promoter sites [5, 20].

In their study, Kazmierczack et al. [21] identified fifty-five genes showing a statistically significant σ^B dependent expression after exposure of *L. monocytogenes* to osmotic stress. In a more recent study by Hain et al. [22], the authors report a significantly higher number of genes that were under the regulation of σ^B. They found 111 genes under negative control of σ^B and 105 genes that showed a positive σ^B dependent regulation. In *L. monocytogenes* σ^B contributes to both stationary- and exponential-phase acid resistance, whereas in *L. innocua* acid resistance is conferred by σ^B only during the exponential-phase of growth [23]. Thus, the function of σ^B seems to be strain and species dependent within the genus *Listeria*. Moreover, it turned out that different serotypes of *L. monocytogenes* reveal differences in their dependency on a functional σ^B regulon [24]. As a consequence, the resulting variations in environmental stress resistance may offer an explanation for the reported differences in the virulence traits and the survival capabilities in the host and in food for different *L. monocytogenes* strains [18]. The obtained results indicate that σ^B contributes to *L. monocytogenes* survival not only in environmental niches but also inside the host [25, 26]. This is emphasized by the finding that σ^B is required for the expression of the manifold genes that are important for the survival of *L. monocytogenes* within the GI tract of a host. In fact, the σ^B regulon comprises important virulence genes, encoding virulence factors such as internalin A and B [21, 22]. In murine and guinea pig models, loss of σ^B function has been shown to result in decreased virulence of *L. monocytogenes* after oral infection but not during systemic infection [27]. Furthermore, *L. monocytogenes* show an up-regulation of the *sigB* gene during passage of the mouse GI tract [5]. Hence, the alternative sigma factor σ^B represents a crucial



(a) (b)

Figure 1: Stages in the intracellular life cycle of *L. monocytogenes*. The cartoon (a) sketches the different stages of *L. monocytogenes* infection: (I) cell entry mediated by invasion factors InlA or INLB, (II) escape from phagolysosom by LLO and PlcA, (III) actin recruitment and replication, (IV) intracellular movement due to polarized actin-polymerization mediated by ActA, (V) cell-to-cell spread by formation of listeriapods, and (VI) subsequent lysis of the two-membrane vacuole by LLO and PlcB. Modified from Tilney & Portnoy [12]. The fluorescence image (b) shows the intracellular movement and cell-to-cell spread of *L. monocytogenes* cells (green) driven by the polarized polymerization of actin tails (red).

prerequisite for the successful infection of a host by *Listeria monocytogenes* via the GI route (Figure 1).

3. Gastroenteritis due to *Listeria monocytogenes*

Listeriosis is a severe foodborne disease characterized by bacteremia and meningoencephalitis in individuals with impaired cell-mediated immunity, including neonates, pregnant woman, elderly persons, and immunosuppressed patients. The incidence of listeriosis is rather low, compared to other common foodborne pathogens such as *Campylobacter* species, *Salmonella* species, *Shigella* species, and *Vibrio* species. However, the outcome is much more severe and often fatal. In fact, it represents one of the most deadly bacterial infections due to its high mean mortality rate of 20%–30%, despite early antibiotic treatment [28]. Ingestion of food contaminated with *L. monocytogenes* is the usual mode of transmission leading to listeriosis. Although many patients experience diarrhea antecedent to the development of bacteremia or meningoencephalitis due to *L. monocytogenes* infection, it was only recently that convincing evidence was obtained that *L. monocytogenes* can cause self-limiting, febrile gastroenteritis in healthy persons [4]. At least seven outbreaks of foodborne gastroenteritis due to *L. monocytogenes* infection have been reported over the last 20 years (Table 1). First evidence was obtained in 1989 when the *L. monocytogenes* strains isolated from blood samples of two febrile pregnant women and those from the stool samples of a person with diarrhea were shown to be identical [29]. All three patients attended the same party, and a total of 17 of the 36 attendees reported gastrointestinal complaints. More convincing evidence that *L. monocytogenes* could cause self-limiting gastroenteritis came from an outbreak of febrile gastroenteritis that was associated with the consumption of contaminated chocolate milk [30]. A total of 45 out of 60 persons who drank chocolate milk served in the course of a picnic developed the symptoms, and identical strains were obtained from 14 symptomatic patients, from unopened cartons of the chocolate milk, as well as from the environment of the dairy that supplied the milk [30]. One of the largest documented outbreaks of febrile gastrointestinal illness comprised 292 persons who had been hospitalized after eating in the cafeteria of two primary schools in northern Italy in 1997. The contaminated food was prepared by the same caterer and cultures from 123 stool samples and 1 blood sample from the hospitalized patients turned out to be indistinguishable to strains isolated from the food, and environmental specimens of the catering plant [31]. Therefore, *L. monocytogenes* should be considered to be a possible etiologic agent in outbreaks of febrile gastroenteritis when routine cultures fail to yield a pathogen.

Common symptoms observed in the effected patients included fever, watery diarrhea, nausea, headache, and pain in joints and muscles. The mechanism by which *L. monocytogenes* causes diarrhea is not entirely clear. However, it is likely that it is the result of direct invasion of the epithelial cells of the intestinal mucosa, as it is not known that *L. monocytogenes* produces enterotoxins [4]. The observed symptoms like fever as well as occasionally bloody diarrhea and bacteremia further support the hypothesis that diarrhea results from direct invasion of *L. monocytogenes* to the intestinal mucosal epithelium. The observation that *L. monocytogenes* could cause self-limiting, febrile gastroenteritis demonstrates that the pathogen induces mucosal inflammation after entering the host intestinal mucosa.

4. Adherence and Invasion of the Gastrointestinal Epithelium

There exist two principle mechanisms by which *L. monocytogenes* can enter into the host through the intestinal mucosa. The first route is direct invasion of the enterocytes lining the absorptive epithelium of the microvilli, leading

Table 1: Outbreaks of gastroenteritis due to *Listeria monocytogenes*.

Year of outbreak	Number of cases	Serotype	Implicated source	Reference
1993	18	1/2b	Rice salad	[41]
1994	45	1/2b	Chocolate milk	[30]
1997	1566	4b	Cold-corn-and-tuna salad	[31]
1998	5	1/2a	Cold smoked trout	[42]
2000	32	1/2	Corned beef and ham	[43]
2001	16	1/2a	Delicatessen meat	[44]
2001	48	1/2a	Cheese	[45]

to infection of the intestinal cells. This entry mechanism occurs only in humans and some susceptible animals (e.g., guinea pigs) that also express the correct isoform of the receptor molecules necessary for recognition by the *Listeria* invasion molecules, termed internalins [32]. The second entry pathway is translocation across the M-cells of Peyer's patches [33]. This mechanism occurs also in hosts that do not express susceptible isoforms of the receptor molecules, such as mice and rats, and represents an unspecific mechanism as nonpathogenic species such as *L. innocua* or *Bacteroides thetaiotaomicron*, a prominent gut symbiont, are translocated equally. However, the latter mechanism seems to be less efficient than direct invasion of enterocytes [34].

As a first step towards invasion of the gastrointestinal epithelium the bacteria need to adhere to the surface of the epithelial cells. To enable contact with the epithelium underlying the mucus layer as the site of invasion many bacteria produce mucinases. This is not the case for *L. monocytogenes* which does not produce mucinases but a number of surface proteins that can bind to a specific type of human mucin [35]. Interaction with the human Muc2 isoform occurs trough the internalin proteins InlB, InlC, and InlJ. This initial interaction is thought to be an important prerequisite for the subsequent events leading to adherence and invasion of the epithelial layer [28, 36]. For various gastrointestinal pathogens, it is known that they use their flagellar structures not only as effectors of motility but also as adhesins or as a secretion apparatus. This is also not the case for *L. monocytogenes* which uses flagella simply for motility thereby increasing the efficacy of host invasion [37, 38].

The initial interaction of internalins with Muc2 seems not to be sufficient and further expression of proteins is necessary to warrant adherence of the pathogen to the epithelium. *Listeria* adhesion protein (LAP) was shown to bind to the host cell heat-shock protein 60 [39] and a specific fibronectin-binding protein (FbpA) of *L. monocytogenes* was identified to interact with cell surface fibronectin in the murine model [40].

Central for the pathophysiology of *Listeria monocytogenes* in the gastrointestinal tract is the ability to cross the intestinal barrier through invasion of enterocytes. This important event is promoted by internalin A (InlA), whereas internalin B (InlB) seems to play no direct role in invasion of cells of the gastrointestinal epithelial layer. Instead, InlB is known to mediate the invasion of hepatocytes and is required for the infection of the fetoplacental unit [46, 47]. The cellular receptor for InlA is human E-cadherin, a protein expressed at the basolateral surface of polarized enterocytes that was identified by affinity chromatography on an InlA-column [48]. The InlA E-cadherin interaction is species-specific, and was shown to rely on a single amino acid residue in the E-cadherin molecule, which is prolin in permissive species such as humans, and glutamic acid in nonpermissive species such as the mouse [34]. Although the E-cadherin of mouse and human show about 85% similarity, InlA does not interact with mouse E-cadherin. For this reason, mice are not an appropriate experimental model for oral infection with *L. monocytogenes* and the investigation of the pathogenic events that enable the organisms to penetrate the intestinal mucosa after ingestion of contaminated food. Consequently, the necessity for further animal models for human listeriosis led to the identification of two novel and complementary animal models. While gerbils turned out to be a natural host for *L. monocytogenes*, a transgenic mouse line was developed that features expression of human E-cadherin in enterocytes [49]. Use of this animal model conclusively demonstrated the role of InlA for crossing the intestinal barrier [34], and the essential and interdependent roles of InlA and InlB in feto-placental listeriosis [46]. The detailed molecular mechanisms of InlA mediated cell entry have been described and reviewed elsewhere [1, 50–53].

Upon uptake, the intracellular pathogen appears surrounded by the membranes of the phagocytic vacuole. Different phospholipases (PI-PlcA and PI-PlcB) are activated by a metalloprotease (Mpl), and cooperate with the pore-forming hemolysin listeriolysin O (LLO), which is most active at the acidic conditions (pH 5.5) of the vacuole, to confer the lysis of the phagosome membrane [54, 55]. Once the bacteria escape into the cytoplasm, they start to replicate while making use of specific transporter systems to gain carbohydrates from the host cell [56].

At the same time the pathogen is released from the phagosome, it induces the expression of ActA, a protein that triggers the nucleation and polymerization of host globular g-actin into f-actin filaments. The polarized polymerization of actin leads to a propulsive force that propels the bacteria through the cytoplasm and occasionally into the cytoplasma membrane of neighboring cells. The resulting pseudopods or "listeriapods" are then taken up (endocytosed) by the adjacent cells, thus, promoting cell-to-cell spread of the pathogen from on cell to another. The bacteria entrapped within the double membrane of the newly infected vacuole

are again released by the combined action of LLO and the phospholipases, in this case the product of the *plcB* gene. This invasion mechanism allows *L. monocytogenes* to safely spread through host tissues without leaving the host cytosolic compartment, thereby protected from the host adaptive immune system. This intriguing strategy has been elucidated and thoroughly reviewed elsewhere [1, 32, 56, 57].

5. Innate Immune Responses to *Listeria monocytogenes*

After infection within the gastrointestinal tract, immediate immune responses are essential for the control of pathogens, such as *L. monocytogenes*. Activation of the innate immune system is triggered when pathogen-associated molecular patterns (PAMPs) engage pattern recognition receptors (PRRs) on intestinal epithelial cells (IECs) [58]. Despite the given name, PAMPS are actually not restricted to pathogens. They are expressed by all bacteria, invasive pathogens as well as noninvasive commensals. Typical PAMPs include bacterial carbohydrates, such as lipopolysaccharide (LPS), mannose, nucleic acids (both DNA and RNA), peptidoglycan components, lipoteichoic acids, and probably many other molecules, and are able to trigger the innate immune response. Innate immunity to *L. monocytogenes* is primarily mediated by two types of pattern recognition receptors, the Toll-like receptors (TLRs), and the nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs). In addition, there is some experimental evidence for the involvement of scavenger receptors and a TLR-9 independent cytosolic sensor system for bacterial DNA [59].

Toll-like receptors (TLRs) are a family of transmembrane glycoproteins, of which 10 members are known to exist in humans, where they are located on the cell surface or within endosomes. Upon recognition of the presence of microbes through sensing pathogen-associated molecular patterns, TLRs can bind any of the 4 known activating adaptors: (i) Myeloid differentiating factor-88 (MyD88), (ii) MyD88 adapter-like (Mal), (iii) TIR domain-containing adapter-inducing IFN-β (TRIF), and (iv) TRIF-related adapter molecule (TRAM), whereas sterile-α and Armadillo repeat-containing molecule (SARM) negatively regulates TRIF signaling [60]. MyD88 appears to be the key adaptor molecule, because it is required for signaling by all TLRs with only one exception: TLR3 uses TRIF [7]. The binding of the activating adaptors results in the subsequent recruitment of IL-1R, associated kinases (IRAKs) and downstream activation of transcription factors including NF-κB and IFN regulatory factor 3 (IRF3), which in turn induces the proinflammatory cytokines and type I IFNs [60].

In the intestinal mucosa, expression and localization of PRRs on IECs and DCs differ significantly from cells of other tissues. Primary human IECs constitutively express TLR3 and TLR5, but only low levels of TLR2 and TLR4. As the location of the TLRs is crucial for their function, TLRs 1, 2, 4, 5, and 6 are expressed on the cell surface to recognize extracellular microbes, whereas TLR3, 7, 8, and 9 are present on premature endosomes [7]. As mentioned above, expression of TLRs on IECs is generally low and some receptors such as TLR5 and TLR9 are located basolaterally, thus possibly preventing an interaction with PAMPs in the intestinal lumen [7]. However, several TLRs such as TLR2, TLR4, TLR5, and TLR9 are expressed on the apical side of the IECs and important for the recognition of molecules from commensal bacteria, which is crucial to trigger innate immune responses that are required to prevent exaggerated adaptive immunity to the intestinal microbiota [61]. This is an important function indicating that low-level recognition by TLRs is necessary for protection from intestinal epithelial injury [62].

Toll-like receptor 2 (TLR2) can interact with several specific ligands, including bacterial lipoproteins, lipoteichoic acids of Gram-positive bacteria such as *L. monocytogenes* and yeast zymosan [7]. TLR2 can form heterodimers with TLR1 and TLR6, thereby improving the recognition of the target lipoteichoic acids [63]. TLR2 is expressed on the cell surface of intestinal epithelial cells and its activation by commensal bacteria is thought to play an important role in the maintenance of the integrity of the intestinal epithelial barrier [64]. TLR2 is also expressed within phagolysosomes, thus, *L. monocytogenes* cells that have escaped into the host cell cytoplasm were not detected by TLR2. The importance of TLR2 signaling for early protection against *L. monocytogenes* is, however, inconclusive. Whereas a first study observed that *L. monocytogenes* infected TLR2 deficient mice were as resistant as wild-type mice, a later study using slightly different experimental settings revealed a protective effect of TLR2 during early *L. monocytogenes* infection [65, 66].

Toll-like receptor 5 (TLR5) can bind to a protein motif common to the flagellin protein making up the flagella from many bacteria, such as *L. monocytogenes*. TLR5 activation induces NF-κB and stimulates TNF production, suggesting that TLR5 may serve as a general alarm system, when the gastrointestinal barrier is compromised by a broad spectrum of motile bacteria. However, activation of TLR5 located on the apical surface of IECs by flagellin leads to an increase in the expression of antiapoptotic genes. This correlates with the observed protective effects of TLR5 signaling in epithelial homeostasis and may suggest that under physiological conditions flagellin ligation of TLR5 located on the apical surface of IECs does not exert an inflammatory response. In contrast, flagellated bacteria that interact with basolateral TLR5 signal an invasion of the epithelium by bacteria, and therefore induce a strong proinflammatory response [7].

On the other hand, flagellin-deficient *L. monocytogenes* revealed no significant differences in virulence in infection experiments. This observation suggests that TLR5 mediated signaling may not be essential for pathogenesis and adaptive immunity after an *L. monocytogenes* infection in immunized animals [67].

Toll-like receptor 9 (TLR9) recognizes the CpG motifs present in bacterial DNA. In immune cells, TLR9 is exclusively localized in the endosomes. In the intestine, TLR9 was shown to be located on both, the apical and the basolateral surface of IECs. Upon activation of TLR9, IκBα is degraded and NF-κB is activated, again resulting in a proinflammatory response. In contrast, stimulation of

apical TLR9 led to the accumulation of polyubiquitinated IκBα in the cytoplasm, preventing NF-κB activation and inflammation [7]. Currently, there is no clear evidence that TLR9 actually contributes to the control of *L. monocytogenes* infection; further clarification may be obtained by animals models deficient in TLR9 [6].

In conclusion, the available experimental evidence suggests that TLR2 is the most relevant TLR for recognition of *L. monocytogenes* cells. However, as IECs show TLR2 commensal ligand-induced activation, TLR2 is also considered to play an important role in maintaining the integrity of the intestinal epithelial barrier [64]. This view is further supported by the observation of an increased expression of both TLR2 and TLR4, in a neonatal rat model of necrotizing enterocolitis-induced mucosal injury, suggesting that TLR2 may promote intestinal inflammation under circumstances where the epithelial barrier has been compromised [68]. In conclusion, there is no doubt that TLR signaling plays an important role for maintaining the integrity and function of the intestinal epithelium during invasion by *L. monocytogenes*.

NOD-like receptors (NLRs) are a group of intracellular pattern recognition receptors, which are structurally composed of an N-terminal effector domain that can comprise caspase recruitment domains (CARDs) like the NODs, or a pyrin domain as in the case of NALPs (NAcht-, Leucine-rich repeat, and Pyrin domain-containing proteins). Known members of the NLR family are NOD1, NOD2, NALP1, NALP3, neuronal apoptosis inhibitory protein-5, and the ICE protease activating factor (IPAF) [69, 70]. In humans, twenty-three NLRs have been identified so far, while in mice 34 NLRs are known [71]. The NLRs are critical for mucosal innate immunity as sensors of microbial components and cell injury in the cytoplasm [72]. They mediate proinflammatory signals through activation of caspase-1 and NF-κB. Activation of caspase-1 leads to cleavage and activation of proinflammatory cytokines, such as IL-1β and IL-18, as well as to apoptotic cell death. Both NOD1 and NOD2 are important for the innate immune response against *L. monocytogenes,* because they represent intracellular sensors of bacterial peptidoglycan components that are thought to enter cells by endocytosis through clathrin-coated pits [73]. While NOD1 is ubiquitously expressed in adult human tissues, NOD2 is expressed only in leukocytes, DCs, and epithelial cells. Activation of NOD1 and NOD2 results in the translocation of NF-κB and mitogen-activated protein kinase into the nucleus, to up-regulate the transcription of proinflammatory genes and mediate antibacterial effects by the up-regulation of another group of small antibacterial peptides, the defensins [7].

The nucleotide-binding oligomerization domain 1 (NOD1) recognizes a diaminopimelic acid-containing dipeptide or tripeptide molecule generated by lysozyme action on the peptidoglycan of many Gram-negative and Gram-positive bacteria, including *L. monocytogenes* [74].

The nucleotide-binding oligomerization domain 2 (NOD2) is activated by muramyl dipeptide (MDP), which is another degradation product of the peptidoglycan produced by lysozyme and other (bacterial) peptidoglycan hydrolases. In intestinal Paneth cells, NOD2-mediated signaling is important for the expression of antimicrobial peptides, the cryptidins, which are able to disrupt the membrane function of most bacteria. NOD2-deficient mice revealed an abnormal development and function of Peyer's patches resulting in increased translocation of microbes across Peyer's patches, and increased concentrations of cytokines such as TNF-α, IFN-γ, IL-12, and IL-14 [75]. As a consequence, NOD2-deficient mice are highly susceptible to *L. monocytogenes* infection via the oral route, but normally susceptible to intravenous challange [76]. This observation demonstrates the importance of NOD2 signaling to prevent infection of the intestinal mucosa by inducing antimicrobial defensins that play an important role in *in vivo* defence against pathogens [77]. The intestinal P glycoprotein also seems to be important for host protection against *L. monocytogenes* GIT infection, most likely by inhibiting absorption of the pathogen into enterocytes [78].

Several NLRs, together with caspase-1, form proinflammatory multiprotein complexes termed the "inflammasomes". After activation, the molecules assemble and lead to multimerization of the adaptor molecule apoptosis-associated speck-like protein containing a C-terminal caspase recruitment domain (ASC). The signaling cascade results in the processing and secretion of mature IL-1β and IL-18, which are mediators for the activation of innate and adaptive immune responses [6]. NLR family members known to form inflammasomes comprise NALP1, NALP2, NALP3, and NALP4.

NALP3 forms an inflammasome complex with ASC, cardinal, and procaspase-1 [79]. The NALP3 pathway is known to be activated by *L. monocytogenes* infection, although the specific ligands that activate NALP3 remain unknown. On the other hand, *L. monocytogenes* DNA in the host cell cytoplasm is known to act as a ligand for a hitherto unknown PRR that mediates induction of IFN-β through activation of interferon regulatory factor 3 (IRF3) [80]. Interestingly, cytosolic *L. monocytogenes* actively increase NF-κB activity by expression of the virulence factors listeriolysin O (LLO) and internalin B (InlB). This strategy leads to an increased proinflammatory response and recruitment of immune cells to the site of infection. An interesting suggestion is that the increased response actually promotes spread of intracellular pathogens, by recruiting more host cells, which can serve as potential vehicles for the pathogen [81]. Furthermore, *L. monocytogenes* induces expression of type I interferon (IFN-$\alpha\beta$) that are known to be essential for the immune system to clear viral pathogens. However, in contrast to the protective effect to virus infections, in the case of *L. monocytogenes* the IFN-$\alpha\beta$ induction results in an increase in host susceptibility to the pathogen [82]. The observed benefit might be due either to direct enhancement of bacterial growth, or more likely, to down-modulation of a part of the immune response that plays an important role in controlling bacterial growth. The latter would be supported by the observation that induction of IFN-$\alpha\beta$ enables *L. monocytogenes* to suppress macrophage activation by IFN-γ [82]. It was also shown that early during *L. monocytogenes* infection type I interferons induce T cell apoptosis, resulting in greater IL-10 secretion by

phagocytic cells which in turn leads to dampening the innate immune response [83].

Autophagy contributes to innate immune defense against various intracellular bacterial pathogens [84]. When autophagy was discovered, it was thought to serve as a pathway for recycling of intracellular organelles and cytoplasmic constituents as part of cellular homeostasis [6]. For this purpose, a double membrane vacuole is formed around the target object in the cytoplasma. This vacuole is then directed to the lysosome pathway, resulting in degradation of the vacuolar content. Similarly, intracellular bacteria can be targeted and destroyed, as it is well known for intracellular pathogens such as *Salmonella*, Group A *Streptococcus*, or *Mycobacterium tuberculosis* [85–87]. However, other bacteria are able to evade or even exploit autophagy during infection [88]. Pathogens adapted to persistence in the cytoplasm, such as *Listeria monocytogenes* and *Shigella flexneri*, have evolved mechanisms to avoid autophagy [89, 90].

For *Salmonella* and *Toxoplasma,* it was shown that the damaged vacuole itself triggers autophagy of the pathogens [85, 91]. However, for *Listeria monocytogenes* infection in *Drosophila melanogaster,* it was demonstrated that a peptidoglycan-recognition protein, acting as an intracellular PRR, plays an essential role for autophagy protection from the pathogen [92]. Results from previous studies indicated that *L. monocytogenes* deploy several mechanisms to evade from autophagy [90, 93, 94], and it was speculated whether this effect is mainly due to actin-based motility, or due to masking of the bacterial cell [93]. An earlier study suggested that active bacterial protein synthesis is required to escape from autophagy in macrophages [90], and results from a more recent study led to the assumption that bacterial phospholipases (PI-PLC and PC-PLC) may play another role [93]. How the bacterial Plc enzymes are involved is not entirely clear, but they are thought to either mediate escape from the autophagosome or prevent their formation [95]. However, in a recent study, Yoshikawa et al. [96] clearly showed that during primary infection *L. monocytogenes* avoids autophagy by disguising itself as a host organelle. Due to the ability of ActA to recruit host cell cytoskeleton proteins such as the Arp2/3 complex and VASP, the pathogens avoid ubiquitination and p62 accumulation. Moreover, it was demonstrated that lack of actin-based motility alone is not sufficient to escape from autophagy.

Although *L. monocytogenes* was previously thought to primarily reside in the cytoplasm, a recent study described the presence of variant *L. monocytogenes* forms that replicate in macrophages, inside large, LAMP1-positive vacuoles designated as spacious *Listeria*-containing phagosomes (SLAPs) [97]. The formation of SLAPs seems to be promoted by inefficient LLO activity that is not sufficient for bacterial escape from phagosomes, which triggers an autophagic response to the damaged phagosome. On the other hand, the LLO leads to disruption of the proton gradient, thereby preventing fusion with lysosomes. Within the SLAPs the bacteria are able to replicate, but the replication rate is low compared to the cytoplasm [97, 98]. The reason for the impaired LLO expression of bacteria in SLAPs is unknown. However, it is known that LLO activity is low in LAMP-1 or alkaline compartments, which are the characteristics of SLAPs [98, 99]. Moreover, function of LLO can be impaired by innate immune responses, such as reactive oxygen and nitrogen intermediates, and cathepsin D [100, 101]. Because the maturation of phagosomes is quite heterogeneous, the bacteria stuck in SLAPs may be effected by other host innate factors, compared to the bacteria that managed to escape from the phagosome [95].

6. Adaptive Immune Responses to *Listeria monocytogenes*

Adaptive immune responses follow the initial innate immune responses and dendritic cells (DCs) represent an important link between the two immunological pathways [102]. DCs respond to different pathogens and initiate the appropriate type of T cell response needed to control the infection. In response to *L. monocytogenes* infection, DCs are critical in priming the T cell response, since mice depleted of DCs are unable to generate a CD8 T cell response [103]. Due to the primarily intracellular localization of *L. monocytogenes*, CD4 and CD8 T cells mediate most of the adaptive immune response, and are crucial for long-term immunity after initial *L. monocytogenes* infection. Other cell subsets may contribute by influencing the CD4 and CD8 T cell responses. Whereas innate immune cells are important for initial control of *L. monocytogenes* infection, T cells are required for final clearance of the pathogen. Almost any cell type that harbors *L. monocytogenes* in the cytoplasm can process the proteins secreted from the pathogen, by degradation and subsequent loading on MHC class I molecules, in order to present them on the cell surface to CD8 T cells. Only professional antigen-presenting cells (APCs) can present antigens derived from lysosomal degradation via the MHC class II pathway to CD4 T cells [6]. The CD8 T cells mediate the anti-*Listeria* immunity by two synergistic mechanisms: first, by secretion of IFN-γ to activate macrophages; secondly, by lysis of infected cells via perforin and granzymes, leading to the exposure of intracellular bacteria to the activated macrophages [104]. IFN-γ is known to be essential for host resistance to intracellular pathogens such as *L. monocytogenes,* as it mediates the activation of resting macrophages that more efficiently restricts the multiplication of intracellular pathogens and promotes long-term protective cellular immunity [105].

The role of CD4 T cells in the course of the control of *L. monocytogenes* infection is much less well understood. *L. monocytogenes* induces a strong T-helper type 1 response and, similar to CD8 T cells, CD4 T cells also secrete IFN-γ. The strong CD8 and CD4 T cell responses results in a stable population of memory T cells specific for *L. monocytogenes* [6].

In the intestine, NKT cells (lymphocytes expressing both NK and T cell markers) play an important role in the control of early infection with *L. monocytogenes* [106]. It was shown that processing and presentation of listerial antigens is mediated by a distinct population of DCs, and strong costimulation is necessary for the development of a local antigen-specific T cell response in the intestinal mucosa.

This strong costimulation seems to be required to activate appropriate antilisterial T cells and to surmount tolerance within the generally immunosuppressive intestinal milieu [107].

In general, adaptive immune responses in the intestine are characterized by high numbers of IgA producing plasma cells, regulatory T cells, and IL-17 producing T cells whose development is closely linked to factors produced by PRRs expressing IECs, DCs, and macrophages. This indicates that PRR mediated recognition of ligands produced by commensal bacteria is involved in positive and negative regulation of both, innate and adaptive immunity in the intestine [7].

7. *Listeria monocytogenes* L-Forms

L-forms are protoplast-like variants of bacteria that lost their ability to maintain a rigid cell wall. They have been first described at the beginning of the last century and were reported for many bacterial species. After their discovery, they have been intensively studied using numerous approaches [108]. However, due to the fastidious nature of L-form bacteria and experimental difficulties using old-fashioned techniques, these studies mainly focused on morphology and physiology of L-form cells and often led to inconclusive data. This was also the case for L-forms of *L. monocytogenes* [109]. It was only recently that L-form research experienced a renaissance, due to new experimental systems and the application of molecular biology and state-of-the-art imaging techniques [110–112]. In a recent study, it was demonstrated that stable L-forms of *L. monocytogenes* are viable bacteria that are not only able to survive, but also able to replicate and multiply using a unique, previously unknown mechanism [110]. Thus, L-forms are unlikely to be just artifacts found under laboratory conditions, but seem to represent a pre-programmed, alternative phenotype of bacterial life. Of particular interest is the observation that *L. monocytogenes* L-forms are able to persist within macrophages, suggesting that they retain at least a part of their pathogenetic traits (Schnell et al., unpublished data). Previous results from tissue culture studies already suggested that L-forms may be able to persist within eukaryotic cells for various time periods [113, 114]. Clinical case reports about the isolation of cell wall-deficient variants in cases of persistent and recurrent bacterial infection also suggested that L-forms may serve as cryptic agents of disease in a variety of human infectious diseases [114–118]. Subsequent reversion to parental forms may lead to a damage of the host cells. In the case of L-forms, when the bacteria have completely shed their cell walls, several proteins that represent important markers for the human immune system are also lost. Therefore, the immune system may no longer be able to discern and recognize these bacteria cells. Lack of the cell wall as an important target for antibiotic treatment represents a further threat, due to the ineffectiveness of cell-wall active drugs such as β-lactams and cephalosporins on L-form cells [110, 119].

8. The Possible Role of *Listeria* in Inflammatory Bowel Disease

Inflammatory bowel disease is a collective term for Crohn's disease and ulcerative colitis, both immune-mediated diseases of the gastrointestinal tract which can develop in genetically susceptible individuals [120]. A potential role of *L. monocytogenes* in the pathogenesis of inflammatory bowel disease (IBD) has been suggested, because interference of the pathogen with NOD2-based signaling [121], and variations of NOD2/CARD15 have been shown to represent a risk factor for Crohn's disease [122]. Especially noteworthy seems the presence of *L. monocytogenes* at the site of colon perforation in a patient with fulminant ulcerative colitis [123]. Another study, however, reported an equal prevalence of *L. monocytogenes* in patients suffering from IBD and non-IBD control patients [124], suggesting a more common occurrence of the pathogen in the gastrointestinal environment. Together with the observation of a low prevalence of *L. monocytogenes* in biopsies from IBD patients the available data do not yet support a role of *L. monocytogenes* in IBD [120]. However, there is still a lot to be done to unravel any potential indirect involvement of *L. monocytogenes* in the pathogenesis of IBD.

Listeria monocytogenes has been employed for decades as a model organism to study host-pathogen interactions and immune responses against intracellular pathogens [125]. The many studies provide significant insight into how *L. monocytogenes* interacts on host mucosal surfaces of the human gastrointestinal tract with the immune system that triggers the antibacterial immune responses. Despite the vast amount of knowledge gathered on the host-pathogen interactions and the bacterial adaptations to mammalian host, it was only recently that *L. monocytogenes* was found to be responsible for induction of local mucosal inflammation in immunocompetent individuals, resulting in febrile gastroenteritis [4]. Hence, the availability of new and improved animal models, such as a humanized mouse model [49], will be an important prerequisite to improve the investigation of the gastrointestinal phase of *L. monocytogenes* infection, in order to further enhance our understanding of the interaction and the interplay of the pathogen with the host intestinal mucosa.

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Review Article

NOD1-Mediated Mucosal Host Defense against *Helicobacter pylori*

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Infection of the stomach with *Helicobacter pylori* is an important risk factor for gastritis, peptic ulcer, and gastric carcinoma. Although it has been well established that persistent colonization by *H. pylori* is associated with adaptive Th1 responses, the innate immune responses leading to these Th1 responses are poorly defined. Recent studies have shown that the activation of nucleotide-binding oligomerization domain 1 (NOD1) in gastric epithelial cells plays an important role in innate immune responses against *H. pylori*. The detection of *H. pylori*-derived ligands by cytosolic NOD1 induces several host defense factors, including antimicrobial peptides, cytokines, and chemokines. In this paper, we review the molecular mechanisms by which NOD1 contributes to mucosal host defense against *H. pylori* infection of the stomach.

1. Introduction

Helicobacter pylori is a microaerophilic, gram-negative bacterium that colonizes human gastric mucosa [1, 2]. Although most individuals with a chronic gastric infection of *H. pylori* are asymptomatic, this bacterium causes peptic ulcer or gastric cancer in a subpopulation of susceptible hosts [1, 2]. The development of gastric disease associated with *H. pylori* infection is determined by the interplay between bacterial virulence factors and host immune responses. The *cag*-pathogenicity island (PAI) is one of the most important virulence factors of *H. pylori*. Infection with strains of *H. pylori* carrying *cag*-PAI is associated with severe gastric disease, including gastric cancer [3].

Chronic infection with *H. pylori* is characterized by a strong T helper type 1 (Th1) response in the gastric mucosa [4, 5]. Th1 cells produce IFN-γ-mediated mucosal host defense against this organism as shown by the fact that IFN-γ-deficient mice fail to eradicate the bacteria from their stomachs upon oral administration of this organism [6]. Although it is well established that the gastric mucosa of patients with *H. pylori* infection is characterized by adaptive Th1 responses, the innate immune responses leading to such Th1 responses are poorly understood. Pattern recognition molecules particularly Toll-like receptors (TLRs) play a crucial role in host defense against mucosal pathogens. TLRs are evolutionarily conserved receptors, which recognize microbial antigens and contribute to host defense by producing proinflammatory cytokines and antimicrobial peptides [7]. The activation of TLRs in antigen-presenting cells (APCs) by TLR ligands associated with *H. pylori* has in fact been shown to be involved in the generation of protective Th1 responses against *H. pylori* [8]. In addition, the neutrophil-activating protein of *H. pylori* induces Th1 responses via TLR2-mediated IL-12 secretion in APCs [9]. Thus, it is clear that the detection of *H. pylori*-associated antigens by APCs expressing TLRs plays a role in the induction of protective anti-*H. pylori* Th1 responses.

Given the fact that most gastric APCs are localized in the submucosal areas and that *H. pylori* adheres to the luminal surface of the epithelium [10], it is likely that innate immune responses by gastric epithelial cells (ECs) directly activated by the organism are involved in the immunopathogenesis of *H. pylori*-associated gastric diseases. TLR signaling may be involved in the development of *H. pylori*-associated gastric disease since patients with gastric adenocarcinoma are more likely to carry the TLR4 polymorphisms [11]. However, on the other hand, TLR signaling may not necessarily play a major role since gastric ECs have been shown to be hyporesponsive to TLR ligands [12]. Another possibility is that epithelial cell signaling occurs through Nucleotide-binding oligomerization domain, Leucine-rich Repeat-containing (NLR) proteins, that is, a rapidly emerging family of innate immune regulatory molecules that function much like TLRs but with different signaling mechanisms [13]. Nucleotide-binding oligomerization domain 1 (NOD1) is of particular relevance in this context since NOD1, a member of the NLR family proteins, recognizes small peptides derived from peptidoglycan (PGN), a component of bacterial cell walls, and is expressed in APCs and gastric ECs [14]. In addition, Viala et al. has shown that eradication of *H. pylori* requires the sensing of PGN by cytosolic NOD1 expressed in gastric ECs [15]. Since this latter discovery, several mechanisms regarding NOD1-mediated mucosal host defense against *H. pylori* have been proposed. In this paper, we focus on these mechanisms with the aim of explaining just how NOD1 signaling contributes to gastric inflammation and host defense against *H. pylori*.

2. Expression of NOD1

NOD1 consists of a C-terminal LRR (Leucine-rich region), a central NOD, and an N-terminal CARD (caspase-activating domain) domain [14]. Whereas TLRs are associated with the plasma membrane or endosomal vesicles, NOD1 is expressed in the cytosol [14]. NOD1 is mainly expressed by cells—APCs and ECs—that are exposed to microorganisms [14]. Importantly, most gastrointestinal cell lines and primary ECs express NOD1 [15, 16].

NOD1 expression is regulated by proinflammatory cytokines. IFN-γ activates the promoter of NOD1 via nuclear translocation of IFN-regulatory factor 1 (IRF1) to up-regulate the expression of NOD1 in intestinal ECs whereas NF-κB activation by TNF does not alter the NOD1 expression in these cells [16, 17].

3. Signaling Pathways of NOD1

It is now established that NOD1 senses a small molecule derived from bacterial cell wall PGN. A minimum motif of NOD1 ligand is γ-D-glutamyl-meso-diaminopimelic acid—called iE-DAP. PGN derived from most gram-positive bacteria lacks iE-DAP. In contrast, PGN derived from most gram-negative bacteria contains iE-DAP. Thus, NOD1 functions as a sensor for gram-negative bacteria. In support of this idea, it has been shown that NOD1 participates in host defense against mucosal infection with gram-negative bacteria such as *Shigella, Escherichia coli, and H. pylori* [18, 19] although no functional mutations have been found to be associated with upper gastrointestinal diseases caused by chronic infection with *H. pylori* [20].

One pathway of NOD1 signaling relates to its ability to activate NF-κB and MAP kinases [14]. Such signaling is initiated by the detection of NOD1 ligands by the LRR domain of NOD1 which is then followed by the recruitment of a downstream effector molecule, RICK [14]. RICK is a CARD-containing serine/threonine kinase that physically binds to NOD1 through a CARD-CARD interaction [14]. RICK then undergoes K63-linked ubiquitination and acquires the ability to recruit and activate TGF-β-activated kinase 1 (TAK1) [21]; the latter, in turn, initiates activation of NF-κB subunits through phosphorylation and K48-linked ubiquitination of IκBα. This sequence of events suggests that the binding of RICK to NOD1 and its K63-linked polyubiquitination is a key step in the NOD1-mediated signaling cascade with respect to responses involving NF-κB and MAP kinases and, as we shall see to other responses as well. This supposition is fully supported by the fact that NOD1 responses are severely curtailed in RICK-deficient cells [14].

The above NOD1 signaling pathway emphasizes the importance of ubiquitination in the signaling cascade. On the one hand, conjugation of K48-linked polyubiquitin chains with the inhibitory protein IκBα leads to proteasomal degradation of IκBα and the nuclear translocation of NF-κB subunits such as p65 and p50. On the other hand, conjugation of K63-linked polyubiquitin chains to RICK, rather than causing RICK degradation leads to the creation of a scaffold that enables recruitment of signaling components, such as TAK1. The ligases that conjugate K63-linked polyubiquitin chains to RICK have recently been identified as the cellular inhibitors of apoptosis proteins (cIAP), cIAP1, and cIAP2 [22]. These proteins bind to NOD1 following the latters' activation by its ligands and have C-terminal RING finger domains with E3 ligase activity which then K63-ubiquitinate the RICK (Figure 1).

As shown in recent studies [19, 23, 24], the stimulation of ECs with NOD1 ligands leads to robust production of proinflammatory chemokines. We confirmed and extended these findings with studies showing that such stimulation induced Th1 chemokines (IFN-γ-induced protein of 10 kDa, IP-10) in gastrointestinal ECs including freshly isolated primary ECs [17] both in the presence and absence of IFN-γ. Initially, we and others attributed such induction to a signaling pathway involving NF-κB, along the lines described above. We noted, however, that the possible involvement of NF-κB was based largely on signaling studies utilizing transfected cells, in which NOD1 and/or an NF-κB reporter gene were overexpressed, rather than on studies employing cells expressing endogenous NOD1 stimulated under physiologic conditions. This introduced the possibility that signaling pathways not involving NF-κB activation play an important role in NOD1 induction of chemokines in EC. In an extensive series of studies to investigate this possibility we found that indeed, stimulation of EC by NOD1 ligand and production of IP-10 was not accompanied by

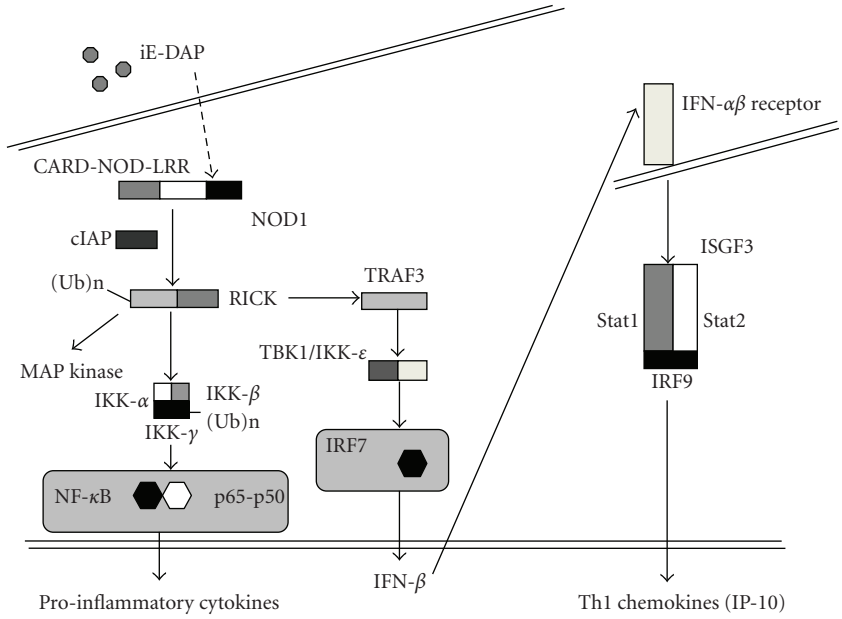

Figure 1: *Signaling pathways of NOD1.* NOD1 activation induces an interaction between RICK and TRAF3 that results in the production of IFN-β through activation of TBK1, IKKε, and IRF7. IFN-β production leads to production of IP-10 through transactivation of ISGF3 (Stat1-Stat2-IRF9 complex). NF-κB activation induced by NOD1 ligand leads to production of proinflammatory cytokines and chemokines such as IL-8.

substantial NF-κB activation, but rather by the induction of type I IFN which leads to IP-10 production by inducing the IP-10 transcription complex, IFN-stimulated gene factor 3 (ISGF3) [17]. This comes about via a unique NOD1 signaling pathway that involves initial interaction of activated RICK with TRAF3, followed by the activation of TANK-binding kinase 1(TBK1) and IKKε and downstream IRF7 to induce the production of IFN-β and, as mentioned ISGF3 (Figure 1). ISGF3, which is a heterotrimer composed of Stat1, Stat2, and IRF9, not only acts as a transcription factor for IP-10, but also for IRF7 which induces further IFN-β production and further rounds of IP-10 production. Overall, these studies showed that, at least with respect to ECs, NOD1 utilizes a signaling pathway more commonly identified with cell signaling by viruses. Whether this signaling pathway also defines NOD1 function in other kinds of cells, such as macrophages, remains to be seen.

4. NOD1 Activation in *Helicobacter pylori* Infection

The majority of patients with *H. pylori*-associated gastritis have a higher NOD1 expression in gastric epithelial cells as compared with controls or *H. pylori*-nonassociated gastritis [20], which suggests the involvement of NOD1 signaling in the development of human gastric inflammation. In addition, animal studies demonstrate that a marked increase of bacterial load in the stomachs of NOD1-deficient mice is observed upon acute infection with *cag*-PAI-positive, but not *cag*-PAI-negative *H. pylori* as compared with NOD1-intact mice [15, 17]. These data are related to the fact that the detection of *H. pylori*-derived PGN by gastric ECs is at least partially dependent on a functional type IV secretion apparatus. Thus, as shown by Viala et al., *H. pylori* expressing functional *cag*-PAI efficiently delivered radio-labeled PGN into the ECs, whereas *H. pylori* strain 251—harboring nonfunctional *cag*-PAI—failed to deliver radio-labeled PGN [15]. In addition, these finding were supported by studies showing that infection with *H. pylori* induced IL-8 production by the gastric epithelial cell line, AGS cells, in an NOD1/*cag*-PAI-dependent manner [25]. It should be noted, however, that Kaparakis et al. have recently provided evidence for the existence of a *cag*-PAI-independent mechanism for NOD1 activation [26]. These authors purified outer membrane vesicles (OMVs) from *cag*-PAI-positive and -negative bacteria. *H. pylori*-derived OMVs containing numerous components of bacterial cell walls including PGN, induced IL-8 production by AGS cells via an NOD1-dependent and *cag*-PAI-independent fashion. OMVs activate the cytosolic NOD1 of ECs through lipid rafts. In addition, NOD1-deficient mice exhibit defective innate and adaptive immune responses to OMVs upon oral challenge with OMVs [26]. Patients infected with strains lacking a functional type IV secretion system still have inflammation and Th1 responses. These data regarding NOD1 activation by OMVs may partially explain the mechanisms by which NOD1-mediated Th1 responses against *H. pylori* are induced in the absence of functional *cag*-PAI. On the basis of this new data, further studies to determine the conditions under which NOD1 activation upon *H. pylori* infection requires functional type IV secretion apparatus are warranted.

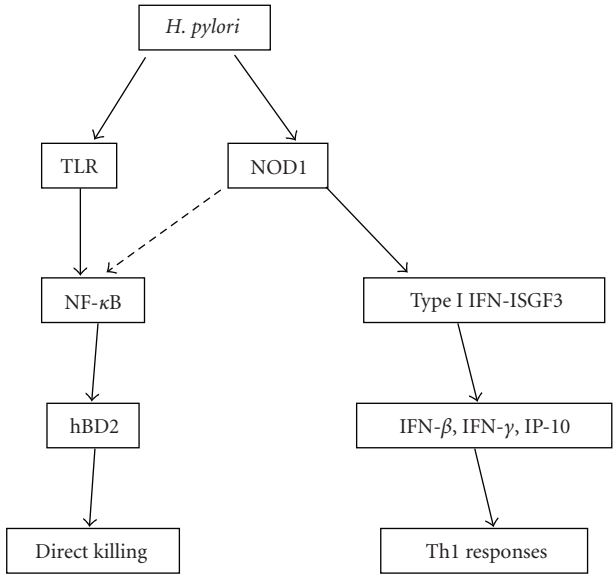

Figure 2: *Molecular mechanisms of NOD1-mediated mucosal host defense against H. pylori.* Infection of gastric epithelial cells with *H. pylori* activates type I IFN signaling, which leads to the generation of protective Th1 responses. *H. pylori* infection induces hBD2 production by epithelial cells mainly via TLR signaling pathways.

5. Molecular Mechanisms of NOD1-Mediated Mucosal Host Defense against *H. pylori* Infection

The generation of Th1 responses is required for mucosal host defense against *H. pylori* infection [4, 6]. Although the mechanism by which recognition of *H. pylori*-derived PGN by cytosolic NOD1 activates the protective response is not completely understood, two main models have been proposed (Figure 2).

The first model for the role of NOD1 activation in *H. pylori* infection is based on the antimicrobial activity of defensins produced by ECs. Grubman et al. showed that *H. pylori* infection induces the production of human β defensin 2 (hBD2) by AGS cells in an NOD1/*cag*-PAI-dependent fashion [25]. Transfection of siRNA specific to hBD2 impairs the killing of *H. pylori*, as shown by the increased number of bacteria in culture supernatants from AGS cells infected with *cag*-PAI-positive *H. pylori*. These authors imply that NOD1 induction of hBD2 upon infection with *cag*-PAI-positive *H. pylori* may be mediated by NF-κB activation, but this is unclear since NF-κB activation is assessed by reporter gene assays that may not reflect endogenous NF-κB regulation [25]. Consistent with these results, the expression of murine β-defensin 4 (an orthologue of hBD2) is markedly reduced in the gastric mucosa of NOD1-deficient mice as compared with NOD1-intact mice [27]. These data regarding hBD2 induction by NOD1 suggest that NOD1 mediates host defense by the direct killing of *H. pylori* through induction of hBD2. However, the contribution of NOD1-induced defensins to the generation of Th1 responses remains unknown.

The second model for the role of NOD1 activation in *H. pylori* infection is based on the production of type I IFN and activation of the ISGF3 signaling mediated by NOD1. As we described in the previous section regarding NOD1 signaling pathways, the stimulation of gastrointestinal ECs including primary cells with NOD1 ligands leads to a robust production of IFN-β and the subsequent induction of ISGF3 [17]. We therefore addressed the role of this new signaling pathway in *cag*-PAI-positive *H. pylori* infection. In *in vitro* studies we found that *H. pylori* infection of AGS cells led to a massive increase of IFN-β and IP-10 production, which was accompanied by the activation of both Stat1 and Stat2, suggesting that cag$^+$ *H. pylori* organisms do indeed activate epithelial cells via the IFN-β-ISGF3 pathway. Although *H. pylori* infection of the AGS cells results in NF-κB activation, NF-κB activation induced by *H. pylori* infection is independent of NOD1. The infection of AGS cells in the presence of siRNA specific for NOD1 resulted in the impaired nuclear translocation of Stat1 and production of IFN-β and IP-10, but unchanged nuclear translocation of NF-κB subunit, p65 [17]. These results are consistent with previous studies of Hirata et al. [28], who showed that *H. pylori* organisms can activate NF-κB in epithelial cell lines through MyD88-dependent mechanisms, but not NOD1-dependent mechanisms, and Viala et al. who reported that primary gastric epithelial cells infected with *H. pylori* produce chemokines in the absence of the nuclear translocation of NF-κB p65 [15].

In further in vivo studies of NOD1 activation during *H. pylori* infection we found a marked increase of bacterial load in the stomachs of NOD1-deficient mice as compared with NOD1-intact mice. Furthermore, this was associated with reduced production of type I IFN-ISGF3-associated cytokines and chemokines such as IFN-β, IP-10, and IFN-γ rather than NF-κB-associated cytokines such as TNF. Thus, the activation of type I IFN and ISGF3 signaling via NOD1 is responsible for the generation of protective Th1 responses against *H. pylori* [17]. This idea was confirmed by the fact that gene silencing of Stat1, a component of ISGF3, increases the bacterial burden in the stomach of NOD1-intact mice due to impaired Th1 and type I IFN responses. Therefore, it is likely that NOD1-mediated type I IFN production and ISGF3 activation provide protective Th1 responses upon infection with *cag*-PAI-positive *H. pylori*. In support of this model, IP-10 expression is observed in the gastric mucosa of patients with chronic *H. pylori* infection [5].

Recent studies addressing the role of NOD1 in mucosal host defense against *H. pylori* have been focusing on acute innate responses as assessed by cell-culture or animal infection models. Thus, it is unclear how NOD1 activation is involved in the development of various gastric diseases associated with chronic *H. pylori* infection. In this regard, IL-17 may play an important role in the chronic inflammatory responses to *H. pylori* infection as shown by the overproduction of this cytokine in *H. pylori*-infected human gastric mucosa [29]. In addition, vaccination of mice against *H. pylori* results in efficient eradication due to enhanced IL-17 production in the gastric mucosa [30, 31]. It is possible that NOD1 activation in response to *H. pylori* infection is

involved in the generation of gastric Th17 responses since NOD1 triggering is required to instruct the onset of both Th1 and Th17 responses [32].

6. Conclusions

Infection with *cag*-PAI-positive *H. pylori* leads to the activation of NOD1 in gastric ECs and to the induction of several host defense factors including hBD2, IFN-β, and IP-10, all of which regulate bacterial growth. Recent studies have shown that NOD1 activation is mediated in ECs largely, if not entirely, by NOD1 induction of IFN-β and thus a signaling pathway ordinarily activated by viral infection. This has important implications for our views on how the mucosal system utilizes innate immune mechanisms to deal with chronic bacterial infections. Interestingly, proinflammatory cytokine responses induced by TLR ligands are markedly enhanced in the presence of NOD1 ligands. Therefore, it is possible that the synergistic activation of NOD1 and TLRs in gastric APCs also plays a role in mucosal host defense against this organism. Thus, future studies addressing whether or not NOD1 expression in APCs contributes to host defense against *H. pylori* will be of great interest.

Abbreviations

APCs: Antigen-presenting cells
CARD: Caspase-activating domain
cIAPs: cellular inhibitor of apoptosis proteins
ECs: Epithelial cells
hBD2: human β defensin 2
IP-10: IFN-γ-induced protein of 10 kDa
IRF: IFN-regulatory factor
ISGF3: IFN-stimulated gene factor 3
LRR: Leucine-rich repeats
NLR: Nod-like receptor
NOD1: Nucleotide-binding oligomerization domain-1
OMVs: Outer membrane vesicles
PAI: Pathogenicity island
PGN: Peptidoglycan
TAK1: TGF-β-activated kinase 1
Th1: T helper type 1
TLRs: Toll-like receptors.

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Review Article

Microbial Sensing by the Intestinal Epithelium in the Pathogenesis of Inflammatory Bowel Disease

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Recent years have raised evidence that the intestinal microbiota plays a crucial role in the pathogenesis of chronic inflammatory bowels diseases. This evidence comes from several observations. First, animals raised under germ-free conditions do not develop intestinal inflammation in several different model systems. Second, antibiotics are able to modulate the course of experimental colitis. Third, genetic polymorphisms in a variety of genes of the innate immune system have been associated with chronic intestinal inflammatory diseases. Dysfunction of these molecules results in an inappropriate response to bacterial and antigenic stimulation of the innate immune system in the gastrointestinal tract. Variants of pattern recognition receptors such as NOD2 or TLRs by which commensal and pathogenic bacteria can be detected have been shown to be involved in the pathogenesis of IBD. But not only pathways of microbial detection but also intracellular ways of bacterial processing such as autophagosome function are associated with the risk to develop Crohn's disease. Thus, the "environment concept" and the "genetic concept" of inflammatory bowel disease pathophysiology are converging via the intestinal microbiota and the recognition mechanisms for an invasion of members of the microbiota into the mucosa.

1. Chronic Inflammatory Bowel Diseases

Two major forms of chronic mucosal inflammation can be discriminated. In Crohn's disease (CD) the whole gastrointestinal tract may be involved. However, the most frequent site of inflammation is the terminal ileum, the last part of the small bowel and the adjacent caecum. In CD the inflammation affects all layers of the gut wall and frequently an alteration of the adipose tissue covering the colon or small bowel at the serosal side is found. Normal and involved areas of the mucosa can be found along the gut (so called skip lesions). This is in contrast to ulcerative colitis (UC). In UC there is a continuous inflammation only of the mucosa that always starts at the rectum. The extent of the disease may vary and sometimes only the rectum or the sigma is involved. In about 1/3 of the patients the whole colon will be inflamed (pancolitis). However, in contrast to CD the small intestine never is affected (the only exception is a so-called back-wash ileitis in severe cases of UC in which some inflammation extends to the last centimeters of the ileum). So from morphological aspects both diseases can clearly be discriminated (which, however, is not always the case in clinical practice). In addition, it has been demonstrated that there are clear differences with respect to pathophysiological mechanisms. In CD a strong genetic susceptibility can be found. A recent study again has shown the impact of genetic factors on the pathogenesis of CD by demonstrating a concordance in 63.6% among monozygotic twins, however, only 3.6% among dizygotic twins [1]. This concordance of monozygotic twins is much lower (around 6%) in UC indicating that a genetic susceptibility plays a minor role in this disease (Figure 1).

Among the environmental factors, diet and host microbiota seem to play the most important roles for the pathogenesis of the diseases. The diet affects the composition of the intestinal flora, which then may influence the disease course [2]. Some studies showed that a high uptake of carbohydrates might be associated with an increased risk for CD as well as UC [3–5]. However, results have not been unequivocally confirmed. Of notice, very high sugar uptake leads to insulin resistance finally resulting in a chronic inflammatory state [6, 7]. Shoda et al. found that the dietary changes in a Japanese population between 1966 and 1985 were associated with a strongly increasing risk for CD. In this population, elevated uptake of total fat, animal fat, and n-6 polyunsaturated fatty acids was paralleled by decreased intake of n-3 polyunsaturated fatty acids [8], which have been shown to exert anti-inflammatory effects [9]. This finding could be confirmed for UC by a prospective study in a European population [10]. Dietary components are also able to affect the intestinal microbiota, which shows a different composition in IBD patients as compared to healthy people [11].

In IBD patients not only the quantity of commensal bacteria in the intestine is reduced (about 10-fold lower compared to control patients), but also the quality and diversity of the commensal composition are altered [11–13]. Especially the number of the major classes of commensals, *Firmicutes* and *Bacteroidetes*, is reduced [11, 12]. On the other hand, the number of mucosal adherent bacteria, such as invasive *E. coli*, or Proteobacteria, such as *Enterobacteriaceae*, is increased, resulting in a so-called state of "dysbiosis" [11, 14, 15]. These pathogenic bacteria may then enhance an inflammatory response of the host intestine and hereby aggravate the intestinal inflammation.

2. The Role of the Microbiota in Animal Models of Chronic Intestinal Inflammation

During the last few years significant advance has been achieved in the understanding of the pathogenesis of inflammatory bowel diseases (IBDs). It became evident that bacteria play an essential role for the initial trigger of the chronic inflammation in Crohn's disease (CD) and ulcerative colitis (UC). Sartor et al. demonstrated that certain bacterial strains such as bacteroides can induce or aggravate colonic inflammation in models such as HLA-B27 rats or IL-10 knock-out mice [16, 17]. Further it could be demonstrated in a number of different mouse models of colitis that these animals were prevented from colitis by raising them under germ-free conditions [18, 19]. In several models, monoassociation with just one bacteria was sufficient to be again able to induce colitis [20]. The cecal bacterial load was clearly correlated with the severity of disease in those animal models [21]. However, in different mouse strains different bacteria proved to be most effective in inducing colitis making it unlikely that one specific microbial pathogen would be the inducing factor of CD or UC. The concept was developed that in IBD the physiologic intestinal flora is no longer tolerated [22, 23].

3. Susceptibility Genes for IBD and the Role of the Microbiota

The insights obtained during genome wide association studies (GWASs) elucidating involved risk genes for IBD have shed new light on the interaction of bacteria with the mucosal immune system and the pathways by which the intestinal microbiota may contribute to chronic mucosal inflammation.

The intestinal mucosa has long been seen as an organ that has mainly the function of nutrient digestion and resorption. However, the mucosa is exposed to a myriad of microbial antigens, uncountable potential pathogens, and even more nonpathogenic bacterial molecules. Due to its enormous surface area the barrier function of the intestinal mucosa may be as important as its function in nutrient absorption. It is obvious that there need to be effective defense mechanisms when the barrier becomes locally leaky. Controlled local inflammation after bacterial recognition may be regarded as crucial component of the mucosal defense system [24]. Mechanisms initiating or limiting inflammation need to be tightly regulated as they themselves might alter the mechanical barrier function [24]. On an intracellular level, pro- and anti-inflammatory signal transducers, regulatory proteins and immune effector genes represent a well-organized orchestra of agonists and antagonists. The interplay between each of the participating components needs to be exactly regulated. Functional deficiency of only one of the respective molecules may have tremendous consequences for the entire organism. During the last years evidence was found that specific single nucleotide polymorphisms (SNPs) within several genes, which may cause dysfunction of their respective protein products, are associated with the risk to develop IBD.

Since 2001, GWAS revealed more than 30 genes that are associated with IBD [25]. Among the identified targets are genes that play an important role for immunological cell-cell interaction and signalling, such as tumour necrosis factor (TNF) [26], TNF-receptor 1 (TNFR1) [27], the interleukin-23 receptor (IL23R) [28], or interleukin-12p40 (IL12B) [29, 30]. Perhaps even more important, there are genes that are involved in the immune response to bacteria, such as the nucleotide oligomerization domain 2 (NOD2) [30, 31], the toll-like receptor 4 (TLR4) [32, 33], as well as the autophagy genes autophagy-related like 1 (ATG16L1) and immunity-related GTPase family M (IRGM) [28, 34, 35]. In addition, regulatory genes, such as the protein tyrosine phosphatase N2 (PTPN2) [29, 36] and the peroxisome proliferation-activated receptor gamma (PPARγ) [37] as well as genes that are involved in cell homeostasis, such as the membrane transporters multidrug resistance gene 1 (MDR1) [38, 39] and the organic cation transporter 1+2 (OCTN1+2) [40, 41] have been found to be associated with the risk of chronic mucosal inflammation.

The functional consequences of the respective SNPs have only been investigated to a limited extent. It is likely of course that these genetic variants alter functional properties of a specific protein resulting in a disturbed function and, finally, in an inadequate immune reaction.

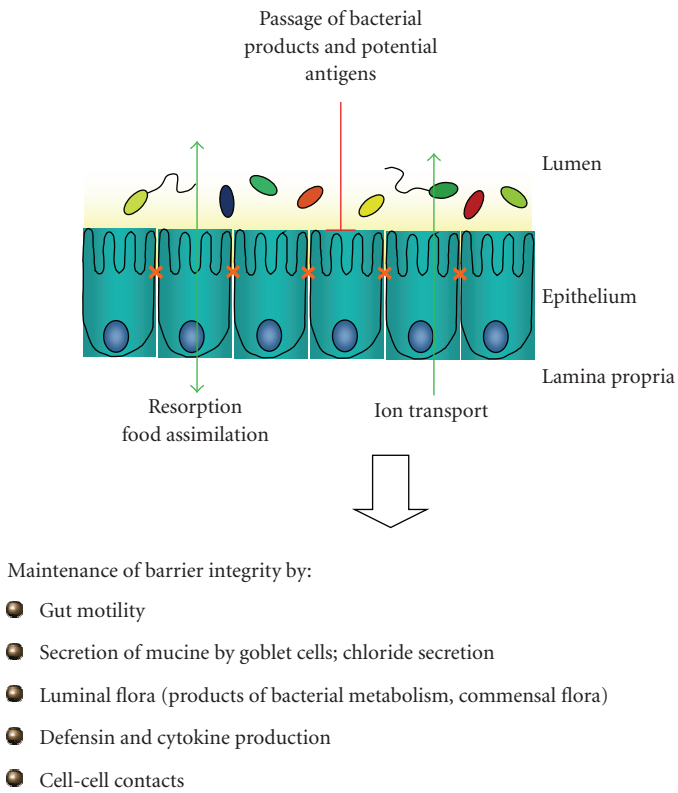

Figure 1: The intestinal epithelial barrier. The human gastrointestinal tract contains myriads of microorganisms. From oral to anal the number of bacteria is increasing tremendously. Especially the colon and the colonic epithelial cells are challenged by a heavily and continuous exposure to bacteria and antigens. The healthy epithelium represents a highly selective barrier that separates the body, especially the cells of the intestinal immune system, from the gut content. Therefore, it inhibits the passage of bacterial products and potential antigens and regulates the nutrient uptake as well as the resorption and secretion of ions and water. The integrity of the intestinal epithelium is maintained by a tightly controlled orchestra of regulatory mechanisms, such as the secretion of mucus, the production of defensins and cytokines, or intercellular connections.

4. Pattern Recognition Receptors—to TOLLerate or NOD

As discussed, bacteria and bacterial components play a crucial role for the onset and perpetuation of chronic intestinal inflammation. Thus, the appropriate response to bacterial stimuli plays a key role for the maintenance of intestinal homeostasis. Two groups of pattern recognition receptors (PRRs), the Toll-like receptors (TLRs), and the nucleotide oligomerization domains (NODs) have been demonstrated to be essentially involved in bacterial recognition, induction of antimicrobial factors, activation and modulation of innate as well as adaptive immune responses, and in the maintenance of intestinal epithelial barrier function.

Though both of the PRR subgroups are ubiquitously expressed within the gastrointestinal tract, TLRs are primarily localised in intestinal epithelial cells (IECs) [42] and intestinal lamina propria macrophages [43, 44]. Most TLRs, such as the lipopolysaccharide (LPS)-receptor TLR4 [32, 43, 45, 46], detect their ligands at the cell surface. In the healthy intestine, TLR4 serves to keep the tolerance of the intestinal immune system to commensal bacteria [42], to maintain mucosal homeostasis [47], and to prevent allergic reaction to food antigens [48]. In active IBD, TLR4 expression is significantly increased in IEC as well as in lamina propria mononuclear cells (LPMNCs) [43, 45]. Several mutations within the TLR4 gene locus have been associated with IBD [32, 33] and an increased susceptibility to IBD has been identified for coexistent mutations within the TLR4 and the NOD2 gene [49]. Activation of TLR4 results in the activation of various signal transducers, such as nuclear factor kappa B (NF-κB), signal transducer and activator of transcription 1 (STAT1), mitogen-activated protein kinases (MAPKs) or PPARγ, with pro- as well as anti-inflammatory effects. As a functional consequence, TLR4 stimulates the expression of cytokines, such as TNF, IL1β, and IL6 via NF-κB or STAT1 [50]. In contrast, increased TLR4-induced PPARγ activity results in subsequent uncoupling of NF-κB target genes as a part of a negative feedback mechanism and therefore limits inflammation [51, 52]. Studies in mice support the hypothesis that TLR4 mutations elevate the receptor function and promote intestinal inflammation via excessively activated cytokine-secretion [53, 54], possibly due to an increased activity of the receptor in response to physiological LPS concentrations. Additionally, mutations within the TLR4 gene locus can also lead to a functional loss of TLR4 that worsens DSS-induced colitis in mice by disturbing the intestinal homeostasis and barrier function [47, 53]. Thus,

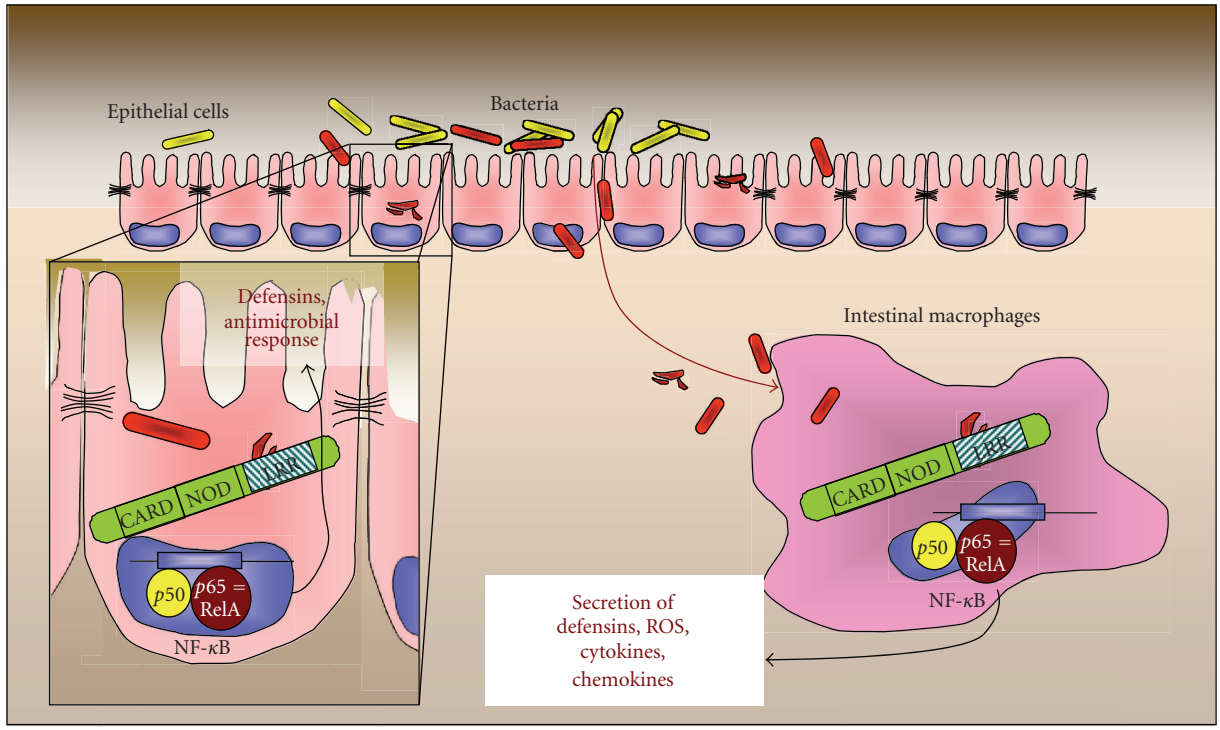

Figure 2: NOD2 and intestinal immune response. The NOD2 contains an effector-binding domain (CARD), a self-oligomerization domain (NOD), and a ligand recognition domain (LRR). The three CD-associated SNPs are located within or near the LRR domain. NOD2 is primarily localised in intestinal epithelial cells and macrophages. Upon binding to its ligand, bacterial MDP, NOD activates the transcription factor NF-κB, what mainly results in the expression of the antimicrobial defensins and various cytokines that trigger the antimicrobial response.

dysfunction of TLR4 in both directions aggravates intestinal inflammation.

A recent study showed that TLR4 specifically activates the transcription factor X-box-binding protein-1 (XBP1), which is part of the unfolded protein response (UPR) cascade that is initiated in response to endoplasmatic reticulum (ER) stress of the cell [55]. The UPR consists of three signalling pathways, namely inositol-requiring enzyme 1 α and β (IRE1α and β) whose activation leads to increased XBP-1 function, protein kinase-like ER kinase (PERK), and activating transcription factor 6 (ATF6). The UPR then is responsible for folding, processing, export, and degradation of proteins during ER stress. An SNP within the XBP-1 gene has also been associated with IBD and loss of the protein is followed by Paneth-cell deficiency and increased levels of TNFα and flagellin in mice [56]. Further, XBP-1 is required for an appropriate response of TLR-4 to its ligands [55]. These observations indicate that ER stress may contribute to the pathogenesis of IBD, that is, by genetically caused XBP-1 dysfunction. On the other hand, ER stress seems also to be a common consequence of chronic inflammatory conditions in the intestine. This latter hypothesis is supported by observations showing that the ER stress response is induced in IL-10 deficient mice and in animals featuring an aberrant mucin assembly [57, 58].

5. NOD2—from Microbiota to Defence

NOD2 represents probably the best investigated and most well-established CD susceptibility gene [30, 31]. NOD2 consists of a C-terminal leucine-rich repeat domain (LRR), which is responsible for the antigen recognition, an intermediate nucleotide-binding domain (NBD) for oligomerization and signal transduction, and two protein-interaction domains, the caspase-activating and recruitment domains (CARDs). NOD2 is strongly expressed in colonic epithelial cells and Paneth cells in the small intestine as well as in intestinal macrophages in the small and large intestine. So far, only one ligand has been identified, namely muramyldipeptide (MDP) [59, 60], a wall component of gram-negative as well as gram-positive bacteria that is transported by the brush border transporter, human peptide transporter 1 (hPepT1), across the apical cell membrane [61]. NOD2 recognizes its ligand in the cytosol and, subsequently, directly interacts with its target molecules causing an activation of the innate immune system [62]. Activation of NOD2 in the uninflamed intestine results mainly in the induction of three different downstream effects. First, the activation of the transcription factor NF-κB, followed by an increased expression of proinflammatory cytokines, such as TNF or IL1β. Secondly, the induction of caspase-mediated apoptosis

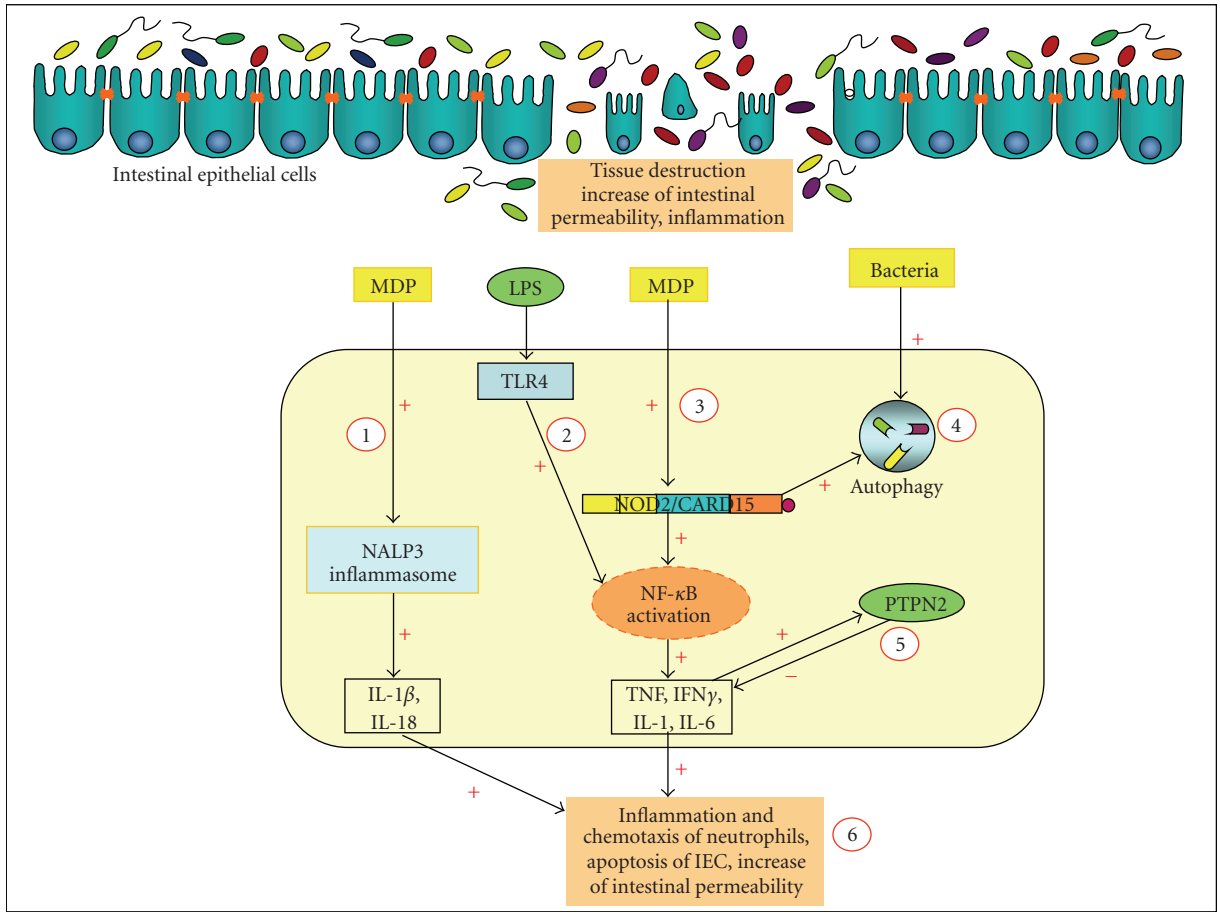

Figure 3: The innate immune system during intestinal inflammation. A defect in the intestinal epithelium, possibly genetically driven, causing tissue destruction, increased epithelial permeability and inflammation permits bacteria and their antigens, such as LPS and MDP, to penetrate through the epithelial monolayer. (1) The bacterial wall component, peptidoglycan, is cut by intracellular endosomes to MDP, that can activate the NALP3 inflammasome. As a consequence, pro-IL1β and pro-IL18 are processed to active molecules, what triggers proinflammatory conditions in the epithelium. (2) LPS binding to its receptor, TLR4, results in the activation of NF-κB and, subsequently, in increased expression of cytokines, such as TNF, IFNγ, or IL6. (3) MDP activates NOD2 directly, causing increased NF-κB activity. In addition to elevated cytokine levels, NOD2 also induces the expression of antimicrobial peptides, such as defensins. (4) Bacteria, such as *E. coli* or *Listeria monocytogenes*, can activate the autophagosome that plays a key role for inactivating invasive bacteria and other pathogenic components. The autophagy machinery is also regulated by NOD2 activity. (5) Cytokines, such as IFNγ, have been shown to increase the activity of PTPN2 that, in turn, downregulates proinflammatory signalling. Dysfunction of PTPN2 results in an impaired epithelial barrier function and elevated secretion of proinflammatory cytokines. (6) Malfunction of the innate immune response mechanisms in the gastrointestinal tract, possibly genetically triggered, causes tissue destruction, increased apoptosis of intestinal epithelial cells, elevated epithelial permeability, and, finally, establishes a chronic inflammatory state in the intestine.

[46], and thirdly, an increase in the expression level of antimicrobial peptides, such as the human defensins [63, 64].

Interestingly, NOD2 expression is increased in intestinal biopsies from CD patients. This may be caused by the proinflammatory cytokines TNF and interferon gamma (IFNγ) that are able to induce NOD2 expression in IEC [65]. This indicates that the innate immune system can increase its alertness for bacterial translocation or invasion. Mutations within the NOD2 gene have only been associated with CD, but not UC, and are present in about 40% of CD patients. Especially three specific mutations have been linked to an elevated susceptibility to CD [30, 31]. In particular, SNP8, SNP12 (both representing missense mutations), and SNP13 (a frame-shift mutation) are independently correlated with an early onset and an ileal localisation of the disease [66, 67]. The CD-associated mutations are located within the LRR domain of NOD2 and surprisingly cause a decreased activation of NF-κB in response to MDP in vitro [59, 60].

These observations suggest that mutant NOD2 is not able to activate NF-κB adequately, what might result in a pathological and insufficient immune response of the intestinal epithelium to microbial contact and stimulation. Interestingly, and contrary, intestinal lamina propria macrophages from CD patients feature a highly induced expression of NF-κB-dependent, proinflammatory mediators, such as IFNγ, TNF, and IL1β [46]. These observations

are corroborated by studies in MDP-stimulated macrophages from NOD2 mutant mice. The animals feature increased and constitutive NF-κB activation, increased IL1β secretion, and elevated apoptosis rates [68]. Additionally, in biopsies from CD patients, far more intracellular and epithelial-adherent bacteria are detectable as compared to biopsies derived from healthy controls [69]. These findings provide a possible mechanism how a defective NOD2-variant could cause a dysregulated immune response in the intestinal epithelium and therefore essentially contribute to the onset of CD; Stimulation by MDP causes overwhelming activation of a defective NOD2 variant in intestinal macrophages. Subsequent overactivation of NF-κB results in increased secretion [46] of proinflammatory mediators, such as TNF and IFNγ that lead to increased expression of NOD2 in IEC. However, due to the mutation, the epithelial isoform of NOD2 is not able to detect and respond to the bacterial stimuli adequately, what results in an inappropriate level of cytokine secretion, uncontrolled inflammatory conditions, a reduced amount of secreted antimicrobial peptides, such as human defensins, and, finally, in an insufficient response to intestinal bacteria (Figure 2).

6. The Microbiota and Graft versus Host Disease

Additionally, recent data also indicate a pivotal role of the IBD-associated NOD2 SNPs in the pathogenesis of graft versus host disease (GvHD), one of the most deleterious complications after allogenic, haematopoietic stem cell transplantation (HSCT). Holler et al. demonstrated an increased risk for the development of GvHD and an elevated treatment-related mortality when the donor or the host was carrying the IBD-associated mutations within the NOD 2 gene [70, 71]. A further study, analyzing the effects of each of the NOD2 SNPs separately, indicated that the clinical manifestation of GvHD might be critically dependent on the presence of SNP13 in the donor [72]. In contrast, it appeared in a different investigation that the risk for the onset of GvHD is reduced when only the donor is carrying the NOD2 variants [73]. A mechanistic explanation, how NOD2 mutations could contribute to increased risk for the development of GvHD and to elevated treatment-related mortality in HSCT patients, could be presented by the finding that NOD2 plays an important role for the regulation of host antigen-presenting cells (APCs). NOD2 dysfunction in host APC causes an increased proliferation and activation of donor T-cells finally resulting in the onset of GvHD, bacteraemia, and, at least in a mouse model, increased intestinal inflammation [74, 75].

7. Human Defensins—The Innate Antimicrobiant

As outlined above, the number of epithelial surface bacteria is increased in CD. These observations lead to the hypothesis that the antimicrobial defence mechanisms in the intestine of CD patients could be impaired. The small intestine, especially the ileum, also represents the home of the Paneth cells with their main innate antimicrobial effector molecules, the human defensins (HDs) 5 and 6. Therefore, it seems plausible that a diminished expression or function of the HD contributes to an impaired innate host defence to bacteria and to the onset of disease. So far, ten HDs have been identified that are separated into two groups, six α-defensins and four β-defensins, being the α-defensins HD5 and HD6 the most important in the intestinal mucosa [76]. On a functional level, the defensins exert bactericidal activity, since they are able to form micropores in the bacterial wall resulting in collapse and death of the bacterium [77]. In the small intestine, HD5 and HD6 are mainly expressed in the Paneth cells at the base of the crypts of Lieberkühn, whereas the α-defensins 1-4 are produced by lamina propria neutrophils [78, 79]. In the colon, the human β-defensins (HBD-1-4) are produced and secreted by IEC and lamina propria plasma cells [63]. The constitutively expressed HD5 and HD6 are believed to contribute essentially to the maintenance of intestinal epithelial barrier function by protecting the intestinal stem cells that are located in the vicinity of the Paneth cells [80]. Wehkamp et al. demonstrated a decreased expression of HD5 and HD6 in patients with ileal CD compared to control patients [81]. They further elucidated that CD patients featuring NOD2 mutations showed even lower levels of HD5 mRNA compared to CD patients with wild-type NOD2. These findings indicate that NOD2 mutants are closely related to altered (decreased) levels of the human defensins and subsequent impaired antimicrobial activity in the small intestine, since NOD2 is also highly expressed in Paneth cells, and NOD2 mutations are also associated with ileal disease. However, the defensin promoter region lacks a binding site for the main transcription factor that is activated by NOD2, NF-κB [82].

8. The Inflammasome—A Sensor for Invasive Bacteria?

One of the biggest advantages of the innate immune system is its ability to respond rapidly and persistently to pathogenic conditions. Though the innate immune system is genetically programmed and reacts always similar to a stimulating agent, it plays a crucial role not only for early host defence but also for the activation of the adaptive immune system and for the induction of acquired immune responses. A key role herein plays the NOD-like receptor (NLR) family. Members of that protein family can form the so-called inflammasomes. These multiprotein complexes activate caspase-1 resulting in the expression and secretion of inflammatory mediators, such as IL1β or IL18. One of the best described members of the respective family is the Pyrin domain containing NLR3 (NALP3). The NALP3 inflammasome is composed of NALP3, caspase-1, and the adaptor molecule ASC [83]. The final assembly of the inflammasome leads to the self-activation of caspase-1 resulting in the activation of the proinflammatory cytokines IL-1β and IL-18 [84–86]. Among the activators of NALP3 are vaccine adjuvants, such as alum [87–90], bacteria, such as Listeria monocytogenes [91, 92] and MDP. In addition to the widely known NOD2-NF-κB-mediated activation of IL1β, MDP is also able to induce the

interleukin level via increasing the activity of caspase-1 and NALP3 in human monocytes, suggesting that NALP3 acts as an additional MDP sensor [93, 94]. Mouse studies revealed that activation and secretion of IL1β is dependent on the activity of both of the regulatory factors, NOD2 and NALP3 [95]. These findings are corroborated by studies using monocytes from CD patients showing that a dysfunction mutation within the NOD2 gene prevents MDP-induced upregulation of IL1β [96, 97], whereas a gain of function mutation within the NALP3 gene in Muckle-Wells patients as well as a respective mutation in mice causes overexpression of IL1β [68, 93]. In addition, in vitro studies showed that the NALP1 inflammasome is also sensitive to MDP and might be involved in MDP-induced IL1β expression [98, 99]. Though the knowledge of the exact role of the NALP inflammasomes in the immune system is just rudimentary so far and needs to be further elucidated, these findings suggest that the NALPs might be essentially involved in the pathogenesis of intestinal inflammation.

9. Autophagy Genes—More Than Just Cleaning the Cell?

Autophagy represents an essential intracellular process that is responsible for the turnover of protein aggregates, the removal of damaged organelles, and the elimination of intracellular microbes. Therefore, autophagy can also be regarded as a part of the innate immune system [100]. Recent GWAS showed a significant association of the autophagy gene, ATG16L1 and IRGM with CD [28, 29, 36, 101]. So far, only little is known about the role and function of the autophagy genes in the intestinal epithelium. The Thr300Ala substitution polymorphism within the ATG16L1 gene is associated with an ileal CD phenotype, similar as the CD-associated NOD2 mutations [65, 102] and a specific E. coli strain, adherent-invasive E. coli [103, 104], which are able to survive and to replicate within intestinal macrophages [105]. A recent study demonstrated that loss of either ATG16L1 or IRGM contributes to increased replication and survival of the specific E. coli strains in vitro [106], a finding that is in good accordance with the results of previous studies demonstrating prolonged survival of Salmonella typhimurium in human epithelial cells [107, 108]. Moreover, ATG16L1 seems to play a major role for the correct function of the intestinal Paneth cells that also represent one of the main intestinal localisations of NOD2 and the main source of the human defensins. Cadwell et al. have recently revealed abnormalities in the Paneth cell granule exocytosis pathway and in the gene transcription profile in ATG16L1-deficient mice as well as in tissue specimen derived from CD patients carrying the CD risk allele [109]. Surprisingly, the ATG16L1 mutation caused, among others, an increased expression of genes involved in PPARγ signalling. Of special interest with respect to the pathophysiology of CD is the finding that the exocytosis pathway of mutant-carrying Paneth cells is disrupted. Since the Paneth cells secrete the important antimicrobial defensins, these findings could essentially contribute to an aberrant innate immune response to microbial stimuli in the gastrointestinal tract and therefore play a pivotal role for the onset of chronic intestinal inflammation. Recent studies also suggest that ATG16L1 is involved in the regulation of inflammasome activity [110] and interacts with NOD2 at the sites of bacterial cell invasion [111, 112].

10. PTPN2—A Likely Key Regulator of Intestinal Inflammation

A recently identified IBD-associated gene locus encodes for PTPN2 [29, 36]. By dephosphorylating and thereby inactivating its targets, the regulatory protein PTPN2 modulates and regulates proinflammatory signal transduction as induced by cytokines such as TNF, IFNγ, or IL6. Among its targets are the signal transducers and activators of transcription 1+3 (STAT1+3) [113–115], mitogen-activated protein kinases (MAPKs) [116], the epidermal growth factor receptor (EGFr) [117, 118], and the insulin receptor [119]. PTPN2 knock-out (PTPN2$-/-$) mice feature excessively high levels of TNF, IL12B, and IFNγ. The importance of PTPN2 for the regulation of inflammation in vivo is further corroborated by the observation that PTPN2$-/-$ mice are not able to survive longer than 3 to 5 weeks, finally dying on a progressive systemic inflammatory syndrome [120, 121]. Of special interest with respect to CD is the fact that PTPN2$-/-$ mice develop severe diarrhoea and weight loss, both of them representing common symptoms in human CD. Additionally, PTPN2$-/-$ mice represent systemic hyperresponsiveness to TLR4 ligand, LPS, resulting in increased production of IFNγ and nitric oxygen (NO) [120, 122] that are also major pathogenetical factors in CD. These observations suggest that PTPN2 might play an important role for the adequate reaction of the innate immune system to bacterial stimuli. The possible importance of PTPN2 for human disease has been underlined by a recent study using IFNγ-treated IEC [123]. Here, it has been demonstrated that PTPN2 downregulates IFNγ-induced proinflammatory STAT1 signalling. From a functional perspective, loss of PTPN2 permitted IFNγ to increase the expression of the pore-forming protein, claudin-2, resulting in a dramatic decrease of the intestinal epithelial barrier function. These data, in addition to the previously identified role for PTPN2 in regulating immune signalling, provide the rationale background for a functional role of the regulatory protein PTPN2 in the pathogenesis of IBD, assumingly by regulating cytokine signalling and innate immune responses as well as in preserving the intestinal epithelial barrier function.

11. Conclusions

The innate immune system plays a pivotal role for the control of the intestinal mcirobiota. On the other hand, the human microbiota regulates the innate immune system (Figure 3). Our increasing understanding of the molecular mechanisms that modulate the innate immune response to bacterial and antigen in the intestine are also raising about the complex signalling and networking. Further understanding of the pathways how the intestinal microbiota contributes to the

pathophysiology of chronic intestinal inflammation will help us to develop new therapeutic strategies.

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Review Article

How Bacteria-Induced Apoptosis of Intestinal Epithelial Cells Contributes to Mucosal Inflammation

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The life cycle of an intestinal epithelial cell is terminated by apoptosis and/or cell shedding. Apoptotic deletion of epithelial cells from the intact intestinal mucosa is not accompanied by detectable inflammatory response or loss of barrier function. But increased permeability of the epithelial barrier and increased apoptotic rates of epithelial cells have been reported for patients suffering from inflammatory bowel disease. Microbiota can both induce or inhibit apoptosis of intestinal epithelial cells thus contribute to mucosal inflammation or support epithelial integrity respectively. Bacteria-mediated cytokine secretion and altered cell signalling are central to epithelial injury. Tumor necrosis factor (TNF) secreted after exposure to invasive bacteria induces both apoptosis and cell shedding. TNF is the major target gene of the transcription factor nuclear factor-kappa B with both pro- and anti-apoptotic effects. Autophagy promotes both cell survival and "autophagic" cell death. If autophagy is directed against microbes it is termed xenophagy. Inhibition of xenophagy has been shown to decrease cell survival. Endoplasmic reticulum (ER) stress causes misfolded proteins to accumulate in the ER lumen. It was suggested that ER stress and autophagy may interact within intestinal epithelial cells. Apoptosis in response to infection may be well proposed by the host to delete infected epithelial cells or could be a strategy of microbial pathogens to escape from exhausted cells to invade deeper mucosal layers for a prolonged bacterial colonization.

1. Introduction

The first boundary between self and nonself is constituted by intestinal epithelial cells. The intestinal mucosa forms a primary phalanx providing both barrier function and immediate effective recognition of bacterial products invading the mucosa. This is of great importance for the prevention of permanent and chronic inflammation as a reaction to the commensal intestinal flora and the multitude of antigens present in the intestinal lumen. Due to its enormous surface area, the barrier function of the intestinal mucosa may be as important as its function in nutrient absorption [1].

Intestinal epithelial cells are generated from stem cells at the lower third of the crypt and reach the intestinal lumen in 3–5 days. Apoptosis is initiated and cells finally lose anchorage and are shed into the lumen [2]. Apoptosis is a conserved genetic program for the removal of malfunctioning or potentially dangerous cells. Intestinal epithelial cells undergo apoptosis when they lose their contact with the extracellular matrix, a phenomenon termed "anoikis" [3]. This special form of cell death is likely to be the main mechanism terminating the physiological life cycle of intestinal epithelial cells, because they gradually lose cell anchorage on their march toward the intestinal lumen. Mechanisms terminating the short physiological life cycle of epithelial cells are supposed to have an impact on these essential functions and have influence on inflammatory reactions in the intestinal mucosa. A disturbance of the epithelial barrier is thought to be a major factor in the pathogenesis of inflammatory bowel disease.

This review focuses on recent significant research findings regarding mechanisms of bacteria-induced apoptosis, probiotic actions which promote host homeostasis, and the potential relevance of these mechanisms to mucosal inflammation and inflammatory bowel disease.

2. Cell Shedding, Sealing, and Healing

The life cycle of a nontransformed, intestinal epithelial cell is terminated by apoptosis or, if cells lose their contact with the matrix, by anoikis. The mechanism of cell shedding is important as abundant shedding must be achieved without loss of intestinal barrier function. An impermeable substance plugs epithelial discontinuities on the villus as a reaction to cell shedding, resulting in 3% of surface area having gaps near the villus tip [4]. Gaps contain no cellular material but are filled with a substance that maintains the epithelial barrier, despite discontinuities in the cellular layer. The source and composition of this "plugging" substance is unknown but could be secreted by neighboring epithelial cells, myofibroblasts, or the shed epithelial cell itself.

Physical wounding in human tissue culture models shows activation of myosin light chain kinase leading to contraction of an actin ring that, like a purse string, closes off the denuded region and restores barrier function [5]. Repair mechanisms after cell shedding in human histological sections have been characterized as similar to wound healing mechanisms of cultured epithelial monolayers [6]. During healing, myosin light chain kinase becomes phosphorylated at the edges of gaps in neighboring cells [6]. An actin ring at the apical pole of cells at the edge of the wound contracts like a purse-string contracture. For 45% of the shedding events, cytoplasmic extensions from the neighboring cells were shown to make a continuous structure beneath the shedding cell but tight junction activity of ZO-1 was only shown in 9% of the shedding events [4]. ZO-1 activity remains at the basal pole of gaps in a V-shaped formation. Thus it was mentioned that tight junctions from neighboring cells can only be used in a fraction of instances to help reseal the breach in the epithelial layer [4, 7]. Watson et al. considered that a number of shed cells do not have an apoptotic morphology or sufficient caspase activation [8]. Cells could be shed as largely intact or during early events in the apoptotic cascade. A forceful cell expulsion after loosening of contacts between neighboring intestinal cells and the basement membrane simultaneous with the secretion of an extracellular substance that fills the void left by the departing cell was mentioned [8]. Extracellular matrix molecules provide essential survival signals to epithelial cells via β1-integrins, focal adhesion kinase, and protein kinase B/Akt-1 pathways [9, 10]. Loss of cell-cell and cell-matrix contacts causes anoikis [3]. Preservation of cell-cell anchorage after *ex vivo* isolation of intestinal epithelial cells maintains survival. Src, PI3-K, and Akt, as well as the adherens junction protein β-catenin, are important elements of cell-cell anchorage-mediated anti-apoptotic signals in intestinal epithelial cells [11]. Apoptotic deletion of epithelial cells from the intact intestinal mucosa is not accompanied by detectable inflammatory response and little, if any, disruptions of the intestinal epithelial barrier integrity are not the major route for bacterial translocation.

3. Implications for Disease

Disturbance of the epithelial barrier and epithelial transport processes is often discussed to be a major factor in the pathogenesis of intestinal inflammation and inflammatory bowel disease. Increased permeability of the epithelial barrier in ulcerative colitis was first reported in 1973 [11]. In a study by Hagiwara et al., epithelial apoptosis in mucosa from patients with ulcerative colitis was found to be considerably upregulated compared with control sigmoid colon [12]. An apoptotic rate of approximately 27.9% in patients ultimately requiring surgery for severe and/or steroid-refractory disease, 17.3% in patients receiving medication alone for more than 3 years, and 13.1% in patients with infectious colitis compared to 2.8% in the control colon. Increased permeability of the epithelial barrier in Crohn's disease was first reported in 1986 [13]. In a study by Zeissig et al., epithelial apoptosis in mucosa from patients with Crohn's disease was found to be considerably upregulated compared with control sigmoid colon [14]. An apoptotic rate of approximately 5.3% was obtained in the inflamed colon compared to 2.1% in the control colon. The apoptotic rate seems to be higher in ulcerative colitis than in Crohn's disease. Direct comparison of the apoptotic rate seems to be objectionable because of unequal experimental conditions. Abnormal gut permeability is characterized by measurably increased rates of apoptosis among epithelial cells. Intestinal bacteria are essential for the development of intestinal inflammation. A number of knockout models of inflammatory bowel disease confirm that bacteria are essential for the onset of inflammation [15–17].

4. Cytokine-Induced Apoptosis

4.1. TNF Increases Cell Shedding. Apoptosis can be induced by mucosal factors secreted by epithelial cells. Several studies have suggested that tumor necrosis factor (TNF) is central to epithelial injury. The epithelial cell lines T84, HT29, and Caco-2 secrete the proinflammatory cytokines interleukin (IL)-8, MCP-1, GM-CSF, and TNF after exposure to the gram-negative invasive bacteria *Salmonella dublin*, *Yersinia enterocolitica*, *Shigella dysenteriae,* and the gram-positive invasive bacterium *Listeria monocytogenes*. Expression of the same array of cytokines has also been shown in freshly isolated human colon epithelial cells [18, 19]. The noninvasive bacteria *Escherichia coli* DH5α and *Enterococcus faecium* had no significant effect on secretion [19, 20]. The cytokine TNF has been found to be elevated in inflammatory bowel disease where epithelial apoptosis is increased [21]. Accordingly, TNF induces both apoptosis and cell shedding when given to mice [22]. In intact intestinal mucosa, local barrier function was preserved during physiological cell shedding but was altered during TNF-induced loss of cells in mice. Approximately 20% of the TNF-evoked gaps were found to present no luminal permeability barrier [8]. TNF substantially increased gap formation and cell loss both individually and in sheets of up to 18 cells [23]. Extensive TNF-mediated apoptosis might be beyond the normal capacity to sustain gap impermeability. Supportive downregulation of epithelial apoptosis was found after TNF antibody treatment in patients with active Crohn's disease [14, 24] and in mouse models of colitis [24, 25]. As soon as microbes or their

products have passed the epithelial barrier, TNF is also secreted by activated macrophages and T lymphocytes [26]. Apart from bacteria-induced apoptosis, dysregulated signals from the intestinal immune system are a major trigger of intestinal epithelial cell apoptosis. Intestinal mucosal biopsies from patients with active Crohn's disease contain increased numbers of TNF-secreting cells [27]. However, TNF initiates both antiapoptotic and proapoptotic signalling through the distinct receptors TNFR1 and 2 in a concentration [28, 29] and time- [30] dependent manner.

4.2. Probiotics Decrease TNF-Induced Cell Shedding. Several probiotics have been shown to prevent cytokine-induced apoptosis in epithelial cells. *Lactobacillus rhamnosus* GG (LGG), a bacterium used in the fabrication of yoghurt, prevents cytokine-induced apoptosis in mouse or human intestinal epithelial cells [31]. Studies on the relationship between LGG and both the murine colon epithelial cell line YAMC and the human colonic epithelial cell line HT-29 demonstrated that LGG inhibits TNF-induced apoptosis. LGG promotes the survival of cells through activation of the antiapoptotic Akt/protein kinase B in a PI3K-dependent manner and inhibition of the pro-apoptotic p38/mitogen-activated protein kinase activated by TNF, IL-1, or IFNγ. LGG produces soluble factors which can be recovered from bacterial culture without previous bacterial-intestinal cell contact [31]. LGG has been shown to reduce chemically induced intestinal epithelial apoptosis *in vitro* in IEC-6 and *ex vivo* in animal experiments [32]. Two novel proteins have been identified in LGG culture supernatant, p40 and p75; the first probiotic bacterial proteins demonstrated to promote intestinal epithelial homeostasis through specific signaling pathways [33]. These two factors are heat- and protease-sensitive [34]. Both p75 and p40 rescue TNF-induced epithelial damage, ulcerative lesions including disruption of epithelial integrity and epithelial apoptosis in cultured mouse colon explants. LGG has been shown to induce remission and prevent recurrence of inflammatory bowel disease in patients [35] as well as in animal models of colitis [36] but therapy in children with Crohn's disease showed no beneficial effect [37].

5. NF-κB Deficiency Leads to Apoptosis in Intestinal Epithelial Cells

Bacteria display pathogen-associated molecular patterns recognized by Toll-like receptors (TLRs) at the surface of enterocytes required for the initiation of an inflammatory response. Intracellularly MyD88 transduces recognition signals for all bacteria. Downstream, inactive transcription factor nuclear factor-kappa B (NF-κB) is complexed with inhibitory protein IκB. NF-κB is activated by dissociation of IκB followed by ubiquitination and proteasomal degradation of the inhibitor. Dissociation of IκB is induced through phosphorylation of inhibitor by IκB kinase (IKK), a complex of two enzymatically active subunits IKKα and IKKβ together with the regulatory subunit IKKγ (NEMO) [38]. NF-κB is activated in intestinal epithelial cells of inflamed mucosa [39] and TNF is one of several prototypic NF-κB target genes [40].

Contrary, physiological importance of TLR-signalling and NF-κB-activation for intestinal homeostasis and maintenance of epithelial barrier has been shown in several recent studies. Inhibition of NF-κB activation results in increased caspase activation and apoptosis [41]. TLR2-, TLR4-, and MyD88-deficient mice exhibited colonic bleeding [42]. Colons from MyD88-deficient mice showed severe and extensive denudation of the surface epithelium [42] but an increased number of proliferating epithelial cells. Dysregulated proliferation suggests the execution of a compensatory mechanism of intestinal epithelial cells as part of the physiological repair response to injury. Also, loss of TLR5 resulted in spontaneous colitis [43]. IKKγ (NEMO) deficiency in intestinal epithelial cells caused spontaneous colitis in mice associated with massive epithelial apoptosis, lost of barrier integrity, and translocation of commensal bacteria in the colon [44]. Analysis of proinflammatory gene expression showed upregulation of TNF in the colon [44]. A similar inflammation phenotype was observed in mice lacking both IKKα and IKKβ.

There are mixed results implicating NF-κB in various models of apoptosis with both pro and antiapoptotic effects observed [41]. Downregulated expression of NF-κB target genes with pro-survival functions in intestinal epithelial cells would be expected to exacerbate disease [38]. Completely absent NF-κB-activation is followed by accelerated epithelial apoptosis and loss of epithelial barrier associated with enhanced exposure to bacteria. Inflammatory response is the total of the activation of both pro- and antiinflammatory signaling pathways in host cells.

6. More Probiotics, More Epithelial Cell Survival

Probiotics-mediated cytoprotection is the opposite of apoptosis. Uptake of probiotics is a therapeutic approach that regulates the homeostasis of intestinal epithelial cells by maintaining cell survival and enhancing barrier function. These nonpathogenic microorganisms have been used for the treatment of gastrointestinal diseases, including ulcerative colitis [45, 46] and Crohn's disease [34, 47]. Proposed mechanisms include bacterial interference, increased cytoprotection and decreased apoptosis of epithelial cells. Probiotic bacteria interact with three components of the gastrointestinal tract including competing bacteria, intestinal epithelial cells and mucosal immune cells [31].

6.1. Bacterial Interference: Microbe versus Microbe. Bacterial interference describes the condition in which a bacterial strain inhibits colonization by competing strains. The most plausible effect of bacterial interference is the antagonistic activity of probiotics against inflammation- and/or apoptosis-releasing pathogens. Probiotics prevent colonization by other pathogenic bacteria by the production of growth inhibitors including bacteriocins [48, 49] and microcins [50]. Probiotics were also shown to compete for

adhesion sites on intestinal epithelial cells thereby replacing intestinal pathogens [51, 52]. Maintaining the integrity of intestinal epithelial cells by bacterial interference is especially relevant during defense against invasive pathogens. The probiotic *Escherichia coli* strain Nissle 1917 (EcN) is already in use for successful treatment of diseases of the digestive tract since several decades. EcN was demonstrated to inhibit invasion of *S. typhimurium*, *Y. enterocolitica*, *S. flexneri*, *L pneumophila* and *Listeria monocytogenes in vitro* [53]. The antiapoptotic effects found were due to soluble factors of EcN. Genomic islands encoding putative fitness factors were suggested as a genetic basis for the dominance of EcN upon other strains [54]. Fitness factors such as different iron uptake systems, adhesins, proteases [55], and the production of microcins [50] may support survival and successful colonization of EcN in the human gut and most likely contribute to its probiotic character. The anti-invasive mechanism of EcN is not dependent on direct physical contact to both epithelial cells and invasive bacteria. By separating EcN from the epithelial cell line with a porous membrane invasion is also effectively inhibited. The invasion systems used by bacterial strains blocked by EcN are diverse. An anti-invasive secreted component of EcN was postulated that acts on a central process or structure on the host [53]. A soluble or shed factor from EcN has been identified to induce β-defensin 2 in Caco-2, mediated through NF-κB- and AP-1-dependent pathways. β-defensin 2 has a broad antibiotic spectrum against gram-negative and -positive bacteria. It may reinforce mucosal barrier by limiting bacterial adherence and invasion. In another study, flagellin has been shown to be a major stimulatory factor of EcN. The signal is transmitted by TLR5 [56].

6.2. Cytoprotective Effects Are Anti-Apoptotic Effects. Next to bacterial interference, more direct cytoprotective effects are described for soluble factors from probiotica. The probiotic mixture VSL#3 (*L. acidophilus*, *L. bulgaricus*, *L. Casei*, *L. plantarum*, *S. thermophilus*, *B. breve*, *B. infantis,* and *B. longum*) has been used as an effective treatment in ulcerative colitis patients [57]. The therapeutic effect of VSL#3 is reflected by increased cytoprotection of epithelial cells.

An early event that occurs immediately after stimulation with VSL#3-conditioned medium *in vitro* is the inhibition of the proteasome [58]. Increase in ubiquitination is restricted to few proteins suggesting that particular features of the proteasome function are specifically inhibited. One of the proteins protected from proteasomal degradation is IκBα. VSL#3-conditioned medium inhibits NF-κB activation by stabilization of IκBα [58]. Diminished NF-κB activation inhibits endogenous immune response genes normally activated by NF-κB, such as MCP-1. VSL#3-conditioned medium confers a degree of protection against oxidant injury through its ability to induce the expression of heat shock proteins (hsps) [58]. Induction of hsps occurs as a late event 12 hours after stimulation with VSL#3. Cytoprotective effects of hsps on epithelial cells are well characterized [59–61]. Hsp25 and 72 are induced by VSL#3 [58, 62]. Hsp25 preserves cytoskeletal and tight junction functions by binding to actin filaments and stabilizing F actin [63]. Hsp72 preserves critical cellular proteins and has been shown to protect epithelial cell lines against oxidant injury [64] and injury by NH_2Cl [60].

7. Xenophagy and ER Stress in Intestinal Epithelial Cells: Novel Links to Apoptosis-Related Disease Mechanisms?

Autophagy is a lysosomal degradation pathway that is essential for both survival and homeostasis [65]. Autophagy constitutes a stress adaptation pathway that promotes cell survival but is also considered to initiate a form of programmed "autophagic" cell death [65]. Autophagy is rapidly emerging as an important component of the innate immune response. Mutations in genes affecting the formation of autophagosomes [66, 67] or mutations in genes affecting lysosomal clearance of autophagosomes [68] are associated with increased frequency of apoptosis (reviewed in [65]). If autophagy is directed against invading microbes, it is termed xenophagy [69]. Xenophagy may have evolved as one of the first eukaryotic cell-autonomous defense mechanisms [70, 71].

Physiological states that increase the demand for protein folding, or stimuli that disrupt the reactions by which protein fold, create an imbalance between the protein-folding load and the capacity of the endoplasmic reticulum (ER), causing unfolded or misfolded proteins to accumulate in the ER lumen—a condition referred to as ER stress [72]. It was suggested that ER stress mechanisms and autophagic pathways may interact within intestinal epithelial cells *in vivo* and may be synergistically related to intestinal inflammation [73, 74].

7.1. Xenophagy. Autophagy directed against intracellular microbes or their products is termed xenophagy [69]. Yet most publications are focused on monocytes [75], macrophages [76, 77], fibroblasts [78, 79], and epithelial cells of nonintestinal origin [80] showing xenophagy-mediated killing of bacteria. Survival might be the benefit for a cell if pathogens are eliminated by xenophagy. Pathogens not eliminated by xenophagy might initiate apoptosis, sometimes accompanied by excessive inflammation.

During the last 3 years, xenophagy has also been investigated in intestinal epithelial cells. A number of single nucleotide polymorphisms (SNPs) showed clear genetic association with susceptibility to Crohn's disease [81, 82]. The strong genetic associations between Crohn's disease and both the autophagy-stimulatory immunity-related GTPase, IRGM1, and the autophagy execution gene, ATG16L, suggest a potential role for autophagy deregulation in the pathogenesis of Crohn's disease [65]. Interestingly, autophagy genes ATG16L1 and IRGM appear to be more specific for Crohn's disease [83] and IRGM risk alleles may predispose even more specifically to the ileal form of Crohn's disease [84, 85]. Deficiency in ATG16L1 had no effect on the overall morphology of the ileum or colon in mice [86] but abnormalities were identified in Paneth cells. Paneth cells are

ileal epithelial cells known to secrete antimicrobial peptides and lysozyme. A lack of lysozyme was determined in the mucus of ATG16L1-deficient mice together with abnormal Paneth cell morphology. No evidence has been found for increased epithelial cell death or proliferation. Deletion of Atg5 in the intestinal epithelium in Atg5flox/floxvillin-Cre mice led to Paneth cell and granule abnormalities similar to those observed in Atg16L1 mice [86], while other epithelial cells appeared normal. This indicates that, within the intestinal epithelium, Paneth cells have a unique sensitivity to autophagy gene disruption. Mucosa from patients homozygous for the *ATG16L1* Crohn's disease risk allele contained Paneth cells with similar alterations to those in ATG16L1 deficient mice including cells with disorganized or diminished granules or cells exhibiting altered cytoplasmic lysozyme staining. Within the intestinal epithelium, the dramatic effect of autophagy protein deficiency on Paneth cells indicates that autophagy can contribute to disease pathogenesis via a highly specific role within a single cell lineage. However, involvement of enterocytes has not been determined so far.

In contrast, xenophagy can indeed act as a cellular defense pathway against secreted bacterial toxins in an apoptosis-dependent manner. Xenophagy targets the noninvasive intestinal pathogen Vibrio cholerae. Intestinal epithelial cells are the main target for the exotoxin Vibrio cholerae cytolysin (VCC). VCC causes extensive vacuolation in epithelial cells and autophagosome formation [87]. In several cell lines, including human intestinal CaCo-2 cells and C2BBe1, VCC-induced vacuoles colocalized with LC3, a clear sign of autophagosomal response. In contrast, LC3 remained cytosolic upon VCC treatment in Atg5−/− mouse embryonic fibroblasts. Autophagy is required to improve cell survival upon VCC intoxication. Both an inhibitor of autophagy and an inhibitor of autophagosome formation have been shown to produce a decrease in cell survival in CaCo-2 cells [87].

Xenophagy deficiency associated with a decrease in cell survival might contribute to a dysregulated immune response to commensal intestinal bacteria, altered mucosal barrier function, and defects in bacterial clearance [88, 89]. It will be essential to further determine whether increased epithelial apoptosis is a pathway induced as a part of xenophagy in human primary cells with different bacterial strains.

7.2. ER Stress. The cell has evolved highly specific signaling pathways, the unfolded protein response (UPR), to deal with ER stress conditions [90]. If UPR is not sufficient, the cell enters apoptosis [91]. Three UPR pathways have been described. The PERK/eIF2α pathway is mediated by a transcription factor called CHOP/GADD153. CHOP forms heterodimers with C/EBPα to induce transcription of Bim in various cell types including kidney epithelial cells, mouse embryonic fibroblasts, thymocytes, a cancer cell line, and a myeloid progenitor *cell line* [92]. Bim is a member of the pro-apoptotic protein family and counteract with the anti-apoptotic protein Bcl-2 to promote apoptosis. Stress stimuli trigger Bim translocation to the mitochondrial surface where it neutralize the anti-apoptotic action of Bcl-2 and induce the release of cytochrome c which finally initiates apoptosis. Bim protein is dephosphorylated by protein phosphatase 2A during ER stress and is thereby rendered resistant to ubiquitin/proteasome-mediated degradation [92]. Other UPR transducers are the activating transcription factor 6 (ATF6) and the inositol-requiring enzyme 1 (IRE1) [91, 93]. Two homologous genes encode IRE1, for which there is a ubiquitously expressed alpha form and a constitutively expressed beta form restricted to the intestinal epithelium [94, 95]. IRE1β has been shown to promote resistance to DSS-induced colitis [96]. The glucose regulated protein-78 (GRP78/BiP) is a central regulator of the concerted cellular response of the three UPR transducers IRE1α, PERK, and ATF6. Several factors are required for optimum protein folding, including ATP, Ca^{2+}, and an oxidizing environment to allow disulphide-bond formation [97]. As a consequence of this specialist environment, the ER is highly sensitive to stresses that perturb cellular energy levels, the redox state, or Ca^{2+} concentration. Such stresses reduce the protein folding capacity of the ER, which results in the accumulation and aggregation of unfolded proteins [93].

Increased expression level of the ER stress chaperone grp-78 in colonic epithelial cells is associated with acute and chronic inflammation in inflammatory bowel disease. Purified human intestinal epithelial cells from patients with Crohn's disease and ulcerative colitis revealed increased expression levels of grp-78 comparing noninflamed and inflamed tissues [98]. As sigmoid diverticulitis patients revealed similar expression levels, grp-78-mediated ER stress may be part of the inflammatory processes rather than a specific feature of inflammatory bowel disease. The regulatory cytokine IL-10 was shown to partly inhibit ER stress responses in intestinal epithelial cells in mice after colonization with *E. faecalis* [98, 99]. TLRs play an important role in the recognition of microbial molecular patterns. It has been shown that ER stress-mediated epithelial apoptosis is associated with the expression of TLRs. A majority of regulated proteins from a comparison between wild-type mice and TLR2-deficient mice have been shown to be involved in energy metabolism and stress responses [100]. Under noninflamed and inflamed conditions, a lack of TLR-dependent signals leads to the induction of ER stress-associated mechanisms in primary intestinal epithelial cells in the large intestine of TLR2 knockout mice.

A number of pathways have been described that induce apoptosis by ER stress (reviewed elsewhere [91]). Indeed microbes can also directly affect ER stress pathways. Trierixin, a triene-ansamycin group compound from a culture broth of *Streptomyces sp.,* has been shown to directly inhibit transcription factor X-box-binding protein 1 (XBP1)-activation [101]. XBP1 deficiency increases susceptibility to colitis in the DSS mouse model [73]. XBP1 level has been shown to be increased in inflamed and noninflamed colonic biopsies from patients with Crohn's disease and ulcerative colitis. SNPs within the XPB1 gene region are associated with inflammatory bowel disease. Similar to deficiency in ATG16L1 Xbp1−/− intestine was completely devoid of

Paneth cells [73]. Increased apoptosis was determined in Paneth cells from Xbp1 deficient mice but no evidence has been found for increased epithelial cell death or proliferation.

8. Conclusion

Induction of apoptosis in pathogenesis remains difficult to understand. Breakdown of the epithelial barrier play a part in the disruption of epithelial defenses and further accelerates mucosal inflammation. The potency of intestinal epithelial cells to identify bacteria through a set of pattern recognition receptors and adequately respond to infection, ultimately with apoptosis of the epithelial cell itself, is not a nonspecific phenomenon that only reflects severity of inflammation. Increased frequency of apoptosis is not synonymic with exceeded histiocytic phagocytic capacity. If human colon epithelial cells undergo exaggerated apoptosis in response to infection with pathogenic bacteria, this may be well proposed by the host itself to delete infected and damaged epithelial cells [102]. Execution of apoptosis is initiated by a proinflammatory program and induced either by immune competent cells or epithelial cells. On the other hand, apoptosis could be a strategy of microbial pathogens to escape from the infected and exhausted host cell to invade deeper mucosal layers for a prolonged bacterial colonization. Anti-apoptotic effects of probiotics may be due to blockade of NF-κB activity and hsp induction.

Balance models are typically discussed to explain the onset of inflammation in the mucosal immune system. Equilibration indeed could be the topic for the mechanisms mentioned above. It will be important to evaluate the complex balance between pro- and anti-apoptotic signals generated by activated transcription factors and cytokines. Impact of xenophagy and ER stress on the molecular network of defence should be analysed in primary intestinal epithelial cells in the future.

Abbreviations

ATG: Autophagy execution gene
ER: Endoplasmic reticulum
GRP78: Glucose regulated protein-78
IKK: IκB kinase
IRGM: Immunity-related GTPase
IL: Interleukin
LGG: *Lactobacillus rhamnosus* GG
NF-κB: Nuclear factor-kappaB
SNPs: Single nucleotide polymorphisms
TLRs: Toll-like receptors
TNF: Tumor necrosis factor
UPR: Unfolded protein response
VCC: Vibrio cholerae cytolysin
XBP1: X-box-binding protein 1.

Conflict

The author has no financial conflicts to disclose.

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Review Article

Peyer's Patches: The Immune Sensors of the Intestine

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The gut-associated lymphoid tissue (GALT) consists of isolated or aggregated lymphoid follicles forming Peyer's patches (PPs). By their ability to transport luminal antigens and bacteria, PPs can be considered as the immune sensors of the intestine. PPs functions like induction of immune tolerance or defense against pathogens result from the complex interplay between immune cells located in the lymphoid follicles and the follicle-associated epithelium. This crosstalk seems to be regulated by pathogen recognition receptors, especially Nod2. Although TLR exerts a limited role in PP homeotasis, Nod2 regulates the number, size, and T-cell composition of PPs, in response to the gut flora. In turn, $CD4^+$ T-cells present in the PP are able to modulate the paracellular and transcellular permeabilities. Two human disorders, Crohn's disease and graft-versus-host disease are thought to be driven by an abnormal response toward the commensal flora. They have been associated with NOD2 mutations and PP dysfunction.

1. Introduction

In the gut, discrimination between pathogens and commensal bacteria is achieved by the interaction of the intestinal epithelium with lymphoid cells. The gut-associated lymphoid tissue (GALT) consists of both isolated and aggregated lymphoid follicles [1] and is one of the largest lymphoid organs, containing up to 70% of the body's immunocytes. Aggregated lymphoid follicles were initially described by Marco Aurelio Severino in 1645 in Italy. They were named Peyer's Patches (PPs) after their detailed description by the Swiss pathologist Johann Conrad Peyer in 1677. PPs are composed by aggregated lymphoid follicles surrounded by a particular epithelium, the follicle-associated epithelium (FAE) that forms the interface between the GALT and the luminal microenvironment. The FAE contains specialized cells named M (for microfold) cells. These M-cells are able to transport luminal antigens and bacteria toward the underlying immune cells that activate or inhibit the immune response leading to either tolerance or systemic immune cell response. The aims of this paper are to describe the different actors and functions of the PP, their implication in the induction of immune tolerance and defense against pathogens and finally their role at the interface between innate and adaptive immunity.

2. Development, Architecture, and Functions of Peyer's Patches

The postnatal development of PPs has been initially investigated by Cornes who reported in 1965 that the number of PPs peaks at ages 15–25 and then declines during the life [2]. Van Kruiningen et al. confirmed these findings [3] and noted that, in addition, the area occupied by PPs in the ileum is maximum in the third decade [4]. In the human small intestine, PPs are oval and irregularly distributed along the antimesenteric side of the gut [2]. At the opposite, in the distal ileum, they are numerous and they form a lymphoid ring [4] (Figure 1). Indeed, at least 46% of PPs are concentrated in the distal 25 cm of ileum in Human [4]. It is to note that there are large variations in size, shape, and distribution of PPs from one individual to another one. The consequences of these variations on the physiological and/or pathological parameters related to PP functions remains to be elucidated [2, 4].

2.1. Development of Peyer's Patches

In Human. The fetal human small intestine contains in average 60 PPs before week 30 of gestation and their number steadily increase reaching a maximum of 240 at puberty [2].

Baginskys and others identified distinct clusters of T and B cells in the small intestine at 14–16 weeks of gestation [2, 5–8]. At week 19, these aggregates mature into recognizable PPs containing follicular dendritic cells (FDCs) and become macroscopically discernable at week 24, even though no germinal centers are present. The latter rapidly develop after birth, when the intestines are exposed to commensal bacteria and antigens [2]. Although macroscopic descriptions of human PP are available, no information concerning the embryonic steps of PP development is actually reported whereas the different steps of PP genesis have extensively been studied in mice.

In Mouse. Three successive steps have been evidenced in PP formation in mouse. The first one, at embryonic day 15.5 (E15.5), marks the beginning of PP development. At that time, VCAM-1 is expressed by distinct clusters of stromal cells located on the antimesenteric side of the small intestine [9]. These VCAM-1 positive cells also express the ligand of the tyrosine kinase receptor RET [10]. During the second step (between E15.5 and E17.5), VCAM-1 positive cells recruit $RET^+CD11c^+cKit^+lymphotoxin^+$ cells and $IL7R^+lymphotoxin^+CD4^+CD3^-$ LTic (Lymphoid Tissue inducer cells) [9–11]. The VCAM-1-positive stromal cells express the lymphotoxin β (LTβ) receptor, and upon ligation of this receptor produce IL7 and homeostatic chemokines such as CXCL13 [12]. This reciprocally leads to increased expression of surface lymphotoxin on LTic, forming a self-sustaining PP primordium [13, 14]. Gene inactivation of CXCL13 and LTβ-receptor interrupts the interaction of LTic with organizer cells and thus abolishes PPs development. Similarly, injection of LTβR fuses to a truncated human immunoglobulin competitively interferes with LTβR signaling by organizer cells and interferes with PP development. Since E17.5, during the third phase of PP genesis, circulating lymphocytes are attracted. They enter into the developing organs and fill up the T and B cell niches [11]. While the embryonic genesis of PPs is largely known, their postnatal development is actually poorly understood (see Section 4.1).

2.2. Architecture of Peyer's Patches. Morphologically, PPs are separated into three main domains: the follicular area, the interfollicular area and the follicle-associated epithelium [1]. The follicular and interfollicular areas consist of the PP lymphoid follicles with a germinal center (GC) containing proliferating B-lymphocytes, follicular dendritic cells (FDCs) and macrophages. The follicle is surrounded by the corona, or subepithelial dome (SED) containing mixed-cells including B-cells, T-cells, macrophages and dendritic cells (DCs). PPs are connected to the body by lymphatic vessels and endothelial venules. Naïve lymphocytes immigrate into the PP via specialized high endothelial venules. Naïve or active lymphocytes leave the PP via efferent lymphatic vessels at the serosal side of the PPs which connect the PPs to the mesenteric lymph nodes (MLN). The arched appearance of PPs is due to the GC forming the core of each follicle (Figure 2).

The follicle-associated epithelium (FAE) differs from the epithelium of the villus mucosa: the production of mucus is weak; the membrane-bound digestive enzymes are lightly expressed and the enterocyte brush border glycocalyx has different glycosylation patterns [15–17]. FAE is also characterized by a large number of infiltrated B-cells, T-cells, macrophages and DCs. Finally, the FAE lacks the subepithelial myofibroblast sheath and, the basal lamina is more porous compared with the regular epithelium [18, 19].

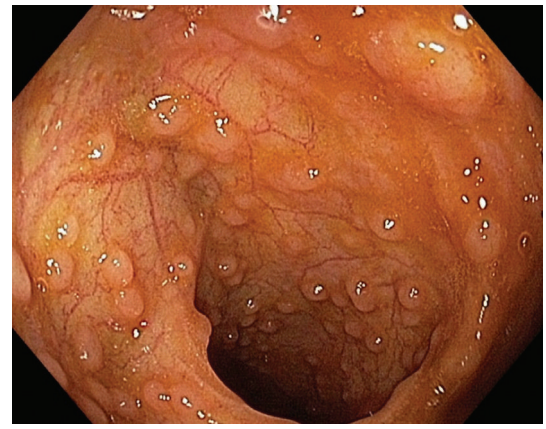

Figure 1: Peyer's patches in the distal ileum. PPs seen in a 20-years-old man during ileocolonoscopy. Note that PPs form a lymphoid ring in the distal ileum.

FAE are constantly renewed from precursor cells located in adjacent crypt zones [20]. The main feature of FAE is the presence of M-cells which are specialized enterocytes. M-cells differentiate from enterocytes under the influence of membrane-bound lymphotoxin (LTα1β2) present on local lymphoid cells, mainly B-cells [21]. The cellular composition of the FAE (i.e., the proportion of enterocytes and M-cells) may be modulated by bacteria present in the gut lumen. For example, the number of M-cells in FAE is increased after transfer of mice from pathogen-free to normal housing conditions [22]. Pathogenic bacteria like *Streptococcus pneumoniae* or *Salmonella typhimurium* may increase the number of M-cells within the FAE [23, 24]. Thus the FAE exhibit an astonishing phenotypical plasticity and can rapidly change its functions depending on host or bacterial stimuli.

M-cells are specialized in the transcytosis of intact luminal material like soluble proteins, antigens, bacteria and viruses [25]. Endocytosis, phagocytosis, pinocytosis, and macropinocytosis are all mechanisms used for the ingestion of the extracellular material. M-cells highly express diverse glyco-signatures which may be exploited as receptors by some microbes [25]. They also express IgA receptors allowing the capture and uptake of IgA trapped bacteria [26]. As a result, luminal IgA not only prevents penetration of bacteria/pathogen into the mucosa but also redirects them to the M cells and PPs [27].

The paracellular permeability is differentially regulated into the FAE [28, 29]. Compared with intestinal mucosa, the FAE exhibits an increased expression of claudin-3 and occludin, which are both described to downregulate the opening of tight junctions. [28]. On the contrary, claudin-2

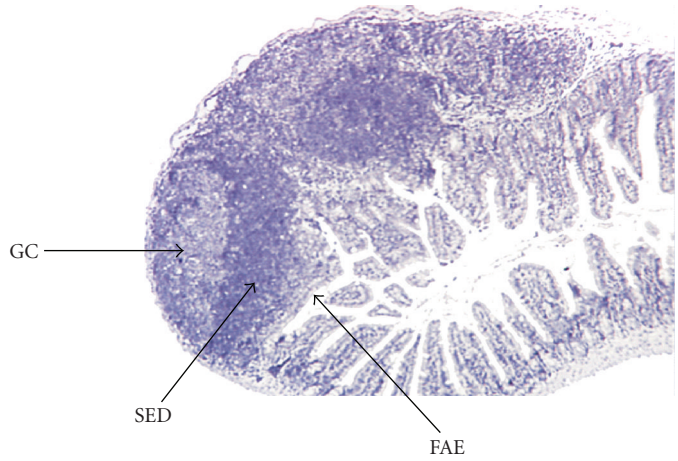

Figure 2: Histological features of a Peyer's patch. Three follicles are individualized. Arrows show the germinal center (GC); subepithelial dome (SED) and follicle associated epithelium (FAE) for one of these follicles.

(which is known to have an opposite effect), is less expressed in FAE than in the villus epithelium [28]. The site of expression may vary within the FAE: claudin-3 and occludin are expressed throughout the dome whereas claudin-4 is preferentially seen in the apex region of the dome [28] and Claudin-2 in the boarding villus epithelium [28]. Moreover, Clark and Hirst found that the adherens junctions of murine M-cells could be recognized by enhanced expression of β-catenin, α-actinin, and polymerized actin [29].

2.3. Cellular Composition of Peyer's Patches

In Human. Because it is difficult to identify and collect PPs during routine endoscopies, studies of human mucosal lymphoid follicles are rare and limited to young patients. In human, among the mononuclear cells (MC), $CD4^+/CD25^+$ (10%) cells and $CD8^+/CD25^+$ (5%) cells are more abundant in PPs than in the peripheral blood [30]. Nagata et al. observed that after incubation with β-lactoglobulin, $CD4^+$ and $CD8^+$ T-cells from PPs were orientated toward a Th1 profile (characterized by the production of IFNγ) but not toward a Th2 profile (characterized by IL-4 secretion) [30]. Junker et al. investigated the cellular subsets within the isolated lymphoid formations (ILFs) [31]. T-cells were found more frequently $CD4^+$ and $CD62L^+$ than $CD8^+$ and $CD103^+$ cells [31]. In addition, antiCD3/CD28 stimulation induced a proliferation of T-cells associated with the secretion of high levels of IFNγ, TNFα and interleukin (IL)-2, but low levels of IL-4, IL-6 and IL-10 [31], confirming that PPs present a Th1 rather than a Th2 profile. Whereas very few papers report human PP's cellular composition, mouse PPs have extensively been studied.

In Mouse. PP exhibit about 60% of B-cells ($B220^+$), 25% of T-cells ($CD3^+$), 10% of dendritic cells ($CD11c^+$) and less than 5% of macrophages ($F4/80^+$) or polymorphonuclear neutrophil ($Ly\text{-}6G^+$). Among T-cells, 45% are $CD4^+$, 35% are $CD8^+$ and 20% are $CD4^-/CD8^-$ T-cell. Among $CD4^+$ T cells, 85% are memory T-cells ($CD25^-CD45RB^{lo}$), 10% are Naive ($CD25^-CD45RB^{hi}$) and 5% are regulatory T-cells ($CD25^+CD45RB^{lo}$) [32]. Distinct subsets of DCs, based on their cell-surface marker expression, together with their location, have been identified in PP [33, 34]. All the subsets express CD11c and major histocompatibility complex class II antigens but differ for their expression of CD8α (lymphoid) and CD11b (myeloid) molecules. Lymphoid $CD11c^+CD8\alpha^+CD11b^-$ DCs are localized within the T-cell–rich interfollicular regions [33]. Myeloid $CD11c^+CD8\alpha^-CD11b^+$ DCs are present under the FAE in the SED [33]. Finally, the "double negative" $CD8\alpha^-CD11b^-$ DCs are found in the SED, the interfollicular region, and within the FAE [33].

In comparison with DCs from spleen (SP), DCs derived from PPs exhibit strong functional differences [35]. PP DCs are more potent in stimulating allogeneic T-cells proliferation compared with DCs from SP, and DCs derived from PPs, but not from SP, are able to prime the production of IL-4 and IL-10 (Th2 anti-inflammatory cytokines) [35]. In addition, PP DCs prime T-cells for the production of much lower levels of IFNγ (Th1 inflammatory cytokine) compared with SP DCs. Finally, stimulation of PP DCs with CD40 molecule resulted in secretion of high levels of IL-10, whereas the same stimulus induced no IL-10 secretion from SP DCs. All DC subpopulations derived from PP secrete a distinct pattern of cytokines upon exposure to T-cells and microbial stimuli. $CD8\alpha^+CD11b^-$ (lymphoid) and double negative DCs share similar functional characteristics as they both orientate the T-cells toward a Th1 profile, notably via IL-12 secretion upon bacterial stimulation [34]. In contrast, only $CD8\alpha^-CD11b^+$ myeloid DCs produce high levels of IL-10 upon stimulation with CD40 ligand, or *Staphylococcus aureus*. In addition, myeloid DCs are particularly capable of priming naive T cells to secrete high levels of IL-4 and IL-10 (Th2 anti-inflammatory cytokines), when compared with those from extramucosal sites, while lymphoid and double negative DCs from all tissues prime for IFNγ (Th1 inflammatory cytokine) production [34].

Very recently, a new subset of myeloid dendritic cells ($CD11c^+CD11b^+$) has been identified in the subepithelial dome of mouse and human PP. These DCs strongly express lysozyme and are able to internalize bacteria and dead cells. Moreover these DCs possess the machinery required to efficiently present antigens to the immune cells—class II major histocompatibility complex and costimulatory molecules—thus actively participating in the first immune defense line within PPs [36].

2.4. Involvement of Peyer's Patches in the Induction of Oral Immune Tolerance. The function of PPs was unknown until 1922, when Kenzaburo Kumagai reported an uptake of *Mycobacterium tuberculosis* inside the epithelial dome of PP. However, as he also observed an uptake of heat-killed bacteria and sheep red blood cells by PPs, he concluded that this uptake was a nonspecific process. Nevertheless, Owen and Jones showed in 1974 that M-cells were able to take up antigens highlighting the role of PPs in the immune system [15].

Immunological tolerance against non-pathogenic bacteria and antigens is a phenomenon observed along the gastrointestinal mucosa [37] which avoids reactions against proteins and commensal bacteria. Oral tolerance is an active process, leading to the generation of antigen-specific T lymphocytes that suppress further immune stimulation. It is defined by the antigen-specific suppression of both cellular and humoral immune responses to orally administered antigens. In addition to the generation of suppressive T cells, anergy and T cell deletion have been described as mechanisms underlying oral tolerance [38]. Consequently, mucosal tolerance protects the mucosa from detrimental inflammatory immune responses. The activation involved in the tolerance induction process to proteins is also important for the maturation of the immune system. As an example, mice feed with a protein-free diet exhibit an underdeveloped GALT with low amounts of immunoglobulin A together with a systemic Th2 profile [39]. A defect in the generation of suppressive T-cells against food or commensal bacterial antigens could lead to food hypersensitivity and celiac disease [40, 41]. Consequently, mucosal tolerance protects the mucosa from detrimental inflammatory immune responses.

Oral tolerance to a broad variety of antigens involves the suppression of different types of immune responses, including delayed hypersensitivity and antibody production. PPs have been extensively studied for their contribution to mucosal tolerance, but their precise role is still unclear. After oral administration of antigens, PPs are the first places of T-cell-specific priming and proliferation in the gut [42]. Mice lacking PPs fail to generate an oral tolerance against ovalbumin but develop an oral tolerance toward small chemical haptens like TNBS suggesting that organized PPs are involved in protein unresponsiveness while epithelial cells modulates the response to smaller molecules [43]. However other observations suggest that this point of view may be too simple: surgical removal of PPs does not interfere with the ability of rats to develop an oral tolerance [44]; an oral tolerance toward proteins has been reported in mice lacking PPs in specific conditions [45, 46]; and the administration of antigens in isolated intestinal loops with or without PPs induced a tolerance in both conditions [47]. Noteworthy, gradual decline in PP immunological functions has been implicated in the lack of oral tolerance in aging mice [48]. Thus, if PPs are clearly very efficient in the uptake and handling of antigens, their exact role in the induction of oral tolerance remains to be clarified.

2.5. Role of Peyer's Patches in the Defense against Pathogens. As previously described, the FAE and M-cell phenotypes are optimized for antigen and microorganism uptake and handling. The mechanisms by which M-cells take up microorganisms and macromolecules vary according to the nature of the biological material. Large particles and bacteria induce phagocytosis, which is often associated with ruffling of the apical plasma membrane of the M cell and rearrangement of the actin cytoskeleton, which permits active formation of pseudopodia-like structures [49, 50]. Viruses and other adherent particles are taken up by endocytosis via clathrin-coated vesicles, whereas non-adherent material is internalized by fluid phase endocytosis [27, 51, 52]. In all these cases, internalization is followed quickly by transport of endocytotic vesicles to the endosomal compartment and then by exocytosis to the basolateral membrane. PP sampling of the lumen is crucial for protective mucosal immune responses. As a counterpart, PPs provide a route of entry into the organism for various pathogenic agents such as bacteria, viruses, protozoa or prion.

Bacteria. Among the pathogenic bacteria with a digestive tropism such as *Escherichia coli*, *Yersinia, Mycobacterium avium paratuberculosis*, *Listeria monocytogenes*, *Salmonella typhimurium* and, *Shigella flexneri*, all of them have been reported to invade the host by adhering with FAE M-cells.

Most of the strains of *E. coli* do not adhere to M-cells but the *Enterohaemorrhagic E. coli* (EHEC) and *enteropathogenic E. coli* (EPEC) show specific adherence to FAE when cocultured with human intestinal biopsies [53, 54]. Infection with the EHEC strain O157:H7 causes diarrhea, hemorrhagic colitis and hemolytic uremic syndrome [55]. This strain selectively adheres to FAE by its intimin-γ protein and binds the β1-integrins expressed on the M-cell apical surface [56, 57]. Other enteropathogenic *E. Coli* strains (like EPEC RDEC-1) adhere to the M-cells but with a mechanism independent of intimin [58, 59]. Finally, some EPEC strains like O127:H7 exhibit a similar rate of translocation across M-cells and enterocytes *in vitro* [60]. In addition, it was observed that translocation rates were significantly increased in the absence of a functioning Type III secretion system [60].

Yersinia enterocolitica and Y. pseudotuberculosis are human foodborne pathogens that cause clinical ileitis or ileocolitis. *Yersinia species* adhere to both enterocytes and M-cells but with a preference for M-cells [61–63]. *Y. enterocolitica* and *Y. pseudotuberculosis* targets the M-cells via the molecular interaction between the β1 integrins present on the host cell and invasin, an outer-membrane *Yersinia* protein [61, 62, 64]. As a result, Yersina causes major damages to PPs and bacterial mutants lacking the invasin

protein display reduced colonization and translocation of PPs *in vivo* [57, 62].

Paratuberculosis or Johne's disease is a chronic enteritis of the cattle and other small ruminant caused by *Mycobacterium avium paratuberculosis (MAP)*. In human, MAP ingestion causes acute and chronic enteritis. MAP are able to invade the intestinal mucosa by interacting with enterocytes [65] and M-cells [66, 67]. *In vitro* studies have shown that the attachment and the internalization of *MAP* by epithelial cells depend on the interaction between Fibronectin attachment proteins and fibronectin [68–70]. In fact β1 integrins are the host cell receptors for fibronectin-opsonized mycobacteria [68, 71]. Because M-cells are the unique intestinal cells expressing β1 integrins at a high density on their luminal surface, they represent the main entrance site for *MAP* [64].

Listeria monocytogenes is the causative agent of human listeriosis, a potentially fatal foodborne infection. Clinical manifestations range from febrile gastroenteritis to more severe invasive forms, including sepsis, meningitis and rhombencephalitis. *L. monocytogenes* invades nonphagocytic cells such as enterocytes and this process is critical for bacterial translocation through the intestinal epithelium [72, 73]. While it is clear that the pathogen interacts with the enterocytes via internalins, several observations suggest that *L. monocytogenes* has also the potential to invade their host via M-cells. First, a rapid localization of *L. monocytogenes* into mouse PPs has been reported [74, 75]. Second, *L. monocytognenes* migrates through differentiated M-cells more efficiently than in non differentiated cells *in vitro* [75]. Finally, *in vivo* analysis of orogastric *L. monocytogenes* infections showed a preferential replication within the PPs with an extremely rapid translocation to internal organs [76, 77]. Moreover, it has been shown that *L. monocytognenes* migrates through differentiated M-cells more efficiently than in non differentiated M-cells [75].

In contrast with *Mycobacterium* [49] or *Yersinia [61, 78]*, which have been shown to specifically attach to and pass through M-cells without modifications or died M-cells, *Shigella flexneri* [79] and *Salmonella typhimurium* [80, 81] are known to alter M-cell homeostasis and functions. *Shigella flexneri* requires both an adhesive and invasive phenotype to efficiently colonize FAE. Following *Shigella* infection, M cells begin to increase in size, which eventually disrupts the integrity of the epithelium [79]. The effect of invasive *Salmonella typhimurium* on M-cells is dramatic [80, 81]. At the earliest stages of *Salmonella* invasion, large membrane ruffles appear on the apical surface of the M-cells, and within a short period of time (30 to 60 min), the cells becomes necrotic and begins to die. Finally, although PPs have been recently involved in the *Helicobacter pylori* induced gastritis, it has been observed that the translocation of *H. pylori* across PPs is performed by DC [82]. Thus, no gastritis is induced in *H. pylori*-infected mice lacking PPs and it has been evidenced that the coccoid form of *H. pylori is* phagocytosed by DC in PPs [82].

Together, these findings indicate that enteric pathogens have evolved distinct mechanisms to interact, invade and destroy PPs. Although the majority of enteric bacteria alter PP homeostasis by interacting and invading M cells from FAE, DCs inside FAE seem to play an alternative pathway.

Viruses. Several viruses like Reovirus type-1, Poliovirus and HIV type 1 are transported by M-cells [83–85].

Reovirus is an orally transmitted murine pathogen, which affects the nervous system, causing encephalitis. Reovirus type-1 selectively adheres to M-cells by interacting with α-2-3-linked sialic acid glycoconjugates expressed by M-cell [86]. The infection causes a depletion of the M-cells from the FAE [87].

Poliovirus is the causative agent of poliomyelitis. It infects humans via the oral route. PPs are the primary sites of virus replication in the gut [83]. In human infected tissues, virions were specifically found on the surface and in intracellular vesicles of M-cells [83].

Transmission of HIV type 1 (HIV-1) infection via anorectal, cervicovaginal, foreskin and urethral epithelia accounts for 80% of AIDS cases [84]. HIV-1 is able to cross the mucosal barrier of the intestinal or genital tracts to infect $CD4^+$ T-cells. HIV-1 can adhere to M-cells—via the chemokine receptor CXCR4 expressed apically on M-cells [88] but not to enterocytes [84].

Prion. Transmissible spongiform encephalopathies (TSE) are characterized by the accumulation of a protease-resistant abnormal isoform of the prion protein (PrP^{Sc}), which is converted from the cellular isoform of the prion protein (PrP^{c}). After oral transmission, PrP^{Sc} can invade the host through PPs [89–91]. In mouse models, reduced PP numbers have been associated with a higher resistance to orally acquired prion infection [91]. Moreover, it has been suggested that the prion protein migration from the gut to the lymphoid system also involve M-cells [92]. Finally, the replication and the accumulation of prion during TSE seem to be located in the FDCs of PP. Altogether, these studies argue for a major role of PPs in TSE pathogenesis [93–95].

3. Peyer's Patches: A Key Organ of the Relationship between Innate and Adaptative Immunity in the Gut

3.1. The Nod2 Sensor in Peyer's Patches. Pathogen associated molecular patterns (PAMPs) present on commensal and pathogenic bacteria are recognized by pathogen recognition receptors (PPRs) present in the host cells. Among the PAMPs, the Toll like receptors (TLRs) and the Nucleotide oligomerisation domain (NODs) are largely expressed in follicle associated cells such as epithelial or dendritic cells. TLRs are mainly extracellular sensors whereas the Nods are cytoplasmic. TLRs and Nods are triggered by a different set of PAMPs. Particularly, Nod2 is able to recognize the muramyl dipeptide (MDP) a component of the peptidoglycan bacterial wall present in most $Gram^+$ and $Gram^-$ bacteria. Common *NOD2* variants have been associated with Crohn's Disease (CD) [96, 97] and graft-versus-host disease (GVHD) [98, 99]. The main CD and GVHD associated variants—R702W, G908R and 1007fs—are located within or

near the Leucin rich repeat domain (LRR) that is supposed to interact with the MDP. [98–100].

While lymphotoxin and IL-7 signalling are essential for the organogenesis of PP during the embryonic stage, studies on germ-free animals argue for a critical role of the gut flora during postnatal development [1]. Germ-free animals have an underdeveloped GALT and are resistant to experimental colitis and to severe GVHD [101], suggesting that bacterial sensors could be implicated in PP development and the Human diseases. Whereas, it has been evidenced a reduction of PP size in TLR deficient mice [102], invalidated mice for *Nod2* gene (*Nod2*$^{-/-}$) are characterized by a hypertrophy and a hyperplasia of the GALT [32, 103]. After birth NOD2$^{mut/mut}$ mice carrying a frameshift mutation homologous to the Human 1007fs variant exhibit a phenotype comparable to that of *Nod2*$^{-/-}$ mice [103].

In fact, *Nod2* seems to play a pivotal role in the GALT homeostasis in response to commensal bacteria [104]. The expression of *Nod2* depends on the presence of commensal bacteria: while its expression in the terminal ileum of mice rederived into germ-free conditions decreased significantly, it is induced by commensal bacteria into germ-free mice [104]. In addition, chronic antibiotic therapy abrogates the overdevelopment of the GALT in *Nod2*$^{-/-}$ mice [103]. Gut microflora exerts a strong stimulation on the *Nod2*$^{-/-}$ PPs mice, inducing a high proportion of CD4$^+$ T-cells, high levels of inflammatory cytokines and high permeability rates for antigens and bacteria [103]. In turn, the terminal ileum of *Nod2*$^{-/-}$ mice exhibits an elevated load of commensal bacteria and its ability to prevent intestinal pathogenic bacteria colonization is diminished [104]. As a result, Nod2 appears to play a key role in the regulation of the interaction between PP and the gut flora.

3.2. Nod2: A Link between Innate Immunity and Adaptative Immunity. *Nod2* appears not only to influence the development of the GALT but it is also able to modulate the immune response toward bacteria, by limiting the development of a Th1 immune response. In wild type mice DCs, MDP acts synergistically with lipopolysaccharid (LPS)—the TLR4 ligand—to promote the proliferation of naïve CD4$^+$ T-cells with a Th2-like cytokine profile. By contrast, DCs carrying *Nod2* mutations are unable to react to MDP, but respond to LPS and promote the development of Th1-orientated cells [105]. As a result, *Nod2* seems to limit the ability of DCs to induce a polarised Th1 response of CD4$^+$ T-cells [105]. Similar data have been evidenced in mice, where Nod2 stimulation by MDP triggers a potent age-specific immune response with a Th2-type polarization profile, characterized by the induction of IL-4 and IL-5 by T cells and IgG1 antibody responses [106]. Nod2 was also found to be critical for the induction of both Th1- and Th2-type responses following costimulation with TLR agonists [106]. Because this synergistic response was recapitulated by DC *in vitro*, it can be supposed that DCs likely play a central role in the integration of Nod2- and TLR-dependent signals for driving the adaptive immune response [106]. Together, these data identify Nod2 as a critical mediator of microbial-induced potentiation and polarization of age-dependent immunity.

In the absence of *Nod2*, PPs present a higher rate of CD4$^+$ T-cells and M-cells in the FAE and increased levels of Th1 (IFNγ, TNFα and IL-12) and Th2 (IL-4) cytokines. These immune alterations are associated with an increased of paracellular permeability and yeast/bacterial translocation [32]. Indeed, PPs from *Nod2* $^{-/-}$ mice exhibit an elevated translocation of *Escherichia coli*, *Staphylococcus aureus* and, *Saccharomyces cerevisiae* [32]. This increase of microbes passage is mediated by an upregulation of myosin light chain kinase expression and activity [103]. CD4$^+$ T-cell depletion and IFNγ-blocking antibodies in *Nod2* deficient mice abrogated this phenotype [103]. Altogether, these data suggest that *Nod2* modulates the adaptive immune response of PPs and may promote the immune tolerance. As a result, Nod2 also regulates the intestinal barrier function, limiting the paracellular and transcellular permeabilities together with bacterial translocation.

Altogether, these data support the contribution of *Nod2* in the immunogenic tolerance toward gut microflora and a key role of *Nod2* in CD4-T cells function. Studies focusing on GVHD also argue for the capacity of Nod2 to regulate the T-cell response. GVHD is a common complication of allogeneic stem cell transplantation, which occurs when donor-derived T-cells are stimulated by host antigen-presenting cells. Acute GVHD is characterized by damages mainly in the skin, the liver, the gastrointestinal tract and other mucosae. Using an experimental model of *Nod2* chimeric mice, *Penack* and coworkers have shown an exacerbated GVHD in case of allogenic transplantation of Nod2$^{+/+}$ mice with *Nod2*$^{-/-}$ bone marrow cells [107]. As expected, this phenotype was associated with an increased activation and proliferation of alloreactive donor T-cells and *Nod2* deficient DCs were involved in the phenotype [107]. At the opposite, allogenic transplantation of *Nod2*$^{-/-}$ mice with Nod2$^{+/+}$ bone marrow cells had no significant impact on the development of GVHD [107]. However, this important role of Nod2 in the T cell function does not seem to be confirmed in human. In human, GVHD proceeded by an allogenic stem cell graft and immunosuppressive prophylaxis, the analysis of biopsies from intestinal GVHD showed a decrease of CD4$^+$-T cells infiltrate when recipient carried *NOD2* GVHD associated variants whereas the donor *NOD2* status had no significant impact on the CD4$^+$ cell infiltrates [108].

Nod2 also plays a role in the immune response to pathogens. For example, *Nod2* deficient mice are more susceptible to *Toxoplasma gondii* infection [109]. This observation was associated with a defect of IFNγ production by Th1 lymphocytes. Interestingly, this phenotype was not due to a lack of CD4$^+$ T-cell activation by DCs. In a model of *Mycobacterium tuberculosis* infection, Divangahi et al., showed that *Nod2* deficient mice exhibited a decreased production of Th1 cytokines—IFNγ and TNFα—as well as a reduced recruitment of CD4$^+$ and CD8$^+$ T cells [110].

If Nod2 modulates the adaptive immune response, its mechanisms of action are probably multiple. In human monocytes-derived DCs *Nod2* is able to induce the autophagy after activation by the MDP. By consequence,

it promotes bacterial handling and activates the major histocompatibility complex class II antigen-specific $CD4^+$ T cell responses [111]. Nod2 activation also enhances the TLR-dependent induction of IL-1 and IL-23, thus promoting Th17 orientated T-cells which have been implicated in antimicrobial response [112]. Finally, the study of Shaw et al. argues for a proper role of *Nod2* in T-cell function independently of DCs and MDP induction. In their model of *T.gondii* infection, DCs from *Nod2* deficient mice were able to activate a normal response of wild type T-$CD4^+$ to *T.gondii* suggesting an intrinsic role of *Nod2* in the generation of an effective Th1 response [109]. Moreover Rick was not necessary to protect against *T.gondii* suggesting the implication of a pathway independent of the *Nod2*-MDP-activation in $CD4^+$ T-cells [109]. Similarly, it has been recently evidenced that NOD2 exerts an important role in the human regulatory T-cells (Treg cells): NOD2 stimulation results in the upregulation of antiapoptotic genes in human Treg cells [113]. In addition, Crohn's disease *NOD2* variants are associated with a deficiency of $FOXP3^+$ Treg cells in the colonic lamina propria [113].

Although the different mechanisms by which *Nod2* promote T-cell response are not fully understood it appears now clearly that *Nod2* has a role not only in innate immunity but also in adaptive immunity.

4. Peyer's Patches and Human Diseases

4.1. Crohn's Disease. Crohn's disease (CD) is an inflammatory disorder characterized by a chronic or relapsing inflammation of the digestive tract. A key role of PPs in CD has been supported by a spatiotemporal relationship between the CD lesions and PPs and by the pathogenesis on CD which is supposed to result of an inappropriate innate and/or adaptative immune response to the bacterial flora.

CD can affect all the digestive tract areas with a preference to the terminal part of the ileum where PPs are more numerous [4]. The number and size of PPs increase from birth to 15–25 years old and then decline with age. This curve is roughly parallel with the age-incidence curve of CD, [114] this is especially true for the ileal presentation of the disease considering that ileal CD is rare in young children and seniors [115, 116]. These observations argue for a temporal relationship between PP development and CD as proposed by Van Kruiningen et al. [2, 4, 115]. Finally, the very early CD lesion, a tiny ulcer called aphtoid lesion has been found by several authors to be centered by lymphoid follicle formations [117–119]. In carefully performed correlative studies with magnifying endoscopy and scanning electron microscopy, *Fujimura* and coworkers demonstrated that the aphtoid lesions of CD are preceded by ultrastructural erosions (150–200 microns in size) in the FAE of hyperemic lymphoid follicles [4, 120].

It is largely admitted that CD is associated with an abnormal T-cell-mediated immune response toward the gut flora. Inflammatory lesions of CD (i.e., aphtoid lesion and ulcers) are more pronounced in the terminal ileum and colon which contain the highest densities of bacteria. The partial efficacy of antibiotics and fecal diversion in CD patients also highlight the fundamental role of bacteria in CD pathogenesis. Now, several genes implicated in bacterial recognition and/or innate immunity including *NOD2* but also the autophagic genes *ATG16L1* and *IRGM* have been implicated in genetic CD susceptibility. Actually, studies on CD microbiota have found evidence for decline in bacterial diversity in CD patients, compared to controls [121, 122]. Because PPs are specialized in sampling and presenting luminal antigens and bacteria to the underlying immune cells, a few authors have studied the role of PPs in CD pathogenesis. Keita et al. have shown an increased translocation of non pathogenic *E. Coli* associated with an increased percentage of *E. Coli* colocalizing with DCs in PPs of ileal CD compared to controls [123]. More recently, these DCs have been characterized by FACS analysis and immunofluorescence microscopy, leading to the identification of a subset of mature $CD83^+CCR7^-$ DC, able to internalize live bacteria [124].

PPs have a pivotal role in the interaction between gut bacterial flora and immune response/tolerance. Their participation in digestive inflammatory disorders such as CD and their interplay with the function and diversity of the gut microbiota is becoming a productive field of research.

4.2. Graft versus Host Disease. Like for CD, in acute GVHD, the interplay between the bacterial flora and the epithelial immune response contributes to inflammatory signals that enhance the donor-derived T cells stimulation by host antigen-presenting cells. The first evidence of the role of GALT in GVHD was provided by *Bekkum* and coworkers in 1974 when they reported that germ-free mice were resistant to enteric GVHD in a model of irradiation followed by incompatible bone marrow transplantation. Using a model of acute GVHD in PP-deficient mice, Murai et al. demonstrated that PPs are the anatomical site for the infiltration of donor $CD8^+$ T-cells and generation of antihost cytotoxic T-cells [101]. However, other authors reported that PPs are not required for the induction of acute GVHD when myeloablative conditioning is applied before bone marrow transplantation [125]. Thus, even if the implication of PP in the pathogenesis of acute GVHD is still in debate, PPs that are at the interface between bacterial flora and immune response have a pivotal role in alloresponse and inflammation.

5. Conclusive Remarks

PPs are key players of the mucosal immune host response toward gut antigens and bacteria. Their function remains to be clarified in many aspects including the regulation of T-cell differentiation after antigen exposure. Nod2 seems to play a crucial role at the interface between innate and adaptive immunity in PPs. It is involved in PP development in response to the commensal flora. It also plays a role in PP permeability, translocation and response toward pathogenic bacteria which exploit PP for their virulence. These findings may be helpful to better understand the mechanisms involved in *NOD2* associated diseases like CD and GVHD.

Abbreviations

CD: Crohn's disease
EHEC: Enterohaemorrhagic *Escherichia coli*
EPEC: *Enteropathogenic Escherichia coli*
FAE: Follicle-associated epithelium
FDCs: Follicular dendritic cells
GALT: Gut-associated lymphoid tissue
GC: Germinal center
GVHD: Graft versus host disease
ILFs: Isolated lymphoid formations
LPS: Lipopolysaccharid
LT: Lymphotoxin
LTic: Lymphoid Tissue inducer cells
M cell: Microfold cell
MDP: Muramyl dipeptide
MLN: Mesenteric lymph nodes
NODs: Nucleotide oligomerisation domains
PAMPs: Pathogen associated molecular patterns
PBMC: Mononuclear cell from peripheral blood
PPs: Peyer's patches
PPMC: Mononuclear cell from PP
PPRs: Pathogen recognition receptors
PrPC: Cellular isoform of the prion protein
PrP^{Sc}: Protease-resistant abnormal isoform of the prion protein
SED: Subepithelial dome
TLRs: Toll like receptors
TSE: Transmissible spongiform encephalopathies.

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Review Article

The Interaction of Large Bowel Microflora with the Colonic Mucus Barrier

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The colonic mucus barrier is the first line of defence that the underlying mucosa has against the wide range of potentially damaging agents of microbial, endogenous, and dietary origin that occur within the colonic lumen. The functional component of mucus is the secreted, polymeric glycoprotein mucin. The mucus barrier can either act as an energy source or a support medium for growth to the intestinal microflora. The mucus barrier appears to effectively partition the vast number of microbial cells from the underlying epithelium. The normal functionality and biochemistry of this mucus barrier appears to be lost in diseases of the colorectal mucosa. Germ-free animal studies have highlighted the necessity of the presence of the colonic microflora to drive the maturation of the colonic mucosa and normal mucus production. A number of by-products of the microflora have been suggested to be key luminal drivers of colonic mucus secretion.

1. Background

The colonic mucosa is constantly exposed to a wide range of luminal agents that have the potential for either mucosal damage, or mucosal protection. These luminal agents can be of microbial, dietary or endogenous origin. "Normal" colonic transit time varies widely in humans but within physiological boundaries would be between 24 and 48 hours, in comparison to transit through the upper GI tract which occurs within a few hours [1, 2]. Therefore, there is a longer exposure time of the colonic mucosa to luminal agents than to the underlying tissues of other areas of the gut. In addition, due to the role of the colon in the salvage of unabsorbed nutrients and absorption of fluid [3], these luminal agents will be concentrated (particularly in the distal bowel), resulting in further increases of mucosal exposure to these agents. While removal of water from the faecal bulk is likely to reduce the diffusion of agents from the majority of the faecal cross-section, direct contact will still occur between the mucus and the outer surfaces of the colonic luminal contents.

The large bowel also plays host to approximately 10^{13} bacteria and other micro-organisms [4] and is thought to include over 500 bacterial species [5]. As such, changes to the prevalent species within the microfloral population or to microfloral functioning and output within the bowel are likely to be intimately linked with colorectal health and disease [6]. While digestion *per se* does not tend to occur in the colon, the colonic microflora acts to degrade dietary fibre and/or other dietary factors that escape digestion to produce further agents that could either harm or protect the underlying mucosa.

The colonic mucus barrier is the first line of defence the underlying mucosa has against the myriad of damaging agents that occur within the colonic lumen [7]. The colonic mucus barrier also acts to greatly reduce the shear stress caused by the passage of the luminal bolus along the colon [8]. This barrier can also act as an energy source or as a niche for bacteria within the large bowel [9, 10]. Despite the relatively high potential for luminal exposure, previous studies in healthy humans suggest that bacteria do not routinely associate with the colonic mucosa and only occur at the luminal side of the intestinal mucus layer [11]. The remainder of this paper will focus on current evidence for how the interplay between microflora and mucus may be a key factor in mucosal health and disease and will also

highlight what areas of research may be considered in the future to further understanding of this topic.

2. Colonic Mucus Production and Secretion

Mucus acts to protect most mucosal surfaces in the gut, airways, and urinogenital tract. The main functional component of mucus is mucin. Disulphide bridges between cysteine-rich areas towards the C- and N-termini of the mucin backbone act to endow mucus with its characteristic viscoelastic gel properties. Up to 85% of the mucin molecule is oligosaccharide side chains by weight. The terminal sugars of these side chains are believed to play a crucial role in the adhesion of mucins to different bacterial cells (e.g., [12, 13]). Changes in both the MUC gene product and glycosylation patterns are believed to be associated with the onset or development of colonic mucosal diseases, such as colorectal cancer and inflammatory bowel disease (IBD) [14].

In humans, there are five polymeric, secretory mucin gene products that are currently known, MUC2, MUC5AC, MUC5B, MUC6 [15], and MUC19 [16]. These genes for MUC2, MUC5AC, MUC5B, and MUC6 are all expressed from the same chromosome locus (11p15.5) [17]. Throughout the small and large intestine, MUC2 is the predominant mucin gene product [18]. Within the mammalian colon, mucins are highly negatively charged, due to the presence of ester sulphate and terminal sialic acids [19]. Reduction of this negative charge in secreted mucins is generally believed to be associated with colorectal disease onset and progression [20, 21].

Following transcription, mucin gene products are firstly N-glycosylated and dimerise (through cysteine-rich regions at the C-terminal of the mucin backbone) in the rough endoplasmic reticulum [22]. This N-glycosylation is also believed to be important in the subsequent transfer of mucins into the Golgi apparatus [23]. Within this compartment, mucins are O-glycosylated [24], prior to N-terminal oligomerisation [25]. Mucin granules are subsequently packaged tightly due to the presence of high levels of calcium ions [26]. Recent studies in this area have noted that the granules of the secreted gel-forming mucin MUC5B (isolated from saliva) appeal to each contain somewhere in the region of 50–100 sub-units of mucin, organised into 10–15 isolated polymers, which are believed to represent the grouping of cysteine-rich C and N-terminal regions [27]. Granules rapidly expand from a diameter of approximately 350 nm to around 1000 nm, with the end products being polymeric chains of 4–8 mucin subunits [27]. Upon their release, mucin molecules become disassociated from the calcium ions and are believed to unfurl in the presence of the aqueous milieu. It has previously been suggested that the rheologically thick mucus secretion seen in cystic fibrosis is a result of incomplete hydration of mucin granules, possibly as a result of defective HCO_3^- transport [28, 29].

The pathways associated with mucus production are highlighted in Figure 1. Triggering of mucin synthesis or secretion, alongside goblet cell/epithelial proliferation and crypt lengthening, may be mediated by a spectrum of neurohumoral, local, and immune factors. Total mucin output from the colon can be elevated as a result of increased mucin biosynthesis, exocytosis rates, and total goblet cell numbers.

Increased MUC2 mRNA was noted by quantitative RT-PCR analysis in human colon cancer cell lines in response to a single stimulation with IL-4 (approximate two-fold increase in comparison to baseline), IL-13, and TNF-α (c.2.5-fold increase in MUC2 mRNA) via MAP kinase pathways [30]. N-glycosylation of MUC2 monomers appears to be necessary to drive further processing of mucin subunits, and is a required step prior to passing into the Golgi apparatus [23]. The expression of mRNA of 3 out of 8 tested isoenzymes governing O-glycosylation of mucins (polypeptide N-acetylgalactosaminyltransferases) in a colon cancer cell line, was also noted to be upregulated by the Th2 cytokine IL-4, resulting in increased incorporation of GalNAc into mucin o-glycans [31]. Within a random mutagenesis model of murine colitis, an increase in both the amounts of Th1 and Th2 cytokines secreted by cultured leukocytes and the increased leukocyte numbers within mesenteric lymph nodes were associated with the accumulation of an unglycosylated Muc2 oligomers precursor in the Golgi apparatus. Within the same study, unglycosylated MUC2 precursors were also noted to occur in human ulcerative colitis, even in noninflamed intestinal tissue [32]. In Il-10-deficient mice, total Muc2 output and synthesis was reduced in germ-free animals. Upon application of a commensal microflora, there was a significant reduction in mucin sulphation compared to sulphation in the germ-free animal [33]. This evidence would therefore suggest a major role for Th2 cytokines in the control of mucin synthesis.

In studies on isolated colonic crypts from macroscopically normal tissues, it was noted that goblet cell exocytosis, as assessed by differential interference contrast microscopy, occurred during cholinergic and histamine-mediated stimulation [34]. Within these studies, prostaglandin E_2 stimulation did not affect mucin exocytosis from goblet cells but appeared to drive fluid loss from columnar cells, which is likely to "wash-out" mucins from the crypts to rest on the luminal surface of the epithelium. Studies on total mucin output from an isolated, vascularly perfused rat colon have noted an increase in the number of cavitated goblet cells following stimulation with cholinergic agonists, prostaglandin E_2, and peptide YY [35]. In further studies using this model, total mucin output (as assessed by ELISA) was also increased in response to the agents used in the previous study [35] as well as serotonin, Vasoactive Intestinal Peptide (VIP), interleukin-1β, and NO precursors [36]. While it is not possible to isolate the effect of these factors on, for example, mucin biosynthesis or granular exocytosis within such studies, these data give a strong physiological indicator of drives for increased colonic mucin discharge.

While mostly associated with goblet cell proliferation in the lung, recent studies have suggested a role for the ETS transcription factor SPDEF in intestinal goblet cell proliferation [37, 38]. Similarly, IL-9 has been linked to the lung inflammatory pathologies. In IL-9-overexpressing mice, Muc2 expression (as well as other goblet cell-related genes) was also elevated in the intestine. Knock-out mouse

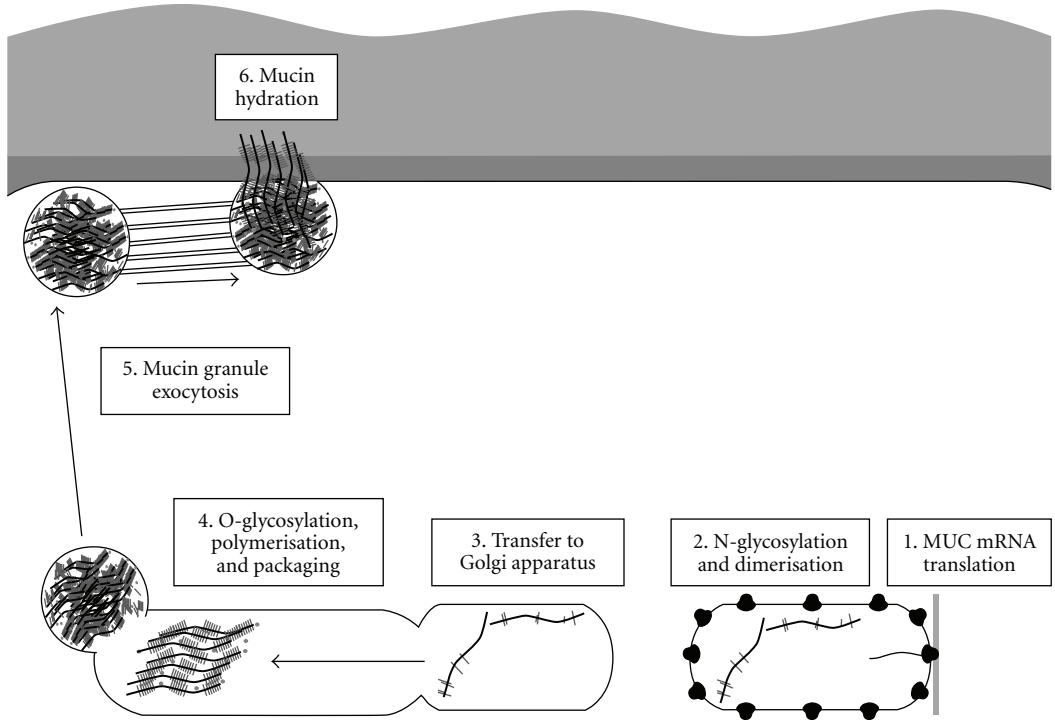

Figure 1: Major posttranscriptional steps involved in colonic mucin synthesis and secretion. (1) MUC gene products are translated at the rough endoplasmic reticulum. (2) MUC gene products are then N-glycosylated and dimerised at the C-terminal. (3) The N-glycosylation is necessary for mucins to be transferred to the Golgi apparatus for further processing. Within the Golgi apparatus, mucins are O-glycosylated and polymerised by disulphide bridge formation between cysteine-rich N-terminal sections of the polypeptide backbone. Polymeric mucins become tightly packed due to the presence of high concentrations of calcium ions. (4) The resulting mucin granules are externalised by the goblet cells via exocytosis. (5) Following release, mucin granules rapidly unfurl into a viscoelastic mucus gel bilayer (adapted from details given in [23, 45, 46]).

studies suggested a necessity for the presence of IL-13 for this hypersecretion and goblet cell hyperplasia to occur [39].

In vivo studies would suggest that the colonic mucus barrier is a functional bilayer [8, 40]. The two layers are rheologically distinct [41]. Upon the application of shear stress, the outer layer of mucus rapidly moves from a gel state to a liquid state. This layer is therefore believed to act as a lubricant and is important in reducing colonic shear stress. As it is constantly removed, this outer layer may also act to return the material back into the centre of the lumen, thereby aiding in the reduction of mucosal exposure to such material [7]. The inner, adherent mucus cannot be removed by suction and is believed to act as a selective physical barrier to the contact of luminal factors with the underlying mucosa, while still allowing absorptive function to occur [42]. Due to the large hydration spheres of mucins in the hydrated mucus gel, it is likely that the mucus gel is imbued with a functional pore size anywhere in the region of 10–500 nm [43]. Diffusion through these pores will be dependent on the charge of the secreted mucins and the properties of the particle crossing the mucus, as well as the thickness of the mucus layer. Studies assessing the secretion dynamics of these mucus layers in an anaesthetised rat model would suggest that the outer, lubricative mucus layer equilibrates to maximal thicknesses of over 600 μm, and the shear-resistant inner layer is maintained at approximately 100–200 μm in rats fed a standard diet [8, 40, 44]. It must be noted that within these studies, the mucus layer is measured in the absence of normal colonic contents.

3. The Colonic Mucus Barrier as a Microbial Niche

The colonic mucus barrier can act as either an energy source, or as a potential support media for growth to the colonic microflora [47]. While a single bacterial species may not possess the necessary enzymes to cleave all the chemical linkages within the mucin structure, it has previously been hypothesised that the ability of each species to thrive as a whole may be dependent on the presence of upstream degradation of mucin by the colonic microbiome. Previous faecal culture studies under anaerobic conditions in mucin-agar gels could suggest that enterobacteria and *Bacteroides* species could be the most predominant within the colonic mucus [48]. However, it must be noted that these studies used gastric mucin as a starting point which has different carbohydrate structures than colonic mucin, which would be expected to affect both bacterial adhesion and degradation.

Due to their proximity to the underlying mucosa, it is likely that the bacteria that populate the colonic mucus

barrier will have the greatest effect on colonic mucosal responses, including mucus secretion, immunity, and inflammatory responses. However, very little is known about the types of bacteria that inhabit the mucus barrier. Recently, a series of experiments outlined by Johansson et al., (2008) [49] have moved the research in this area forwards. Perhaps the major finding of this work was that within the normal mouse colonic mucus bilayer, bacteria were only found to occur within the outer, lubricative layer and did not occur within the inner adherent layer (as evidenced by 16s ribosomal RNA *in situ* hybridisation of histological sections). This would therefore suggest that under normal conditions, the adherent layer is impenetrable to colonic bacteria and that the outer layer could be a major habitat for commensal bacteria.

While colonic mucus acts as both a barrier and a potential niche for the microflora to exploit, it also appears as if the presence of bacteria in the colon is a major drive of both mucus secretion and normal colonic morphology. This is highlighted by the classic histological observation that germ-free animals have a thinner colonic musculature, with shorter colonic crypts alongside a lack of goblet cells and thin mucus layer [50–53]. In normal human development, the colonic microflora begins to develop from parturition due to indirect maternal inoculation [3]. While germ-free conditions are unlikely in either human physiology or pathophysiology, large-scale changes to bacterial numbers or content could greatly affect mucosal protection. In the case of an already deficient mucus layer, increasing numbers of bacteria that are able to degrade proteins would be more likely to cause mucosal damage/infiltration. Temporary reduction of colonic bacterial numbers (e.g., during antibiotic therapy) would be unlikely to cause unwanted mucosal side effects, but in the long term could lead to a less protective mucus barrier.

Studies in knock-out mice have suggested that deletion of the murine MUC2 orthologue Muc2 results in the onset of "spontaneous" (i.e., not chemically induced) colorectal cancer and colitis [54, 55]. Within studies on human colorectal adenoma progression, changes to mucin gene product expression and glycosylation patterns have been noted. The MUC5AC gene product, which is normally secreted in the stomach, but is absent from normal colon, is frequently found in colorectal adenomas and in the area surrounding the adenoma [20]. Mucinous and nonmucinous carcinomas exhibit separate phenotypic changes to mucin gene expression. In the mucinous adenocarcinoma, there is an increased expression of both MUC5AC and MUC2 in comparison to the nonmucinous form [56]. In the nonmucinous carcinoma, there is a reduction in total mucus output, accompanied by a shortening of the mucin oligosaccharide chains [21], particularly through the increased presence of two-residue long GalNAc -sialic acids (Sialyl-Tn antigens) [57]. The change in mucin oligosaccharides is also characterised by a reduction in sulphation levels versus normal mucosa [58] and reduced sialic acid content [59]. The loss of these factors from oligosaccharides results in the reduction of negative charge from the secreted mucin.

Similar losses in sulphation (as well as fucosylation) have also been noted to occur in ulcerative colitis [21, 60, 61]. A reduction in total MUC2 secretion also seems evident in active ulcerative colitis [62, 63]. As with colorectal cancer, there appears to be an increased expression of MUC5AC in ulcerative colitis [64, 65], which both animal and patient studies would suggest be linked to pre- or early neoplastic changes [66, 67].

The above evidence highlights that changes to both the protein and carbohydrate portions of secreted mucins occur in the diseased state. Such changes are likely to reduce the protective potential of the colonic mucus gel and may lead to an altering of the available microfloral niche within the secreted mucus, thereby potentially changing the bacterial population.

Recent preliminary metabolic profiling studies have suggested that an increased appearance of cysteine and proline occur in the faecal water extracts of individuals with colorectal cancer compared to controls [68]. Both of these amino acids are found in high amounts in mucins (cysteine is found in globular terminal structures and is necessary for polymerisation whereas proline is found in high amounts in the glycosylated variable number of tandem repeat structures, where it is thought to act as a "spacer" between glycosylated residues (serine or threonine) that imbue the molecule with a greater degree of flexibility). The increased presence of these amino acids would be suggestive of elevated mucolysis, yet would more broadly predict an increase in protein degradation in the colorectal lumen.

Certain bacterial strains appear to have the ability to preferentially target human colonic mucins [69, 70]. Adhesion to mucins is believed to be driven by the interaction between external bacterial structures and mucin carbohydrate structures. Proteomic analysis of mouse colonic mucus gels demonstrated that Fc-gamma-binding protein was found covalently bound to isolated mucins [71].

16s ribosomal RNA analysis has previously been used to assess global bacterial make-up of the human colonic microflora [72]. In some cases, this technology has been used to assess the occurrence of bacteria within mucosal biopsies (referred to as mucosa-associated bacteria but likely to be a mixture of any bacteria adhered to the mucosa and those associated with the outer and inner mucus layers). Such studies would suggest that the faecal microflora is distinct from that found in mucosal biopsies, with differences occurring in the mucosal biopsy microflora along the length of the large bowel [73], with marked intra-individual variations being also noted [74]. The mucosal biopsy microflora of ulcerative colitis moving from remission to relapse patients was noted to be considerably less stable over time than healthy control individuals in a small cohort study [75]. A wider diversity of the colonic biopsy TM7-bacteria was also recently noted in Crohn's Disease compared to ulcerative colitis patients or controls [76].

It is possible that there is either a direct bacterial degradation of colorectal mucins by colonic bacteria in colorectal mucosal disease or that the presence of certain bacterial species or by-products alters the pathways of mucin biosynthesis and secretion. While the relative ratios of

constituent bacterial species within the colonic microflora (particularly the relative proportion of Bifidobacteria and Lactobacilli) have been postulated to be of importance to colonic health and disease [77, 78], it should be noted that the overall enzymatic spectrum [79] of the microflora or the by-products thereof [80] may be of greater relevance to human health. A number of studies have suggested that there is an increase in the numbers of mucosa-associated bacteria, as reviewed by Strober et al., (2007) [81], although these levels did not necessarily appear to correlate well with mucosal inflammation [82]. There is no consensus on large-scale changes of bacterial populations or obvious mucosal infections that occur within IBD [81] or colorectal cancer. Within the case of epithelial damage and/or infiltration, it is likely that bacterial contact with Toll-like receptors could trigger inflammatory and immune responses, including increased mucin secretion [83].

4. Bacterial By-Products and the Colonic Mucus Barrier

Although the colonic mucosa is surrounded by a wide variety of potentially damaging agents, the physiological response could be described as a "dampened inflammation" or general tolerance. As previously discussed, an absence of colonic microflora tends to result in a reduction in the standard maturation of the colonic epithelium, as seen in atypical histology. As very few bacteria cross the colonic epithelia (and possibly even the inner adherent mucus barrier) outside of major mucosal trauma, it is unlikely that bacterial presence is having a direct effect on colonic physiology until end-stage mucosal infection. Therefore, many of the effects of bacteria on mucus secretion may be elicited indirectly by bacterial by-products. Previous evidence from experimental models (see below) would support this hypothesis although it must be noted that only a fraction of bacterial by-products have been tested for their potential to affect such processes.

Upon bacterial cell death, lipopolysaccharides (LPS) are shed into the colonic lumen. In germ-free rats, colonic goblet cell numbers increased five days after LPS was applied orally [84]. The level of interleukin-8 (IL-8) and total mucin mRNA levels have also been shown to be significantly elevated in a mucus-producing colonic cell culture study in response to LPS stimulation [85].

Both increased mucin release and increased goblet cell numbers have been reported with direct instillation of low levels of butyrate (5 mM) into a vascularly perfused rat colon model. Higher levels of butyrate (up to 100 mM) actually lowered the level of mucin secretion whereas the same concentration range of acetate (5–100 mM) increased mucin secretion in a dose-dependent manner and propionate had no effect [86]. Previous studies on mucus secretion dynamics have suggested that low luminal concentrations of butyrate (7 mM) resulted in an increased rate of mucus barrier secretion following removal, but also resulted in a decrease in the maximal mucus thickness attained (see Figure 2) [87]. Similar effects were noted in more recent studies where long-term administration of high butyrate concentrations (100 mM) directly into the mouse colon over a 7-day period

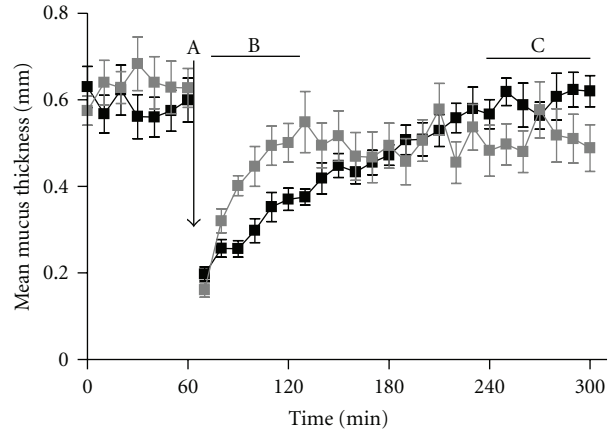

Figure 2: Rat colonic mucus secretion dynamics assessed in the presence of 7 mM butyrate in saline (grey line) and isotonic saline (black line). $N = 5$ animals for each treatment. After 60 minutes, the loosely adherent mucus layer is removed by suction (A). Over the subsequent 60 minutes (B), the mucus replenishment rate was approximately three times higher in the presence of butyrate versus the saline control ($P = .0313$ when compared by paired, nonparametric t-test). Over the last hour of assessment when the mucus barrier had reached equilibrium (C), the total maximal mucus thickness in the presence of butyrate was significantly lower than the saline control group ($P = .0023$ when compared by unpaired nonparametric t-test).

resulted in an upregulation of Muc2 gene expression, but a reduction in the histologically assessed adherent mucus layer was noted [88]. Mechanistic studies have suggested that mucin output [89] and upregulation of MUC2 gene expression [90] are dependent on cholinergic pathways and myofibroblast-derived prostaglandins, respectively.

Reactive oxygen species (ROSs) are a by-product of aerobic respiration that have been shown to occur to millimolar levels in human colonic luminal contents [91]. ROS have been shown to increase mucus secretion rates at low luminal concentrations (5 mM H_2O_2 in the presence of Fe^{++}), but lead to mucus degradation and mucosal reddening at higher concentrations (25 and 50 mM H_2O_2 [42]. These data would suggest that the presence of ROS could be sensed by the colonic mucosa, with the low levels driving the secretion of a more protective mucus barrier. At higher levels, the degradation of mucus, and potential damage of the underlying mucosa, results in degradation of the mucus barrier that outweighs increased secretion.

5. Summary

The colonic mucus bilayer acts to reduce shear stress and protect the underlying mucosa from damaging luminal entities while still allowing colonic salvage to occur. As such, the mucus barrier is a key to innate immunity. The colonic mucus barrier represents a window of colorectal health. There is evidence that the MUC gene products secreted are different in the normal state compared to colorectal pathophysiology, such as adenoma formation and ulcerative colitis.

There are uniquely high numbers of resident bacteria within the large intestine. However, the inner layer of colonic mucus appears to be generally impermeable to this resident microflora and maintains a physical barrier with an exclusion limit of 100 microns between the overlying bacteria and the underlying epithelium. The outer, lubricative mucus layer appears to act as a niche for bacterial population. 16s ribosomal RNA analysis would suggest qualitative differences between the bacterial population that resides within the mucus and that occurring within the lumen (approximated by faecal sampling). It is likely that the bacteria that reside within the mucus will have the greatest impact on the physiology and pathophysiology of the colonic mucosa.

Development of normal colonic morphology, including production of a functionally relevant mucus barrier, is largely driven by the presence of the resident colonic microflora. As bacteria rarely appear to interact directly with the colonic epithelium under normal physiological conditions, it appears likely that the diffusion of bacterial by-products across the colonic mucus barrier to the underlying mucosa acts as a major drive for mucosal maturation and hence affects the processes that govern mucus secretion (mucin synthesis, mucin granule exocytosis, and goblet cell proliferation) Previous evidence notes particular roles for LPS and SCFA in driving mucus secretion.

There is a need for further studies into how fluctuations in specific bacterial populations affect mucin synthesis and secretion, as well as how such populations adhere to or degrade mucus/mucins in a mixed culture. Coassessment of faecal mucins and bacterial populations/bacterial by-products could be utilised as a noninvasive screening technique in human participants. This methodology may be an indirect route of testing (a) whether changes in the microflora drive changes to mucin secretion/degradation and (b) whether such changes are associated with disease incidence or onset.

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Research Article

Suppression of Tumorigenesis: Modulation of Inflammatory Cytokines by Oral Administration of Microencapsulated Probiotic Yogurt Formulation

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The objective of this study was to examine the ability of a novel microencapsulated probiotic yogurt formulation to suppress the intestinal inflammation. We assessed its anticancer activity by screening interleukin-1, 6, and 12 (IL-1, 6, 12), secretory levels of tumor necrosis factor-alpha (TNF-α), interferon-gamma (IFN-γ), prostaglandin E_2 (PGE_2), and thromboxane B2 in the digesta obtained from the duodenum, jejunum, proximal, and distal segments of the ileum of C57BL/6J-Apc^{Min}/J mice. Formulation-receiving animals showed consistently lower proinflammatory cytokines' levels when compared to control group animals receiving empty alginate-poly-L-lysine-alginate (APA) microcapsules suspended in saline. The concentrations of IL-12 found in serum in control and treatment group animals were significant: 46.58 ± 16.96 pg/mL and 158.58 ± 28.56 pg/mL for control and treatment animals, respectively. We determined a significant change in plasma C-reactive protein: 81.04 ± 23.73 ng/mL in control group and 64.21 ± 16.64 ng/mL in treatment group. Western blots showed a 71% downregulation of cyclooxygenase-2 (COX-2) protein in treatment group animals compared to control. These results point to the possibility of using this yogurt formulation in anticancer therapies, in addition to chronic gut diseases such as Crohn's disease, irritable bowel syndrome (IBS), and inflammatory bowel disease (IBD) thanks to its inflammation lowering properties.

1. Introduction

The burden of colon cancer in Western countries is overwhelming, amounting to 50 000 deaths per year in USA alone [1]. Much effort is being devoted to the development of effective therapies for this disease as well as to its prevention.

Inflammation plays a major role in pathogenesis of colorectal cancer, and its evaluation is a powerful tool in screening and understanding the key components that lead to this complex disorder. Normally, the intestinal microflora is effectively confined to the lumen by the epithelium. However, intestinal epithelial barrier defects, for instance, disrupted epithelial tight junctions (leaky gut) may contribute to the chronic inflammation as bacteria that translocate through the epithelium may expose submucosal immune cells to inappropriate antigenic stimulation and incite an inflammatory response towards the commensal microflora [2].

The recognition of the compelling association between intestinal inflammation leading to such disorders as Crohn's disease (CD), ulcerative colitis (UC), and colon cancer has led to an abundance of studies investigating the therapeutic potential of altering luminal bacteria using probiotics.

Probiotics are defined as living organisms in food and dietary supplements which, upon ingestion, improve the health of the host beyond their inherent basic nutrition [3].

Probiotic bacteria have beneficial effects on the intestinal epithelia both directly and indirectly, including enhanced barrier function, modulation of the mucosal immune system, production of antimicrobials, and alteration of the intestinal microflora [4].

The story of probiotics reaches far into the 1850s when Louis Pasteur identified the first probiotic bacteria, *Lactobacillus*, and in 1900 Dr. Henry Tissier was the first person to attempt using bacteria to treat intestinal diseases. By the 1920s, *Lactobacillus acidophilus* was successfully used by the United States to treat diarrhea and constipation. Today, performance and efficacy testing of probiotic bacteria in gastrointestinal disorders, both in animal studies and clinical trials, continues to be of great interest [5, 6].

Furthermore, specific strains of bacteria have been implicated in the pathogenesis of colon cancer [7, 8], in particular, *Lactobacillus acidophilus* and *Bifidobacterium longum* have been shown to reduce incidence of colonic tumors and aberrant crypt foci, respectively, in animal models [9, 10]. Although many studies suggest that probiotics are capable of preventing relapse of chronic intestinal inflammation and have beneficial contributions in disorders such as diarrhea, gastroenteritis, irritable bowel syndrome, and inflammatory bowel disease [11, 12], their therapeutic potential has been hampered by inherent limitations in their use, for example, poor survival during gastric transit, concerns regarding the production, cost, storage, and safety. Microencapsulation is a technique which offers protection to live bacteria from the harsh gastrointestinal environment during transit by use of specialized ultrathin semipermeable polymer membranes [13]. It also limits stimulation of the host immune response as well as minimizes risks of systemic infections, the replacement of the normal intestinal flora and gene transfer [14].

There are many tumor markers which can be measured whose measurement or identification is useful in patient diagnosis or clinical management in cancer. However, as no single marker has been established yet as a practical cancer screening tool either in a general healthy population or in most high risk populations, a set of tests needs to be performed in order to draw conclusions. Cyclooxygenase (COX) is an enzyme which catalyzes the first step in the formation of prostaglandins (PGs), the conversion of arachidonic acid to PGH_2, followed by the metabolism of PGH_2 to biologically active end-products, PGD_2, PGE_2, $PGF_{\alpha 2}$, PGI_2, or thromboxane A_2 (TxA_2) via specific synthases [15]. Two cyclooxygenase isoforms, COX-1 and COX-2, have been identified. COX-2 is critical for the development of colorectal neoplasia [16]. COX-2 inhibitors can reduce intestinal inflammation leading to tumorigenesis [17] and are therefore used to treat familial adenomatous polyposis (FAP) patients [18] and patients with colorectal cancer [19]. Accumulating evidence has shown that prostaglandin E_2 (PGE_2), the main product of cyclooxygenase-2 (COX-2) activity, can promote a number of molecular mechanisms involved in colorectal carcinogenesis [20] in particular tumor cell proliferation and angiogenesis [21–24]. C-reactive protein (CRP) is an acute-phase systemic protein produced primarily in the liver in response to stimulation by interleukin-6 (IL-6) [25]. In addition to studies which show consistency in demonstrating an increased risk of mortality due to inflammation and subsequent cancer development, CRP and IL-6 have been shown to be associated with total and noncardiovascular mortality [26–28].

Tumor necrosis factor-α (TNF-α) and interleukin 1-β (IL1-β) seem to play an important role in ulcerative colitis (UC) in relevant experimental models [29] and are linked to colorectal cancers via inducing the expression of vascular endothelial growth factor (VEGF) [30, 31] or causing a significant increase in the release of soluble B7-H3 in colon cancer cell lines [32].

In this study, we investigated the potential of microencapsulated probiotic bacterial cells contained within our yogurt formulation in reducing intestinal inflammation in an animal model for colorectal cancer, C57BL/6J-Apc^{Min}/J mice. We provide evidence that the daily gavage of the probiotic formulation to mice reduces expression of COX-2 as well as lowers plasma C-reactive protein (CRP) levels. We have quantified the levels of secretory cytokines IL-1β, IL-6, IL-12, PGE_2, TNF-α, IFN-γ, and Thromboxane B2 in ileal contents.

2. Materials and Methods

2.1. Chemicals. Sodium alginate (low viscosity), poly-L-lysine hydrobromide (MW = 27,400), and calcium chloride (A.C.S. reagent) were purchased from Sigma-Aldrich, Canada. Difco Lactobacilli MRS AGAR and Difco Lactobacilli MRS BROTH were purchased from Becton, Dickinson and Company Sparks, USA. Liberty plain yogurt 2% M. F. containing bacterial cultures *Streptococcus Thermophilus, Lactobacillus Acidophilus, Bifidobacterium bifidum,* and *Lactobacillus bulgaricus* was procured from a local grocery store. It contained Calories: 110 kcal, fat: 3.5 g, carbs: 9 g, protein: 9 g, vitamin A 6%, calcium 30% vitamin C 4%, and iron 0% per 175 g serving.

2.2. Bacteria and Culture Conditions. Bacterial strain of *Lactobacillus acidophilus* no. 314 ((Moro) Hansen and Mocquot deposited as *Bacillus acidophilus* Moro, Designation 43) used in this study was obtained from ATCC (Manassas, VA) and was cultivated and serially propagated in the MRS medium before experimental use. Incubations were performed at 37°C in a Professional Sanyo MCO-18 M Multi-Gas Incubator under anaerobic conditions (1-2% CO_2, Atmosphere Generation System AnaeroGen; Oxoid Ltd., Hampshire, England). Bacteria were harvested after 20 hours of the 3rd passage for encapsulation.

2.3. Microencapsulation Method. The bacterial strains were microencapsulated into alginate-poly-L-lysine-alginate (APA) membranes. All membrane components were filter sterilized through a 0.22 μm Sterivex-GS filter (Millipore, Bedford, MA, USA) prior to use. Grown cultures were centrifuged at $3000 \times$ g for 15 minutes at 25°C, and the supernatant broth was decanted. The pellet of wet cells was weighed and suspended in 0.85% saline, pooled, and slowly added to a gently stirred sterile 3.3% sodium alginate solution (final concentration adjusted to 1.65% with 0.85% saline). The entire procedure was performed under sterile conditions in Microzone Biological Containment Hood (Microzone Corporation, ON, Canada) and all solutions were autoclaved with the exception of poly-L-lysine which was 0.22 μm sterile filtered prior to usage. APA microcapsules

were prepared aseptically using an Inotech Encapsulator IER-20 (Inotech Biosystems Intl. Inc., Switzerland). Freshly prepared microcapsules were washed twice with 0.85% saline and stored at 4°C. Parameters for microencapsulation were as follows: gelation time in $CaCl_2$—30 minutes, coating time—10 minutes, nozzle diameter—300 μm, vibrational frequency—918 Hz, voltage >1.00 kV, and current 2 amp.

2.4. Preparation of Probiotic Formulation. APA microcapsules loaded with *L. acidophilus* bacterial cells were carefully mixed with Liberté plain yogurt 2% M.F. and suspended in sterile 0.85% saline to 80% (vol/vol) final concentration. The bacterial cell count was kept constant at 10^{10} cfu/mL throughout the experiment. Empty APA microcapsules were suspended in 0.85% saline using the same formulation and stored at 4°C until further use.

2.5. Animals. Male heterozygous C57BL/6J-Apc^{Min}/J [33] mice, 5 or 6 weeks old, were obtained from The Jackson Laboratory (Bar Harbor, ME). Multiple intestinal neoplasia (Min) mice are heterozygous for *Apc (*Min/+), a germline truncating mutation at codon 850 of the *Apc* gene, and spontaneously develop pretumoric numerous intestinal neoplasms. They are a popular animal model for studies on human colorectal cancer [33]. The animals were housed in the McIntyre Medical Sciences Building Animal Care Facility in a room with a 12-hour light-dark cycle and controlled humidity and temperature. The mice were maintained in a barrier facility. They were allowed sterile water and the laboratory rodent diet 5001 from Purina Land O'Lakes *ad libitum*. Overall health of the animals was monitored daily. The animal use protocol was approved by the Animal Care Committee of McGill University, and animals were cared for in accord with the Canadian Council on Animal Care (CCAC) guidelines.

2.6. Experimental Design. Upon arrival, animals were kept in a sterile environment in individual ventilated cages (IVCs) which filter the air with HEPA filters. The cages, food, water bottles, and so forth were autoclaved. Animals were randomly placed in the cages and allowed one week of acclimatization. The animals were ranked and assigned to groups according to a randomized block design. The mice were separated into two experimental groups: control (n = 24) animals were gavaged 0.3 mL of 0.85% saline solution and treatment animals (n = 24) were gavaged 0.3 mL of APA microencapsulated *L. acidophilus* bacterial cells blended in 2% M.F. yogurt for the total of 0.66×10^{10} cfu/mL of encapsulated bacterial cells per mouse per day. The caloric content of each gavage (0.125 kcal) was considered to be an insignificant factor in potential animal weight gain and therefore not taken into account. Animals were weighed individually every week, and their food consumption was weighed per cage of 4 animals. Blood collection from the saphenous vein was performed every 4 weeks. Blood was separated using 5000 × g at 4°C for 10 minutes.

2.7. Luminal Digesta. At the time of sacrifice, the small intestine of each animal was measured and cut into 4 equal segments, each approximately representing a distinctive part, namely, duodenum, jejunum, and proximal and distal ileum. Each segment was flushed with cold D-PBS buffer (Gibco), its contents were collected and flash frozen at −85°C. Before analyses, the digesta samples were thawed and treated with 1.0% BSA-50 mM Tris buffer (pH 7.5) for 60 min at room temperature to separate the food matrix from cellular material. The samples were then centrifuged at 50,000 × g for 15 min. The supernatants were stored at −85°C and used later for measurements.

3. Analytical Methods

3.1. Quantification of IL-1β, IL-6, IL-12, PGE_2, Thromboxane B2, TNF-α, IFN-γ, and CRP Expressions Using ELISA. IL-1β, IL-6, and IL-12 were quantified using kits from Biosource, Invitrogen, USA according to manufacturer's recommendations. PGE_2 was measured using a competitive enzyme immunoassay (Cayman Chemical, Ann Arbor, MI) as described previously in [34]. Briefly, 96-well plates were precoated with the capture Ab (goat antimouse Ab). 100 μL enzyme immunoassay (EIA) buffer was loaded to nonspecific binding (NSB) wells. 50 μL EIA buffer was loaded to maximum binding (B_0) wells. 50 μL PGE_2 standards were loaded into appropriate wells. 50 μL of samples or standards were incubated with 50 μL of PGE_2 tracer and 50 μL of PGE_2 mAb overnight at 4°C. After three washes in wash buffer, 200 μL of Ellman's reagent was added to the plate and allowed to incubate for 1 h for the color to develop. The optical Density (OD) was determined using the Perkin Elmer Victor microtiter plate reader at 405 nm, and PGE_2 production was expressed as picograms per milligram.

Thromboxane B2 (Express EIA kit-monoclonal, Cat.No. 10004023, Cayman Chemical, Ann Arbor, MI) was measured according to manufacturer's instructions. TNF-α was measured using a competitive enzyme immunoassay (Cat.No. KMC3012, Immunoassay Kit, Biosource Int., Inc, USA) according to manufacturer's instructions. Biotin gamma rabbit antimouse interferon-γ was purchased from Cedarlane, (Hornby, ON, Canada) and reconstituted from sterile form to 50 μg/mL with PBS solution containing 0.1% BSA. Murine IFN-γ ELISA kit was purchased from PeproTech (Rocky Hill, NJ) and used as recommended by the manufacturer. CRP was measured in plasma using a mouse CRP ELISA kit (Life Diagnostics, Inc., USA).

3.2. Immunoblotting of COX-2. Intestinal tissue samples (flushed with cold PBS) were flash frozen in liquid nitrogen before storing at −85°C. Frozen samples were weighed, and 3 mL of RIPA buffer (Santa Cruz Biotech, CA) (with PMSF in DMSO, protease inhibitor cocktail, and sodium orthovanadate) was added per gram of tissue. The samples were homogenized, pooled, sonicated, and centrifuged at 4°C for 10 minutes at 10,000 × g. The protein content was determined using Quant-iT protein assay kit (Invitrogen, Burlington, Canada) with bovine serum albumin (BSA) as the standard. Twenty micrograms of total proteins, as evaluated by Quant-iT protein assay, from tissue were used for Western

blot. Aliquots containing protein were fractionated on 4–12% Bis-Tris Gel (Invitrogen, Carlsbad, CA) at 120 V for 2 hours. After electrophoresis, proteins were transferred from the gel to a nitrocellulose membrane (Whatman, Maidstone, Kent, UK) using Novex Semi-Dry Blotter (Invitrogen, Carlsbad, CA). COX-2 mouse monoclonal antibody (1 : 1,000; Santa Cruz Biotechnology, Santa Cruz, CA) was used as the primary antibody. Horseradish peroxidase-conjugated goat antimouse IgG was used as the secondary antibody (1 : 1,000; Santa Cruz Biotechnology, Santa Cruz, CA). The membrane was then developed with chemiluminescent agents (ECL, BM Chemiluminescence Blotting Substrate (POD), Roche Diagnostics, IN) and visualized in a Versa Doc Imaging System using software Quantity One-4.5.1 (Model 5000, Bio-Rad Laboratories (UK)). Western blot images were analyzed using Image J software (http://rsb.info.nih.gov/ij/ (accessed in December 2005, NIH, USA)).

3.3. Adenoma Classification and Enumeration. The number of adenoma, low-grade dysplasia, high-grade dysplasia, and gastrointestinal intraepithelial neoplasias (GIN) was scored by a blinded veterinary pathologist to the treatment in the small and large intestine. The standards for the histological assessment were established from the MMHCC-sponsored symposium and are detailed on the MMHC web site (http://emice.nci.nih.gov/emice/mouse_models/organ_models/gastro_models/murine_intestinal_neoplasia/models_colorectal_cancer).

3.4. Statistical Analyses. All results in this paper are means calculated using Excel and expressed as means ± SEM or SD. Student t-test was used to assess the statistical significance of the differences between test and control groups. Data was considered significant at $P < .05$.

4. Results

Artificial cell microcapsules containing *L. acidophilus* were prepared using the multistep preparation methods described and were stored at 4°C for use in experiments. Sterile conditions and procedures were strictly adhered during the process of microencapsulation. Results show that the bacterial cells were able to survive the encapsulation process and grow normally when obtained supernatant was plated after breaking of the microcapsule membrane. The microcapsules contained, on average, 10^{10} cfu/mL of bacteria. Freshly prepared microcapsules were spherical and opaque on account of bacterial density. Morphological studies by microscopic analysis revealed that the mean capsule diameter was 433 ± 67 μm, and they exhibited high homogeneity. Bacterial cells were able to survive during the encapsulation process and grow normally (data not shown).

4.1. Food Intake and Body Weights. Results show (Figure 1) that all animals gained weight steadily up to week 16 of age in control group (25 ± 1.1 g) and in treatment group (25 ± 1.2 g). All the animals maintained constant weight until 17 weeks of age after which the weight in control group animals decreased to 20 ± 1.2 g whereas treatment group animals continued to increase in their weight up to 27 ± 1.2 g at the time of sacrifice. The food consumption was consistent with body weight gain and loss. Every week after week 14 of age, mice in control group had a lower consumption than treatment group animals. The potential weight gain from the caloric intake originated from yogurt fat was not taken into account due to its insignificant value of (0.125 kcal) per gavage.

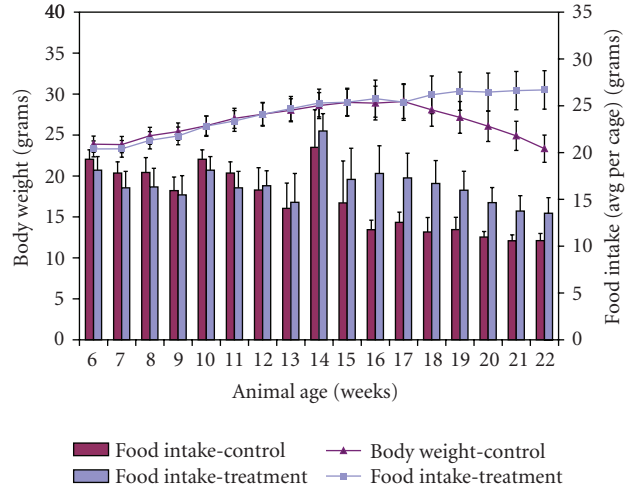

Figure 1: The effect of daily gavage of microencapsulated *L. acidophilus* cells in 2% M.F. yogurt in *Apc*Min mice on animal body weights and food intake (weekly food intake averaged per cage (4 animals)). Data represent the mean ± SEM per group; $n = 24$.

4.2. Serum IL-12. During the 17-week experimental period animal serum was used to measure the levels of inflammatory interleukin 12. Results show the concentration levels were significantly higher (weeks 5, 13, and 17, $P < .05$) in treatment animal group compared to control group animals (Figure 2). At the time of sacrifice the average levels were 46.58 ± 16.96 pg/mL and 158.58 ± 28.56 pg/mL for control and treatment animals, respectively.

4.3. Luminal IL-12. Concentrations of luminal IL-12 were measured in 4 distinct parts of the small intestine: duodenum, jejunum, and proximal and distal ileum. In the control group similar concentrations were found in all 4 intestinal sections (12.35±5.55 pg/mL) (Figure 3(a)). Among the treatment group, luminal IL-12 concentrations were the lowest in the duodenum, 35.79 ± 16.13 pg/mL, and the highest in proximal ileum, 53.74 ± 14.29 pg/mL. All measurements were significant when compared to control ($P < .05$).

4.4. Luminal IL-6. The concentration of luminal IL-6 was measured in the same sections of the small intestine as IL-12. Control group animals had statistically higher levels of IL-6 in all intestinal sections when compared to treatment group animals ($P < .05$). The IL-6 concentration was especially high in the duodenum and jejunum of control group animals

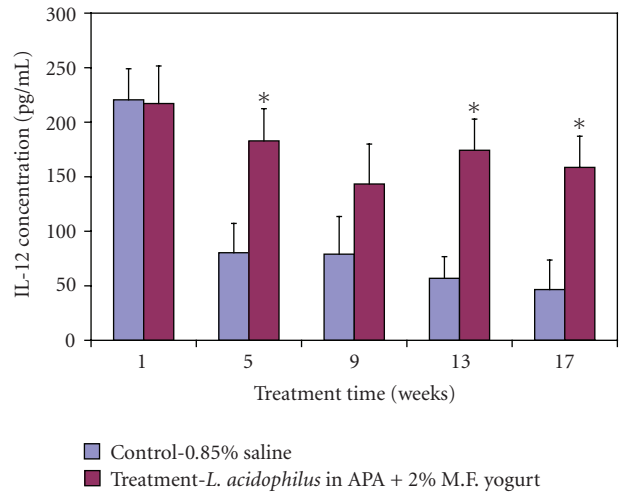

Figure 2: The effect of treatment on IL-12 concentrations in serum. Data represent the mean ± SD of concentration levels per group; $n = 24$. Asterisks: statistical differences ($P < .05$) when compared to control.

115.07 ± 27.12 pg/mL and 116.29 ± 38.92 pg/mL, respectively (Figure 3(b)).

4.5. Luminal TNF-α. The concentration of luminal TNF-α was measured in the same manner as described for IL-6 and IL-12. Higher concentrations were detected in control group animals and were relatively comparable in all intestinal sections (Figure 3(c)). The highest concentration was in proximal ileum, 24.08 ± 10.59 pg/mL. The concentrations of TNF-α in proximal ileum in treatment group animals were statistically the lowest, 9.38 ± 4.23 pg/mL.

4.6. Luminal IFN-γ. There were significantly higher concentrations of luminal IFN-γ in control group animals when compared to those of the treatment group (Figure 3(d)). The highest concentration of luminal IFN-γ was found in the jejunum of the treatment group, 168.55 ± 11.55 pg/mL. The lowest concentration was found in the jejunum of control group animals, 51.08 ± 24.59 pg/mL.

4.7. Luminal IL-1β. The difference in concentration of luminal IL-1β was statistically significant in jejunum, $P < .05$ (Figure 4(a)). In control group animals it was found to be 460.4 ± 68.45 pg/mL whereas in the treatment group it was found to be 180.09 ± 43.56 pg/mL. In the duodenum, proximal and distal ileum the concentration levels did not differ statistically from control.

4.8. Luminal Thromboxane B2. The thromboxane B2 concentration was especially high in all intestinal sections in control animal group, and its range was from 51.27 ± 23.53 pg/mL in duodenum to 35.50 ± 13.16 pg/mL in distal ileum (Figure 4(b)). On the contrary, the levels of thromboxane B2 found in treatment animal group were relatively low, ranging from 3.31 ± 1.75 pg/mL to 1.52 ± 0.62 pg/mL. All the concentration levels between groups in each intestinal section were statistically significant ($P < .05$).

4.9. Luminal PGE_2. The concentration of luminal PGE_2 was the highest in control animals in all intestinal sections, especially in duodenum 1836.55 ± 389.88 pg/mL (Figure 4(c)). The concentration of PGE_2 correlated positively with the total number of adenomas, adenoma burden, and the relative proportion of medium-sized and large adenomas. PGE_2 correlated negatively with the relative proportion of small adenomas in treatment group. The lowest concentration of PGE_2 was found in treatment group in distal ileum 248.57 ± 126.88 pg/mL.

4.10. C-Reactive Protein (CRP). C-reactive protein concentrations were measured using ELISA kit from plasma stored at −85°C obtained from animals by cardiac puncture at the time of sacrifice. The levels between control and treatment group animals were not significant (Figure 5). They were found to be 81.04 ± 23.73 ng/mL in control group and 64.21 ± 16.64 ng/mL in treatment group.

4.11. COX-2 Expression. Intestinal lysates obtained from distal ileum were analyzed by Western blotting employing antibodies specific for the COX-2 isoform. Figure 6 shows representative Western Blot bands of the 72 kD COX-2 protein. A higher expression level of COX-2 was found in control group animals. Using Image J software, the bands were analyzed and relative intensities for control and treatment group animals measured. It was found that the COX-2 in treatment group animals was 71% lower than that in control group animals.

4.12. Adenoma Classification and Enumeration. There were on average 4.5 ± 1.46 tumors found per animal in control group and 2.5 ± 1.60 tumors in treatment group. Most found lesions were small GIN: a total of 66 in small intestine of control group and 42 in large intestine of treatment-receiving animals. In general, adenomas found in colon of control group (4) and treatment group (2) animals were less numerous. This is 44% decrease in total number of lesions in treatment-receiving animals when compared to control group animals.

5. Discussion

Cancer is a chronic pathologic process. Inflammation is considered to be a particularly important factor in the pathogenesis of the colorectal cancers. A period of time is required for a cancer to develop, invade or metastasize, and eventually kill the host. Diet and disease development are strongly related in disease incidence. For instance, the so-called "Western diet", containing red meat, is considered to be the leading cause of higher colorectal cancer development. The main objective of this study was to suppress the occurrence of spontaneously developing polyps in animals ensuring that the levels of inflammatory cytokines are as low as possible. The immunoenhancing effect of microencapsulated probiotic bacterial cells may be an important

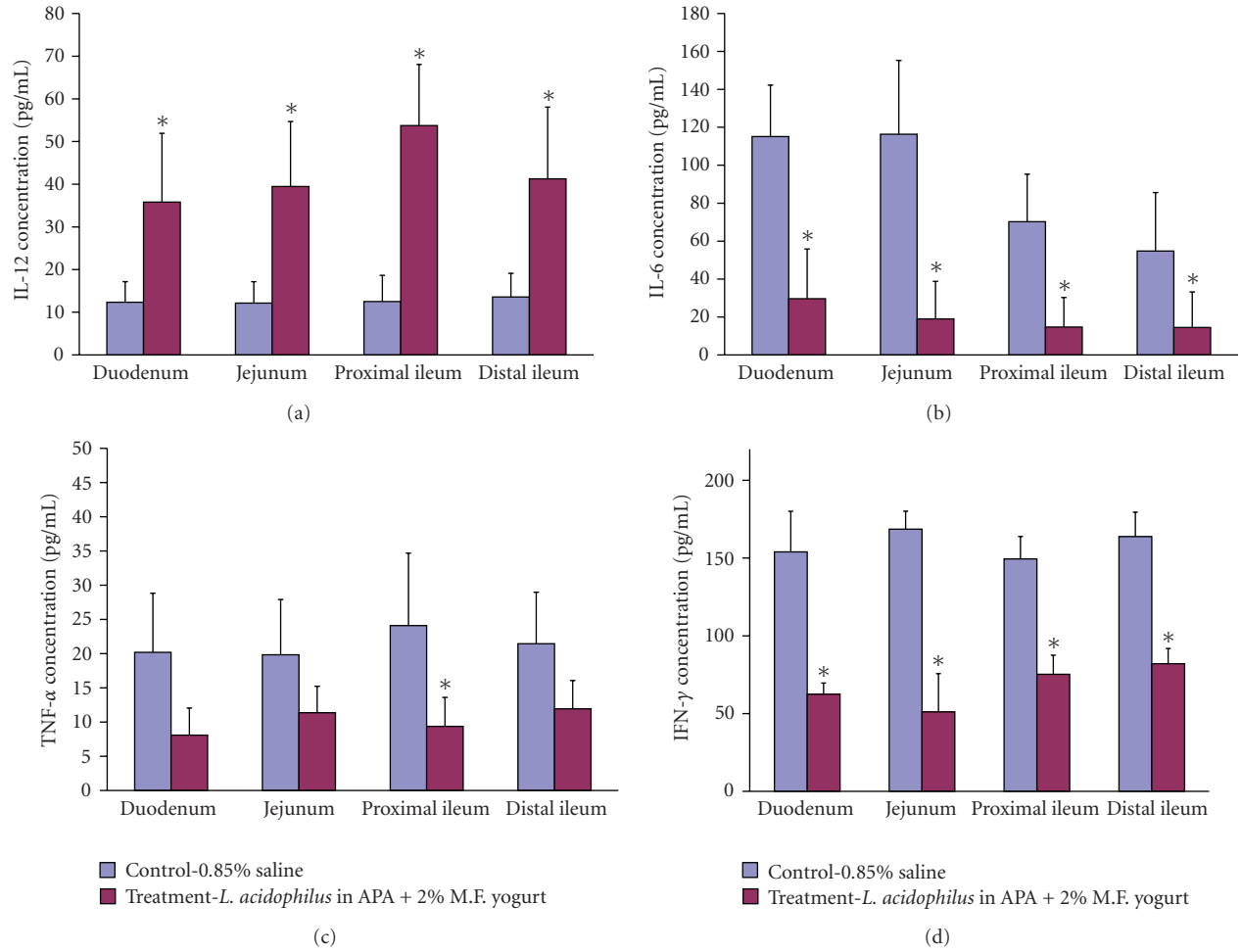

Figure 3: The effect of treatment on luminal cytokine concentration levels found in duodenum, jejunum, and proximal and distal ileum: IL-12 (a), IL-6 (b), TNF-α (c), and IFN-γ (d). Data represent the mean ± SD of concentration levels per group; $n = 24$. Asterisks: statistical differences ($P < .05$) when compared to control.

mechanism that reduces the growth of malignant tumors. Thus the animals were administered with microencapsulated probiotic bacterial cells daily. The assessment of animal health was achieved by measuring the various inflammation biomarkers at the time of sacrifice. Animals which received daily treatment with probiotic yogurt formulation were able not only to maintain their body weight but also to slightly increase it, which is a general indication of an overall health. They also had a higher food intake comparing to control animals. This may further imply that the rate of disease progression was slower.

Biomarkers are very beneficial to identify pathological processes before individuals become symptomatic or to identify individuals who are susceptible to cancer [35]. Luminal digesta obtained from the intestines were used to give an indication of IL-1β, IL-6, IL-12, PGE_2, Thromboxane B2, TNF-α, IFN-γ, and CRP levels in the gastrointestinal tract and CRP in the plasma at the time of sacrifice. Our results showed an overall trend indicating notably lower inflammation in the small intestines in the animals receiving daily treatment. As *Apc*Min mice develop spontaneous neoplasia predominantly in the small intestine as opposed to the colon, this study investigated and validated the inflammatory state of that organ. In the intestine, IL-1 has been shown to be an important inflammatory mediator whose levels are increased in inflammatory bowel disease [36, 37]. IL-1β is solely active in its secreted form whereas IL-1α is mainly active in cell-associated forms (intracellular precursor and membrane-bound IL-1α). IL-1α is only rarely secreted in a limited manner [38]. The assay showed higher concentrations of IL-1β in all sections of the small intestine in control group animals. In addition, IL-6 lower concentration and IL-12 higher levels were indicative of a slower progress of the disease in treatment group animals. Similar results were obtained for TNF-α and IFN-γ.

These findings are consistent with those from previous studies [39], which further proves that inflammation plays an important role in the development of cancer. Tumor invasiveness can be assessed by measuring thromboxane B2 and PGE_2. Therefore, we compared the levels of thromboxane B2 and PGE_2 in the small intestines. PGE_2 is one of the primary prostaglandins formed from the

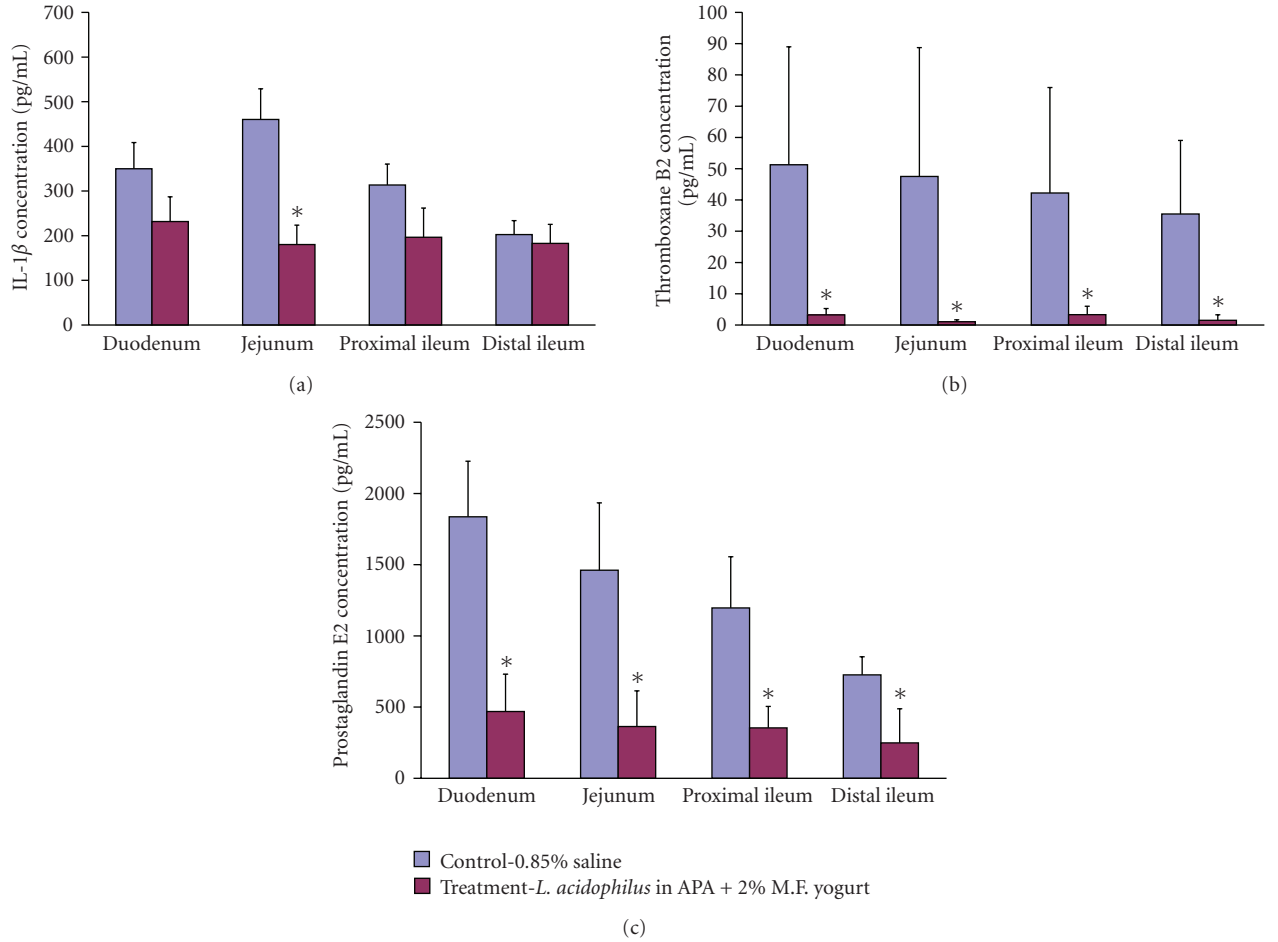

Figure 4: Luminal IL-1β (a), thromboxane B2 (b) and prostaglandin E_2 (c) concentration levels found in duodenum, jejunum, and proximal and distal ileum. Data represent the mean ± SD of concentration per group; $n = 24$. Asterisks: statistical differences ($P < .05$) when compared to control.

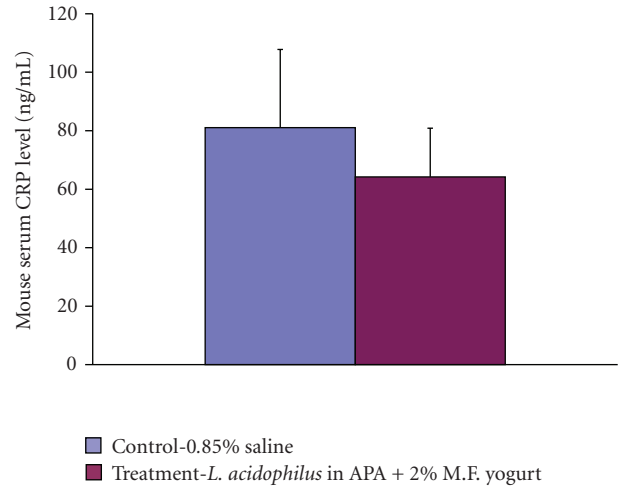

Figure 5: Comparison of plasma C-reactive protein (CRP) levels between control and treatment mice measured by enzyme-linked immunosorbent assay at the time of sacrifice; $n = 24$; error bars represent SD. Asterisks: statistical differences ($P < .05$) when compared to control.

coupled metabolism of arachidonic acid by the COX-1 and COX-2 and PGE synthases (microsomal and/or cytosolic). Moreover, its activity influences inflammation, fertility and parturition, gastric mucosal integrity, and immune modulation [40, 41]. Accumulating evidence suggests that PGE_2 has direct effects in enhancing colonic epithelial cell survival by stimulating cell proliferation and survival, tumor cells invasiveness, and production of angiogenic agents [42]. Higher concentrations of PGE_2 in control group animals further show intestinal inflammation of a greater extent when compared to treatment group animals. As it was postulated before, the inducible cyclooxygenase isoenzyme, COX-2, is significantly overexpressed at sites of inflammation and in various malignant tissues, with concomitant overproduction of the major arachidonate metabolite, PGE_2 [43].

Increased expression of CRPs has been described in several different malignancies, including colorectal [44], gastric [45], lung [46], renal [47], and breast [48] cancers. We have measured plasma concentrations of C-reactive protein from animals at the time of sacrifice. Elevated levels of CRP were detected in control group animals when compared

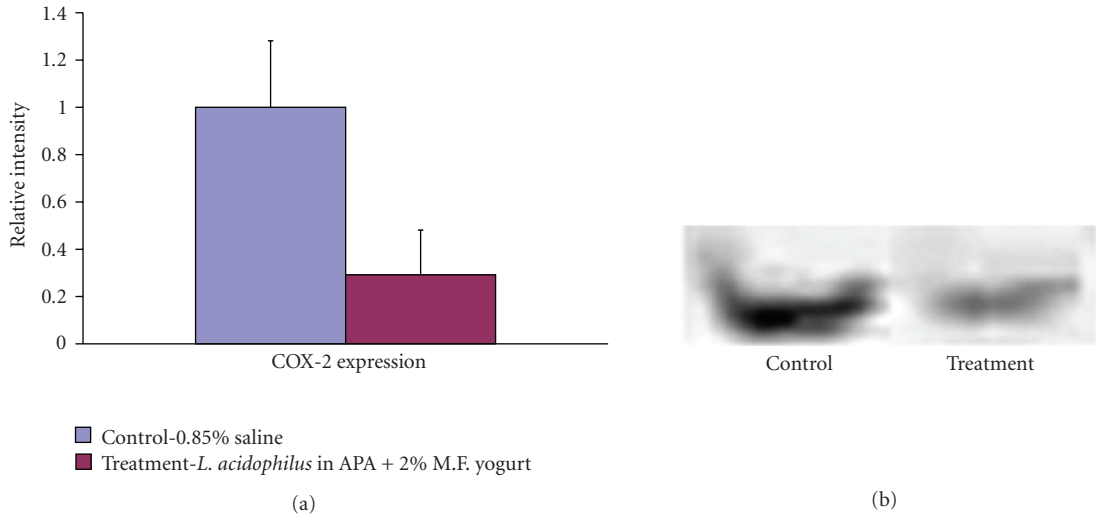

Figure 6: Western blot showing COX-2 expression found in homogenized distal ileum of small intestine tissues in control and treatment, animals, MW of COX-2-72 kD. Samples were pooled per treatment and the average expression was obtained for the total of 24 per group. The relative band intensities were calculated with Image J software ($P = .018$).

to animals receiving treatment. C-reactive protein remains significantly associated with a higher risk of colon cancer in *Apc*Min mice. Nonetheless, CRP is a nonspecific marker of inflammation, and additional studies of specific cytokines or factors that regulate acute-phase response are necessary to elucidate the mechanisms by which inflammation increases the risk of colon cancer.

COX-2 expression has a large impact on adenoma growth in *Apc*Min mice, where treatment with a COX-2-specific inhibitor is known to markedly reduce both the numbers and growth of adenomas [49]. Marked upregulation of COX-2 occurs in various cells including endothelial cells during stress and in inflammatory conditions such as sepsis. As COX-2 expression is induced by a number of cytokines including TNF-α and IL-1, mitogens or growth factors, lipopolysaccharide (LPS), and other inflammatory stimuli, it was of crucial importance to verify its expression levels. COX-2 levels were different between control and treatment groups, (we obtained 71% reduction of averaged inflammation level in treatment-receiving animals) as expected, further providing evidence that the animals in treatment group have lower intestinal inflammation when compared to control group animals.

As gastrointestinal intraepithelial neoplasias (GINs) are the precursors of adenomas and later of intestinal carcinoma, it was interesting to note the highest polyp counts of these lesions. Although *Apc*Min mice provide a genetically valid model for studying and understanding intestinal tumorigenesis, its major drawback is that it differs from the cancer development in humans. For instance, in human, carcinogenesis is a complex multistep, often including metastasis process. The polyps found in *Apc*Min mice do not undergo the process of metastasis [50]. Furthermore, adenomas in *Apc*Min mice occur primarily in the small intestine whereas tumors in human are generally restricted to the colon and rectum. To improve the effectiveness of polyp enumeration and classification, one needs to design an automated system (protocol) that would allow consistency by implementing universal guidelines and standards.

Several factors should be considered in the interpretation of our findings. A major strength of the current study is that it is a prospective study, and, thus, we can more confidently infer a temporal association between inflammation and the occurrence of colon cancer. Compared to other studies [51–53], we obtained greater reduction of inflammation in *Apc*Min mice due to oral treatment with formulation of microencapsulated probiotic bacterial cells and yogurt. Based on the results of the present study, it is not possible however, to explain the tumorigenesis in the *Apc*Min mouse model by immunological responses. Although confirmation of these results is clearly warranted, this finding, if true, could have implications for prevention strategies. Additional studies are needed to clarify the mechanism of bacterial activity and its impact on immunomodulating gastrointestinal tract.

6. Conclusions

In conclusion, daily oral administration of the microencapsulated probiotic formulation results in an overall decrease in total number of intestinal lesions and general functioning which leads to increasing host protection against various pathologies. This study supports the role for supplemental probiotics as a strategy both for suppressing inflammation and for preventing colon cancer.

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Review Article

The Bowel Microbiota and Inflammatory Bowel Diseases

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The human bowel contains a large and biodiverse bacterial community known as the microbiota or microbiome. It seems likely that the microbiota, fractions of the microbiota, or specific species comprising the microbiota provide the antigenic fuel that drives the chronic immune inflammation of the bowel mucosa that is characteristic of Crohn's disease and ulcerative colitis. At least twenty years of microbiological research have been expended on analysis of the composition of the bowel microbiota of inflammatory bowel disease patients in comparison to that of control subjects. Despite extensive speculations about the aetiological role of dysbiosis in inflammatory bowel diseases, knowledge that can be easily translated into effective remedies for patients has not eventuated. The causes of this failure may be due to poorly defined and executed bacteriological studies, as well as the overwhelming complexity of a biome that contains hundreds of bacterial species and trillions of bacterial cells.

1. Introduction

The large bowels of mammals comprise a biome (ecosystem) that includes a bacterial community that is biodiverse and numerous. It can be estimated that there are a total of twenty trillion bacterial cells per human colon if it is assumed that the colonic contents weigh on average about 200 grams. The majority of these bacteria are obligate anaerobes. They are commonly referred to as commensals or symbionts because they form long-lasting, interactive associations with their animal hosts [1, 2]. These associations are generally regarded to involve mutually beneficial interactions, although some commensals are opportunistic pathogens when appropriate predisposing events occur [3]. The bacterial communities (microbiota, microbiome) of human bowels contain predominantly representatives of four phyla: *Actinobacteria*, *Proteobacteria*, *Bacteroidetes*, and *Firmicutes* (Table 1). The *Firmicutes* and *Bacteroidetes* are numerically dominant in the community. Numerous genera and species of bacteria are represented in each of these broad phylogenetic groups and, at least in the case of humans, there is person-to-person variation in the composition of the bowel microbiota [4–6]. Descriptions of a "normal range" of bowel inhabitants of humans in terms of genera and species has recently begun to emerge, sometimes referred to as the "core" microbiota (5, 6; Table 2). The bowel microbiota is, like bacterial communities in general, self-regulating and this provides temporal stability in composition. At least in health, the proportions of broad phylogenetic groups of bacteria within the community remain much the same over time [4]. Inducible biochemical pathways that mediate hydrolysis of exogenous (dietary plant cell wall polysaccharides) and endogenous (mucins) substrates enable the bacteria to obtain an uninterrupted supply of carbon and energy despite daily changes in the composition of the human diet [7, 8]. The fermentation of hydrolytic products by the bacteria produces short chain fatty acids, amines, phenols, indoles, and gases [9]. Knowledge of the nutritional niches of specific bacterial groups is sparse but a detailed description and importance of the butyrate-producing species in the ecology of the bowel is emerging due to work at the Rowett Institute, Scotland [10, 11]. Partitioning of bacteria within the contents of the bowel occurs. Some bacterial types are more likely to be associated with particulate (plant residues) material in the digesta than in the liquid fraction [12]. The mucus associated with the mucosal surface has a layered conformation and minimizes bacterial contact with the surface of enterocytes. The outer mucus layer is loosely structured and contains bacterial cells that are perhaps living there or are trapped cells that will eventually be swept away by the mucus flow. The

inner mucus layer is dense and devoid of bacteria in healthy people [13]. Nevertheless, bacteria-enterocyte signaling has been demonstrated in laboratory animal models [14]. This suggests that at least bacterial cell components or products pass through the mucus and reach receptors on the surface of enterocytes. These signaling events have been demonstrated to affect physiological processes even in systemic organs [15], as well as affecting mucosal immunology in which enterocytes and dendritic cells act as front-line mediators [16, 17]. Thus, the complex, numerous, and interactive microbiota of the bowel cannot be overlooked in considerations of the aetiology of inflammatory bowel diseases.

Crohn's disease (CD) and ulcerative colitis (UC) are chronic immune inflammatory conditions of the alimentary tract referred to collectively as inflammatory bowel diseases (IBD). There are strong genetic associations with susceptibility to CD in particular [18]. Environmental factors such as stress and diet are probably also important in the aetiology of IBD, but remain poorly delineated [19]. CD lesions can occur in upper regions of the alimentary tract but are usually located where there are microbial residents (ileum and colon), whereas UC is limited to the large bowel. In both diseases, therefore, a case can be made that the sheer antigenic load represented by trillions of bacterial cells will have an important role in driving chronic inflammation of the bowel mucosa [20, 21]. Experimental animal models of colitis do not exactly mimic CD or UC but can be used to examine the role of specific bacteria in the etiology of colitis in general terms. The results of this work provide the clearest evidence that bacteria resident in the bowel have an essential role in the pathogenesis of colitis because, when maintained germfree, the animals do not develop disease [22]. Two major reviews of publications relating to bacterial involvement in inflammatory bowel diseases have been published recently [23, 24]. Therefore this paper provides an appraisal of current views rather than a definitive coverage of the whole scientific literature.

2. Analysis of the Bowel Microbiota

Total microscope counts of the bacteria in human faces are at least two-fold greater than colony counts obtained by nonselective agar cultures [25]. Experiments using molecular exclusion probes show that the majority of the bacterial cells in feces are alive [26]. The results of nucleic acid-based analytical methods applied to bulk DNA extracted from stool or bowel samples (Table 3) have provided evidence that the discrepancy between total and colony counts is due to the presence of not-yet-cultivated bacteria [27]. In addition to providing a means of analysis of bacterial communities containing noncultivable members, culture-independent methods based on bacterial nucleic acids are useful because faecal and other samples can be collected, frozen, and dispatched to an analytical laboratory that may be far distant from the location in which the human subjects reside. Bacterial culture, on the other hand, requires that samples be processed without freezing within a few hours of collection. Thus, nucleic acid-based analyses of the bowel microbiota have held sway during the past two decades and have enabled extensive phylogenetical analysis of bowel communities to be made [6, 28]. The major weakness of nucleic acid-based analytical methods, however, is that they do not differentiate between DNA from bacteria that are actually active members of the community and transients or relatively inactive cells; DNA sequences from dead, quiescent, and metabolically active bacterial cells are all detected by methods based on the extraction of bulk DNA from human faeces [29]. Further, bacterial metagenomic sequences garnered from the bowel biome show the metabolic potential of the microbiota and its phylogenetic composition, but cannot reveal the temporal changes in expression of bacterial genes in the bowel, although this will be achievable through metatranscriptomics studies [8, 28]. While culture-independent studies have been indispensable in modern microbial ecological studies and have yielded a vast amount of information about the phylogeny and metabolic potential of the bowel microbiota, there is a need to return to culture-dependent studies. Such studies have been largely abandoned in recent times because so many of the bacterial inhabitants of the bowel were considered to be unculturable. New enrichment culture methods hold hope that the so far uncultivated bacteria will soon be cultured. Culture of bowel bacteria that have specific metabolic or antigenic properties would greatly enhance gene knockout or transgenic, gnotobiotic animal studies. Ranking bowel bacteria in terms of proinflammatory potential might be a worthwhile exercise and could probably only be achieved with cultivated species in combination with in vitro or in vivo immunological systems. The immune system of CD and UC patients has been clearly shown to be dysregulated and that this is associated with mutations in distinct genetic loci [20, 21]. Therefore, the balance between the bacterial inhabitants of the bowel and the mucosal immune system has to be considered to be different to that prevailing in healthy subjects. This assumption must underpin all considerations of the role of the bowel microbiota in inflammatory bowel diseases.

3. Confounding Factors in Analysis of the Microbiota

Bacteriological analysis of the bowel microbiota is fraught with several difficulties that often confound valid interpretation of results.

3.1. Sampling. Bacterial communities in stool reflect the bacteriology of the rectum and do not offer much ecological knowledge of other regions of the digestive tract [30]. Mucosal biopsies, unlike stool, provide samples collected from regions of the intestinal tract where inflammation occurs. They are not perfect specimens for bacteriological analysis, however, because they consist of only a few milligrams of tissue and have been collected from subjects that have usually undergone bowel cleansing prior to endoscopy. Residual bowel cleansing solution pools in the large bowel and can be collected by aspiration. The aspirate, essentially

Table 1: Common terms used in bowel microbial ecology.

Phylogeny	The history of organismal lineages as they change through time. It implies that different species arise from previous forms via descent, linking all forms of life.
Dysbiosis	A term generally used in relation to the bowel biome indicating an imbalance in the composition of the microbiota.
Firmicutes	A phylum of bacteria, most of which have gram-positive cell wall structure. The principal genera detected in human faeces are *Clostridium*, *Eubacterium*, *Anaerostipes*, *Coprococcus*, *Dorea*, *Lachnospira*, *Roseburia*, *Faecalibacterium*, *Ruminococcus*, *Subdoligranulum*, and *Coprobacillus*.
Bacteroidetes	A phylum of bacteria that have gram-negative cell wall structure. The principal genera detected in human faeces are *Bacteroides*, *Parabacteroides*, and *Alistipes*.
Actinobacteria	A phylum of gram-positive bacteria that includes, amongst others, the genera *Bifidobacterium* and *Collinsella* that are often detected as members of the bowel microbiota of humans.
Proteobacteria	A phylum of bacteria that includes *Escherichia coli*, a common facultatively anaerobic species in the bowel.
Bacteroides-Prevotella cluster	A broad phylogenetic classification comprising gram-negative, anaerobic species forming a major portion of the bowel microbiota.
Clostridial cluster XIVa.	A broad phylogenetic classification comprised of several genera and species of gram-positive bacteria, not exclusively clostridia.
Clostridial cluster IV	A broad phylogenetic classification comprised of several genera and species of gram-positive bacteria, not exclusively clostridia.
Enrichment culture	An understanding of the environmental conditions favored by an organism, together with genetic clues about the microbe's abilities is used to guide the design of culture media and conditions.
Probiotic	Live microorganisms which when administered in adequate amounts confer a health benefit on the host.
Prebiotic	A dietary supplement of nondigestible carbohydrate (inulin and fructo-oligosaccharides are the best known) that can be metabolized by particular bacteria in the human colon.

a faecal solution, bathes the mucosal surface of the intestine and in all likelihood contaminates it, as well as contaminating the endoscope and its mechanical parts that collect the tissue sample. Despite washing of the biopses immediately after collection, the TTGE profiles of biopsy-, aspirate-, and faecal bacteria have been reported to be highly (about 80% on average) similar [31]. This result supports the view that bacteria detected in association with biopsies are mostly contaminants from a faecal solution (aspirate) that pools in the bowel and bathes the mucosal surface after bowel cleansing.

3.2. Individuality and Nationality of Subjects. The composition of bowel communities, as judged by the results of stool and biopsy analysis, is individualistic, extending even down to the level of bacterial strains [32, 33]. Adding to the complexity of the situation, it has been shown that TTGE profiles generated from bacteria associated with biopsies were influenced by nationality of the donors: Mexican biopsy-associated profiles could be differentiated from those of Canadians [31]. This might be a particularly important observation because Canada has the highest incidence and prevalence of Crohn's disease yet reported, whereas this disease is rare in Mexico [34, 35]. Thus, the bowel community of Canadians may contain commensals critical to fueling chronic immune inflammation, whereas most Mexicans may lack them. It was noteworthy that TTGE profiles of Canadians commonly contained DNA fragments originating in members of the *Bacteroidetes*, a bacterial phylum that has been detected more commonly in association with biopsies of Crohn's disease patients, as well as producing bowel inflammation in an experimental animal model of colitis [36, 37].

3.3. Choice of Subjects. Most studies involving bowel commensals have been weak in terms of statistical power because only small numbers of patients are studied [24]. Carefully matched patients and controls need to be recruited, and sampling of patients and controls on several occasions would provide more reliable results. Previous or concurrent therapeutic drugs administered to patients, although not antimicrobial, may nevertheless alter bowel physiology, and hence community composition, relative to that of controls.

4. The Bowel Microbiota and Inflammation

There are several ways in which the microbiota might be linked to Crohn's disease or ulcerative colitis: the microbiota as a whole could act as a surrogate pathogen; specific members of the microbiota could be overt pathogens and incite mucosal inflammation; changes in the proportions of phylogenetic groups comprising the microbiota could

Table 2: Common species in human faeces. Seventy five bacterial species with >1% genome sequence coverage in >50% of 124 adult humans. After Qin et al. [6]

Faecalibacterium prausnitzii	*Bacteroides fragilis*
Roseburia intestinalis	*Eubacterium biforme*
Dorea formicigenerans	*Bacteroides eggerthii*
Bacteroides vulgatus	*Streptococcus thermophilus*
Clostridium sp	*Bacteroides capillosus*
Bacteroides uniformis	*Holdemania filiformis*
Eubacterium hallii	*Clostridium leptum*
Bacteroides sp.	*Prevotella copri*
unknown sp	*Clostridium sp.*
Coprococcus comes	*Bacteroides plebeius*
Eubacterium rectale	*Butyrivibrio crossotus*
Ruminococcus sp.	*Bacteroides coprocola*
Dorea longicatena	*Bacteroides finegoldii*
Bacteriodes xylanisolvens	*Clostridium bartlettii*
Bacteroides sp.	*Clostridium sp.*
Bacteroides sp.	*Escherichia coli*
Ruminococcus torques	*Parabacteroides johnsonii*
Bacteroides sp.	*Subdoligranulum variabile*
Alistipes putredinis	*Bacteroides intestinalis*
Collinsella aerofaciens	*Catenibacterium mitsuokai*
Parabacteroides distasonis	*Clostridium bolteae*
Eubacterium siraeum	*Bifidobacterium pseudocatenulatum*
Bacteroides ovatus	*Anaerotruncus colihominis*
Bacteroides sp.	*Bifidobacterium catenulatum*
Bacteroides sp.	*Ruminococcus gnavus*
Bacteroides thetaiotaomicron	*Bacteroides coprophilus*
Bacteroides dorei	*Bacteroides pectinophilus*
Parabacteroides merdae	*Gordonibacter pamelaeae gen. nov. sp.*
Bifidobacterium longum subsp. infantis	*Clostridium asparagiforme*
Ruminococcus obeum	*Clostridium nexile*
Bifidobacterium adolescentis	*Blautia hansenii*
Bacteroides caccae	*Clostridium scindens*
Ruminococcus bromii	*Enterococcus faecalis*
Ruminococcus lactaris	*Mollicutes bacterium*
Eubacterium ventriosum	*Bryantella formatexigens*
Coprococcus eutactus	*Clostridium methylpentosum*
Akkermansia muciniphila	
Bacteroides stercoris	
Bacteroides cellulosilyticus	

initiate or perpetuate the inflammation by providing a pathogenic, antigenic fuel. Alternatively, changes in composition could remove members of the microbiota that normally inure the mucosal immune system to the presence of commensals in the bowel.

4.1. The Unaltered Microbiota Acts as a Surrogate Pathogen. This hypothesis concerning the pathogenesis of inflammatory bowel diseases does not invoke changes to the bacteriology of the bowel. It is supported by the observation that the bacterial profiles associated with biopsies are not different between inflamed and noninflamed mucosa [36, 38]. In this proposition, genetic predisposition of patients to abnormal permeability of the bowel mucosa allows entry of commensal antigens into subepithelial tissues. Cells of the immune system are activated as if infection by an invasive pathogen had occurred. Impaired regulation of the subsequent immune inflammation, again due to genetic predisposition, results in a chronic immune inflammation; the immune response is poorly regulated and the permeable epithelium allows constant movement of antigens into the tissue so as to resemble a continuing infection [39]. In this

Table 3: Commonly used nucleic acid-based (culture-independent) analytical methods.

PCR/denaturing electrophoretic polyacrylamide gels (PCR/DGGE, PCR/TTGE)
DNA is extracted directly from faecal samples. Hypervariable gene sequences (most often 16S rRNA) are amplified using PCR primers that anneal with conserved sequences that span the selected hypervariable regions. One of the PCR primers has a GC-rich 5′ end (GC clamp) to prevent complete denaturation of the DNA fragments during gradient gel electrophoresis. Using 16S rRNA gene sequences as example, amplified fragments from different types of bacteria and present in the PCR product are separated using polyacrylamide gel electrophoresis. In DGGE (denaturing gradient gel electrophoresis), the double-stranded 16S fragments migrate through a polyacrylamide gel containing a gradient of urea and formamide until they are partially denatured by the chemical conditions. The fragments do not completely denature because of the GC clamp, and migration is radically slowed when partial denaturation occurs. Because of the variation in the 16S sequences of different bacterial species, chemical stability is also different; therefore, different 16S "species" can be differentiated by this electrophoretic method. Similarly, in TTGE (temporal temperature gradient electrophoresis), the 16S sequences can be separated by gradually increasing the temperature of the polyacrylamide gel during electrophoresis. Separation is achieved on the basis of differing temperature stability of the 16S fragments. These methods generate a profile of the numerically predominant members of the bacterial community. Individual fragments of DNA can be cut from DGGE/TTGE gels, further amplified and cloned, then sequenced. The sequence can be compared to those in gene databanks in order to obtain identification of the bacterium from which the 16S sequence originated. Depending on the length of the sequence, identification to at least bacterial genus can be made.
Fluorescent in situ hybridization/fluorescence-activated cell sorting (FISH/FC)
DNA (oligonucleotide) probes target specific rRNA sequences (16S or 23S) within ribosomes to which they hybridize. The probes are 5′ labelled with a fluorescent dye which permits both detection and quantification of specific bacterial populations. Bacterial cells within which hybridisation with a probe has occurred fluoresce and hence can be detected and counted by epifluorescence microscopy (preferably automated) or fluorescence-activated flow cytometry. Continual reassessment of the specificity and coverage of FISH probes is essential in order to update and confirm their continuing specificity and hence reliability. This is because new 16S rRNA gene sequences are constantly added to databases. Epifluoresence microscopic detection is laborious and time consuming, and manual microscopic enumeration requires careful attention by the operator. A lower detection limit of about 10^6 bacteria per gram of faeces can be achieved. An automated method of counting fluorescent bacterial cells has been developed by coupling fluorescence microscopy to a computerized system of image analysis. Using this automated counting device, the lower detection threshold has been estimated to be 10^7 bacteria per gram of faeces. Therefore, only the more numerous members of the bacterial community can be detected. Nevertheless, identification of individual bacterial cells, as well as morphological and topographical information are valuable characteristics of fluorescence microscopy. Combined with flow cytometry, FISH provides a high throughput quantitative and qualitative method of analysis. Flow cytometry combines quantitative and multiparametrics analysis (size, internal granularity, fluorescence signal). A lower threshold of detection of 0.4% relative to the total number of bacteria determined with the universal bacterial probe EUB338 has been demonstrated.
Quantitative PCR
PCR primers and fluorescent probes targeting nucleic acid sequences, usually 16S rRNA gene sequences, which are unique to particular bacterial species, are used to quantify the specific sequences in DNA extracted from faeces. Real-time quantitative PCR can be used to quantify specific populations or phylogenetic clusters using specific PCR primers and fluorescent probes. Target sequences in DNA are amplified and simultaneously quantified (as absolute number of copies, or relative amount when normalized to DNA input, or by reference to additional normalizing genes). The procedure follows the general principle of PCR but its key feature is that the amplified DNA is detected as the reaction progresses in *real time* in contrast to standard PCR where the product of the reaction is detected at its end. Two common methods for detection of products in real-time PCR are: non-specific fluorescent dyes that intercalate with any double-stranded DNA, or (2) sequence-specific DNA probes consisting of oligonucleotides that are labelled with a fluorescent reporter which permits detection only after hybridization of the probe with its complementary DNA target.
16S rRNA gene phylogeny
Older studies utilised PCR amplification of 16S rRNA genes from bulk DNA extracted from faeces followed by cloning the 16S rRNA gene sequences in a plasmid vector in an *Escherichia coli* host, prior to sequencing. More recently, high throughput bead/emulsion-based sequencing of PCR-amplified DNA or random sequencing of DNA fragments derived from bacterial communities in faeces (metagenome) has been used. These approaches provide catalogues of the constituent bacterial types (usually broad phylogenetic groups) of the community when analysed in relation to a databank of 16S rRNA gene sequences (such as the Ribosomal Database Project pyrosequencing pipeline tools).
Metagenomics
A microbial community is studied in terms of its collective genomes. Nowadays, this approach involves shotgun genome methods to sequence random fragments of DNA from microbes in a sample collected from a biome of interest. DNA is directly extracted from the sample, is broken into small fragments, and portions of these fragments are sequenced. Searches of DNA sequence databases permit collation of the sequencing information in terms of 16S rRNA genes (biodiversity), genes associated with metabolic pathways including their potential regulation, and cell structural molecules. This methodology can reveal novel and fundamental insights about the biodiversity and metabolic impacts of microbial life in biomes.
Metatranscriptomics
Measuring the transcriptomics (gene expression) of microbial communities in the wild. RNA (which includes mRNA) is extracted directly from samples. Ribosomal RNA, which forms the major portion of the total RNA is removed. Then, remaining RNA is converted to cDNA by reverse-transcription PCR. Random sequencing of the cDNA reveals the transcripts produced in the ecosystem. This approach has mostly been used with oceanic samples but application of the methodology to bowel samples is possible.

scenario, it is the antigens that pass to the subepithelial tissues that are relevant, rather than phylogenetic issues. It may be more useful to identify bacterial antigens against which the immune cells of Crohn's disease and ulcerative colitis patients react rather than to determine shifts in community composition. Knowledge of these antigens might aid diagnosis, as in the case of the CBir1 flagellin against which Crohn's disease patients with complicated conditions produce high antibody titres, or be included in assays useful in the development of anti-inflammatory drugs [40].

4.2. Specific Members of the Microbiota Act as Overt Pathogens and Incite Mucosal Inflammation. The concentration of enterobacteria in CD may be increased [24] and they may be phenotypically different from commensal *Escherichia coli*. Darfeuille-Michaud et al. isolated *E. coli* from resected chronic ileal lesions and from neoterminal ileum (with and without CD recurrence) after surgery [41]. Many of the isolates from diseased ileum adhered to Caco-2 cells. These authors confirmed, in a subsequent study, that an adherent-invasive type of *E. coli* (reference strain LF82) was specifically associated with ileal mucosa in some CD patients [42].

Bifidobacterium animalis has been shown to be more prevalent in colitic mice. In this study, the colonic microbiota of formerly germfree interleukin 10 (IL-10)-deficient mice that had been exposed to the faecal microbiota of specific-pathogen-free animals was screened using PCR/DGGE. The composition of the large bowel microbiota of IL-10-deficient mice changed as colitis progressed. DNA fragments originating from four bacterial populations ("*Bacteroides* sp.", *Bifidobacterium animalis*, *Clostridium cocleatum*, enterococci) were more apparent in PCR/DGGE profiles of colitic mice relative to noncolitic animals, whereas two populations were less apparent (*Eubacterium ventriosum*, Acidophilus group lactobacilli). Specific DNA:RNA dot blot analysis showed that bifidobacterial rRNA abundance increased as colitis developed [43]. In a subsequent experiment, monoassociation of IL-10-deficient mice with *Bifidobacterium animalis* subspecies *animalis* resulted in bowel inflammation, thus fulfilling Koch's postulates [44]. In this study, a bacterial species belonging to a genus (*Bifidobacterium*) generally regarded as harmless in the bowel and possibly even "beneficial", was shown to have pathogenic potential in immunologically dysregulated animals.

Chronic or recurrent pouchitis (CP) is the most important long-term complication leading to poor function following ileal pouch-anal anastomosis for ulcerative colitis. Antibiotic administration reduces symptoms of pouchitis indicating that bacteria have a role in pathogenesis [45, 46]. The stool microbiota of patients with pouchitis and of familial adenomatous polyposis patients has been shown to be markedly different [47]. PCR/TTGE profiles of the stool microbiota of familial adenomatous patients ($n = 14$) clustered at the 80% level of similarity, whereas those of pouchitis patients ($n = 17$) were disparate. The results of FISH analysis showed that bacteria not commonly present in human faeces, nor in the stool of familial adenomatous polyposis patients, comprised about 50% of the stool microbiota of untreated pouchitis patients. Antibiotic treatment reduced the proportion of these unknown bacteria in the stool of pouchitis patients. Therefore, chronic or recurrent pouchitis was found to be associated with a microbiota that contained bacteria not commonly associated with human faeces or FAP pouches. *Clostridium perfringens* was detected (comprising about 30% of the total bacterial community), by quantitative PCR, among these less common bacteria in symptomatic pouches of 10 out of 17 (58.8%) CP patients. This species was not detected in the same pouches when asymptomatic. *C. perfringens* was detected in some normal pouches (no inflammation), but in numbers about 30-fold lower than in pouchitis. [47; Tannock and Thompson-Fawcett, unpublished data]. Other studies have also reported the detection of *C. perfringens* in pouchitis samples from UC patients. For example, Ruseler-van Embden et al [48]. analyzed the bacterial composition of the ileal reservoir from patients that had undergone a restorative proctocolectomy either for ulcerative colitis ($n = 12$) or familial adenomatous polyposis ($n = 2$). The study was carried out at least one year after the surgery and five patients were diagnosed with pouchitis. Two fecal samples were collected from each subject of the pouch control group ($n = 9$) with an interval of at least two months. Plate counts showed large differences in the anaerobic bacterial composition between two samples taken at different times suggesting the non-inflamed pouch had a bacterial community of unstable composition. Compared to the control group, stool from pouchitis patients contained a larger number of *Clostridium perfringens.* Gosselink et al [49]. Monitored the fecal microbiota of patients diagnosed with ulcerative colitis and having undergone a pouch construction ($n = 13$). The aim of this study was to compare the effect of two antibiotics, metronidazole and ciprofloxacin, on the fecal microbiota at different times. The bacteriological content of the pouch was analyzed at the beginning of an inflammatory episode before antibiotic treatment, during treatment with ciprofloxacin or metronidazole, and during pouchitis-free periods. Higher numbers of *C. perfringens* and hemolytic strains of *E. coli* were observed during pouchitis episodes in 50% of the patients. Administration of metronidazole eradicated the *Clostridium perfringens*. Treatment with ciprofloxacin inhibited not only the growth of *Clostridium perfringens* but also that of coliforms, including hemolytic strains of *Escherichia coli*. Taken together, these results offer the possibility that *C. perfringens* is the aetiolgical agent of pouchitis in some patients. The absence of *C. perfringens* in non-inflamed pouches (antibiotic administration) but its presence in inflamed pouches (no antibiotic) probably satisfies Koch's third postulate ("the isolated microbe, when administered to humans or animals must cause disease") in modified form.

Duffy et al. [50] compared the pouch bacterial content from ulcerative colitis ($n = 10$) and familial adenomatous polyposis ($n = 7$) patients. None of the patients had had a previous episode of pouch inflammation. Sulphate-reducing bacteria were exclusively detected in pouches of ulcerative colitis patients. Ohge et al. [51]) have also shown an

association between sulphate-reducing bacteria and pouches. Sulphate-reducing bacteria were detected in higher numbers in active pouchitis patients ($n = 8$) in comparison to patients without a history of pouchitis ($n = 8$), patients with past episode(s) of pouchitis ($n = 18$), patients having an ongoing antibiotic treatment for pouch inflammation ($n = 11$) and familial adenomatous polyposis patients ($n = 5$). The authors observed that this particular group of bacteria was sensitive to metronidazole or ciprofloxacin treatment.

4.3. Changes in the Proportions of Phylogenetic Groups Comprising the Microbiota. A decrease in the number of phylotypes (reduced biodiversity) has been reported in relation to the fecal community of CD patients [52–56]. Scanlan et al.[53], for example, reported that they failed to detect members of clostridial cluster IV in 27% of CD fecal samples (PCR) but all control samples contained these bacteria ($P < .0001$). Taken together, the results of these studies showed a quantitative and a qualitative (biodiversity) reduction in representation of the *Firmicutes* phylum, and particularly clostridial cluster IV members in the feces of CD patients. This phylogenetic group contains several butyrate producing bacteria, such as *Faecalibacterium prausnitzii* [55]. Butyrate and other short chain fatty acids are believed to be important sources of energy for colonic epithelial cells and may have anti-inflammatory properties, as well as improving barrier function of the bowel epithelium [57–60]. Hence, the decrease in butyrate-producing bacteria in the colon might have an overall detrimental effect on the colonic mucosa. The major finding of metagenomic studies to date has been, therefore, the reduced biodiversity of the microbiota. It is not clear whether the reduced biodiversity initiates inflammatory bowel diseases, perpetuates the diseases, or is a result of the diseases. Nor can a change in the composition of the faecal microbiota (representing the rectum) explain CD lesions in the small bowel. A reduction in biodiversity has been reported for both CD and UC patients which suggests that the changes in microbiota composition are due to the inflamed state of the bowel. The pathology and immunology and pathogenesis of CD and UC are distinctly different and it will be surprising if the bacterial aetiology, should there be one, is the same for both diseases. Much ingenious experimentation will be required to end speculation about these changes and to produce knowledge of benefit to medical practitioners and their patients.

5. Conclusions

Improvements to the quality of microbiological investigations of Crohn's disease and ulcerative colitis patients will rely on the more careful selection of patients (perhaps aided by human genotyping because of the variety of genetic polymorphisms associated with Crohn's disease), the recruitment of newly diagnosed and untreated patients, and greater attention to the way in which specimens to be used in microbiological investigations are collected. Overall, more thoughtful planning of studies aimed at analysis and comparison of commensal communities are needed in order to improve microbiological studies in Crohn's disease and ulcerative colitis. Analysis of the microbiota of CD and UC patients has so far resulted in diverging views of the importance of particular bacteria in the pathogenesis of inflammatory bowel diseases. Nevertheless, strong cases for the involvement of adherent/invasive *E. coli* in ileal lesions of CD are building, and for the role of *C. perfringens* in some cases of pouchitis in ulcerative colitis patients.

Reduced biodiversity of the microbiota in CD seems, however, to be the current popular choice of aetiological significance, especially since it provides the opportunity to develop probiotic strains of *F. prausnitzii* or other butyric acid-producing bacteria. Implantation or enrichment (by concurrent prebiotic administration) of a cultivated strain in the inflamed bowel seems an unlikely proposition since the prevailing intestinal conditions associated with disease have apparently led to the observed reduction or elimination of the species. Moreover, inflamed mucosa seems to have impaired ability to utilize butyrate [61]. Culture supernatants of *F. prausnitzii* and some other butyrate-producing bacteria appear to contain anti-inflammatory substances that might be purified and used to treat patients. Detection and testing of these presumptive anti-inflammatory substances relies on the use of experimental animal models of colitis and it is not clear yet whether the research can be translated to treat human patients. Meta-analysis of seven studies that tested the effect of maintenance treatment with standard probiotics (*Lactobacillus rhamnosus* GG, *Escherichia coli* Nissle 1917, VSL#3, *Saccharomyces boulardii*) among patients with Crohn's disease in remission did not demonstrate any benefit of probiotic treatment [62]. Elahi et al. [63], conducted a meta-analysis of five trials on the effect of probiotics on pouchitis (acute, chronic, and recurrent remission). Four of these studies (conducted by the same research group in each case) utilized the multi-strain (*Lactobacillus casei*, *Lactobacillus plantarum*, *Lactobacillus acidophilus*, *Lactobacillus delbrueckii* subspecies *bulgaricus*, *Bifidobacterium longum*, *Bifidobacterium breve*, *Bifidobacterium infantis*, and *Streptococcus thermophlus*) probiotic product VSL#3. The outcome of interest in the meta-analysis was for pouchitis defined as a pouchitis disease activity index of >7.0. Pooling of the results from five trials yielded an odds ratio of 0.04 (95% confidence interval 0.01–0.14, $P < .0001$) in the probiotic group relative to the placebo group. These kinds of results continue to encourage the continuing development of probiotic therapies.

An alternative approach may be to gain information about potent antigens associated with the bowel microbiota that drive the inflammatory response. Treatment options would then be to selectively use novel or extant antibiotics to reduce the fraction of the microbiota producing the most potent antigens, or to derive drugs that would effectively block signaling pathways stimulated by these antigens. After at least twenty years interest in the microbiology of inflammatory bowel diseases without significant accrual of knowledge that can be translated to improved treatments for patients, new thinking and new research approaches are clearly indicated.

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Review Article

Inflammatory Bowel Diseases: When Natural Friends Turn into Enemies—The Importance of CpG Motifs of Bacterial DNA in Intestinal Homeostasis and Chronic Intestinal Inflammation

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From numerous studies during the last years it became evident that bacteria and bacterial constituents play a decisive role both in the maintenance of intestinal immune homeostasis as well as in the development and perpetuation of chronic intestinal inflammation. In this review we focus on the role of bacterial DNA which is a potent immunomodulatory component of the bacterial flora. Bacterial DNA has been shown to be protective against experimental colitis. In contrast bacterial DNA essentially contributes to the perpetuation of an already established chronic intestinal inflammation in a Toll-like receptor (TLR)9-dependent manner. This dichotomic action may be explained by a different activation status of essential regulators of TLR signaling like Glycogen synthase kinase 3-β (GSK3-β) depending on the pre-activation status of the intestinal immune system. In this review we suggest that regulators of TLR signaling may be interesting therapeutic targets in IBD aiming at the restoration of intestinal immune homeostasis.

1. Introduction

Chronic intestinal inflammation as found in Inflammatory Bowel Diseases (IBD) is common in developed countries with a prevalence of about 0.5% and, according to the chronicity and the early onset of disease, has an enormous socioeconomic impact. Despite great advances in the understanding of IBD in the past ten years the aetiology of both Ulcerative Colitis (UC) and Crohn's disease (CD) remains elusive.

The spectrum of possible explanations reaches from the autoimmune hypothesis which seems to be supported by the existence of different autoantibodies against epithelial cells in IBD (reviewed in [1]) to the statement that IBD should be considered as an infectious disease [2] which may develop based on a defective immune response [3, 4].

Although some truth may lay in every specific hypothesis the most likely pathophysiologic explanation according to the current knowledge is that commensal luminal bacteria with which we normally live in friendly or even symbiotic coexistence are mistakenly recognized as possible pathogens resulting in a chronic inflammatory response.

Obviously, both complex environmental [5] and genetic factors [6] contribute to the development of IBD. The most important susceptibility gene described for CD, the NOD2 gene [7, 8], participates in the innate immune response to the bacterial wall component muramyl dipeptide (MDP). Recently, strong genetic interactions between polymorphisms in CD-associated variants of NOD2, IL23R, DLG5, and Toll-like receptor 9 (TLR9, coding for the intracellular receptor which recognizes bacterial DNA) have been demonstrated [9]. Taken together, these data argue for a central role of interactions between bacteria and the mucosal immune system in the pathogenesis of CD. Supporting this view, studies from several murine IBD models underlined the importance of the bacterial flora in the pathogenesis of chronic intestinal inflammation [10].

2. Proinflammatory Potential of Bacterial DNA: CpG-Motif-Containing DNA Contributes to the Perpetuation of Chronic Colitis

During the last years, several groups aimed to characterize the role of numerous bacterial constituents in murine colitis models. These studies were put on a new basis by the identification of pattern recognition receptors for microbial constituents including the Toll-like receptors (TLRs). We and others focused on the role of bacterial DNA as a specific bacterial constituent based on its ability to mount a strong Th_1-skewed immune response resulting in extraordinary high production of tumor necrosis factor (TNF), interferon (IFN)-γ, or Interleukin (IL)-6 [11–14], which is also found in experimental and human IBD. Ten years ago, unmethylated cytosine-guanosine (CpG) sequences were identified as the immunostimulatory component of microbial DNA [15], and five years later, TLR9 was identified as the receptor responsible for CpG recognition [16].

In a first approach, we could demonstrate in different models of murine colitis that treatment with CpG-containing oligodeoxynucleotides (CpG-ODN) results in an exacerbation of established intestinal inflammation [17]. Vice versa, TLR9-deficient mice which do not respond to CpG motifs showed a more than 50% reduced chronic intestinal inflammation two months after the induction of chronic dextran sodium sulphate- (DSS-) induced colitis. The latter finding further indicates an important contribution of commensal-derived CpG motifs to the perpetuation of chronic intestinal inflammation. This was further confirmed by a strong therapeutic effect of adenoviral-derived inhibitory motifs in different colitis models, including chronic DSS-induced colitis, the T cell-dependent SCID transfer model of colitis, and IL-10-deficient mice, which develop spontaneous colitis [18]. Together these results support the hypothesis that bacterial constituents and specifically bacterial DNA essentially contribute to the perpetuation of established chronic intestinal inflammation [17].

3. Anti-Inflammatory Potential of Bacterial DNA: Protective Qualities of Bacterial DNA in Intestinal Inflammation and Possible Mechanisms

In sharp contrast to the results arguing for a proinflammatory role of bacterial DNA in established intestinal inflammation, we and others found that CpG-ODN exposition had strong protective effects in different models of intestinal inflammation when used in a prophylactic approach. Pretreatment with CpG-ODN resulted in a strong protection from acute DSS colitis as well as from acute Oxazolone- and TNBS-induced colitis [19–21]. At first sight these surprising anti-inflammatory effects of a potent immune stimulating, proinflammatory bacterial compound seem to be confusing.

One important contribution to these protective effects seems to be made by intestinal epithelial cells.

Anti-inflammatory effects of bacterial DNA on intestinal epithelial cells with an inhibition of TNF, and IL-8 secretion as well as NFκB activation were demonstrated in several reports [22, 23]. It was further shown that the anti-inflammatory effects of bacterial DNA/TLR9 interaction on intestinal epithelial cells depend on the origin of the bacterial DNA. In contrast to DNA from pathogenic bacterial strains, DNA isolated from Lactobacillus rhamnosus GG and other probiotic bacteria attenuated TNF-induced NFκB activation and NFκB-mediated IL-8 expression [22–24]. Interestingly, in contrast to the apical stimulation of epithelial cells, basolateral stimulation resulted in a strong NFκB activation and a proinflammatory response [25]. The protective, anti-inflammatory response of epithelial cells upon apical stimulation by DNA derived from commensal or probiotic strains seems reasonable as this is exactly what occurs under physiologic conditions in our intestinal tract. Therefore, apical stimulation of intestinal epithelial cells by bacterial DNA may essentially contribute to the control of intestinal homeostasis.

There, however, seems to be no sufficient explanation for the strong protective qualities of TLR9 activation in colitis models as not only orally administered CpG-ODN but also systemic CpG-ODN application protects from intestinal inflammation. Another important epithelial cell-dependent mechanism might be the CpG/TLR9-induced secretion of bactericidal proteins [26] and/or induction of prostaglandin E_2 [19, 27] both of which are considered as protective for the intestinal mucosa. In this case, the activation of epithelial cells may even occur from the basal membrane after systemic administration of CpG motifs.

Apart from these possible epithelial cell-mediated mechanisms we could identify a CpG/TLR9-mediated modulation of T-cell function in the $CD4^+$ T-cell-dependent SCID transfer model of colitis in which colitis is induced in SCID recipients by adoptively transferred $CD4^+CD62L^+$ cells. Pretreatment of donor mice with CpG-ODN completely abolished colitis development in SCID recipients. Even more important, $CD4^+CD62L^+$ cells from donors which were exposed to CpG motifs had the capacity to inhibit the development of colitis when cotransferred with colitis-inducing $CD4^+CD62L^+$ cells from untreated donors, indicating a regulatory potential of $CD4^+CD62L^+$ cells from CpG-ODN treated donors [28]. In fact $CD4^+$ T-cells from germ-free CpG-ODN-treated donor mice displayed increased PD-1 and FoxP3 expression as potential markers of regulatory T-cells [29].

Vice versa, transfer of cells from TLR9-deficient mice resulted in a much more severe intestinal inflammation compared to cells from wildtype controls [28]. This does not only confirm that CpG motifs can attenuate proinflammatory lymphocyte function but also underlines that the physiological TLR9/CpG interactions are necessary for immune homeostatic mechanisms such as controlling the proinflammatory potential among $CD4^+$ T cells.

Concerning the further characterization of $CD4^+$-dependent prophylactic effects of bacterial DNA we asked three main questions.

(1) Is there a direct TLR9/CpG-interaction on $CD4^+$ T-cells or is the effect mediated indirectly via dendritic

cells or Bcells known to be constitutively TLR9-positive?

(2) In the case of indirect effects; which cellular and/or molecular mediators play a role in the modulation of T-cell functions?

(3) Is TLR9 activation alone sufficient to induce prophylactic effects or is it dependent on the coexistence of other bacterial products (as they are endogenously found in all colonized mice)?

Our recent results indicated that dendritic cells (DC) are essential for the mediation of prophylactic TLR9-dependent effects on T-cells. When DC-depleted donor mice were treated with CpG-ODN, the colitis-attenuating properties of transferred T-cells were absent in SCID hosts. In addition, CpG-ODN preincubation of purified $CD4^+$ T-cells with dendritic cells but not with B cells or alone resulted in an effective reduction of their colitogenic potential after transfer in SCID hosts [30]. Important molecular mediators conferring protective CpG effects of bacterial DNA to T cells seem to be type-I interferons. When type-I interferons were neutralized in CpG-ODN-treated donor mice, transferred T-cells again induced severe colitis in SCID hosts. Vice versa IFN-β treatment of donor mice strongly reduced the colitogenic potential of transferred $CD4^+$ T-cells. In addition to these T-cell dependent protective effects of CpG-ODN induced type-I interferons, IFN-β has been shown to mediate colitis-protective effects in a model in which inflammation is only dependent on innate immune responses [31].

The detailed mechanisms by which T-cells modulated by CpG motifs prevent colitis are still unclear. According to recent results it seems likely that increased secretion of IL-10 participates in protection from colitis after transfer of $CD4^+$ T cells from CpG-ODN-treated donors [29].

4. Bacterial DNA in Colitis: Good, Bad, or Both?

Despite increasing insight in intestinal immune-modulating properties of TLR9 activation, it is still unclear which mechanisms explain the dichotomic effect in intestinal inflammation, including both protective and proinflammatory qualities. According to our results we favour the hypothesis that the preactivation status of the intestinal immune system essentially contributes to the outcome of TLR9 activation, being protective under healthy physiologic conditions but contributing to a further exacerbation in already established chronic colonic inflammation. A recent publication underlines that the outcome of TLR9-activation fundamentally differs depending on the anatomical location in the gastrointestinal tract. Hall et al. described a proinflammatory role of DC activated by TLR9 engagement even under healthy conditions. Such activated DC inhibited regulatory T-cells and activated Th_1 and Th_{17} T-cells [32]. These proinflammatory effects, however, were restricted to the small intestine in which the number of resident bacteria strongly differs from that in the colon altering the physiologic microbial/immune system interaction and, thus, the status of immune activation.

It is important to note that these paradoxical effects are not restricted to TLR9-activation but have also been described for other TLR ligands and the bacterial flora in general.

It is well documented that commensals have the potential to trigger and perpetuate intestinal inflammation in various spontaneous colitis models like IL-10 deficient mice [33] which do not develop colitis when kept under germ-free conditions. This seems to be in contrast to recent results which indicate that the physiologic colonization of the gut with commensals is a necessary prerequisite for a properly working (mucosal) immune system [34–36]. In fact, the permanent interaction of commensals and their constituents with the mucosal immune system has been shown to exert strong protective functions concerning intestinal inflammation. Genetically engineered mice which are unable to respond to TLR4 or TLR2 ligands such as LPS or lipoteichoic acid developed a strikingly more severe intestinal inflammation after chemically induced colitis [37]. This has been explained by a reduced barrier function due to a lack of bacteria-induced mucosal secretion of defensins and mucins by paneth cells and intestinal epithelial cells. Beyond this impaired production of protective mucosal molecules, we could demonstrate—in line with the results in TLR9-deficient mice described above—that commensals/immune system interactions also contributed to a less aggressive behaviour of transferred naïve $CD4^+$ T-cells in the SCID transfer model of colitis. Transfer of naïve $CD4^+$ T-cells from germ-free donor mice to SPF-housed SCID recipients resulted in a much more severe intestinal inflammation than transfer of such cells from conventionally housed donors with a normal bacterial flora [29, 38].

The key helping to resolve these paradoxical effects may be a differential regulation of TLR signalling as shown in Figure 1. Recently, several molecules were identified which decide—depending on their state of activation—whether TLR-activation results in a strong proinflammatory response or a more tolerizing response [39]. For example, activated glycogen synthase kinase 3-β (GSK3-β) led to a strong TLR-dependent TNF-, IL-6-, and IL-12-production in DC whereas TLR-activation in DC with low levels of activated GSK3-β resulted in a predominant IL-10 production and production of only low levels of proinflammatory cytokines [40]. We could demonstrate that inhibition of GSK3-β ameliorated chronic intestinal inflammation in chronic DSS-induced colitis and even abrogated the proinflammatory effects of an additional CpG-ODN exposition seen in established chronic colitis [41]. Interestingly, human lamina propria cells displayed an increased production of proinflammatory cytokines upon TLR9-activation compared to non-IBD patients which could be abolished by blockade of GSK3-β [41]. This underlines a possible relevance for this TLR-regulating molecule also in the human situation.

Targeting key modulators of TLR signalling such as GSK3-β may represent a promising option for future therapeutic approaches. While current therapeutic principles just more or less effectively inhibit proinflammatory immune responses at the cost of an increased risk for serious infections, the restoration of the physiologic equilibrium

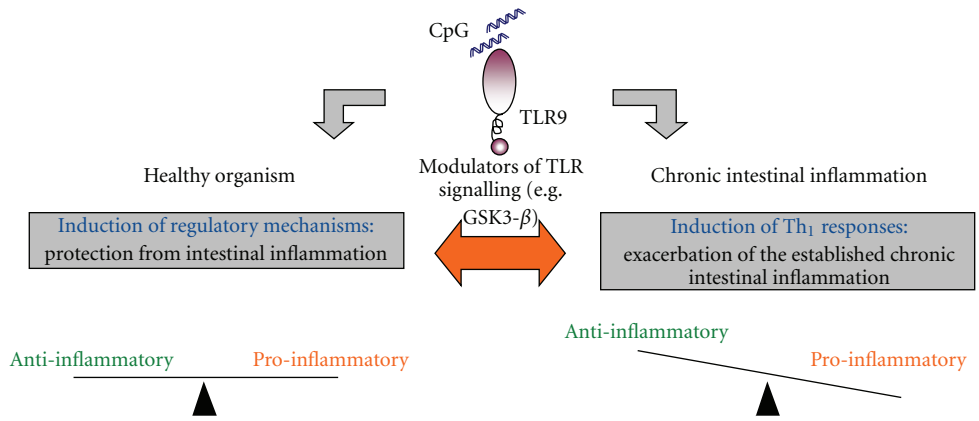

Figure 1: Whereas in a healthy organism under steady-state conditions CpG DNA/TLR9 activation favours the induction of regulatory mechanisms in chronic intestinal inflammation CpG/TLR9 interaction contributes to the perpetuation of chronic intestinal inflammation. The identification of possible regulators of TLR9 activation (e.g. GSK3-β) which have the potential to decide whether TLR activation results in a more tolerizing or proinflammatory response may help to explain these dichotomic effects. These regulators are interesting targets for therapeutic approaches.

between effectors and inhibitors may help to reestablish the friendly coexistence of luminal bacteria and the intestinal immune system. This should not only result in reduced side effects but may even have the potential to achieve long-lasting remission.

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Review Article

Intestinal Host-Microbe Interactions under Physiological and Pathological Conditions

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The intestinal mucosa is unique in that it can be tolerant to the resident, symbiotic microbiota but remaining, at the same time, responsive to and able to fight pathogens. The close interaction between host-symbiotic microbiota at the mucosal level poses important challenges since microbial breaches through the gut barrier can result in the breakdown of gut homeostasis. In this paper, hosts-integrated components that help to preserve intestinal homeostasis including barrier and immune function are discussed. In addition global alterations of the microbiota that can play a role in the initiation of an exaggerated inflammatory response through an abnormal signaling of the innate and adaptive immune response are briefly described.

1. Introduction

The close association of dense bacterial communities of the gut with the mucosa at different habitats of the distal intestine are the basis of symbiotic interactions between the host and its microbiota. Host factors encourage the establishment of the microbiota preserving at the same time tissular homeostasis. The magnitude and the diversity of intestinal microflora represent yet a threat of microbial break through the single-cell epithelial layer that covers the intestinal surface [1]. The invasion of host tissue by resident bacteria would certainly result in the breakdown of the symbiotic host-microbiota interactions.

Commensal invasion of the intestinal tissues is a rare event during the homeostatic situation or occurs in a very limited and controlled manner [2–4]. In some major disturbances of gut balance and integrity it results in severe clinical conditions such as bacteraemia, necrotising enterocolitis in the newborn period, or chronic local or systemic inflammatory conditions.

A diversity of components contributes to the preservation of the barrier integrity. Cellular and extracellular host components at the intestinal mucosa participate in the prevention of bacterial leakage from the lumen. Working in concert with the barrier function, the immunological tolerance to commensals sustains the symbiotic microbiota-host interactions.

The intestinal immune system is tolerant to the components of the commensal microbiota, however immunological tolerance is not the result of immunological ignorance. In fact commensals stimulate host reactions that do not promote host tissue damage but rather cytoprotective mechanisms at the mucosal environment [5, 6]. Thus, systemic immune tolerance concurs with local host responses to commensal bacteria that strengthen the barrier function maintaining the microbiota within the intestinal environment through mechanisms that do not stimulate inflammation. This is the result of permanent interactions between the host and its commensal microbiota [7].

A basic functional feature of the intestine immune system is to avoid tissue-damaging overreactions to commensals that would unnecessarily damage intestinal tissues by way of inflammatory processes, at the same time the intestinal barrier and the innate immune defense needs to be effective regarding the distinct recognition of commensals from pathogens.

Microbial-associated molecular patterns (MAMPs) or "infectious nonself" [8–11] are broadly shared molecular motifs expressed on most bacteria either commensal or pathogens. They interact with pattern recognition receptors (PRRs), expressed on epithelial cells or innate mucosal immune cells such as macrophages and dendritic cells [12]. PRRs are germ-line encoded molecules that are expressed on the plasma membrane or in intracellular endosomal compartments, that is, Toll-like receptors (TLRs) [11], or cytosolic molecules such as nucleotide-binding oligomerization domain (NOD) [13].

The best characterized PRRs are the family of TLRs but, as mentioned above, other families of PRRs have been described. Together they can sense the presence of bacteria in the extracellular and intracellular compartments [11, 13, 14].

Several MAMPs that are ligands for PRRs are common to pathogens and commensal microorganisms and yet, the intestine initiates a protective response to the former while allowing an important and complex microbiota to establish itself at the intestinal surface without any detrimental effect on homeostasis. Discrimination between pathogens and commensals clearly involves other mechanisms. The expression and engagement of PRRs in different cellular or different plasma membrane compartments [15], as well as the presence of additional "danger signals" from stressed or damaged tissues, may be important determining factors [16]. Certainly, most pathogens express a number of virulence determinants such as adherence to and invasion of host cells, and the production of toxins. An important virulence trait of pathogens like *Salmonella* and *Shigella* is their capacity to access the intracellular environment where they interact with cytosolic PRRs that are part of the inflammasome molecular complex [14, 16].

In this paper we will discuss the mechanisms that prevent immune/inflammatory reactions against commensals as well as evidences of deviations to this that contribute to IBD. We will focus on (1) mechanisms that limit direct bacterial contact with epithelial cell surfaces either by a secreted mucus layer or the fast clearance of bacterial cells, (2) bacterial dysbiosis commonly associated to pathological conditions, and (3) the modulation of the innate/inflammatory reaction to commensals.

2. Intestinal Mucosal Factors Involved in Host-Microbiota Homeostasis

Large quantities of bacteria reside in the gut lumen without initiating any detrimental inflammatory response [17] whereas low numbers of bacteria in blood or tissues trigger an energetic inflammatory response.

The intestinal homeostasis is achieved through robust cellular and molecular mechanisms that contribute to reinforce the intestinal barrier function through secreted products, promote cytoprotective epithelial responses, and innate immune reactions to commensals for clearance of physiological passage of bacteria and modulation of detrimental inflammatory response (Figure 1).

2.1. Mucosal Secreted Barrier. On the luminal side of the epithelial cells, a series of interacting factors work together for the prevention of mucosal bacteria close juxtaposition to host tissues.

One of the first physical contacts between the host and the luminal bacteria is the intestinal mucus layer, which covers the mucosal surface. This mucus is a product of goblet cells that actively secrete the mucin glycoproteins. The secreted barrier is composed primarily by highly glycosylated multimeric glycoproteins produced by goblet cells which are responsible for the viscosity of the mucus [18], but also by other secreted compounds like phospholipids, lectins, immunoglobulins, and antimicrobial peptides (defensins, lysozyme, and cathelicidins).

2.1.1. Mucins. Increasing evidences from animal models show that intestinal inflammation could result from defects in this physical interface (both the secreted and the cellular barriers), even in the presence of normal microbiota and normal innate and adaptive immunity [19–21]. Therefore, it is not surprising that an important area of research on inflammatory bowel diseases has focused on failures in the intestinal barrier function in order to elucidate whether these dysfunctions are primary contributors to the inflammation or a consequence of the inflammatory reaction [22]. This, however, has not been an easy task. The investigation of the secreted barrier is troubled with technical sampling problems; most studies fail to preserve the architecture of the mucus layer [23]. Similarly, the definition of the mucosa-associated bacteria and the location of the microbes in the mucus layer remain controversial because the procedure to collect the biopsies lacks consistency from one study to another, as it has been previously indicated [24]. For instance, the preparation of the subject for the colonoscopy can impact the composition of the bowel community [25], the preparative fluid may still be present in the bowel making difficult to distinguish what is actually being collected [26], bacteria from the outer mucus layer can be easily dislodged and lost during the preparation of the sample [25], and so forth. It seems that at least the inner mucus layer remains quasisterile [27, 28] with most of the microbes residing at least 800 μm from the surface of the mucosal epithelial cells [29].

It has been suggested that the contribution of the intestinal bacteria to the pathogenesis of inflammatory bowel disease could be by increased penetration in the mucus, increased adherence to epithelial cells, or invasion of the epithelium. Schultsz et al. [30] studied the spatial distribution of bacteria in the mucosa of rectal specimens from IBD patients and controls. They observed that bacteria were localised within the mucus layer but did not adhere to epithelial cells and were not present in the lamina propria. They conclude that the intestinal mucus in IBD patients is less protective against endogenous bacteria than in healthy individuals, which could explain the increased association of luminal bacteria with the mucus layer [31]. The reduced protection of the mucus could be a result of a genetically determined alteration, for example, in the glycosylation of glycoproteins that renders the mucus prone to degradation,

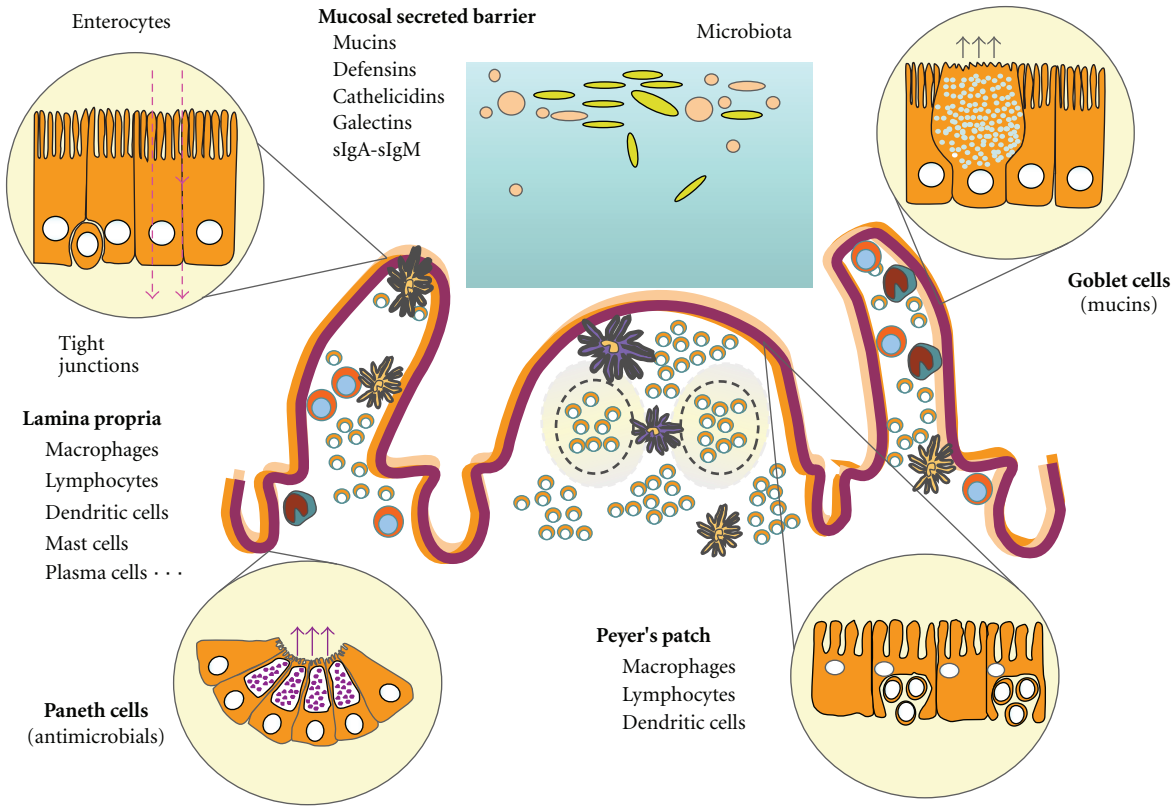

Figure 1: Components of the gut barrier.

or a depletion of specific mucin subspecies [32, 33]. Various changes of the mucus properties have been documented since mid 80s [28, 34–37]. Jacobs and Huber observed that ulcerative colitis patients had an altered glycosylated mucin [38]. Fyderek and colleagues showed that the thickness of the mucus layer of adolescents with IBD was three times thinner in both CD and UC patients compared to controls [39]. In ulcerative colitis, there is a decreased mucin sulfation [34].

2.1.2. Antimicrobial Peptides. The antimicrobial peptides provide protection from intestinal infections and contribute to the maintenance of enteric homeostasis; the concentrations of the different products show a decreasing gradient within the mucin gel from the epithelial side to the luminal side [22].

Paneth cells and other mucosal cell types such as enterocytes, colonocytes, and goblet cells are the source of antimicrobial peptides [40]. The importance of these compounds in the susceptibility to mucosal infections has been demonstrated using experimental models [41]. There are two major groups of antimicrobial peptides in humans and other mammals: defensins and cathelicidins [42] which are also active in cell signaling.

The α-, β-, and θ-defensins kill bacteria by membrane disruption [42]. The cysteine-rich α-defensins also known as cryptdins [43] are produced as an inactive precursor that requires activation by matrilysin in the small intestine [44]. Experimental models show that α-defensins contribute to host defence by influencing the composition and limiting the numbers of resident microbes [45] and in rodents have microbicidal activity against *Escherichia coli*, *Staphylococcus aureus,* and *Salmonella typhimurium*. The nucleotide-binding oligomerization domain-containing protein 2 (NOD2) controls the expression of a distinct subsets of α-defensins and defensin-related cryptdins by Paneth cells [46] upon bacterial ligand recognition. It has been shown that some patients with IBD suffer from an impaired synthesis of α-defensins [47] associated with NOD2 variants [13].

Expression of β-defensins HBD 2 and 3 is induced in the case of inflammation or infection [48]. The induction is mediated by proinflammatory cytokines like IL-1β and bacterial signaling through the activation of TLRs. Thus, extracellular and intracellular cell signaling are both involved in the stimulation of bactericidal product secretion by Paneth and superficial epithelial cells.

Cathelicidins, the other main family of antimicrobial peptides, are characterized by an N-terminal signal peptide (cathelin prosequence) and a structurally variable cationic peptide at the C-terminus [41, 42, 49]. The human mature cathelicidin is called LL-37, and it is originated from a precursor molecule that requires proteolytic activation. The processed peptide has antimicrobial activity against Gram-negative and Gram-positive bacteria [50]. It is expressed by

gastric epithelial cells, distal small bowel enterocytes, and throughout the colon.

Another homeostatic product that controls microbiota overall size and composition is the production of regenerating islet 3 gamma (Reg III γ), a C-type lectin with bactericidal properties [51]. In fact, Reg III γ and the human homolog HIP/PAP bind to peptidoglycan component of bacteria particularly of Gram-positive bacteria resulting in bacterial killing. Reg III γ expression depends on epithelial stimulation through TLRs ligands to epithelial [5]. It is possible that Reg III γ fills a particular antibacterial niche because it targets Gram-positive bacteria such as *Enterococcus fecalis* [51].

The maintenance of intestinal homeostasis depends also on host-microbial interactions that involve protein-carbohydrate recognition [52]. Some animal lectins function as PRR, in the same way that TLRs and NODs do. They cover a wide range of host-microbial interactions. Soluble- and membrane-associated lectins mediate interactions with microorganisms that may lead to mutualistic interactions (commensalism or symbiosis), host colonization, immune recognition by the host, or "subversion" of the nonself recognition functions [52].

Galectins, a subtype of lectins, appear to play important functions in the innate and adaptive immune response at the intestinal mucosa. They are expressed by dendritic cells, macrophages, mast cells, natural killer cells, gamma/delta T cells, and B1 cells, as well as cells from the adaptive immune system (activated B and T cells) [53]. Although galectins lack a typical secretion signal peptide, they are present not only in the cytosol and the nucleus, but also in the extracellular space. Galectin 1 displays anti-inflammatory activities by blocking or attenuating signalling events that lead to leukocyte recruitment, migration, and infiltration [53]. It has been shown that galectin-1 can drive the differentiation of DCs with a regulatory phenotype and function; in fact they can induce T cell tolerance, blunt TH-17 and TH1 responses and suppress autoimmune inflammation through mechanisms involving IL-27 and IL-10 [54].

Galectins can interact directly with bacterial surface glycans in the lumen of the gut or more specifically in the mucus environment. A recent observation has underlined the capacity of galectins to kill bacteria in the lumen of the gut [55]. Both Gram-positive bacteria, such as *Streptococcus pneumoniae*, and Gram-negative bacteria, such as *Klebsiella pneumoniae*, *Neisseria meningitidis*, *Neisseria gonorrhoeae*, *Haemophilus influenzae*, and *Pseudomonas aeruginosa*, display surface carbohydrate galectin ligands [52].

Many human pathogens express on their surfaces diverse carbohydrate structures, and many of these structures have similarities to human antigens, a mechanism utilized by both commensal and pathogens to render themselves immunologically inert. However galectins give the host the opportunity to overcome the pathogenic strategy. *In vitro* and *in vivo* results demonstrate that Gal-4 and Gal-8 possess the ability to specifically kill bacteria expressing blood group antigens. For example, they recognize and kill *Escherichia coli* expressing human blood group antigens while failing to alter the viability of other *E. coli* strains or other Gram-negative or Gram-positive organisms [56]. The ability of Gal-4 and Gal-8 to also kill α-Gal-expressing bacteria shows that galectin-mediated killing is not limited to human blood group antigen-expressing bacteria and suggests that galectins may affect the composition of multiple populations of intestinal bacteria, thereby modulating the intestinal microbiota.

2.1.3. Secretory Antibodies. The third mechanism for the luminal sequestering of symbiotic bacteria involves secretory antibodies in particular secretory immunoglobulin A (sIgA). sIgA-coated bacteria have lower chances to become associated with the intestinal epithelial surface. Suzuki et al. have shown that secretions of IgA are as or more important than innate antimicrobial peptides in the regulation of commensal bacterial flora. In fact in induced cytidine deaminase (AID) deficiency in mice, the absence of hypermutated IgA results in two different consequences: (a) a production of large amounts of unmutated IgM and (b) higher colonization of segmented filamentous bacteria of the intestine. In turn this abnormal level of colonization results in a strong and abnormal stimulation of the mucosal immune system [57].

Hypermutated IgA specific for components of the microbiota involves bacterial sampling by DC and a limited migration of DC up to the mesenteric lymph nodes where B cells are induced to differentiate into plasma cells [7, 58]. Lamina propria plasma cells produced dimeric IgA that is shuttled to the apical side of the enterocytes by the polymeric immunoglobulin receptor or secretory component.

2.2. The Cellular Barrier. Despite the multiple components of the secreted barrier to prevent bacterial contact with the epithelial layer occasional breaches are inevitable. Thus a second layer of the intestinal immune protection is the rapid detection and clearance of bacteria that penetrate the epithelial layer or go beyond it and into the lamina propria.

Bacterial invasion of epithelial cells or breach of the epithelial barrier provides a signal to epithelial cells to initiate responses that are of inflammatory nature in the majority of the cases and whose final goal is the clearance of invading microorganisms. The epithelial layer seems to play a critical role in the recognition between commensals and pathogens. It is essential that the immune system recognizes and reacts to bacterial associated "danger signals" but remains tolerant to nonthreatening microbes and host cells [12], and the epithelial layer plays an important role.

Nonpathogenic bacteria induce a limited immune reaction on enterocytes, with only a transient innate component [15] that may contribute to the physiological, low-level inflammation in the intestine. In addition they induce the secretion of homeostatic cytokines in the mucosal microenvironment [59]. In contrast, true pathogens induce a rapid and more aggressive response that involves intracellular signaling pathways that detect cellular injury, also called "danger signals" [12]. Taken together, the host response at the epithelial layer can thus be considered as a two-tiered process which in a first instance, involves proinflammatory genes that are triggered by most bacteria, pathogenic or not. Subsequent activation of a second cluster of genes is defined by specific virulence traits of the microorganism

[10]. In fact the epithelium has a central role in the initiation of the inflammation in response to bacterial pathogens. For example IL-8 derived from epithelial cells initiates the inflammatory response and tissue damage. However this is necessary for the clearance of invading microorganisms [60] by neutrophils.

A limited number of commensals can physiologically breach the epithelial barrier. When this happens they are taken up and killed by lamina propria macrophages. LP macrophages can clear bacteria without triggering a strong inflammatory reaction [61]. In addition upon bacterial breaching of the epithelial barrier, LP macrophages migrate and produce growth factors for the epithelial restitution [62]. The lamina propria macrophages that express low CD14 are a major cellular component of the mucosal homeostasis.

Another cell type that interacts with commensal bacteria are the dendritic cells [63]. A population of DC—CX3CR1+ DCs—are closely associated with the epithelial lining. In fact CX3CR1+ DCs extend transepithelial dendrites into the gut lumen to sample and process luminal Ags including components of the microbiota [3, 4]. In addition CX3CR1+ DCs initiate the host defence to intestinal pathogens, such as *Salmonella*, as shown by the enhanced susceptibility of CX3CR1-deficient animals to Salmonella infection [64].

The interactions of DCs with T cells mediate the initiation of the adaptive immune response. In addition to lamina propria DC, a rare population of white blood cells have a crucial role in determining the nature of immune reactions and in fine-tuning the balance between immunologic tolerance or induction of inflammation upon recognition of commensals or pathogens, respectively. This functional dualism is crucial at the intestinal level where the immune system does not react against commensals but should be still reactive to clear pathogens upon challenge. A long-standing question has been how dendritic cells drive these distinct immune outcomes required for the clearance of pathogens. Recently, CD103+ (the α E chain of the αEb7 integrin) small intestine lamina propria (siLP) DCs are potent inducers of homing receptors (CCR9 and the α4b7 integrin) on (CD4 and CD8) T and IgA+ B cells. These subsets have the enhanced capacity to induce the differentiation of Foxp3-expressing regulatory T (Treg) cells. In part, this process is driven by vitamin A metabolite retinoic acid [63], and it is crucial in the immunological tolerance to preserve tissue homeostasis.

CD4+ regulatory T cells are essential components of the host-microbiota symbiosis. The two main subtypes of T Reg cells are CD4+FoXP3+ T Reg cells that are found in the colon and small intestinal lamina propria and CD4+FoXP3– Il-10+ T Reg cells that are found in the small intestinal intraepithelial and lamina propria compartments [65]. Colonic FoXP3+ T Reg cells express Il-10, which reciprocally inhibits TH17 and TH1 cells.

2.3. Dysbiosis. The essential role of bacteria in the pathogenesis of colitis has achieved a general consensus. Although animal models of colitis do not mimic exactly Crohn's disease or ulcerative colitis, they provide one of the best evidences of the direct or indirect etiological role of bacteria in IBD. Human studies, however, have not been so conclusive. Even if these studies have failed to reveal categorically a specific altered composition on the microbial makeup of IBD patients versus control subjects, either in the stools or associated to biopsies, they support the hypothesis that a general "dysbiosis" underlies IBD. This term has been largely used since the late 50s and 60s to define deviations of the "normal" bacterial flora under antibiotic treatment in infants, adults, and patients in intensive care [66, 67]. In a healthy individual, the fecal bacterial community has a remarkable stability. This has been largely reported during the last years [68–71]. In contrast, a relatively unstable microbiota has been shown in Crohn's disease patients [72–74]. The presence of "unusual" bacteria was associated to the disease, for instance, low proportions of Firmicutes particularly *C. leptum*, high proportions of Gram-negative bacteria [75], conflicting information about *B. vulgatus* [74, 76], reduced concentration of *F. prausnitzii* [77], and increased numbers of Enterobacteria [72, 78]. Little information is available with regards to ulcerative colitis, but it seems to follow the same trend in that UC patients have a less diverse bacterial community [75, 79]. Pyrosequencing data of healthy and IBD patients show that the distribution of bacterial species differed only slightly between disease states (i.e., Crohn's disease versus ulcerative colitis) with regards to anatomic sites. The IBD group had marked decreases in the main representatives of the gut community: Bacteroidetes and Firmicutes [80].

Similarly, a dysbiosis was described on mucosal biopsies from IBD patients. Bacteria from these sites have been shown to differ from the luminal bacteria [81, 82].

The group of Schreiber [83] reported a reduction in the microbial diversity in mucosal specimens from Crohn's disease patients reflected in a reduction in the number of DGGE bands and in the diversity indices. Notwithstanding some contradictory results reported by different laboratories, a common finding seems to be an increased concentration of total bacteria both in CD and UC [30, 84–87].

Some reports argue against a localized dysbiosis to explain the patchy distribution of mucosal lesions [26, 88] or crypt abscesses [89]. One report suggests the contrary, but in this case biopsy samples from inflamed and noninflamed sites were pooled before the analysis [90].

In conclusion, despite the exponential evolution of technological approaches, the complex gut ecosystem still remains enigmatic. Efforts in deciphering its impact on IBD suggest that restoring shifts from the "normal" commensal microbiota rather than focusing on one particular member may improve these conditions.

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Review Article

The Role of Bacteria and Pattern Recognition Receptors in GvHD

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Graft-versus-Host Disease (GvHD) is the most serious complication of allogeneic stem cell transplantation (SCT) and results from an activation of donor lymphocytes by recipient antigen-presenting cells (APCs). For a long time, it has been postulated that the intestinal microflora and endotoxin exert a crucial step in this APC activation, as there is early and severe gastrointestinal damage induced by pretransplant conditioning. With the detailed description of pathogen-associated molecular patterns and pathogen recognition receptors single nucleotide polymorphisms of TLRs and especially NOD2 have been identified as potential risk factors of GvHD and transplant related complications thus further supporting the crucial role of innate immunity in SCT, related complications. Gastrointestinal decontamination and neutralization of endotoxin have been used to interfere with this early axis of activation with some success but more specific approaches of modulation of innate immunity are needed for further improvement of clinical outcome.

1. Introduction

Graft-versus-Host Disease (GvHD) in its acute and chronic form is the major cause of mortality and morbidity following both, experimental and clinical allogeneic stem cell transplantation (SCT). Donor T-cells activated by major or minor histocompatibility antigens on host antigen presenting cells (APCs) are the essential players in the pathophysiology of GvHD [1], and T-cell depletion of the graft is able to abrogate both, GvHD and the beneficial graft-versus-leukemia (GvL) effect. However, it has been well known since the fundamental experiments of van Bekkum that activation of innate immunity by the gastrointestinal microflora is a crucial and initiating step in induction of alloreactions. Mice grown under germ-free conditions and receiving bone marrow as the only stem cell source (containing a limited number of T-cells) failed to develop acute GvHD whereas, mice grown under conventional conditions or reconventionalized early after transplantation died from acute GvHD. If spleen cells containing a high number of T-cells were added, germ-free conditions could not prevent but still delayed the onset of GvHD [2]. Since this first observation substantial progress has been made in understanding the exact pathways how bacteria and their ligands interact with specific pattern recognition receptors (PRRs), thus activating and modulating APCs and targets of GvHD. In the present paper we summarize current evidence on the impact of microbia and microbial patterns on pathophysiology of GvHD and clinical outcome following allogeneic SCT.

2. Indirect Evidence: Gastrointestinal Damage and GvHD

Major target organs of acute GvHD are the skin, the liver, especially bile duct epithelia, the gastrointestinal tract and still controversially discussed, the lung. In chronic GvHD, oral manifestations and again, the pulmonary involvement of bronchiolitis obliterans (BOs) are frequent. A common denominator of these organs is that they present epithelial surfaces with strong immunological interactions between commensal and pathogenic bacteria, epithelial barrier and defence mechanisms and the immune system which are usually in a perfect balance to maintain a status of immunological tolerance [3].

These epithelial defence mechanisms are heavily disturbed by epithelial damage through pretransplant

conditioning which is the first step in the SCT procedure and includes high-dose cytotoxic therapy or total body irradiation (TBI). Although conditioning aims to eliminate the recipient's leukemia and achieve myeloablation, in both, experimental and clinical SCT, it has become clear that increasing the dose of TBI from 900 to 1300 cGy in mice and from 12 to 15,5 Gy clinically was associated with increased epithelial damage, more severe GvHD and inferior outcome [4–6]. Similarly, an increased area under the curve (AUC) following exposure to intravenous busulfan was also associated with increased gastrointestinal toxicity and acute GvHD [7].

In 2 clinical trials, gastrointestinal toxicity was directly assessed by either monitoring diarrhea during the aplastic phase [8] or direct analysis of intestinal permeability [9] and could be correlated with severity of subsequent acute GvHD. In line with this, prophylactic treatment of mice with the rhu Keratinocyte Growth Factor (KGF) protected from apoptosis of intestinal epithelial cells, LPS mediated TNF release, and finally lethal GvHD [10] while maintaining GvL effects [11]. However, in a randomized clinical trial KGF reduced severity of mucositis in patients receiving TBI but did not affect GvHD and outcome as expected from murine data [12].

3. Indirect Evidence: The Role of SNPs of Innate Immunity

In murine GvHD, endotoxin has been identified as a major mediator of inflammation involved in initiation of intestinal and systemic GvHD. Genetic susceptibility as well as direct antagonisms against endotoxin has a major impact on the occurrence and severity of experimental GvHD [5, 13] and an LPS-TNFα axis has been postulated as a major mechanism of acute GvHD. In humans, TLR4 is considered to represent the classical endotoxin receptor. With the characterization of the human genome, single nucleotide polymorphisms (SNPs) for many genes have been described which translate into altered functions of these genes. In the setting of allogeneic SCT, TLR4 SNPs have been assessed by 2 groups which however, reported opposing effects, either protection or enhancement of GvHD in the presence of TLR4 SNPs [14, 15].

Beyond TLR4, there is a large set of further TLRs recognizing other microbial patterns including further bacterial and viral ligands. SNPs have been described for most of these TLRs, and recently, presence of the homozygous TLR9 variant in the patient has been associated with improved survival and a reduced relapse rate following allogeneic SCT [16]. In addition to TLRs, the NOD-like receptors are a family of highly conserved intracytoplasmatic receptors involved in activation of an important inflammatory cascade, the inflammasome, which finally results in activation of NF-κB and/or activation and cleavage of IL-1β [17]. Based on pathophysiological similarities between inflammatory bowel disease and intestinal GvHD, we speculated that SNPs involved in the pathogenesis of Crohn's disease might be also of relevance in GvHD and outcome following allogeneic SCT [18]. We and others have therefore tested SNPs of NOD2, a receptor sensing muramyl-dipeptide derived from Gram-positive and Gram-negative bacteria, as well as SNPs of other innate immunity molecules such as the autophagy-related gene 1 (ATG16L1) involved in autophagy of bacteria [18], in large cohorts of patients receiving HLA-identical sibling SCT and their donors. Presence of any NOD2 SNPs or the ATG16L1 variants in either the recipient or the donor increased the risk of GvHD and subsequent treatment related mortality, and this effect was further increased if variants were present in both, donor and recipient (Figure 1).

Since our first description of association of NOD2 variants with GvHD and mortality [19] several further groups addressed the role of this important receptor. In HLA-identical sibling transplants, most of the studies were confirmatory [20–24]; in unrelated donor transplantation, the association either was absent [25], present only for SNP13 [26] or even contradictory as one group reported association with less GvL and more relapse [27–29] (Table 1). This may be explained by the fact that in unrelated donor SCT HLA-differences are much more likely to occur and dominant against the SNPs of innate immunity or by the more intense immunosuppression which usually includes in vivo T-cell depletion with monoclonal or polyclonal sera. In addition, our European cohort analysis nicely demonstrated that differences in transplant specific strategies, especially with regard to antibacterial decontamination, had a strong impact on the prognostic significance of NOD2. Thus, immunoregulatory SNPs may be specifically sensitive to interaction with transplant or center-specific strategies and it may be therefore difficult to establish these SNPs as risk factors allowing exact prediction of complications.

However, a further and even more important aspect of these observations is the implication of these molecules in pathophysiology. Our observation of an association of bronchiolitis obliterans syndrome with NOD2 SNPs suggest that this interference of SNPs with epithelial defence mechanisms applies to all epithelial tissues [30], and it will be of major interest to learn more about antibacterial peptides released like defensins released under the control of NOD2 [31]. Recently, NOD2$^{-/-}$ mice and bone marrow chimeras were used as recipients in experimental BMT models. These data confirmed an accelerated mortality from GvHD if hematopoietic cells were NOD2 deficient, and O' Penack and his group identified the antigen-presenting cell (APC) as the target of NOD2 deficiency [32]. APCs from NOD2$^{-/-}$ mice induced a much stronger alloreaction as compared to wild-type mice pointing to a deficiency of immunoregulatory APC molecules. Recently, our group addressed the immunohistopathology of skin and gut GvHD in relation to absence and presence of NOD2 SNPs: In both tissues, skin and gut, recipient NOD2 SNPs had no impact on the extent of apoptosis as the hallmark of GvHD nor on the CD8 and macrophage infiltrate in the biopsies. However, we observed a uniform reduction of CD4 cells in the presence of NOD2 SNPs suggesting that GvHD especially in these patients is characterized by a loss of protective CD4 population including a loss of regulatory T-cells [33]. NOD2 has been shown to be involved in release of chemokines attracting T-helper cells as well as in recruitment

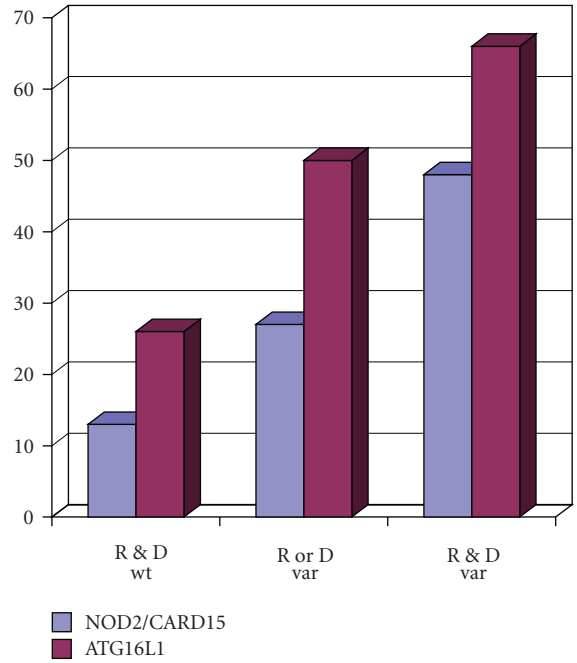

Figure 1: Treatment-related mortality and SNPs of innate immunity. SNPs 8,12 and 13 of NOD2 ($n = 358$) and the T300A. SNP of ATG16L1 ($n = 127$) were assessed by PCR in patients receiving allogeneic SCT and their respective HLA-identical sibling donors. Cumulative treatment related mortality as calculated by Kaplan-Meier method is shown in relation to absence of any SNPs (wt, wild-type) or presence of SNPs (variant, var) in recipients (R) and/or donors (D). Both associations were significant by log rank tests ($P = .003$ for NOD2, $P = .03$ for ATG16L1).

of TH17 cells, and ongoing studies try to address these questions. Thus, a dysregulated APC function is the most likely explanation for this loss of CD4 cells as it has been observed in mice.

4. Direct Evidence: The Role of Bacteria and Bacterial Ligands

van Bekkum's work elegantly elaborated the role of the intestinal microflora and is up to now the basis for the gnotobiotic approach to modulate GvHD. In his early experiments he did not only show protection from GvHD induced by bone marrow transplantation in gnotobiotic mice. He also reported that subcutaneous fetal gut implants revealed attenuated histopathological GvHD if the carrier mice were germ-free prior to allogeneic transplantation indicating a systemic effect of decontamination [34]. Similar observations pointing to a systemic modulation [35] were reported by Lampert et al. [35], as they reported attenuation of both gut and skin GvHD after oral decontamination in mice. In the same year, Veenendaal et al. published strain-dependent effects of decontamination, as C3H/He recipients were protected from delayed type GvHD by decontamination whereas C57Bl/6 were not [36]. Later on, a debate started whether selective decontamination versus decontamination including anaerobic bacteria conferred greater protection from GvHD. In both, experimental BMT [37] and in clinical trials published by the Essen group [38, 39] suppression of the anearobic flora seemed to have a stronger effect than suppression of enterobacteriaceae alone.

Further evidence for a role of the intestinal flora can be derived from attempts to neutralize endotoxin. In 1987, Cohen reported protection of mice by passive immunization with anti-*E.coli* sera, and an increased antibody titer against a certain *E. coli* strain in patients was associated with a lower incidence of severe GvHD [40, 41]. In the 90s, IgM enriched immunoglobulin preparations were thought to reduce GvHD due to their potent antiendotoxin effects [42].

Based on positive reports in inflammatory bowel disease, our group tested the use of probiotic bacteria (*Lactobacillus rhamnosus*) in an experimental BMT model. Indeed, feeding of lactobacilli reduced severity of experimental GvHD and improved survival. The systemic effect of probiotic bacteria could be demonstrated by a reduction of splenic donor T-cell proliferation further demonstrating that activation of innate immunity sets the state for subsequent adaptive alloreactions [43].

Besides endotoxin modulation, only rare reports have addressed the role of other bacterial and TLR ligands. In an elegant study, Chakraverty et al. proved the checkpoint function for innate immunity by applying a TLR7 activator locally to the skin before inducing GvHD by donor lymphocyte infusion in mixed murine chimeras. Whereas there were almost no cellular infiltrates and signs of GvHD in untreated control skin, massive T-cell infiltrates and histopathological damage was observed in the TLR7ligand pretreated skin [44]. Similar processes of activation should occur in the intestinal tract and in other epithelial target tissues of GvHD after TLR4 and TLR2 binding of endotoxins. In line with the checkpoint function of innate immunity, binding of CPG-oligodeoxynucleotides to host antigen-presenting cells accelerated GvHD in a recent murine study [45], whereas murine $TLR9^{-/-}$ recipients were protected from GvHD [46]. Interestingly, other TLR-ligands seem to induce opposing and even silencing effects. Pretreatment of mice with the TLR5 ligand flagellin reduced severity of GvHD indicating that TLR5 may be more involved in dampening activation of antigen presenting cells (Gerwitz, ASBMT 2010, abstract).

5. Perspective: Modulation of PRRs to Avoid GvHD While Preserving GvL Effects

Since many years, attempts to reduce GvHD frequently also affected the major therapeutic principle of allogeneic SCT, the graft-versus-leukemia effect. This was most obvious for direct T-cell depletion [47] but can also be observed for classical immunosuppressants like cyclosporin. Especially in acute leukemias, occurrence of mild acute GvHD grade I-II and chronic GvHD confers the best antileukemic effect. In our studies on the role of NOD2 SNPs, however, we observed a strong impact of recipient and combined donor/recipient SNPs on GvHD, and GvHD-related mortality, however, there was no difference in relapse rates between the different

Table 1: Summary of published studies on NOD2 SNPs and outcome following SCT.

	Type of SCT	Association	Comment	Refereces
Holler 2004	Related	GvHD, TRM	Single centre	[17]
Holler 2006	Related	GvHD, TRM, OS	Multicentre; Impact of decontamination	[18]
Granell 2006	Related	TRM, pulmonary compl.	CD34 selected grafts	[19]
Sairafi 2008	Related	No association	Low frequency of NOD2 variants	[22]
Hanssen 2008	Related	Weak with GvHD		[20]
Van Velden 2009	Related	Strong with GvHD	Partially T depleted Grafts	[21]
Hildebrandt 2009	Related and Unrelated	Bronchiolitis obliterans		[28]
Mayor 2009	Unrelated	Strong with relapse, not with GvHD	Majority received T-cell depletion With MabCampath	[25]
Holler 2009	Unrelated	Only SNP13 with TRM		[24]
Ngyen 2010	Unrelated	No		[23]

groups. Patients with wild-type NOD2 had a cumulative incidence of relapse of 41%, patients with either recipient or donor variants of 29% and patients with combined donor and recipient variants 33% [20]. This observation and pathophysiological considerations suggest that modulation of epithelial inflammation in the gut or the bronchial epithelial system thus might reduce GvHD but should not interfere with antileukemic immunity which is located in the central lymph nodes or in the marrow.

6. Conclusions

Although our current understanding of the interplay between intestinal microbes, activation of innate immunity, and specific alloreactions explains some of the long standing preclinical and clinical findings such as the potential protective effect of intestinal decontamination, there are still a variety of issues to be solved. Comparable to the situation in IBD, SNPs of innate immunity alone by far do not explain the individual susceptibility for intestinal GvHD or allow even prediction of intestinal GvHD which would be extremely helpful to tailor immunosuppressive prophylaxis and treatment suggesting that intestinal homeostasis is far more complex. In addition, the role of the diversity of the intestinal microbiota and the impact of immunological memory against these antigens in GvHD has not been addressed so far. In addition, pathophysiology has focussed on excess inflammation so far. As suggested by our findings on a reduction of intestinal regulatory T-cells in patients with NOD2 SNPs and GvHD, GvHD may be far more a loss of intestinal immunoregulation, and mechanisms of immunoregulation need to be investigated in detail. Recent data indicate that the balance of regulatory to TH17 cells is strongly regulated by the enzyme Indolamine-2,3 dioxygenase (IDO) in intestinal antigen-presenting cells. Experimental data suggest a strong impact of this enzyme in GvHD pathophysiology [48], and ongoing studies address this new player in the clinical setting. In the long term, these findings should help to substitute nonspecific immunosuppression for treatment of intestinal GvHD by strategies aiming at reconstitution of immunoregulation.

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Review Article

Antimicrobial Peptides in Gastrointestinal Inflammation

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Acute and chronic inflammations of mucosal surfaces are complex events in which the effector mechanisms of innate and adaptive immune systems interact with pathogenic and commensal bacteria. The role of constitutive and inducible antimicrobial peptides in intestinal inflammation has been investigated thoroughly over the recent years, and their involvement in various disease states is expanded ever more. Especially in the intestines, a critical balance between luminal bacteria and the antimicrobial peptides is essential, and a breakdown in barrier function by impaired production of defensins is already implicated in Crohn's disease. In this paper, we focus on the role of antimicrobial peptides in inflammatory processes along the gastrointestinal tract, while considering the resident and pathogenic flora encountered at the specific sites. The role of antimicrobial peptides in the primary events of inflammatory bowel diseases receives special attention.

1. Introduction

Although a host of different bacteria colonizes the gut from the oral cavity to the rectum, translocation of bacterial agents through the intestinal walls remains limited to highly pathogenic bacteria or predisposing disease states in which the natural defense mechanisms are compromised.

In the healthy individual, the physical barrier created by the thin layer of epithelium forms the basis of the mucosal defense. In addition, the production of an array of antimicrobial peptides by secretory epithelial cells limits the invasion and adherence of pathogenic and commensal bacteria. Salient examples of antimicrobial peptides are the defensins and cathelicidin LL37, the two major classes of AMPs in mammals, yet other molecules like elafin or secretory leukocyte protease inhibitor (SLPI) complement the effector mechanisms of innate and adaptive immune systems. Equally important, in the small and large intestine, goblet cells are responsible for the production of highly glycosylated proteins, which form a gel-like layer over the surface epithelium. The outer portion of this layer is heavily colonized by bacteria, whereas the inner stratum's low bacterial load results from the high local levels of antimicrobial peptides [1].

Recent years have seen a steadily rising interest in antimicrobial peptides, and their implication in the pathogenesis of intestinal processes like Crohn's disease [2] or necrotizing enterocolitis [3] as well as their role in psoriasis and atopic dermatitis, cystic fibrosis and otitis media has garnered the attention of an increasing group of scientists.

In this paper, we would like to focus on the role of antimicrobial peptides in inflammatory processes along the gastrointestinal tract, while considering the resident and pathogenic flora encountered at the specific sites. The role of antimicrobial peptides in the pathogenesis of the idiopathic inflammatory bowel diseases receives special attention.

2. Antimicrobial Peptides of the Gastrointestinal mucosa

2.1. Defensins. Defensins serve as endogenous antibiotics with microbicidal activity against Gram-negative and Gram-positive bacteria, fungi, viruses, and protozoa [4]. One of their fundamental characteristics is the presence of three intramolecular disulfide bonds. The pattern of linkage between the cystein residues allows the classification into two major groups, the α-defensins and the β-defensins (the cyclic octadecapeptide called θ-defensin were not found

in humans so far). The former are linked by a 1–6, 2–4, 3–5 pattern, the latter 1–5, 2–4, 3–6 pattern, yet their three-dimensional structure is similar [5, 6]. The total of six α-defensins includes human neutrophil peptides 1–4 (HNP1–4) produced by granulocytes and human defensin 5 and 6 (HD5 and HD6) produced by Paneth cells. It should be noted that the Paneth cell defensins are stored as propeptides and require cleavage by trypsin, which is stored in Paneth cell granules as a zymogen as well [7, 8].

β-defensins are mainly produced by epithelial cells [9], and four subtypes, designated hBD-1 to hBD-4, have been identified in the human mucosa so far. hBD-1 is ubiquitously expressed at all surfaces of the human body including the skin, the respiratory, urogenital and the gastrointestinal tract [10–15]. hBD-2 and hBD-3 are inducible antimicrobial peptides expressed by enterocytes throughout the intestinal tract on demand.

Biochemical properties of the human defensin family include a low molecular mass from 3 to 6 kDa and a cationic charge, which allows these molecules to bind to negatively charged phospholipid groups on microbial surfaces. The exact mechanism by which defensins exert their bactericidal effect has still not been identified, but it has already become clear that they do not act with a uniform mechanism. One model, the "Shai-Matsuzaki-Huangh" model, proposes that after integration of defensins into the cell membrane, its outer layer expands and strains the inner leaflet of this bilayer, leading to disruption or formation of toroidal pores [16]. On the other hand, hBD-3 has been shown to function rather by inhibiting steps of the biosynthesis of the bacterial cell wall [17].

Defensins were also noted for their chemotactic properties. The chemoattractant effect on immature dendritic cells and CD4+ T cells has been shown to act through chemokine receptor CCR6 [18]. Chemoattraction of macrophages and monocytes has been observed as well, but these cells do not express CCR6. A recent publication now reported that hBD-2 and hBD-3 are chemotactic for these cell lines in a CCR2-dependent manner [19]. Other investigations have shown that hBD-2 induces the migration of mast cells by activating G-protein-phospholipase C-coupled receptors and is a specific chemoattractant for human neutrophils [20, 21].

In a broader concept, Peyrin-Biroulet and Chaimallard position defensins at the interface between innate and adaptive immunity, proposing that NOD2-mediated microbial recognition leads to secretion of defensins, which in turn attract immature dendritic cells, help in their maturation and promote the subsequent activation of T cells [22].

Also, HD-5 may influence the intestinal inflammatory response by binding to the cell membrane of intestinal epithelial cells. A subsequent induction of interleukin-(IL-) 8 was observed in a concentration- and structure-dependent fashion [23, 24].

2.2. Cathelicidins. The second major group of AMPs in mammals are the cathelicidins. While a signal peptide called "cathelin prosequence" can be found at their N-terminus, the C-terminal part is formed by a more variable cationic region that has antimicrobial activity once cleaved from the holoprotein. The only cathelicidin identified in humans was termed LL-37/h-CAP18. Its constitutive expression is found in various immune cells, in salivary glands, and in epithelia of respiratory, digestive and reproductive tracts while keratinocytes and intestinal cells can be induced to enhance expression. LL-37's antimicrobial properties are supplemented by its chemotactic effect on blood cells, activation of histamin release from mast cells, or induction of angiogenesis [25].

2.3. Other Antimicrobial Peptides. Antimicrobial activity has been noted in a multitude of other small molecules. For example, the chemokines CCL14 and CCL15 are constitutively expressed at high levels in human intestinal epithelium and display potent antibacterial effects [24]. CLL20/macrophage-inflammatory-protein-3α and an additional 17 chemokines function as antimicrobials as well [26]. Elafin and secretory leukocyte protease inhibitor (SLPI) also exhibit broad spectrum antimicrobial activity against Gram-positive and Gram-negative bacteria, selected fungi and viruses [5], though in their principal role, these antiproteases serve to maintain tissue integrity by antagonising aggressive serine proteases like human neutrophil elastase (HNE) [27]. Yet another epithelial antimicrobial peptide is bactericidal/permeability-increasing protein (BPI), which is involved in lipid-mediated killing and the attenuation of proinflammatory signalling by bacteria. Its sphere of action covers mostly Gram-negative bacteria [28, 29]. For a quick overview, Table 1 lists the abovementioned antimicrobials along with their properties.

3. Antimicrobials in Gastrointestinal Diseases

3.1. Esophagus. Microbial infections of the esophagus represent a rather uncommon event in healthy individuals. Nevertheless, the immunocompromised host quite frequently suffers from infections with *C. albicans*, CMV or HSV, while bacterial infections remain rare.

Fittingly, despite a high expression of numerous antimicrobial peptides, assays with oesophageal tissue showed a weakened potency to kill *C. albicans* [30], a fact which could help explain the susceptibility of esophageal tissues to infections with this yeast. Kiehne et al. [31] observed that *Candida* colonization induced a high expression of a subset of antimicrobial peptides, especially hBD-2 (shown in Figure 1) and hBD-3. In a subsequent mechanistic study the group showed that polymorphonuclear leukocytes (PMNs) reinforce the defensin expression in the epithelium. The authors speculate that individuals suffering from neutropenia lack this stimulus for the expression of epithelial antimicrobial peptides and thus, a pathophysiologic explanation for the high incidence of *Candida* esophagitis and *Candida*-related deaths in neutropenic patients can be proposed [32]. Furthermore, even in esophageal reflux disease, an induction of β-defensin expression (hBD-2 and hBD-3) could be found, although to a minor degree [31].

Table 1: Antimicrobials in the gastrointestinal tract.

Antimicrobial peptide	Chromosomal location	Molecular mass (kDa)	Secretory stimuli	Distribution in gastrointestinal tract	Biological function	Changes in inflammatory bowel disease
hBD-1	8p23.1	3.5–4.5	Constitutive in epithelial cells, IFN-γ and LPS in monocytes	Ubiquitous in epithelial cells of small and large intestine, monocytes, monocyte-derived dendritic cells	Antimicrobial, chemotactic	Reduction in colonic IBD
hBD-2, 3, 4	8p23.1	3.5–4.5	LPS, flagellin mediated by NF-κB and AP-1	Epithelial cells, monocytes	- Antimicrobial, chemoattractant for macrophages and monocytes, - hBD-2: mast cells and neutrophils	- Attenuated induction observed in colonic CD - Reduced copy numbers for hBD-2 in colonic CD
HD-5 and HD-6	8p23.1	3.5–4.5	NOD2 activation (MDP, LPS) TLR	Granules of ileal Paneth cells (also metaplastic Paneth cells in other areas of intestinal tract)	Antimicrobial, induction of IL-8	- Reduction in ileal CD, more pronounced in patients with NOD2 mutation - HD-5 and HD-6 expression due to metaplastic Paneth cells in UC and CD colon
Cathelicidin ("LL-37")	3p21.3	18	Butyrate, vitamin D, bile acids, MDP	Epithelial cells, leukocytes	Antimicrobial, chemotactic	- Attenuated induction in colonic CD - Ileal CD and UC show regular induction
Elafin	20q13.12	9.8	IL-1, TNF-α	Epithelial cells, leukocytes	Antiprotease with antimicrobial and chemotactic properties	Attenuated induction in colonic CD
Secretory phospholipase A2	16p13.1–p12	14	LPS	Epithelial and inflammatory cells, Paneth cell granules	- Acute phase protein involved in eicosanoide metabolism - Small intestinal mucosal defense	?
Lysozyme	12q15	16.5	?	Gastric, pyloric and duodenal glands, small intestine, macrophages and monocytes, not in colonic tissue	Antimicrobial against Gram-positive bacteria, chemotactic	- Small intestine: no changes observed - Increased colonic expression due to metaplastic Paneth cells
BPI (bactericidal/ permeability-increasing protein)	20q11.23	50	LPS	Epithelial cells, neutrophils	Antimicrobial, binds LPS-compounds	No changes observed, regular induction in IBD

3.2. Stomach. The high prevalence and morbidity resulting from colonization by the Gram-negative bacterium *Helicobacter pylori* has captured much interest in the role of antimicrobial peptides in the stomach. Though the mucosa exhibits a strong inflammatory response against *H. pylori* bacteria, clearance of the pathogen is unsuccessful in many cases.

Helicobacter infection is known to lead to a significant induction of hBD-2 (see Figure 1), while the defensin gene expression caused by non-*Helicobacter* gastritis is much less pronounced [33], a finding which was confirmed in a pediatric cohort [34]. In a recent study, it could be demonstrated that *H. pylori* induces gastric epithelial cells to upregulate the endogenous production of hBD-2 [35],

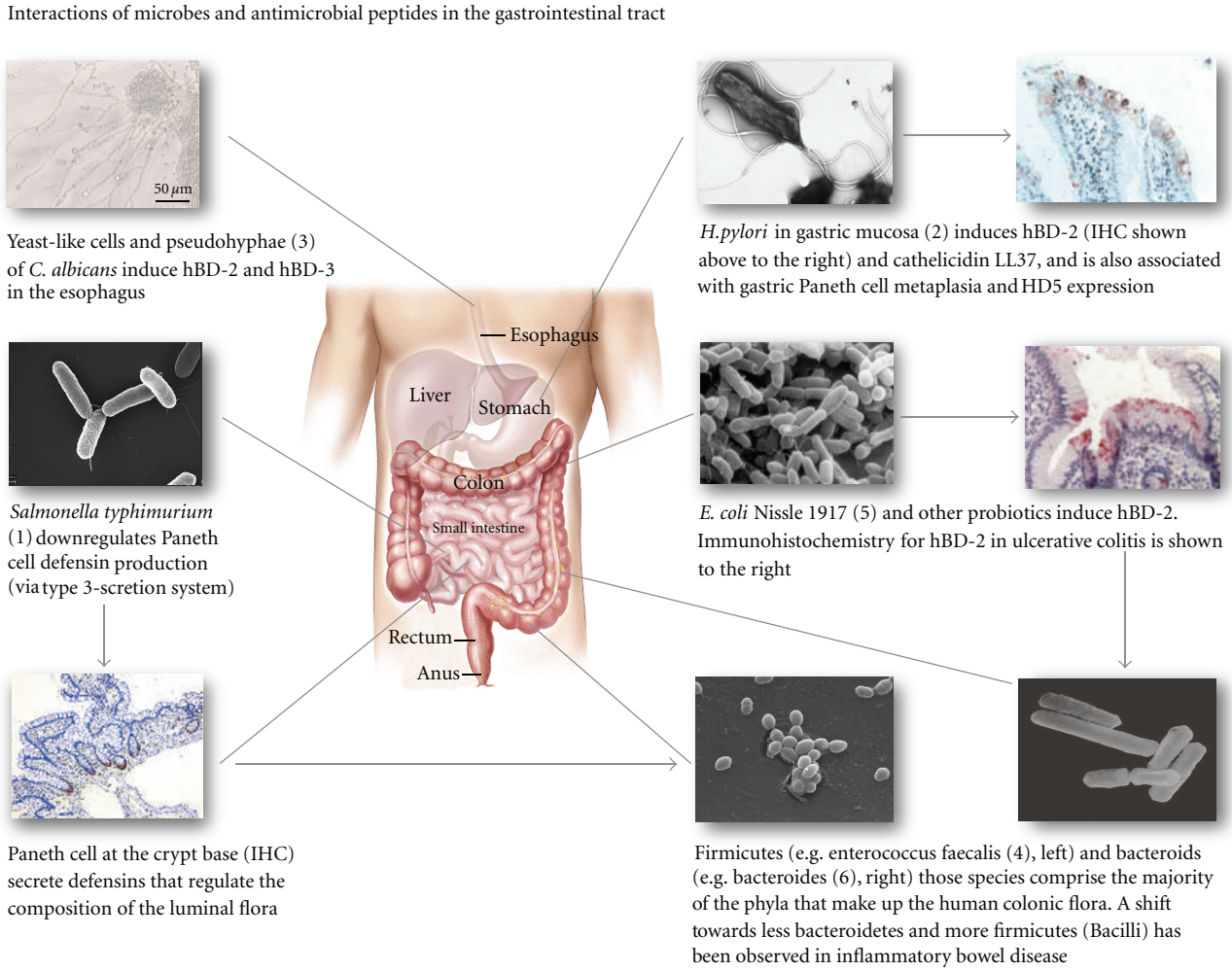

Figure 1

furthermore the authors showed that this is mediated by the cytosolic pattern recognition receptor NOD1 (nucleotide-binding oligomerization domain 1).

Also, an analysis of single nucleotide polymorphisms in the DEFB1 gene correlated patients with chronic active *H. pylori*-induced gastritis with the SNP G-52A, suggesting an involvement of the constitutive expressed hBD-1 in susceptibility to this form of gastritis [36]. In the setting of chronic *H. pylori* induced gastritis, intestinal metaplasia (replacement of the normal mucosa by a columnar epithelium with characteristics of intestinal epithelia, e.g., goblet cells, Paneth cells), is a frequent event. A high HD-5 expression has been observed by Shen et al. [37], suggesting that in intestinal metaplasia, where α-defensin producing Paneth cells are present, this metaplastic change may strengthen the antibacterial response via production of HD-5. Aside from the defensins, *H. pylori* is reported to induce Cathelicidin LL-37 in gastric epithelial cells [38].

3.3. Inflammation of the Biliary Tree. 10%–20% of adult populations in developed countries suffer from cholelithiasis (gallstones). Though more than 80% of patients remain asymptomatic, infections of the gallbladder or the biliary tree are common diseases, which require antibiotic treatment in many cases. The normal sterility of bile is maintained by the bactericidal effect of bile salts and immunoglobulin A, and a notable expression of hBD-1 and hBD-2 is documented in biliary tract epithelium and in the liver [39]. Similarly to other anatomic sites, hBD-1 expression is constitutive, while in the large intrahepatic bile ducts, hBD-2 was induced by biliary obstruction or hepatolithiasis, where these peptides contribute to the local antimicrobial defense.

Interestingly, in the epithelium of four of five patients with primary sclerosing cholangitis and in all controls with normal histology [39], hBD-2 expression remained low. Furthermore, in all bile samples which were analysed, hBD-1 could be found constitutively, while hBD-2 was confined to those with hepatolithiasis [39]. Patients with primary sclerosing cholangitis, especially following endoscopic manipulation, suffer from frequent bouts of infection. Although further studies are needed, the observed lack of induction of hBD-2 and possibly other antibacterial peptides could be implicated in the disease mechanism.

D'Aldebert et al. found an intense immunostaining for cathelicidin in human liver biliary epithelium, and showed that bile salts (chenodeoxycholic acid and ursodeoxycholic acid), which also possess intrinsic bactericidal properties, induce cathelicidin expression through different nuclear receptors. According to their results, either farnesoid X receptor or vitamin D receptor is involved and upon activation, promote cathelicidin expression in the biliary tract [40].

3.4. Intestine. The microbial colonization of the lumen increases along the intestine, though the number of bacteria is still very low from the duodenum to the proximal ileum. The distal ileum contains up to 10^8 primarily anaerobic bacteria per gram of luminal contents [41], whereas up to 10^{11}–10^{12} bacteria per gram colonize the colon. The bacterial microflora is crucial for the maintenance of human health and the development of the mucosal immune system. Moreover, its contribution to the pathogenesis of the chronic idiopathic inflammatory bowel diseases is widely acknowledged. In these entities, a shift in microbial composition towards less Bacteroidetes and more Firmicutes (Bacilli) has already been observed (see Figure 1).

3.4.1. Small Intestinal Inflammation. On the one hand, the scarcity of bacteria in the ileum can be attributed to the hostile environment created by acid, bile, and pancreatic secretions as well as to the phasic propulsive motility of this part of the gut [42]. On the other hand, adaptive and innate branches of the immune system contribute as well to maintain a low microbial density. Paneth cells, which are a characteristic epithelial lineage of the small intestine and localize to the bottom of the intestinal crypts, secrete α-defensins in response to bacterial antigens including lipopolysaccharide and muramyl dipeptide [43]. A constitutive expression of exceptionally high levels of α-defensins HD-5 and HD-6 could be demonstrated in human small intestines [44]. Interestingly, expression of HD-5 exceeds expression levels of other AMPs produced by the Paneth cell (lysozyme and sPLa2) by a factor up to 100 [45].

In studies with knockout animals, intestinal extracts from mice deficient for the cryptdin-processing enzyme matrilysin and thus lacking functional mature mouse α-defensins (the mouse homologs to defensins are called cryptdins), show decreased antimicrobial activity [46], and the authors furthermore observed that these mice are more susceptible to orally administered bacterial pathogens as well as to DSS-induced colitis. Other findings from a transgenic animal study revealed that human α-defensin HD-5 transgenic mice are resistant to infection from orally administered *S. typhimurium* [47]. Interesting in this context is the fact that *S. typhimurium* can downregulate HD-5 expression via a type-3 secretion system (see Figure 1).

In addition, the Paneth cell defensins can shape the composition of microbial species present in the small intestinal lumen, while the total number of bacteria remains unaffected [45, 48]. In a mouse model with transgenic expression of DEFA5, Salzman et al. demonstrated that the colonization with segmented filamentous bacteria (termed SFB, from the genus Clostridia) was dramatically decreased when the mice produced the human α-defensins HD-5. Interestingly in this context is the fact that mice colonized with SFB were shown to be more resistant to infection with *Citrobacter rodentium*, a close relative to the well-known *Escherichia coli*. Paneth cells also exert control over intestinal barrier penetration by commensals and pathogenic bacteria [49], apparently mediated by TLR (Toll-like receptor) recognition and a subsequent induction of antimicrobial peptides. The signalling was shown to be dependent on the expression of the MyD88 adaptor protein inside the Paneth cell. The release of Paneth cell secretions into the intestinal lumen thus follows stimulation of pattern recognition receptors (PRR, e.g., Toll-like receptors, NOD-like receptors, RIG-I-like receptors) with pathogen-associated molecular patterns, termed PAMPs, which are provided by resident and pathogenic bacteria. Corroborating the concept of a host driven composition of the microbial flora, Petnicki-Ocweija et al. showed that in the mouse model, the bactericidal activity of crypt secretions of the terminal ileum was severely compromised by NOD2 deletion, and that *NOD2* expression depends on the presence of commensal bacteria [50].

The human NOD2 protein (nucleotide-binding oligomerization domain/caspase recruitment domain (NOD/CARD) is a cytoplasmic receptor for bacterial molecules which is predominately expressed in Paneth cells [51]. NOD2 received great attention after it was identified as a susceptibility gene for Crohn's disease in 2001 [52, 53]. Structural changes in the leucine-rich repeat region of NOD2 result from two single nucleotide polymorphisms (SNPs) and an insertion mutation that leads to a frameshift mutation at Leu1007 (L1007fsinsC). The authors found that homozygosity or compound heterozygosity increases the relative risk for Crohn's disease by as much as 40-fold compared with individuals without mutation. Approximately one third of patients affected by ileal Crohn's disease show mutations in the NOD2 status [22]. Of note, the three common allelic variants of the NOD2 gene were correlated with an increased susceptibility only in Caucasians and studies have shown remarkable differences in the genetic variability of the NOD2 gene in different ethnical populations. The three common variations could not be found in Asian populations [54, 55] and in African Americans mutation frequency as well as the attributable risk were much lower [56]. These findings could partially explain variations in the frequency of Crohn's disease in different world populations.

A link among Crohn's disease, NOD2, and α-defensins is strongly suggested by observations made in NOD2-knockout mice which exhibit a decrease in Paneth cell defensins (cryptdins) alongside an impaired mucosal immune response to orally delivered but not intraperitoneally administrated *L. monocytogenes* [57]. Also, a decreased α-defensin mRNA expression in biopsy specimens of ileal Crohn's patients, which was even more pronounced in patients carrying NOD2 mutations [58, 59], was observed. The decrease in α-defensins was independent of inflammation in the specimens and not observed in ulcerative colitis or pouchitis, an inflammatory control of non-Crohn's ileitis. Of note,

patients with colonic Crohn's showed unchanged levels of α-defensins in biopsies from their ilea [60], Wehkamp 2005).

In contrast to these results, Simms et al. dispute the notion that reduced α-defensin mRNA expression is a primary effect. The authors propose that this finding is due to epithelial loss, as they did not observe reduced α-defensin levels in noninflamed ileal mucosa in CD patients [61]. Furthermore, the association between NOD2 mutation (L1007fsinsC) and particularly low α-defensin levels could not be reproduced in their cohort.

Yet, in an assessment of luminal HD-5 levels in ileostomy fluids, significantly lower defensin levels in Crohn's patients than in controls were observed, and especially in those with homozygous/compound heterozygous NOD2-mutations [62]. Elphick et al. reported furthermore that in Crohn's disease, the processing of pro-HD-5 to mature HD-5 by trypsin is impaired. HD-5 in the ileostomy fluid of Crohn's patients is predominantly present in complexes with trypsin or chymotrypsin, suggesting an additional way by which epithelial defense might be compromised in CD.

Other mechanisms leading to diminished Paneth cell α-defensin function in patients with ileal CD are even more complex. The differentiation of crypt stem cells into mature secretory cells is governed by the so-called Wnt pathway. Disruption of this signaling cascade leads to impaired Paneth cell differentiation, an event which manifests as a disordered localization of these cells within the crypts [63]. One of the Wnt signaling transcription factors, TCF-4, shows a reduced expression in patients with ileal CD, independent of the extent of inflammation in the biopsies [64]. Further investigations revealed that in the ileal subset of Crohn's disease, a SNP in the TCF-4 promotor region (res3814570) was significantly more frequent than in colonic Crohn's or in ulcerative colitis [65].

A genomewide association study from 2007 identified ATG16L1 as susceptibility locus for ileal Crohn's disease [66]. ATG16L1 protein is involved in autophagy, a process which is essentially responsible for the degradation of intracellular structures, but also mediates degradation of phagocytosed or invasive bacteria. Moreover, Cadwell et al. provided evidence that in ATG16L1 knockout mice, granule exocytosis is abnormal [67, 68], and recently it has been shown that recruitment of ATG16L1 to the site of bacterial entry in the plasma membrane is dependent on activation of NOD2 by bacteria ([69]). As the Paneth cell's foremost activity is the secretion of huge amounts of defensins, an attractive interrelation can be proposed for the decreased defensin functionality in Crohn's disease and the mutation in ATG16L1.

Genomewide screening could also identify X-box binding protein 1 (XBP1) as a risk factor for Crohn's disease and ulcerative colitis [70]. XBP1 deletion in mouse intestinal epithelial cells leads to an increased susceptibility to DSS-induced colitis and even spontaneous enteritis occurred [70].

Furthermore, an association study from Australia implicates KCNN4, a calcium-mediated potassium channel, in ileal Crohn's disease. This channel is involved in the secretory mechanisms in Paneth cells, and mRNA levels are reduced with NOD2 mutations [71].

Aside from these numerous associations between Paneth cells and defensins in ileal inflammation of CD, the involvement of antimicrobial peptides in active celiac disease, an inflammatory disorder of the small intestine as well, has been investigated. Vordenbäumen et al. assessed a panel of β-Defensins (hBD-1 to 4) and α-defensins (HD5-6) in duodenal biopsies of pediatric celiac disease patients and found a decreased hBD-1 and hBD-4 expression, while the remainder of the antimicrobial peptides did not show differences to healthy controls [34]. Although this observation confirmed previous investigations, the pathophysiologic significance of this expression pattern has yet to be determined.

3.4.2. Colon. The composition of the extensive colonic microflora has been characterized more thoroughly by sequencing of 16S ribosomal DNA of fecal contents. Among the approximately 400 different species harboured by the human colon, two phyla clearly dominate: anaerobic Gram-positive firmicutes (Clostridium, Bacillus, Lactobacillus) and anaerobic Gram-negative bacteroidetes (Bacteroides, Flavobacteria) [72]. A high interindividual diversity has been noted, though at any time, each individual carries a stable "fingerprint" pattern [73]. Considering that an epithelium of only a single cell layer separates the bowel from the microbe-laden lumen, this barrier is remarkably effective. In addition to secreted immunoglobulins provided by the adaptive immune system, the innate branch offers a wide variety of antimicrobial peptides.

The first defensin identified in the human large bowel was the β-defensin hBD-1, and in the noninflamed colon, it is the major β-defensin. A recent publication reported that the peroxisome proliferator-activated receptor (PPAR) gamma is playing a major role in the constitutive expression of hBD-1 [74] and confirmed an earlier finding of a reduction of hBD-1 expression in inflamed mucosa of IBD patients [75]. Strongly supporting an important role of hBD-1 in colonic IBD, Kocsis et al. have reported a genetic association of hBD-1 SNPs with colonic Crohn's disease in a Hungarian cohort [76]. These findings challenge the perspective that reduced defensin expression is merely the result of epithelial loss in inflammatory states [77].

In the healthy colon, hBD-2 and hBD-3 are absent and only induced during inflammation or infection. Stimuli for hBD-2 induction comprise both bacteria and cytokines, like *Campylobacter jejuni* [78] or the bacterial component flagellin from the E. coli strain Nissle 1917 (shown in Figure 1), which is used as probiotic in the maintenance treatment of ulcerative colitis [79]. On the cytokine level, the induction is mediated by proinflammatory cytokines such as IL-1β (through NF-κB-dependent and AP-1-dependent pathways) and TNF-α [80] or IL-17 [81].

Different defensin mRNA expression in the different forms of inflammatory bowel diseases has been noted, as in patients with ulcerative colitis, hBD-2 and hBD-3 are strongly induced in the event of inflammation. In comparison, the induction is attenuated in Crohn's disease [14, 82, 83] and the colonic mucosa of Crohn's disease patients is compromised in the killing capacity towards different commensal bacteria [84]. The mechanism behind

the reduced hBD-2 expression in inflamed colonic Crohn's has not been elucidated up to now. In a European and US cohort, gene copy numbers for hBD-2 [85] were reduced, but results from a New Zealand Cohort were inconsistent with this finding [86].

As NOD2 mutations have been generally associated with Crohn's disease, researchers also investigated the effects of NOD2 mutations on the expression of β-defensins. Voss et al. demonstrated that the expression of hBD-2 is mediated by NOD2 activation [87], but a subanalysis stratified for NOD2 mutation status could not identify differences in colonic hBD-2 expression (Wehkamp, unpublished observation). Further investigations revealed that 1,25-dihydroxyvitamin D3 and MDP induce expression of hBD-2 and cathelicidin through stimulation of NOD2 expression [88].

Many lines of evidence thus point to a major role of β-defensins in inflammatory processes of the colon. New data for the *α-defensins* from a mouse model show that the Paneth cell cryptdins synthesized in the ileum retain their structure and functionality till the colonic lumen [89], suggesting a role for α-defensins in the large bowel as well. As has been mentioned above, Paneth cell metaplasia is noted on different sites of inflammation along the gastrointestinal tract, including the colon [90]. This metaplastic response could therefore represent a mechanism that provides additional protection by α-defensins at these sites. Furthermore, an interesting observation by Langhorst et al. showed a significant elevation of hBD-2 peptide in fecal samples from patients with irritable bowel syndrome, a condition which demonstrates no macroscopic visible inflammation on colonoscopy [91].

The antimicrobial peptide elafin shares a similar expression pattern with the inducible β-defensin, LL37 and secreted leukocyte protease inhibitor. Its additional function as an antiprotease balances the proteolytic effects of HNE (human neutrophilic elastase) from polymorphonuclear cells in healthy tissues. Moreover, it has been found to be reduced in colonic Crohn's disease, which could point to an involvement of protease-antiprotease disbalance explaining in part the penetrating, transmural type of inflammation [92].

Cathelicidin (LL37) shows induction in inflamed tissues of ulcerative colitis, while in active Crohn's disease the induction seems to be attenuated [93]. In mutant mice, Cathelicidin restricts colonization with epithelial adherent bacterial pathogens like *Citrobacter rodentium* [94], confirming its vital role in the armamentarium of the innate immune system.

The large intestine harbors a complex ecosystem, where classical immune cells and colonic epithelial cells interact in concert with the dense resident microflora [95]. After recognizing the importance of the microbiota in chronic intestinal inflammation too, the characterization of the enteric luminal flora in inflammatory bowel disease revealed differences in the composition compared to healthy controls. Swidsinski et al. [96] among others demonstrated that mucosa-associated bacteria are dramatically increased in IBD mucosa. Anaerobic *Bacteroides* species and aerobic *Enterobacteriaceae* (*E. coli*) were most prevalent and furthermore early disease recurrence seemed to be accompanied by increased numbers of *E. coli*, *Bacteroides,* and *Fusobacterium*.

4. Therapeutic Consequences

Taken together, defensins seem to be attractive targets for pharmacologic intervention in a range of diseases. In the case of inflammatory bowel disease, Kubler et al. examined the effect of the currently available treatments (immunomodulators like azathioprine, corticosteroids or aminosalicylates) on the expression of main antimicrobial defensins, but no significant changes could be observed [97]. Treatment with the anti-TNF antibody infliximab was reportedly associated with normalization of defensin mRNA expression [77], but this was interpreted as a general effect stemming from epithelial regeneration. New insights in the genetics of antimicrobial peptides and their respective pathways of induction, regulation, and secretion could lead to therapeutic strategies which aim to strengthen barrier defense on epithelial surfaces. As detailed above, in the Paneth cell, numerous mechanisms leading to defective α-defensin function have been identified and can offer sweet spots for directed therapies in the future. Probiotics are effective as a maintenance treatment in colonic IBD, which has already been shown in a placebo controlled, double-blind study with the bacterium *E. coli* Nissle 1917 [98]. A recent meta-analysis confirmed the beneficial effect of probiotic treatment in the maintenance of ulcerative colitis [99]. A possible mechanism is the induction of hBD-2, which has been demonstrated for *E. coli* Nissle, as well as for other therapeutic probiotic *E. coli* strains and *Lactobacilli* [100–102].

Moreover, induction of antimicrobial peptides by agents like worm eggs, vitamin D, specific bacteria, food, artificial components, or possibly prebiotics may also be helpful. A larger, dose finding phase II clinical trial with live ova from *Trichuris suis* (porcine whipworm) is about to be initiated in autumn 2010, as previous small studies have been shown to improve the clinical outcome in ulcerative colitis (a double-blind clinical study) and in Crohn's disease (an open-label study) [103, 104]. Iatrogenic infection with these parasitic worms (Helminths), which are not able to survive in the human intestine for longer than 12 days, is thought to modulate the immune response. Evidence for these findings come from the observation that children with helmintic infections have reduced atopy [105], and peripheral blood mononuclear cells increase the production of anti-inflammatory mediators IL-10 and TGF-β [106]. Whether the therapeutic effect is mediated by a shift in adaptive immune function, or whether stimulation of the production of antimicrobials is significantly involved, is an intriguing question. We hope to address this within the context of the aforementioned study. However, recent animal studies have provided evidence that infection with *Hymenolepis diminuta*, also known as rat tapeworm, can cause a significant disease exacerbation as well [107]. Thus, the therapeutic use of helminths in IBD has to be considered carefully and its risk potential has to be assessed meticulously.

In any case new treatment alternatives for inflammatory bowel diseases are eagerly anticipated by patients and physicians alike, and most probably, advances will come from the field of innate immunity.

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