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*J Immunol* 2009; 183:7514-7522; Prepublished online 16 November 2009; doi: 10.4049/jimmunol.0900063

http://www.jimmunol.org/content/183/11/7514

**Supplementary Material**

http://www.jimmunol.org/content/suppl/2009/11/16/jimmunol.0900063.DC1

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G Protein-Coupled Receptor 43 Is Essential for Neutrophil Recruitment during Intestinal Inflammation

Christian Sina,*† Olga Gavrilova,* Matti Förster,* Andreas Till,* Stefanie Derer,* Friederike Hildebrand,* Björn Raabe,† Athena Chalaris, † Jürgen Scheller,† Ateequr Rehmann,* Andre Franke,* Stephan Ott,*† Robert Häslers,* Susanna Nikolaus,*‡ Ulrich R. Fölsch,‡ Stefan Rose-John, † Hui-Ping Jiang,§ Jun Li,¶ Stefan Schreiber,2*,§ and Philip Rosenstiel2*,3*

Molecular danger signals attract neutrophilic granulocytes (polymorphonuclear leukocytes (PMNs)) to sites of infection. The G protein-coupled receptor (GPR) 43 recognizes propionate and butyrate and is abundantly expressed on PMNs. The functional role of GPR43 activation for in vivo orchestration of immune response is unclear. We examined dextran sodium sulfate (DSS)-induced acute and chronic intestinal inflammatory response in wild-type and Gpr43-deficient mice. The severity of colonic inflammation was assessed by clinical signs, histological scoring, and cytokine production. Chemotaxis of wild-type and Gpr43-deficient PMNs was assessed through transwell cell chemotactic assay. A reduced invasion of PMNs and increased mortality due to septic complications were observed in acute DSS colitis. In chronic DSS colitis, Gpr43−/− animals showed diminished PMN intestinal migration, but protection against inflammatory tissue destruction. No significant difference in PMN migration and cytokine secretion was detected in a sterile inflammatory model. Ex vivo experiments show that GPR43-induced migration is dependent on activation of the protein kinase p38α, and that this signal acts in cooperation with the chemotactic cytokine keratinocyte chemoattractant. Interestingly, shedding of L-selectin in response to propionate and butyrate was compromised in Gpr43−/− mice. These results indicate a critical role for GPR43-mediated recruitment of PMNs in containing intestinal bacterial translocation, yet also emphasize the bipotential role of PMNs in mediating tissue destruction in chronic intestinal inflammation. The Journal of Immunology, 2009, 183: 7514–7522.

Inflammatory bowel diseases (IBD),4 Crohn’s disease (CD) and ulcerative colitis, are disorders of unknown etiology characterized by chronic relapsing-remitting inflammation of the gastrointestinal tract. Pathophysiological and genetic evidence points to an important role of intestinal barrier function in the initiation and perpetuation of the disease (1–7). Impaired epithelial permeability and loss of the integrity of the primary immunological barrier may lead to an increase of physiological and pathogenic bacteria and their components, which in turn may trigger intestinal inflammation (8). A pathological hallmark of active IBD is a strong migration of neutrophilic granulocytes (polymorphonuclear leukocytes (PMNs)) into the mucosa, which can be characterizedly found in the lamina propria and in the epithelial layer in IBD patients (9, 10).

Physiologically, phagocytic cells, including PMNs, build up an evolutionary ancient first line of mesenchymal defense. The cells migrate to sites of invaded pathogens guided by a variety of chemotactic molecules exerting an important role in the containment and eradication of pathogens. Genetic deficiency in neutrophil oxidative defense has been discovered as the cause of chronic granulomatous diseases, rendering the affected individuals susceptible to bacterial and fungal infections (11, 12). Conversely, increased and deregulated PMN recruitment and overactivation have been accused to pivotally contribute to tissue damage in chronic inflammatory disorders and are key pathological features of both human IBD and experimental colitis models (13–15).

It has been shown that migration of PMNs into inflamed tissues in general depends on several factors, including the presence of various chemokines, up-regulation of integrins, and reorganization of the cellular cytoskeleton (16–18). Recently, short chain fatty acids (SCFA) that are mainly produced by the commensal flora of the gut have been described as potent activator molecules of PMNs by binding to the membrane-associated Gg/Gi protein-coupled receptor (GPR) 43 (19–22). In vitro experiments revealed a SCFA-dependent induction of chemotaxis in human PMNs in the...
dose-dependent manner (20). Furthermore, it was shown that GPR43-dependent signaling leads to phosphorylation of p38 MAPK and activation of the MAPK pathway in PMNs (23).

Phosphorylation of p38 MAPK, which has been described as a major determinant contributing to chemotaxis (24), is, among other immune cells such as macrophages, also significantly enhanced in PMNs (25) and contributes to chemotaxis of PMNs in vitro (26). Inhibition of p38 MAPK has been propagated as an experimental IBD therapy in clinical trials.

Importantly, the relative role of SCFA-induced GPR43-dependent neutrophil migration versus migration induced by other chemotactants in intestinal inflammatory responses is completely unclear. The aim of this work was thus to dissect the molecular role of GPR43 for PMN migration in acute and chronic dextran sulfate colitis as an established murine model of human IBD.

Materials and Methods

Generation of Gpr43 knockout mice

The Gpr43 knockout mice were generated in collaboration with Lexicon Pharmaceuticals. The Gpr43 targeting vector was derived using the Lambda KOS system. Gene-specific arms were appended by PCR to a yeast selection cassette containing the URA3 marker. The yeast selection cassette and a positive pKOS genomic clone were cotransfected into yeast, and clones that had undergone homologous recombination to replace a 890-bp region of the single coding exon 3 with the yeast selection cassette were isolated. The yeast cassette was subsequently replaced with a LacZ/Neo selection cassette to complete the Gpr43 targeting vector. The NorI-linearized targeting vector was electroporated into 129/SvEvBrd (Lex-2) embryonic stem cells. Two targeted embryonic stem cell clones were identified and microinjected into C57BL/6 (albino) blastocysts to generate chimeric animals that were bred to C57BL/6 (albino) females; the resulting heterozygous offspring were intercrossed to produce homozygous Gpr43-deficient mice and littermate controls. Gpr43 gene expression was measured by quantitative RT-PCR (mGPR43F, 5′-AAATTCTCTGTTGTCCTTTGG-3′; mGPR43R, 5′-ACCACGACCAACTTCTGCGTG-3′) and Western blot. For the Western blot, 10 μg of total protein was separated by SDS-PAGE and transferred to polyvinylidene difluoride membrane by standard techniques. GPR43 was detected with an anti-gpR43 Ab (Santa Cruz Biotechnology) in a dilution of 1/500 (supplemental Fig. 1). 3

Homozygous mice were followed up for over 2 years. No macroscopic and histological evidence for spontaneous pathologies, such as sepsis, other immune cells such as macrophages, also significantly enhanced in PMNs (25) and contributes to chemotaxis of PMNs in vitro (26). Inhibition of p38 MAPK has been propagated as an experimental IBD therapy in clinical trials.

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Histopathological and immunohistochemical analyses of mouse colon tissue

Paraffin sections from colon segments were cut and stained with H&E. Histological scoring was performed on at least five transverse sections per each animal in a blinded fashion as described (28). The PMN infiltrating score was obtained by counting all extravascular PMNs per view. The PMN infiltrating score represents the average of PMNs in four random mucosal and submucosal views of three different sections from the descending colon at a magnification of ×600. Immunohistochemical stainings were performed by using standard methods. Sections were incubated with the primary Abs (anti-Ly6G (1:100; Abcam) and anti-phospho p38 (1:200; Cell Signaling Technology)) for 1 h at room temperature. Tissue-bound Ab was detected using biotinylated goat anti-rabbit or goat anti-mouse IgG Abs (Vector Laboratories, followed by HRP-conjugated avidin. Controls were included using irrelevant primary Abs as well as omitting the primary Abs using only secondary Abs and/or HRP-conjugated avidin. Bound Ab was detected by standard chromogen technique (Vector Laboratories) and visualized by an AxioImager Z1 microscope (Zeiss). Pictures were captured by a digital camera system (Axioxiom HrC; Zeiss).

For immunofluorescence staining, cryosections were fixed in ice-cold acetone (10 min), followed by sequential incubation with blocking reagent (DakoCytomation) to eliminate unspecific background staining. Slides were then incubated (1 h, room temperature) with primary Abs against Chromogranine A (1:200; Abcam) and peptide YY (1:100; Enzo Life Sciences). After three times washing with PBS, slides were incubated (45 min, room temperature) with Cy3-labeled secondary Abs (Jackson Immunoresearch Laboratories). Before examination, nuclei were counterstained with 4,6-diamidino-2-phenylindole.

Colon organ culture and ELISA

A segment of the distal colon was removed, cut open longitudinally, and washed in PBS containing penicillin and streptomycin. The colon was then further cut into segments of 1 cm, placed, and incubated in 24 flat-bottom well culture plates containing 1 ml of fresh RPMI 1640 medium supplemented with penicillin and streptomycin at 37°C for 24 h. Culture supernatants were then harvested and assayed for cytokines. ELISA using commercially available components was applied to quantify murine keratinocyte chemoattractant (KC), TNF-α, and IL-6 (CytoSet kit; BioSource International), as well as MCP-1 (Ready-SET-Go/mouse MCP-1 ELISA Kit; eBiosciences), according to the manufacturers’ protocols.

Myeloperoxidase (MPO) activity measurement

The activity of granulocyte-specific MPO was measured in each of three freshly isolated distal colonic segments per mouse. Murine tissue sections were homogenized, and equal amounts of precleared homogenates were assayed for MPO activity using a commercially available kit (Hbt Bio-technology). Absolute values were normalized to weight of the applied tissue sections (in mg).

Bacterial studies

DNA from liver and spleen tissue was extracted, and the universal bacterial 16S primer set COMI/COM2 (30) was used to amplify 16S RNA gene V regions. Cloning and sequencing of V3/V4 was performed, as described (31), to generate clone libraries. Resultant sequences were checked for possible chimera artifacts (32) and were eliminated from further analysis. Nonchimeric sequences were subjected to National Center for Biotechnology Information blast analysis (http://www.ncbi.nlm.nih.gov/BLAST) and sequences match program at RDP II release 9.53 (33) for phylogenetic affiliation.

Chemotaxis assay

Chemotaxis was assessed using a transwell cell chemotactic assay as described previously (34). PMNs for the chemotaxis assay were obtained from the peritoneal lavage of WT and Gpr43−/− mice (n = 3 per each group) 4 h after i.p. injection of 1 ml of 3% thioglycollate (35),
PMN counts were determined by morphological analyses after staining with Meyer’s hemalaun. During treatments and chemotaxis assay, no significant cellular toxicity using various agents, including KC, SB203580, propionate, and butyrate was observed, as assessed by trypan blue exclusion.

Air-pouch model

The air-pouch model was performed with Gpr43−/− and WT mice, according to Edwards et al. (36). Six days after s.c. pouch generation, 1 ml of 1% carrageenan (Sigma-Aldrich) in sterile PBS was injected into the pouches. The corresponding WT mice received sterile PBS. Twelve hours after treatment, the animals were killed and the pouches were washed with 3 ml of PBS. The lavage fluid was immediately cooled on ice and centrifuged at 5000 rpm (Eppendorf desktop centrifuge) for 10 min at 4°C. Aliquots of the air-pouch lavage fluid were analyzed by FACS. For PMN detection, we used the mAb Ly6G (BD Biosciences).

L-selectin quantification assay

To quantify L-selectin levels on the cellular surface of granulocytes, splenocytes were isolated from WT and Gpr43−/− mice using standard preparation techniques as described elsewhere (37). Cells were stimulated with propionate (10−2 M) or butyrate (10−2 M) for 1 h in the absence or presence of a broad-spectrum hydroxamic acid inhibitor of matrix metalloproteinases (GM6001, catalog 364206; Calbiochem) at a concentration of 25 nM or left untreated. As control, PMA (100 nM) was added to provoke maximal shedding of L-selectin. Afterward, cells were stained for cell surface expression of L-selectin using FITC-coupled anti-L-selectin Ab (Acris Antibodies; 1:1000). Unstained cells were used as negative control. Fluorescence was detected using a FACSCalibur flow cytometer (BD Biosciences) after gating for the granulocyte population in forward/side scatter mode. To confirm identity of the granulocytes, cells were stained for specific marker protein Ly6G using anti-Ly6G Ab (Hbt Biotechnology; 1:500). Unstained cells were used as negative control. Fluorescence was detected using a FACSCalibur flow cytometer (BD Biosciences) after gating for the granulocyte population in forward/side scatter mode. To confirm identity of the granulocytes, cells were stained for specific marker protein Ly6G using anti-Ly6G Ab (Hbt Biotechnology; 1:500) in a parallel setup. All measurements were done in triplicate; all experiments were repeated independently three times. Cell surface levels of L-selectin were calculated from FACS data as described before (38). In brief, 100% cell surface expression was set as the percentage of cells positive for L-selectin in an untreated control sample. Minimal L-selectin expression (0%) was set at the appropriate value for a PMA-treated sample. Relative L-selectin expression was calculated as percentage expression in relation to these controls.

Statistical analysis

Normality of the data was checked by calculating Lilliefors probabilities based on the Kolmogorov-Smirnov test. Results are expressed as means ± SEM. Values of p were calculated using a multifactorial ANOVA test and independent Student’s t test. Values of p < 0.05 were considered statistically significant. Experiments and measurements were replicated at least three times.
RESULTS

Attenuated colonic tissue damage coincides with high mortality in Gpr43−/− mice in acute DSS colitis

To study the relevance of Gpr43 receptor deficiency under acute inflammatory conditions, we induced acute DSS colitis by adding 4% DSS to the drinking water of Gpr43−/− and littermate control mice (WT) for 6 days. Up to day 4, all mice showed identical clinical symptoms (weight, stool consistency, and rectal bleeding) resulting in a relatively similar DAI (Fig. 1A). From day 5 on, Gpr43−/− mice developed significantly more weight loss (p < 0.05), whereas rectal bleeding and stool consistency were not attenuated compared with WT mice (data not shown). Finally, all Gpr43−/− mice died between days 6 and 8, whereas all WT mice survived until day 10 when the experiment was stopped (Fig. 1B).

Endoscopical examination of mice (n = 20) revealed lesser signs of inflammation in Gpr43−/− animals on day 6 (Fig. 1C) with a significantly reduced endoscopic score (Fig. 1D). To rule out a possible bias from effects of Gpr43 on gastrointestinal motility (39), we studied the spontaneous intestinal phenotype, including histology, colonic transit time, weight curves, and numbers of enterochromaffine cells. No significant differences could be detected (supplemental Figs. S1, S3, S4, and S6).

Histological examination of the distal colon showed reduced tissue damage and inflammatory cell infiltration of Gpr43−/− mice (Fig. 2, A and B). Multiple PMN-laden small vessels were observed, whereas the amount of PMN in the submucosal tissue was significantly reduced compared with WT mice (Fig. 2, C–E). All together, these findings resulted in a significantly decreased corresponding histological score in Gpr43−/− mice (Fig. 2F).

To quantify the amount of colonic PMN, we performed additionally MPO-ELISA in homogenized colonic tissue. As indicated, MPO levels were significantly reduced in Gpr43−/− mice (Fig. 3A and B). Additionally, the level of TNF-α and KC in splenic tissue of WT and Gpr43−/− mice are summarized by the endoscopic score (Fig. 3C and D). Multiple PMN-laden small vessels were observed in all WT animals, whereas Gpr43−/− mice are protected against chronic DSS colitis.

Because the clinical presentation and the highly elevated KC and TNF-α serum level of the Gpr43−/− mice suggested a septic complication, we performed bacteriological studies in liver and spleen tissue of WT and Gpr43−/− mice. Indeed, we found 16S rDNA bacterial signatures of Clostridium spp. in all three examined animals from the Gpr43−/− group, whereas nontreated Gpr43−/− and treated WT mice liver and spleen were sterile (n = 3 per genotype; Fig. 3E).

Gpr43−/− mice are protected against chronic DSS colitis

To further dissect this function of Gpr43 in chronic intestinal inflammation, we exposed mice to repeated administrations of 2% DSS for 30 days, as described (28). We did not observe any lethality in both animal groups. Importantly, the analysis of the clinical data revealed less diarrhea, less weight loss, and less rectal bleeding in Gpr43−/− mice represented by a significantly milder DAI (Fig. 4A). Colonoscopy examinations of all animals on day 30 showed less inflammatory mucosal signs in Gpr43−/− than in
WT mice (Fig. 4B). This is also depicted by a significantly decreased endoscopic score (Fig. 4C) and increased colon length in Gpr43<sup>−/−</sup> mice (Fig. 4D).

On day 30, cross-sections of the descendent colon in DSS-fed Gpr43<sup>−/−</sup> mice revealed diminished mucosal lesions and especially lower neutrophil infiltration (Fig. 5, A and B), quantitatively demonstrated by a reduced PMN infiltrating score and histological score (Fig. 5, C and D). MPO and TNF-α concentrations were diminished in Gpr43<sup>−/−</sup> animals (Fig. 5, E and F). In contrast to the acute DSS model, signs of sepsis were absent in the 2% group, and no bacterial 16S rDNA signature (n = 3 per each genotype) could be detected in tissue samples from spleen or liver (data not shown).

In summary, Gpr43 receptor deficiency leads to reduced colonic inflammation in the chronic DSS model.

Sterile inflammatory responses and neutrophil migration per se are not altered in Gpr43<sup>−/−</sup> mice

To functionally address the SCFA-induced chemotactic responses of PMN via Gpr43, we isolated neutrophils from the peritoneal lavage fluid of thioglycolate-treated Gpr43<sup>−/−</sup> and WT mice (n = 3 per each group). This was followed by chemotaxis assay, as described in Materials and Methods. After 45 min of propionate or butyrate stimulation (each 10<sup>−4</sup> M), a significant increase of chemotactic migration was observed in WT, but not in Gpr43<sup>−/−</sup> PMN (Fig. 6A). We next used the air-pouch model to investigate the migratory properties of Gpr43<sup>−/−</sup> PMN in nonbacterial inflammation. One percent carrageenan solution was injected into the preformed air pouch of Gpr43<sup>−/−</sup> and WT mice (n = 10 per each group), as described in Materials and Methods. After 12 h, mice were sacrificed, and the cellular contents of the air pouch were analyzed by FACS and ELISA. As indicated in Fig. 6B, the migratory properties of PMNs were not significantly affected by Gpr43 deficiency. Furthermore, no difference in KC, MCP-1, and IL-6 secretion was detected (see supplement, Fig. S2). This finding suggests that PMN migration does not differ significantly between Gpr43<sup>−/−</sup> and WT mice under sterile inflammatory conditions.

To assess a SCFA-independent migration ability of Gpr43<sup>−/−</sup> PMN, we further investigated the chemotactic response to KC, a strong inducer of chemotactic activity in PMN. As indicated in Fig. 6C, WT and Gpr43<sup>−/−</sup> PMN showed a similar increase of migrated PMN represented by an elevated chemotactic index in response to KC stimulation (100 ng/ml). Costimulation with KC and propionate (10<sup>−3</sup> M) led to a cooperative significant increase in PMN migration, as indicated by the attenuation of the chemotactic index (Fig. 6D).

**FIGURE 5.** Gpr43<sup>−/−</sup> mice exhibit less severe colonic inflammation. Histological assessment of colon sections obtained of the descendent colon of WT and Gpr43<sup>−/−</sup> mice on day 30 (×200 magnification; A and B). Black arrows indicate different amounts of PMNs in the submucosa. Histologic score was performed in a blinded fashion, as described in Materials and Methods (C). PMN infiltrating assay was obtained by counting PMN cellularity in four random mucosal and submucosal views of three different sections from the descendent colon (D). Activity of MPO was measured in colon tissue homogenates (E). TNF-α levels were quantified after 24-h incubation time in the supernatant of COC isolated of 10 WT and 10 Gpr43<sup>−/−</sup> mice (F). Data are mean ± SEM. **, *p* < 0.01 (n = 10 per group).
GPR43 deficiency led to reduced SCFA-induced p38 MAPK-dependent chemotactic activity and modulates shedding of L-selectin

We next examined the presence of phosphorylated p38-positive cells in colonic tissue of WT and Gpr43−/− mice on day 30 after three cycles with 2% DSS. As shown in Fig. 7A, phosphorylated p38 staining of submucosal PMN revealed positive staining in almost all PMN isolated from the WT, whereas Gpr43−/− PMN were mostly negative. The ratio between positive and the total amount of PMNs represents the average of four random mucosal and submucosal views of three different sections from the descending colon at a magnification of ×600 (Fig. 7B).

To test the influence of p38 MAPK activation on Gpr43-mediated SCFA-induced chemotaxis, we used the selective p38 MAPK inhibitor SB203580. As demonstrated in Fig. 7C, pharmacological inhibition of p38 MAPK (10 μM SB203580) nearly abolished chemotactic activity of propionate in WT PMNs.

Given the fact that p38 MAPK is an important regulator of TNF-α converting enzyme (TACE; ADAM17), a sheddase of L-selectin (38, 40–42), we further asked whether SCFA could potentially modulate L-selectin shedding. To study this, we analyzed genotype-dependent L-selectin staining in PMNs by FACS analysis after stimulation with butyrate and propionate, as described in Materials and Methods and supplement (supplemental Fig. S5). Interestingly, detection of L-selectin protein levels on the cell surface of PMNs was significantly reduced after 1 h of stimulation with SCFA. This effect was significantly impaired in PMNs isolated from Gpr43−/− mice as compared with WT mice (Fig. 7D and supplemental Fig. S5). A broad-spectrum inhibitor of metalloproteinases (GM6001), which prevents TACE-induced L-selectin shedding, served as positive control.

Discussion

The salient finding of the present study is the demonstration that GPR43 is required for neutrophil recruitment in a barrier lesion model of intestinal inflammation. The data demonstrate the bipotential pathophysiologic role of PMNs being a protective factor against acute bacterial transmigration, but also having a detrimental role in chronic inflammatory responses. We could clearly demonstrate a protective role of Gpr43 receptor deficiency against chronic lesions after several cycles of low-dose DSS administration. Gpr43−/− mice exhibited less severe mucosal inflammation, as indicated by the clinical course, endoscopy, histology, and levels of proinflammatory cytokines in organ-specific cultures. The data raise the questions as to which direct or indirect Gpr43-mediated molecular pathways are involved in activation and migration of PMNs in intestinal inflammation and whether these mechanisms could potentially be safely exploited as a therapeutic principle in IBD. Although the data discussed in the following paragraphs argue for a direct role of Gpr43 on PMNs, it must be noted that at least theoretically an indirect effect of Gpr43 on other cell types (e.g., CNS neurons) may contribute to the observed phenotype.

We could demonstrate an activation of neutrophilic migration along a gradient of SCFA in Boyden chamber chemotaxis assays in vitro, which was abolished in neutrophils derived from Gpr43-deficient mice. Non-SCFA stimulation with the chemotactic cytokine KC or the formylated peptide fMLP (data not shown) led to similar migration properties in WT and KO mice. The results from the sterile inflammation model also demonstrate in vivo that Gpr43 receptor deficiency does not alter the migratory and proinflammatory properties per se, but points to a specific role for SCFA recognition for PMN recruitment after a barrier lesion in the intestinal tract (20). p38 MAPK has been described as an important mediator of chemotactic activity in immune cells (43, 44). Our data clearly show that SCFA-mediated p38 MAPK phosphorylation and chemotaxis are completely dependent on the presence of Gpr43, and demonstrate a remarkable difference of in vivo p38 MAPK phosphorylation levels in neutrophils between WT and KO mice in chronic DSS colitis. The question remains why migration of neutrophils in experimental intestinal inflammation critically depends on GPR43 signaling, although other chemotactic stimuli such as KC are present and responses to these stimuli are unaltered in Gpr43−/− PMNs.

Data from the KC knockout mouse demonstrated a pivotal role of KC (murine IL-8) for PMN migration. In contrast, the recruitment of PMNs is not solely dependent on the genetic integrity of
the \( Kc \) gene (45, 46). One of the factors involved in the recruitment of PMN is L-selectin (47). Shedding of L-selectin is observed shortly after adhesion to endothelial cells (40) and may be essential for PMN migration (48). Furthermore, shedding of L-selectin depends on TACE (ADAM17) (40) and is regulated among other things by the MAPK p38 (41, 42). Given the fact that p38 MAPK phosphorylation is inducible by SCFA via GPR43, as shown in this study (Fig. 7, A–C) and by others (23), we asked whether SCFA could potentially influence the shedding of L-selectin via Gpr43. Indeed, our experiments display a significant influence of SCFA on L-selectin surface levels of PMNs, which is abolished in PMNs isolated from \( Gpr43^{-/-} \) mice (Fig. 7D and Fig. S5). Of note, shedding per se (induced by phorbol esters) remains unaffected, and the study does not imply a critical role of Gpr43 on neutrophil migration in the absence of SCFA. The conclusion is in line with the data from the carrageenan-induced inflammation in the air-pouch model in which similar migratory properties were observed in WT and \( Gpr43^{-/-} \) knockout animals. We suggest that SCFA can potentially influence the emigration of PMN from the vessels via the endothelium into the tissue by modulation of L-selectin levels through activation of shedding. Given the fact that PMN migration in vivo is a complex biological process and can only be sufficiently explained by the cellular integration of many simultaneous signals, we furthermore postulate the concurrence of different effects, as follows: 1) We demonstrate a cooperative effect of SCFA and KC in vitro, which is abolished in the \( Gpr43^{-/-} \) mice. SCFA-induced signals may act as a master switch in vivo to reach a critical threshold for other stimuli. 2) The initially small difference between WT and KO in the chronic model is potentiated in the later DSS cycles, pointing to a vicious circle in which long-term PMN infiltration may lead to additional tissue damage and barrier dysfunction and additional PMN influx. Furthermore, one of the pivotal effects of neutrophils in intestinal inflammation is the down-regulation of epithelial intercellular junction proteins; thus, sustained neutrophil infiltration would further contribute to a weakened intestinal barrier function (14). Interestingly, in the acute model with 4%, there is a clear protective effect of \( Gpr43^{-/-} \). Because it is generally assumed that DSS is directly toxic to gut epithelial cells of the basal crypts and affects the integrity of the mucosal barrier, we argue that the change in concentrations is enough to explain the phenotypic differences during the first days. In this scenario, the larger barrier defect induced by 4% DSS leads to a higher transmigration of bacteria into the submucosal layers. With emigrated PMNs as the first line of defense being reduced in the \( Gpr43^{-/-} \) animals, development of septicemia is highly facilitated. 3) It is very likely that the SCFA-dependent effect is not restricted to the intestinal system, but may also contribute to PMN recruitment in other pathological conditions, e.g., bacterial abscesses, where Gpr43 ligands are present. However, it must be noted that physiological concentrations of SCFAs are highest in the intestinal...
lumen, which argues for accentuated role of Gpr43 for neutrophil recruitment in the gut mucosa.

The question remains whether excess intraluminal SCFA concentrations induced by diet can induce recruitment of neutrophils and thus contribute to onset of inflammation. There are several observations in the literature that argue against a proinflammatory role of nutritional modulation of luminal SCFA concentrations in rodents and humans. In rats, no histological evidence for inflammation after a fiber-rich diet was observed previously (49); in a surgical stress model in rats, a similar diet led to enhanced colonic crypt length as a marker of epithelial restoration (50). Kataoka et al. (51) reported a beneficial effect of Brown rice fermented by Aspergillus oryzae on the course of DSS colitis, which is regarded as a traditional fiber-rich diet, but also contains other metabolites, including antioxidants. Butyrate itself has been shown to suppress NF-κB activity in intestinal epithelial cells (52–54). The results clearly underscore the multipotential roles of SCFAs in intestinal homeostasis and furthermore point to the importance of an intact intestinal barrier.

The decrease of mucosal PMNs is a goal of several therapeutic approaches in human IBD. Whereas anti-TNF therapy in CD indirectly lowers PMN concentrations in the intestinal mucosa (55), direct Ab-mediated integrin neutralization has resulted in promising efficacy profiles in both CD and ulcerative colitis (56, 57). A small clinical trial using a small molecule inhibitor of c-Raf, a critical upstream kinase of p38 MAPK and JNK, showed beneficial effects in CD and included a reduction of activated PMNs in the inflamed mucosa of the responders (58, 59). In line with this suggestive data, F-actin content, motility, and chemotaxis of PMNs are reduced upon treatment with this compound (60). However, albeit this strong rationale for MAPK inhibitors in intestinal inflammation, most compounds had dose-limiting adverse effects unrelated to immunosuppression in the subsequent clinical trials (61), which points to the limits of inducing promiscuous pleiotropic signaling pathways for specific therapies.

Thus, inhibiting GPR43 receptor-mediated signaling could prove as a promising specific way of inhibiting PMN migration in intestinal inflammation, but the data also demonstrate the critical balance between protective properties of PMNs and their tissue-destructive abilities. Similarly, mice deficient for IL-12 p40 are highly susceptible to sepsis induced by cecal ligation puncture in inflammatory diseases.

Acknowledgments

We gratefully appreciate the technical assistance of Maren Reffelmann, Yasmin Brodtmann, Tanja Kaacksteen, Lynn Pantages-Torok, and Mary McFarland. We thank Jens-Michael Schröder for helpful discussions and advice on chemotactic assays.

Disclosures

H.-P.J. and J.L. are employees of Boehringer Ingelheim. The remaining authors have no financial conflict of interest.

References


Baseline characteristics of the GPR43<sup>−/−</sup> mice
Histologies (HE staining) of WT and GPR43<sup>−/−</sup> colonic tissue without DSS treatment at a magnification of x200 (A and B). RT-PCR (C) and Western Blot (D) assessment of GPR43 transcript and protein levels in the intestinal mucosa of WT and GPR43<sup>−/−</sup> (see supporting material and methods)
Sterile inflammatory responses per se are not altered in Gpr43⁻/⁻ mice.
Levels of MCP-1, KC and IL-6 in the supernatants of air-pouch lavage fluid were measured by ELISA (A-C) (see supporting material and methods). Data are mean +/- SEM. **, p<0.01.

Figure S2
Weight development and gastrointestinal transit time are not altered in GPR43\(^{-/-}\) mice

Weight development at different time points for WT and KO mice (Figure A); gastrointestinal transit time is expressed in relation to the intestinal length (Figure B, see supplement); Laboratory values were obtained by hematology analyzer Sysmex XE-2100 (Table 1); Genexpression of GPR43 by RT-PCR is predominantly detected in PMN, only low levels of GPR43 cDNA are detectable in colonic tissue (CT). Generally no visible GPR43 expression in KO mice and in primary intestinal epitheliel cells (pIEC) of WT mice.

Table 1: Laboratory values (median +/- SEM)

<table>
<thead>
<tr>
<th>Hematology</th>
<th>wt</th>
<th>Gpr43(^{-/-})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leukocytes (cells/(\mu)l)</td>
<td>3.5 (+/− 1.12)</td>
<td>4.67 (+/− 0.83)</td>
</tr>
<tr>
<td>Neutrophils (%)</td>
<td>11.2 (+/− 2.09)</td>
<td>14.75 (+/− 2.3)</td>
</tr>
<tr>
<td>Erythrocytes (cells/(\mu)l)</td>
<td>8.42 (+/− 0.51)</td>
<td>8.85 (+/− 0.31)</td>
</tr>
<tr>
<td>Hemoglobin (g/dl)</td>
<td>13.5 (+/− 1.13)</td>
<td>14.3 (+/− 0.55)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Clinical chemistry</th>
<th>wt</th>
<th>Gpr43(^{-/-})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Creatinine (mg/dl)</td>
<td>0.27 (+/−0.08)</td>
<td>0.26 (+/− 0.08)</td>
</tr>
<tr>
<td>ASAT (U/l)</td>
<td>140 (+/− 36.18)</td>
<td>133 (+/− 42.62)</td>
</tr>
<tr>
<td>ALAT (U/l)</td>
<td>33 (+/− 9.65)</td>
<td>26.5 (+/− 10.66)</td>
</tr>
<tr>
<td>Bilirubine (mg/dl)</td>
<td>0.14 (+/− 0.01)</td>
<td>0.11 (+/− 0.008)</td>
</tr>
</tbody>
</table>
**Numbers of enterochromaffine cells are not altered in Gpr43<sup>−/−</sup> mice**

Representative immunofluorescence pictures of Chromogranine A- (CgA) and PYY-positive cells in WT and GPR43<sup>−/−</sup> mice (n=6 per each genotype) (Figure A); statistical analysis of the cell counts (obtained at a magnification of x400) revealed no significant difference between WT and GPR43<sup>−/−</sup> mice.

**Figure S4**
Flow cytometry measurement for detection of L-selectin on granulocytes

(A-H) Freshly isolated spleenocytes were treated as described in the Material and Method section. Cells were stained for granulocyte marker protein Ly6G (green, Figure B,D) or for L-selectin (red, E-H) using specific antibodies and analyzed by FACS. Cells were gated for granulocytic phenotype in FSC/SSC mode, and gated cells were checked for expression of Ly6G (A-D). Using the same gate, L-selectin cell surface level were detected on granulocytes (E-H). L-selectin surface levels were calculated from Facs data as described (Borland et al., 1999). Unstained cells were used as controls (black hairline). All measurements done in triplicate, all experiments were repeated three times.

Figure S5
Ratio of colonic wet/dry weight and the concentration of PYY in serum and colon are not altered in GPR43<sup>−/−</sup> mice. Ratio of colonic wet and dry weight was obtained as described by Nosál'ová et al., J. Physiol Res. 1999;48(1):65-72. No significant genotypic differences could be detected (Figure A); PYY concentration were measured in serum and colon organ culture (COC) samples by Fluorescent Immunoassay Kit (Phoenix Pharmaceuticals, Inc, Karlsruhe, Germany) according to the manufacturers’ protocols. No significant genotypic differences could be detected (Figure B).