Specific changes of gut commensal microbiota and TLRs during indomethacin-induced acute intestinal inflammation in rats

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KEYWORDS
Bacterial adherence; FISH; Gut commensal microbiota; Gut inflammation; Host–bacterial interactions; Inflammatory bowel disease; TLR; Toll-like receptors

Abstract

Background and aims: Gut microbiota is a contributing factor in the development and maintenance of intestinal inflammation, although precise cause–effect relationships have not been established. We assessed spontaneous changes of gut commensal microbiota and toll-like receptors (TLRs)-mediated host–bacterial interactions in a model of indomethacin-induced acute enteritis in rats.

Methods: Male Spague–Dawley rats, maintained under conventional conditions, were used. Enteritis was induced by systemic indomethacin administration. During the acute phase of inflammation, animals were euthanized and ileal and ceco-colonic changes evaluated. Inflammation was assessed through disease activity parameters (clinical signs, macroscopic/microscopic scores and tissue levels of inflammatory markers). Microbiota (ileal and ceco-colonic) was characterized using fluorescent in situ hybridization (FISH) and analysis of 16s rDNA polymorphism. Host-bacterial interactions were assessed evaluating the ratio of bacterial adherence to the intestinal wall (FISH) and expression of TLRs 2 and 4 (RT-PCR).

Results: After indomethacin, disease activity parameters increased, suggesting an active inflammation. Total bacterial counts were similar in vehicle- or indomethacin-treated animals.

Abbreviations: FISH, Fluorescent in situ hybridization; GCM, gut commensal microbiota; IBD, inflammatory bowel disease; MPO, myeloperoxidase; RT-PCR, reverse transcription polymerase chain reaction; TLR, toll-like receptor; t-RFLP, terminal restriction fragment length polymorphism.

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1. Introduction

Gut commensal microbiota (GCM) is a dynamic factor in gastrointestinal homeostasis and can mediate both beneficial and harmful effects for the host.\(^1\)\(^-\)\(^3\) Accordingly, GCM has been suggested to play a major role in the pathogenesis of inflammatory bowel disease (IBD), a pathological condition characterized by strong activation of the mucosa-associated immune system due to a complex interaction of genetic, immunological and environmental factors.\(^4\) According to this, several reports have established that the total microbial charge as well as the composition of the intestinal microbiota have significant impact on host immunity and influence the course of mucosal inflammation.\(^5\)\(^,\)\(^6\) For instance, germ-free rats and animals treated with antibiotics (with reduced bacterial charge) develop reduced inflammatory responses within the gut.\(^5\)\(^,\)\(^6\) On the other hand, in humans, an increase of IBD incidence in industrialized countries has been related to the improvement in standards of hygiene and the subsequent changes in GCM.\(^7\) Overall, these changes generate alterations in microbiota recognition and altered host-microbial interactions, leading to the appearance of abnormal gut immune responses and, as a result, an increased susceptibility to gut inflammation and the generation of persistent gut inflammatory states.\(^1\)\(^,\)\(^2\)\(^,\)\(^7\) Further supporting a role for microbiota in the development and maintenance of intestinal inflammation, the addition of some bacterial strains, with probiotic properties, has shown beneficial effects on different experimental models of intestinal inflammation.\(^8\) Although these evidences, a clear cause–effect relationship has not been established for GCM in intestinal inflammation. In the present study we assessed spontaneous changes in GCM in a model of indomethacin-induced ileitis in rats. This model has been previously characterized by us and represents a valid IBD model in which a local inflammation of the ileum appears after systemic treatment with indomethacin.\(^9\)\(^-\)\(^11\) Here, we assessed the indomethacin-induced acute inflammatory response, both in the primary site of inflammation (ileum) as well as in a distant area (cecum–colon) in animals maintained in standard microbiological conditions, with the objective of characterizing potential inflammation-associated changes in GCM and alterations in host-bacterial interactions. For this, we characterized (qualitatively and quantitatively) the luminal microbiota and determined the incidence of bacterial adherence to the intestinal epithelium and the changes in the expression of Toll-like receptors 2 and 4 (TLR-2 and TLR-4), both implicated in the development of intestinal inflammation.\(^11\)\(^,\)\(^12\)

2. Materials and methods

2.1. Animals

Adult male Sprague–Dawley OFA rats (300–350 g) were obtained from Charles River Laboratories (Lyon, France). Animals were acclimatized during a 3-week period to the new housing conditions before starting any experimental protocol. All animals were housed in standard plastic cages with stainless steel grid roofs in an environmentally controlled room (20–21 °C, 40–70% humidity, 12 h light/dark cycle), and received a commercial diet (15.4% protein, 2.9% fat and 3.9% fiber; SASE, Panlab S.L., Barcelona, Spain) and tap water ad libitum. All procedures were approved by the Ethical Committee of the Universidad Autónoma de Barcelona and the Generalitat de Catalunya.

2.2. Experimental procedures

Intestinal inflammation was induced by administration of two injections of indomethacin (7.5 mg/kg, subcutaneous, n = 7) 48 h apart, as previously described by us.\(^9\)\(^-\)\(^11\) Control animals received similar treatment with saline (0.3 ml/rat, subcutaneous, n = 6). Animals were euthanized at day 4 after the first injection of indomethacin, corresponding to the acute phase of inflammation.\(^9\)\(^-\)\(^11\) During this time, clinical signs were monitored every other day (see below, clinical assessment of inflammation).

2.3. Clinical assessment of inflammation

Clinical assessment of inflammation included appearance of feces, state of hydration and general condition (including hunch posture, piloerection, motor activity and state of mucous membranes). A total score of 0–9 was assigned to each animal, corresponding to the addition of separate scores (0–3) for each parameter (0: normal, 1: mild alteration; 2: moderate alteration; 3: severe alteration). Body weight changes were also monitored. In addition, a macroscopic score (0–9) was also assigned to each animal during necropsy. The macroscopic score was based on: presence or absence of abdominal distension (0: absent; 1: present), presence and characteristics of edema in the abdominal cavity (0: absent; 1: moderate/localized; 2: clear and generalized), presence of adherences (0: without adherences; 1: some local adherences; 2: extensive and generalized adherences), appearance of the intestine (ileum and cecum–colon) (0: normal; 1: moderate sings of congestion and/or distension; 2: clear and generalized adherences).
altered appearance and appearance of bowel (ileal and ceco-colonic) contents (0: normal; 1: reduced content with some fluid and/or mucus; 2: no content, only fluid or mucus, bloody appearance).

2.4. Samples collection

Rats were euthanized by CO₂ inhalation. Immediately, a medial laparotomy was performed and the inflammatory status assessed macroscopically as detailed above. Thereafter, tissue samples of the ceco-colonic region (cecum and proximal part of the colon) and ileum and fecal content of the same areas (about 0.5 g) were obtained under sterile conditions and immediately frozen with liquid nitrogen. All samples were stored at −20 °C (fecal samples) or −80 °C (tissue samples) until analysis. A set of tissue samples from the ileum was fixed overnight in 4% paraformaldehyde and thereafter processed following standard histological procedures.

2.5. Histological score

Paraffin sections (5 μm) of ileal samples were stained with hematoxylin-eosin following standard histological procedures. Two to four coded sections for each animal were scored for inflammation. A histological score based on the epithelial structure (0: normal; 1: mild alterations; 2: moderate alterations; 3: severe alterations), the presence of edema (0: absent; 1: minor and localized; 2: minor generalized; 3: severe generalized), the presence of ulcerations (0: absent; 1: one ulcer observed; 2: several small ulcers observed; 3: numerous small and large ulcers observed), and the inflammatory infiltrate (0: absent; 1: mild localized inflammatory infiltrate; 2: moderate diffuse infiltrate; 3: severe generalized infiltrate) was assigned to each animal (maximal score of 12). Histological evaluation was performed, in a blinded fashion, by two independent investigators, to avoid bias.

2.6. Myeloperoxidase levels

To determine myeloperoxidase (MPO) content frozen tissue samples from the ileum and cecum colon were powdered, weighted and homogenized in lyses buffer (Mini complete protease inhibitor, HEPES 1 M, Triton X-100, PMSF 100 mM). Thereafter, the homogenates were incubated for twenty minutes at 4 °C, centrifuged at 14000 rpm for 10 minutes at 4 °C and the supernatant collected and fixed overnight (4% paraformaldehyde, 4 °C). Fixed aliquots were stored (−20 °C) until use. At the time of analysis, samples were diluted in PBS and disposed on 10-well gelatin-covered slides, air-dried at room temperature and fixed with ethanol (10 min). For hybridization, slides were incubated overnight in a dark moist chamber with the corresponding probe (5 ng/μl in hybridization buffer: 20 mM Tris–HCl, 0.9 M NaCl, 0.1% sodium dodecyl sulfate pH 7.2, 50 °C; with 20% formamide for the LAB 158 and NON 338 probes). Except for the BAC 303 probe which was hybridized for only 3 h at 47 °C and for the LAB 158 probe which was incubated overnight at 47 °C. Samples to be hybridized with the LAB 158 probe were pre-treated with lysozyme (1 h, 37 °C) prior to the hybridization. After incubation, slides were rinsed in preheated washing buffer (20 mM Tris–HCl, 0.9 M NaCl, pH 7.2, 180 mM NaCl for the LAB 158 and NON 338 probe; 30 min, 50 °C), briefly rinsed with milli-Q water, air dried and mounted with Vectashield (Vector Laboratories, Peterborough, UK).

Slides were viewed under oil immersion, using a Nikon Fi 60 epifluorescence microscope equipped with a filter for Cy3. Twenty five randomly selected fields were counted for each sample (in duplicate).

2.8. Terminal restriction fragment length polymorphism (t-RFLP)

T-RFLP analysis of bacterial community was performed according to previously published procedures. Briefly, a 1497-bp fragment of the 16S rDNA gene was amplified using a 6-carboxy-fluorescein-labeled forward primer: 5'-D-Bact-0008-a-S-20 (5'-6-FAM-AGAGTTGATCMTGGCTCAG-3') and reverse primer PH1552 (5'AAGGAGGTGATCAGCCGCA-3'). Fluorescent-labeled PCR products were purified (QIAquick PCR purification kit columns; Qiagen, West Sussex, UK) and eluted in a final volume of 30 μl of Milli-Q water. The resultant PCR product was subjected to a restriction with Hhal (20,000U/μl) (Biolabs Inc., New England, USA) and fluorescent-labeled terminal restriction fragments (TRF) analyzed by capillary electrophoresis (ABI 3100 Genetic Analyzer, PE Biosystems, Warrington, UK) with a 25-U detection threshold. Determination of the TRFs sizes in the range 50–700 base pairs (bp) were performed with the size standard GS-1000-ROX (PE Biosystems). Data was analyzed and standardized following the method described by Kitts (2001). Microbial richness was considered as the number of peaks in each sample after standardization. For pair-wise comparisons of the profiles, a Dice coefficient was calculated and dendograms were constructed using the Fingerprinting II software (Informatix, Bio-Rad, CA, USA) and an unweighted pair-group method with averaging algorithm (UPGMA).

Because of the limited availability of ileal contents, the t-RFLP analysis was only carried out in ceco-colonic samples.
2.9. Assessment of bacterial wall adherence

Bacterial wall adherence was assessed following techniques described elsewhere. Briefly, a sample of ileal tissue (about 2 cm in length, corresponding to an area of inflammation) was rinsed in saline solution, sonicated twice for 60 s and then fixed in 4% paraformaldehyde for 16 h at 4 °C. Thereafter, tissues were paraffin embedded using standard protocols, sectioned at 5 μm and sections hybridized following the general FISH procedures described above. Slides were viewed under oil immersion, using a Nikon Fi 60 epifluorescence microscope equipped with a filter for Cy3. 2 to 3 sections (in duplicate) per animal were observed. Observation of hybridized bacteria in contact with the epithelium in at least one section was taken as prove of bacterial attachment.

2.10. Semi-quantitative reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was extracted from ileal and ceco-colonic tissue samples using RNAwiz (Ambion, Madison, WI) and treated with DNA-free (Ambion, Madison, WI) for 30 min at 37 °C. cDNA was synthesized from 5 μg total RNA in a reaction mixture of 50 μl containing 0.5 μg of oligo 18 (DT) primer (Ambion, Madison, WI), 2 mM dNTP (Ecogen, Barcelona, Spain), and 10 Units Moloney Murine Leukemia Virus (MMLV) (Ambion, Madison, WI). The resulting cDNA was amplified in a total volume of 50 μl with 1 unit of taqDNA, 1 mM dNTP mixture, and 0.5 μM primers (Proligo-Sigma, Madrid, Spain) (Table 1). The PCR amplification protocol was as follows: 35 (GAPDH) or 40 (TLR-2 and TLR-4) cycles with 1 min (GAPDH) or 30 sec (TLR-2 and TLR-4) of denaturation at 95 °C, 1 min (GAPDH) or 30 sec (TLR-2 and TLR-4) of annealing at 50 °C, and 1 min of extension at 72 °C on a thermal cycler (Techno Cambridge Ltd.). Amplified products were electrophoresed on 2% agarose gel in TAE buffer, stained with ethidium bromide, photographed under ultraviolet light, and quantified using image-analyzing software (Quantity-One, Bio-Rad laboratories). For semiquantification, the ratio of the optical density of each PCR product and GAPDH was determined.

2.11. Statistical analysis

Data are expressed as mean ± SEM or median (interquartile range) ± SD (for bacterial counts). When assessing differences between two groups data were analyzed using a Student’s t test or a Mann–Whitney test, as appropriate. When assessing differences in bacterial counts a non-parametric analysis of variance (ANOVA), followed when necessary by a Kruskal-Wallis multiple comparisons test, was used. Bacterial wall adherence was analyzed using the Chi-square test. In all cases, results were considered statistically significant when P < 0.05.

3. Results

3.1. Clinical indices and macroscopic assessment of inflammation

During the 4-day period after treatment, vehicle-treated animals showed no clinical signs of inflammation and a linear increase in body weight (Fig. 1A). On the other hand, during the same period of time, indomethacin-treated animals showed a progressive reduction in body weight (Fig. 1A) and an increase in their clinical scores (reflecting a worsening in the general state of the animals: piloerection, reduced activity, hunch posture and presence of chromodacryorrhea) (Fig. 1B).

At necropsy, macroscopic assessment of the abdominal cavity and the gut in vehicle-treated animals revealed no signs of intestinal inflammation. However, in indomethacin-treated animals, macroscopic scores increased significantly over control values (Fig. 1C). Main alterations observed included abdominal distension, adherences and distension/congestion of the intestine.

3.2. Microscopic assessment of inflammation

Vehicle-treated rats showed no histological changes consistent with the presence of intestinal inflammation. In contrast, indomethacin-treated animals showed microscopic signs of inflammation, with histopathological scores significantly higher than those in control animals (Fig. 1D). The more common histopathological changes observed were alterations of the epithelial structure, with variable degree of destruction of the villi, including the presence of ulcers (observed in 3 out of 7 indomethacin-treated animals), and the presence of local or generalized inflammatory infiltrate characterized by the presence of lymphocytes and neutrophils.

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Characteristics of the primers for rat TLR-2, TLR-4 and GAPDH.</th>
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<tbody>
<tr>
<td><strong>Primer</strong></td>
<td><strong>Sequence</strong></td>
</tr>
<tr>
<td>TLR-2</td>
<td></td>
</tr>
<tr>
<td>LEFT</td>
<td>5′-CTGACCTCTCTCAACGAACT-3′</td>
</tr>
<tr>
<td>RIGHT</td>
<td>5′-CGCTGAGGTCTAAGACTTCT-3′</td>
</tr>
<tr>
<td>TLR-4</td>
<td></td>
</tr>
<tr>
<td>LEFT</td>
<td>5′-CATACGAGATTTCTCTAACG-3′</td>
</tr>
<tr>
<td>RIGHT</td>
<td>5′-GGAGTCTGTAGTGCTGTA-3′</td>
</tr>
<tr>
<td>GAPDH</td>
<td></td>
</tr>
<tr>
<td>LEFT</td>
<td>5′-ATGAGGCCTCTTCCATGAGGC-3′</td>
</tr>
<tr>
<td>RIGHT</td>
<td>5′-CCGCCCCCCTTCCGCTGAC-3′</td>
</tr>
</tbody>
</table>

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3.3. Myeloperoxidase levels

In control conditions, MPO levels were similar, and relatively low, in ileal or ceco-colonic tissues (Fig. 1E). Indomethacin treatment resulted in a 40-fold \( (P < 0.001) \) and 4-fold \( (P < 0.01) \) increase over control values in MPO levels in the ileum and ceco-colonic area, respectively (Fig. 1E).

3.4. Characterization of the luminal microbiota by FISH

In vehicle-treated animals, total bacterial counts, determined by FISH as EUB338-positive cells, were higher in ceco-colonic vs. ileal contents \( (P < 0.05) \) and oscillated between \( 1.8 \times 10^9 \) and \( 3.7 \times 10^9 \) cells/ml in the ileum and \( 17.3 \times 10^9 \) and \( 41.0 \times 10^9 \) cells/ml in the ceco-colonic area (Fig. 2). In indomethacin-treated animals, total bacterial counts were within the same range, although a slight increase in bacterial counts was observed in the ileum, reaching in some cases values similar to those observed in the ceco-colonic region. Nevertheless, during inflammation, a dysbiotic state, manifested as an alteration of the relative composition of the luminal microbiota, was observed both in the ileum and ceco-colonic area.

In control conditions, luminal ileal microbiota was dominated by \textit{Lactobacillus}/\textit{Enterococcus} spp. (LAB 158 probe), which represented by 43% of the total bacterial counts.
detected in the ileum. Enterobacteriaceae, Bifidobacterium spp. and Bacteroides spp. were very low (overall by 1% of the total bacterial counts) and the Clostridium coccoides–Eubacterium rectale group were undetectable (below 10^6 cells/ml, considered the FISH detection limit) (Fig. 2).

During inflammation the counts of Bacteroides spp. and Enterobacteriaceae augmented and Clostridium coccoides–Eubacterium rectale group appeared at significant levels; while Lactobacillus and Enterococcus spp. had a tendency towards a reduction, although statistical significance was not reached (Fig. 2).

Luminal ceco-colonic microbiota was characterized by the relatively high counts of Bacteroides spp and Enterobacteriaceae augmented and Clostridium coccoides–Eubacterium rectale group appeared at significant levels; while Lactobacillus and Enterococcus spp. had a tendency towards a reduction, although statistical significance was not reached (Fig. 2).

Fig. 3 shows the relative abundance of the Individual bacterial groups assessed. In control conditions, relative composition of the microbiota was clearly different between the ileum and the ceco-colonic area (see also Fig. 2 for exact bacterial counts). However, during inflammation, the relative distribution of the microbiota indicated a state of dysbiosis with similar microbial pattern in both regions (Fig. 3).

3.5. Ecological characterization of the luminal microbiota: t-RFLP analysis

The dendogram representation of the similarity indexes of the t-RFLP profiles of the ceco-colonic microbiota showed a clear clustering of the two experimental groups (control vs. inflamed; Fig. 4A). Table 2 summarizes the main bacterial groups, as detected by t-RFLP analysis, with the differential presence in controls or indomethacin-treated animals. The overall prediction for the major bacterial groups was similar in both groups of animals, thus suggesting a similar bacterial biodiversity in control or inflamed conditions. Indeed, the number of t-RFs, taken as a measure of biodiversity, varied from 22 to 34 among the different animals, with similar
mean values in control conditions or after the treatment with indomethacin (Fig. 4B).

3.6. Bacterial wall adherence

In control conditions, only *Lactobacillus–Enterococcus* spp. (LAB 158 probe) were adhered to the ileal wall (by 40% incidence; **Table 3**). However, during inflammation the incidence of bacterial adherence increased and all bacterial groups assessed, with the exception of Clostridia, were found adhered to the ileal wall. In particular, adherence of Enterobacteriaceae was observed in all the animals assessed, and *Bacteroides* spp. in 50% of the cases (**Table 3**).

3.7. Expression of TLR-2 and TLR-4 in ileum and ceco-colonic area

TLR-2 and TLR-4 expression was detected, with different intensities, in ileal and ceco-colonic tissues from all animals. In control conditions, levels of expression of TLR-2 and TLR-4 were slightly lower in the ceco-colonic area than in the ileum, but statistical significance was not found. During inflammation, expression of TLR-2 and TLR-4 was up-regulated both in the ileum and ceco-colonic region (Fig. 5). Relative changes in receptor expression were larger in ceco-colonic (by 2.6-fold increase) than in ileal tissues (by 1.5-fold increase), regardless the TLR subtype considered.

4. Discussion

In the present study we assessed inflammation-related changes of GCM in a model of indomethacin-induced acute intestinal inflammation in rats. The primary site of inflammation (ileum) and the cecum/colon were evaluated. Results obtained show that during a primary ileitis an inflammatory-like response can also be detected in the ceco-colonic area. Simultaneously, a dysbiotic state, characterized by qualitative and quantitative changes in the ileal and ceco-colonic luminal microbiota was evidenced. Alterations in host–bacterial interactions, as suggested by increased bacterial adherence to the intestinal epithelium and changes in TLRs' expression, were also observed.

Systemic administration of indomethacin in rodents is a well-known model of acute intestinal inflammation, coursing with a primary ileitis. Here we show that, in addition to the ileal response, an inflammatory reaction can also be observed in the ceco-colonic region, although attenuated when compared with the ileum, as indicated by the changes in MPO levels. Extended inflammatory-like responses and disturbed function are common to several models of intestinal inflammation and are likely to derive from complex neuro-immuno-endocrine interactions originated at the primary site of insult, leading to a pan-inflammatory-like state. Results obtained here show that similar widespread changes can also be observed for GCM.

In control conditions, GCM showed qualitative and quantitative differences between the ileum and the ceco-colonic area. Total bacterial counts were higher in the ceco-colonic region. This agrees with well-established data showing that the intestinal microbial charge increases aborally, with the higher counts in the ceco-colonic area. In general, Gram-positive firmicutes were predominant. Ileal commensal microbiota was dominated by the *Lactobacillus* and *Enterococcus* spp. group, while the clostridia group was undetectable. However, clostridia represented the dominant group in the ceco-colonic region. Regardless the area considered, enterobacteria counts were low. Overall, this relative distribution of the GCM is consistent with previous reports in rats and mice.

Evidences suggest that GCM plays a key role in the development of intestinal immune and inflammatory responses and is an important pathogenic component of IBD. Gut microbiota seems to be particularly critical for the induction of intestinal inflammation in the indomethacin model. In fact, microbiota seems to be necessary for indomethacin-induced...
intestinal damage, because both germ-free rats\textsuperscript{31–33} and animals treated with antibiotics, with reduced microbial charge,\textsuperscript{34,35} develop minimal intestinal lesions after the administration of indomethacin. Similarly, colonization of germ free animals with conventional bacteria restores the susceptibility to non-steroidal anti-inflammatory drugs-induced intestinal ulceration.\textsuperscript{36} However, the potential changes that a standard GCM might experience during indomethacin-induced intestinal inflammation have not been characterized in detail.

The present report shows that commensal microbiota changed both qualitatively and quantitatively during indomethacin-induced inflammation in rats, in agreement with previous reports.\textsuperscript{9,10,29–31} Similar microbial changes were observed both in the ileum and the ceco-colonic region; although they were more prominent in the ileum, corresponding to the primary site of inflammation. Indeed, the t-RFLP analysis showed a separate clustering of control and indomethacin-treated animals, thus indicating that, during inflammation, animals experienced a similar change in the composition of their microbiota. However, biodiversity was not changed, thus suggesting a redistribution of the existing microbiota rather than the disappearance of existent bacterial groups or the apparition of new ones. This, contrasts with data from mice in which indomethacin-induced enteritis was linked to a reduction in bacterial diversity.\textsuperscript{30}

Changes in the microbiota were, overall, comparable to those observed in the ileum. Again, total bacterial counts remained fairly stable, contrasting with data indicating that during colitis the total microbial charge is reduced.\textsuperscript{37–39} Nevertheless, there was a dysbiotic state characterized by a heavy increase in the counts of enterobacteria and a moderate increase in the counts of Bacteroides spp. Interestingly, both experimental data derived from rodent models of colitis and human clinical
data implicate members of these two bacterial groups in the pathogenesis of intestinal inflammation. Overall, these observations suggest that indomethacin-induced gut inflammation does not imply an indiscriminate bacterial overgrowth, or bacterial disappearance, but a specific state of dysbiosis.

Enterobacteriaceae, and specifically *Escherichia coli*, have been directly associated with the development of intestinal inflammation.
indomethacin-induced enteritis and with the aggravation of colitis.42,43 Interestingly, enterobacteria counts increased during inflammation. Moreover, all animals with intestinal inflammation were positive for Enterobacteriaceae adherence, indicating an increased direct interaction with the epithelium.43

The rates of bacterial adherence to the ileal wall and the expression of TLRs suggest that host–bacterial interactions were also altered during inflammation. First, as mentioned above, the incidence of bacterial attachment increased during inflammation, as previously suggested by us.10 This can be of importance at a pathogenic level because adhered microbiota has been suggested to be the one directly interacting with the host’s bacterial recognition systems and, thus eliciting beneficial or harmful responses within the gut.45,46 Increased rate of adherence might be regarded as a direct consequence of the increases in bacterial luminal counts, thus rising the possibilities of an interaction with the epithelium. However, not all bacterial groups behave in this manner. In particular, the clostridia group was significantly increased during inflammation, but epithelial attachment was not observed. This, together with previous studies, suggests that a direct relationship between incidence of attachment and bacterial counts cannot be established.14 In addition, ileal and ceco-colonic expression of TLR-2 and TLR-4 was up-regulated during inflammation. TLRs, and particularly TLR-2 and TLR-4, have been largely implicated in the initiation, course and resolution of intestinal inflammation.11,12,47,49 TLR-2 recognizes several Gram-positive bacteria-derived products; while TLR-4 is activated by lipopolysaccharides (LPS) from Gram-negative bacteria.48 Up-regulation of these receptors might be associated to the dysbiosis observed during inflammation; characterized by increases in both, Gram-positive (Bifidobacterium spp. and clostridia group) and Gram-negative counts (Bacteroides spp. and enterobacteria). These observations support an alteration in host-bacterial interactions during indomethacin-induced inflammation and agree with previous data showing that non-steroidal anti-inflammatory drug-induced small intestine damage is TLR-4-dependent50 or data showing upregulation of TLR-2 and –4 in IBD patients.51,52

**Table 3** Incidence of bacterial adherence to the ileal wall during indomethacin-induced inflammation.α

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Inflamed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enterobacteriaceae</td>
<td>0 (0/5)</td>
<td>100 (5/5)**</td>
</tr>
<tr>
<td>Bacteroides spp.</td>
<td>0 (0/5)</td>
<td>50 (3/6)</td>
</tr>
<tr>
<td>Clostridium coeoides Cluster XIVa</td>
<td>0 (0/5)</td>
<td>0 (0/5)</td>
</tr>
<tr>
<td>Bifidobacterium spp.</td>
<td>0 (0/6)</td>
<td>20 (1/5)</td>
</tr>
<tr>
<td>Lactobacillus and Enterococcus spp.</td>
<td>40 (2/5)</td>
<td>20 (1/5)</td>
</tr>
</tbody>
</table>

α Results are the incidence of bacterial attachment (percentage) for the different bacterial groups assessed. Values between brackets represent the positive cases/total number of rats assessed.

**P < 0.01 vs. control.**

*P = 0.06 vs. control (Chi-square test).

Figure 5 Relative expression of TLR-2 and TLR-4 in ileal and ceco-colonic tissues from controls and indomethacin-treated animals. Data are mean ± SEM of 6–7 animals per group. *: P < 0.05 vs. respective control group. +: P = 0.05 vs. respective control group.
increased host–bacterial interaction (up-regulation of TLR-2 and -4 and increased bacterial adherence). All together, these observations support the view that gut microbiota an host–bacterial interactions are important pathogenic factors in intestinal inflammation. Microbiological conditions of the experimental animals should be taken into consideration in all animal models of IBD.

Conflict of Interest

None.

Acknowledgements

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ETV designed and performed experiments, analyzed data and drafted the manuscript. MA analyzed data and drafted the manuscript. PV designed experiments, participated in data analysis and drafted the manuscript. VM designed and performed experiments, analyzed data and drafted the manuscript. All authors read and approved the final manuscript.

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