ORIGINAL CONTRIBUTION

Lipocalin 2 protects from colonic inflammation and tumorigenesis through its microbiota modulating properties

Short title: Lcn2 in intestinal inflammation

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SUMMARY

The siderophore-binding peptide Lipocalin–2 (Lcn2) is involved in host defense against pathogens. Lcn2 function in mucosal immunity remains largely unknown. Here, we demonstrate that Lcn2 protects from early–onset colitis and spontaneous emergence of right–sided colonic tumors in Il10−/− mice. Bone marrow chimeric experiments indicate that hematopoietic and non-hematopoietic Lcn2 is protective. Exacerbating inflammation in Lcn2−/−/Il10−/− mice is driven by IL-6 which also controls tumorigenesis. Lcn2+/−/Il10−/− mice exhibit profound alterations in gut microbial composition which contributes to inflammation and tumorigenesis as demonstrated by the transmissibility of the phenotype by microbiota transfer and by protection conferred by antibiotics. Facultative pathogenic Alistipes spp. utilize enterobactin as iron source, bloom in Lcn2−/−/Il10−/− mice, and induce colitis and right–sided tumors when transferred into Il10−/− mice. Our results demonstrate that Lcn2 protects against intestinal inflammation and tumorigenesis through modulation of the microbiota.

(150 of 150 words)
INTRODUCTION

The mammalian intestinal tract harbors the highest density of microbial organisms in the body (Ley et al., 2006). The host–microbe relationship is based on a mutualism that is essential for host nutrient acquisition, immune development, and pathogen defense (Lozupone et al., 2012). This intimate juxtaposition necessitates a sophisticated spatial compartmentalization between commensal bacteria and the mucosal immune system, which allows a graduated response to various challenges such as dietary factors, toxins, or invasive pathogens (Maynard et al., 2012). Resilience to host inflammation and infection is a fundamental property of the healthy gut microbiota, which can remain stable for years in humans (Lozupone et al., 2012). Persistent deviations from a healthy composition, particularly low species richness and low gene count, are increasingly recognized as a common feature in various gut-related and extraintestinal disorders although cause–effect relationships remain unclear (Le Chatelier et al., 2013). As such, the knowledge of host factors released by the mucosal immune system that regulate the host–commensal relationship and drive gut microbial community structures is of great clinical and scientific interest.

Lipocalin 2 (Lcn2), also known as 24p3 or neutrophil gelatinase–associated lipocalin (Ngal) is a member of the lipocalin superfamily comprised of more than 20 secreted small molecules (Skerra, 2000). These differ substantially in terms of protein sequence and function but share a 3-dimensional β-barrel structure with eight anti-parallel β-sheets that encircle a central binding groove capable of accommodating ligands of different chemotypes, sizes, and shapes (Akerstrom et al., 2000; Chakraborty et al., 2012). Lcn2 has been implicated in several biologic processes such as acute phase
response, kidney morphogenesis, tissue involution, erythropoiesis, iron metabolism, and immune functions (Liu et al., 2013).

Lcn2 is produced by various cell types including myeloid and epithelial cells and is strongly up–regulated upon IL-1β, IL-22, Toll-like receptor (TLR) TLR2, TLR4, TLR5 ligation, and ischemia-reperfusion injury (Behnsen et al., 2014; Chakraborty et al., 2012). It acts as an anti–microbial defense mediator by binding a subset of bacterial siderophores, thereby preventing bacterial iron acquisition and growth of siderophore–dependent strains (Goetz et al., 2002). Accordingly, Lcn2–deficient animals are prone to infection and sepsis from enterobactin–dependent bacteria (Berger et al., 2006; Flo et al., 2004). Furthermore, Lcn2 has been implicated in cellular iron metabolism. By interacting with its surface receptor 24p3R, iron-containing Lcn2 (24p3) increases and iron-lacking Lcn2 decreases intracellular iron concentrations respectively (Devireddy et al., 2005).

Based on the observation that Lcn2–deficient animals are also susceptible to siderophore–independent pathogens such as Streptococcus pneumoniae or Listeria monocytogenes, additional immune–modulating mechanisms have been proposed. As a neutrophil secondary granule protein Lcn2 is required for adequate neutrophil functions such as chemotaxis, extravasation, migration and phagocytosis (Liu et al., 2013; Schroll et al., 2012). Furthermore, Lcn2 has been implicated in macrophage deactivation via induction of IL-10 (Warszawska et al., 2013).

Lcn2 accumulates in the gut lumen during intestinal infections and Lcn2–resistance owed to specific siderophores confers growth and survival advantages for certain pathogens such as Salmonella enterica serovar typhimurium which thrives in the inflamed gut despite the host’s attempts to limit iron availability (Raffatellu et al., 2009).
However, high concentrations of Lcn2 are also observed in the mucosa and the feces in animal models of intestinal inflammation and patients with inflammatory bowel disease (IBD) which is why Lcn2 was increasingly appreciated as an inflammatory biomarker in the intestine (Stallhofer et al., 2015). However, the functional role of Lcn2 in the context of chronic intestinal inflammation remains unclear (Nielsen et al., 1996).

Here we report that Lcn2 acts as an antimicrobial protein that limits inflammation and colitis–associated cancer emerging from IL-10 deficiency. $\text{Lcn}2^{-/-}\text{IL10}^{-/-}$ animals exhibited a markedly altered microbial community structure and expansion of certain species particularly $\text{Alistipes}$ spp. The disease phenotype was dependent on IL-6–signaling, was transmissible by cross–fostering and co–housing, could be prevented by antibiotics, and was mimicked by $\text{Alistipes}$ infection. Our data indicate a protective role of colitis–induced Lcn2 through modulation of the microbiota.
RESULTS

Lcn2−deficiency results in increased colitis severity and spontaneous proximal (right-sided) tumors in the IL-10 knockout model of colitis

To decipher the functional role of Lcn2 in colonic inflammation we first generated mice double−deficient in Lcn2 and IL-10 (Lcn2−/−/Il10−/−). The histological severity of colitis was elevated in Lcn2−/−/Il10−/− mice compared to Il10−/−, Lcn2−/−, and wildtype (Wt) animals (Figures 1A and 1B).

Macroscopic analysis of 12−week−old Lcn2−/−/Il10−/− mice, age− and gender−matched to respective controls, revealed early onset rectal prolapse (Figure S1A) and signs of severe colitis including shortened colonic length (Figure 1C), pronounced thickening of the bowel wall (Figures 1B and 1D), along with mesenteric lymphadenopathy and splenomegaly (Figure 1D).

Macroscopic features were mirrored histologically by an intensified lympho−/histiocytic, mononuclear and neutrophilic infiltrate, marked epithelial hyperplasia, and various grades of epithelial injury in Lcn2−/−/Il10−/− compared to Il10−/− mice (Figure 1B). Cryptitis and crypt abscesses were also common features in Lcn2−/−/Il10−/− mice (Figure S1B).

Increased histological disease severity was paralleled by high expression levels of inflammatory cytokines including IL-1β, IL-6, IFNγ, IL-17A, and IL-22 both at mRNA levels in epithelial scrapings (Figure 1E) and protein release from colonic organ cultures (Figure 1F). Notably, Lcn2−/− mice were both macroscopically and histologically indistinguishable from Wt animals.

We investigated the composition of infiltrating leukocytes by immunohistochemistry. As expected from H&E histology, the total number of infiltrating leukocytes (CD45+ cells) was higher in Lcn2−/−/Il10−/− compared to Il10−/− mice and in both groups greater than in
Wt and Lcn2−/− animals. This was mainly due to expansion in F4/80+ macrophages and CD3+ T cells, but not B220+ B cells (Figure 1G and Figure S1C).

To distinguish between the contributions of hematopoietic versus non–hematopoietic Lcn2, we generated bone marrow chimeric mice. Chimerism was verified by IL-10 and Lcn2 release from LPS–stimulated blood leukocytes (Figure S2A) and further corroborated by immunohistochemistry for Lcn2 on colonic tissue sections (Figure S2B). Histological severity of colitis was significantly attenuated in Lcn2−/−/Il10−/− mice reconstituted with Il10−/− bone marrow compared to control animals (Lcn2−/−/Il10−/− transplanted with Lcn2+/−/Il10−/− bone marrow, Figure 1H). Reciprocal chimerism, i.e. Il10−/− mice reconstituted with Lcn2−/−/Il10−/− bone marrow, resulted in deteriorated histology compared to Il10−/− mice reconstituted with Lcn2–sufficient Il10−/− bone marrow (Figure 1H). Histology scores were comparable between Lcn2+/−/Il10−/− mice reconstituted with Il10−/− bone marrow and Il10−/− mice reconstituted with Lcn2+/−/Il10−/− bone marrow suggesting a biological relevant contribution of both hematopoietic and non–hematopoietic Lcn2.

To investigate the natural history of disease, we assessed the severity of intestinal inflammation in 4- and 8-week old mice. Lcn2−/−/Il10−/−, compared to Il10−/− mice, exhibited colitis based on histology and cytokine profiles as early as 4-weeks of age that progressed over time (Figure S2C to S2F).

In light of early-onset inflammation, we noted that 8 out of 8 (100%) Lcn2−/−/Il10−/− mice developed multiple spontaneous right-sided tumors by 12 weeks of age (Figures 2A to 2C). Polyps were confined to the cecum and the first segment of the proximal colon (Figure 2A), which will be referred to as right-sided tumors throughout the manuscript. In contrast, in Il10−/− animals detection of minuscule polyps at 12 weeks was only observed
in 1 out of 6 animals (17%, Figures 2A to 2C). The macroscopically hemispheric, exophytic appearance of tumors in \( \text{Lcn2}^{-/-}/\text{Il10}^{-/-} \) mice translated histologically into adenomatous polyps with low-grade dysplasia (Figure 2A). In \( \text{Lcn2}^{-/-}/\text{Il10}^{-/-} \) mice only invasive cancer was observed in 2 out of 17 mice at 20 weeks of age (Figure S3A).

Together, our findings indicate that Lcn2 is key in the control of colonic inflammation and tumor formation consequent to IL-10 deficiency. Our data further indicate that both hematopoietic and non-hematopoietic Lcn2 is relevant for this protective effect.

**Lcn2-deficiency instigates an IL-6-dependent pathway that drives inflammation, hyper-proliferation, and tumor formation**

We hypothesized that formation of colonic tumors may be fueled by excessive inflammation in our model. Various cytokine networks including TNFα–NF-κB, IL-6–STAT3, IL-17A and IL-22 have been established as important promoters of colitis–associated cancer (Greten et al., 2004; Grivennikov et al., 2009; Huber et al., 2012; Punkenburg et al., 2015). In 12–week–old \( \text{Lcn2}^{-/-}/\text{Il10}^{-/-} \) mice several pro-inflammatory cytokines were strongly elevated. As we particularly observed increased IL-6 and IL-22 early in the natural history of colitis in \( \text{Lcn2}^{-/-}/\text{Il10}^{-/-} \) mice (Figure S2E and S2F), we next analyzed whether IL-6 signaling was activated in intestinal epithelial cells (IECs). Consistent with this, we found increased total and phosphorylated STAT3 in \( \text{Lcn2}^{-/-}/\text{Il10}^{-/-} \) mice (Figures 2D and 2E), which was mainly of epithelial origin (Figure 2F and 2G). The pro-inflammatory transcription factors C/EBP-β and NF-κB were not different between \( \text{Lcn2}^{-/-}/\text{Il10}^{-/-} \) and \( \text{Il10}^{-/-} \) mice (Figure S3B to S3D). We next determined intestinal epithelial cell (IEC) proliferation within colonic crypts, 2 and 24 hours after injection of bromodeoxyuridine (BrdU). Per crypt
BrdU–immunopositive cells were more frequent in $Lcn2^{-/-}/Il10^{-/-}$ than in $Il10^{-/-}$ mice, yet comparable in $Lcn2^{-/-}$ and Wt controls (Figures 2H and 2I).

To examine the functional relevance of these findings, we crossed Wt, $Lcn2^{-/-}$, $Il10^{-/-}$, and $Lcn2^{-/-}/Il10^{-/-}$ mice with $Il6^{-/-}$ mice. Loss of IL-6 had multiple effects on the phenotype. Firstly, the severity of colitis was minimized in $Il6^{-/-}/Lcn2^{-/-}/Il10^{-/-}$ triple–deficient compared to $Lcn2^{-/-}/Il10^{-/-}$ double–deficient mice (Figure 3A). Secondly, epithelial hyperplasia was strongly attenuated from a mean mucosal thickness of 468μm to 287μm (Figure 3B and 3C). Finally this was paralleled by a significant reduction in the number of right–sided tumors (Figures 3D and 3E) and overall tumor area (Figure 3F) in $Il6^{-/-}/Lcn2^{-/-}/Il10^{-/-}$ triple–knockout compared to $Lcn2^{-/-}/Il10^{-/-}$ double–knockout mice.

Taken together, these data demonstrate that IL-6 promotes inflammation, epithelial hyperplasia, and tumor formation in our model.

$Lcn2^{-/-}/Il10^{-/-}$ mice are characterized by a defective mucus layer, deteriorated mucosal barrier, and an altered microbial composition

Since anti–bacterial properties are attributed to Lcn2 (Flo et al., 2004; Goetz et al., 2002), we next investigated whether Lcn2–deficiency results in barrier defects and/or alterations in microbial community structures.

To obtain insights into structural attributes at the colonic mucosa surface, we performed fluorescence in situ hybridization (FISH) on Carnoy–fixed tissue sections (Figure 4A). The overall, juxtamucosal bacterial density was comparable between all genotypes (Figure 4B). In contrast, 100% of $Lcn2^{-/-}/Il10^{-/-}$ mice exhibited profound mucus–related deficits resulting in a complete loss of the interlaced mucus layer, bacteria attaching to IECs and crypt invasion (Figures 4A and 4B). Again, no significant differences were
detected between *Wt* and *Lcn2* animals which lacked signs of inflammation including absence of fecal leukocytes and a preserved spatial segregation (Figure 4A and 4B). To confirm a relevant barrier defect, we determined numbers of 16S gene copies in mesenteric lymph nodes and characterized intestinal permeability using FITC dextran assays. Compared to *Wt, Lcn2*, and *Il10* mice, *Lcn2*/*Il10* animals had 4.2-fold higher 16S copy numbers in mesenteric lymph nodes (Figure 4C) and FITC dextran administration resulted in 4.3-fold higher serum concentrations (Figure 4D).

The commensal microbiota plays a key role in the pathogenesis of intestinal inflammation and drives tumor development (Arthur et al., 2012). In a first step we examined gut bacterial composition using culture-independent analyses based on the 16S rRNA gene. The microbial community structure was comparable between *Wt* and *Lcn2* animals yet strikingly different in *Il10* as well as *Lcn2*/*Il10* mice both at the phylum and genus level (Figure 4E). Significant differences between major taxa (defined as average abundance ≥ 1% in the whole dataset) were found between several groups as well as between the microbiota of *Il10* and *Lcn2*/*Il10* mice in post-hoc analyses (for details see Table S1). Furthermore, we observed significant differences in species evenness (Shannon's index, Figure 4F) and richness (Chao1 index, Figure 4G) between animals of different genotypes. Both indices were significantly decreased in *Il10* versus *Wt, Lcn2*, and *Lcn2*/*Il10* mice, with the latter three groups being in the same range (Figures 4F and 4G).

*Lcn2*/*Il10* mice develop a colito- and tumorigenic microbiota that is transmissible to cross-fostered or co-housed *Il10* mice

The above results indicate that *Lcn2*/*Il10* mice harbor a unique colonic microbiota. To assess the impact of this microbiota on colitis severity and tumor prevalence, we first
treated mice with broad–spectrum antibiotics. Strikingly, ciprofloxacin and metronidazole strongly attenuated the severity of colitis in \textit{ll10}^{-/-} and particularly in \textit{Lcn2}^{-/-}/\textit{ll10}^{-/-} mice (Figure 5A) and abrogated tumor formation (Figure S4A).

In line with a critical role of the microbiota, newborn \textit{ll10}^{-/-} mice were cross–fostered (CF) at birth with \textit{Lcn2}^{-/-}/\textit{ll10}^{-/-} mothers (Figure 5B) and developed more severe colitis than non–CF littermates (Figure 5C). Conversely, \textit{Lcn2}^{-/-}/\textit{ll10}^{-/-} pups CF with \textit{ll10}^{-/-} mothers demonstrated milder colitis than their non–CF littermates (Figure 5C). Notably, \textit{ll10}^{-/-} mice CF with \textit{Lcn2}^{-/-}/\textit{ll10}^{-/-} mothers had comparable tumor counts and densities to non–CF \textit{Lcn2}^{-/-}/\textit{ll10}^{-/-} mice (Figures 5D to 5F). In contrast, \textit{Lcn2}^{-/-}/\textit{ll10}^{-/-} animals CF with \textit{ll10}^{-/-} mothers did not develop right-sided tumors during the observation period (Figures 5D to 5F). Analyses of the 16S data revealed that fostering conditions drove major differences in the composition of microbial communities between littermates of similar genotypes. In essence, \textit{ll10}^{-/-} and \textit{Lcn2}^{-/-}/\textit{ll10}^{-/-} mice adopted a comparable microbiota when raised by the same mother, which was reflected mainly on the the on the genus level (Figures 5G and 5H and Table S2), and further on species evenness and richness levels (Figures 5I and 5J). Using Bray-Curtis and weighted Unifrac as measures of community differences, we detected an influence of genotype and foster status in particular experimental groups (Figures 5K and 5L). Again, the gut microbiota became indistinguishable between CF mice and their nursing mother. In a sense cross–fostering overrode the genetic background of cross–fostered mice and drove microbial variations primarily through the genotype of the nursing mother (p=0.001, “adonis” test; see Materials and Methods). Taken together, penetrance and severity of vertical transmission in CF animals was 100 percent and bi–directional. Cross–fostering and the maternal microbiota had strong effects on the establishment of the suckling’s
microbiota and the development of both inflammation and polyps, overcoming the effect of the individual genotype.

To affirm that disease phenotypes were driven by the intestinal microbiota, we next co–housed $\text{Il10}^{-/-}$ and $\text{Lcn2}^{-/-} / \text{Il10}^{-/-}$ mice age and gender–matched and in a 1–to–1–ratio (Figure S4B). As expected, genotype–specifically housed $\text{Il10}^{-/-}$ and $\text{Lcn2}^{-/-} / \text{Il10}^{-/-}$ littermates either did or did not develop excessive mucosal inflammation and polyps (Figure S4C to S4E). In contrast, $\text{Il10}^{-/-}$ mice co–housed with $\text{Lcn2}^{-/-} / \text{Il10}^{-/-}$ animals developed more severe colitis (Figure S3C). With respect to tumor formation, co–housed $\text{Il10}^{-/-}$ and $\text{Lcn2}^{-/-} / \text{Il10}^{-/-}$ animals developed comparable tumor numbers, yet significantly higher than observed in genotype–specifically housed $\text{Il10}^{-/-}$ and significantly lower than in genotype–specifically housed $\text{Lcn2}^{-/-} / \text{Il10}^{-/-}$ littermates (Figure S4D). Differences in tumor numbers were not reflected in tumor areas which were comparable to genotype–specifically housed $\text{Lcn2}^{-/-} / \text{Il10}^{-/-}$ littermates (Figure S4E). 16S sequencing data from co–housed animals revealed that co–housed $\text{Il10}^{-/-}$ mice acquired a microbiota highly similar to that of their $\text{Lcn2}^{-/-} / \text{Il10}^{-/-}$ “cagemates” (Adonis testing showed no significant differences between $\text{Lcn2}^{-/-} / \text{Il10}^{-/-}$ single–housed and any of the co–housed $\text{Il10}^{-/-}$ and $\text{Lcn2}^{-/-} / \text{Il10}^{-/-}$ animals) (Figures S4F and S4G).

To test for a potential impact of coprophagy–delivered $\text{Lcn2}$, which is stable at low pH (Paragas et al., 2014), we determined Lcn2 concentrations in the stool of co–housed animals. Co–housing with $\text{Il10}^{-/-}$ mice did not result in a relevant increase in stool Lcn2 in $\text{Lcn2}^{-/-} / \text{Il10}^{-/-}$ mice relativing the importance of such a mechanism (Figure S4H).

Inflammation is a well–known modulator of the gut microbiota (Lupp et al., 2007). We therefore assessed younger, i.e. 4–week–old $\text{Il10}^{-/-}$ and $\text{Lcn2}^{-/-} / \text{Il10}^{-/-}$ mice. These mice already exhibited colitis, although to a lesser extent (Figure S2C). Already at 4
weeks Il10−/− and Lcn2−/−/Il10−/− mice clearly segregated regarding their community structures in the MDS plots (Figure S4I and S4J).

Together, these observations indicate that in a state of colonic inflammation Lcn2−deficiency contributes to the selection of a colitis– and tumor–promoting microbiota that is transmissible by cross–fostering and co–housing and responsive to broad–spectrum antibiotics.

**Ablation of Lcn2 during intestinal inflammation creates a niche for the expansion of pathogenic *Alistipes* species.**

To understand the contribution of specific bacteria to colonic inflammation we next sought to identify microbial species overrepresented in Lcn2+/−/Il10−/− mice and to test their impact on the development of colitis. Therefore, the above-mentioned 16S rRNA data set was subjected to representational difference analysis (RDA) and BioPlot (Figure 6A). RDA at the genus level indicated that *Alistipes* as well as unclassified *Lachnospiraceae* might be relevant drivers of community differences. At the species level, we identified 3 OTUs significantly enriched in Lcn2+/−/Il10−/− mice, namely OTU 3 and 12 (both from unclassified *Lachnospiraceae*) and OTU 808 from *Alistipes* (Figure 6B). We confirmed by qPCR that *Alistipes* spp were only present in in the microbiota of Lcn2−/−/Il10−/− mice (Figure S5A).

To determine a potential direct growth inhibitory effect of Lcn2 particularly on *Alistipes* spp., we cultured *Alistipes putredinis* (CCUG 45780 T) and *Alistipes finegoldii* (CCUG 46020 T) anaerobically with or without increasing concentrations of recombinant Lcn2. *In vitro*, recombinant Lcn2 had a strong effect on *Alistipes* spp. in terms of reduction in numbers of colony–forming units (CFU) (Figure 6C). As Lcn2 has been well documented
to control iron homeostasis by binding catechol siderophores such as enterobactin (Goetz et al., 2002), we hypothesized that the underlying mechanisms by which Lcn2 impacts on Alistipes growth involved the limitation of iron availability. We did not detect a relevant production of endogenous Alistipes–derived siderophores (Figure 6D). However, according to the KEGG database, Alistipes finegoldii encodes at least two different Fe$^{3+}$–siderophore transporters (http://www.genome.jp/kegg-bin/show_organism?org=afd). The potent iron chelator deferoxamine strongly attenuated bacterial growth underlining the importance of iron as a regulatory factor for Alistipes finegoldii growth (Figure 6E). Notably, Fe$^{3+}$ supplementation did not affect Alistipes growth. Conversely, addition of iron–free enterobactin and particularly iron–laden enterobactin markedly promoted the numbers of CFUs suggesting a relevant role for siderophore–bound iron for Alistipes growth. Again, addition of recombinant (siderophore–free) Lcn2 resulted in a strong reduction in numbers of CFUs (Figure 6E).

These findings suggested that Alistipes spp. thrives in an inflamed environment that lacks Lcn2.

To test whether Lcn2 targets Alistipes in vivo, 5–week–old Il10$^{-/-}$ mice, which produce abundant concentrations of colonic Lcn2 (Figure S5B to S5D), and Lcn2$^{+/+}$/Il10$^{-/-}$ mice, which lack Lcn2 but synthesize high levels of alternative antimicrobial peptides (Figure S5E to S5H), were gavaged with $3 \times 10^5$ CFU 5-ethyl-2´-deoxyuridine (EdU)–labeled Alistipes finegoldii which were then quantified time–resolved in the fecal pellets by flow cytometry (Figure S5I). After 24 hours, the number of EdU–positive Alistipes was higher in Lcn2$^{+/+}$/Il10$^{-/-}$ compared to Il10$^{-/-}$ mice (Figure 6F). This was confirmed by confocal microscopy (Figure S5J) which indicated that beside EdU–positive Alistipes counts, particularly numbers of mucosa–adherent cells were higher in Lcn2$^{+/+}$/Il10$^{-/-}$ animals,
which was also manifested in a diminished mucus layer (Figure S5K to S5N). These data were in support of a direct Alistipes–targeting effect of Lcn2 in vivo.

To test for a pathogenic potential of Alistipes spp. we low–dose challenged Wt, Lcn2−/−, and Il10−/− mice orally with 3 × 10⁵ CFU of Alistipes finegoldii. Strikingly, Alistipes finegoldii induced intestinal inflammation in all three genotypes after only one week (Figure 6G). Histological analyses revealed distinctive parallels between Alistipes−treated Il10−/− and Lcn2−/− / Il10−/− animals regarding the infiltrate and epithelial hyperplasia, respectively (Figure 6H). Long–term treatment of Wt, Lcn2−/− and Il10−/− mice receiving 3 × 10⁵ CFU Alistipes finegoldii once per week over 8 weeks resulted in comparable histologic inflammation (Figure 6I). Strikingly, Il10−/− animals developed right–sided polyps in 62% compared to mock–treated littermates (Figures 6J to 6L). No polyps were observed in Wt and Lcn2−/− animals (data not shown).

Together, these results indicate that specific species and particularly Alistipes spp., are enriched in Lcn2−/− / Il10−/− mice, thrive in an inflamed Lcn2–deficient environment, and promote inflammation and tumor formation in our model.

**Right–sided tumors are associated with site–preferential Alistipes colonization, STAT3 activation and induction of “cancer”–related gene ontology pathways.**

The restriction of tumors to the cecum and proximal colon (right–sided tumors) in Lcn2−/− / Il10−/− mice prompted us to further investigate mechanisms underlying this unilateral localization. With respect to right–sided tumors it has been appreciated previously that in the genetically susceptible host an interplay between an inflammatory responses and the microbiota might be particular relevant (Bongers et al., 2014). As inflammation represents a critical driver of tumorigenesis, we first considered that an inflammatory
disequilibrium between the proximal and distal colon may be relevant. Thus, we performed per-segment histological analyses by studying “swiss rolls” of formalin–perfused Il10^+/− and Lcn2^−/− / Il10^−/− animals (Figure 7A). However, the histologic sub-analysis of the four colonic segments (cecum, proximal, middle, and distal) in Lcn2^−/− / Il10^−/− mice indicated a sheet-like pancolitis with negligible differences in histological inflammation on the longitudinal axis of the colon (Figure 7B).

As site–specific colonization of bacterial pathogens to host surfaces represents a well–established virulence mechanism (Stecher and Hardt, 2011), and an increasing number of species–specific commensal colonization factors are identified (Lee et al., 2013), we next assessed the possibility of a site–preferential colonization by Alistipes using FISH in Il10^−/− mice three days after oral gavage. We detected reasonable numbers of Alistipes finegoldii in the ceca and proximal colons. In contrast very few FISH-positive Alistipes were found in the middle and distal colon segments (Figure 7C) suggesting that site–preferential colonization of Alistipes might determine tumor localization.

We have shown that in our model IL-6 represents an important driver of inflammation and tumor formation in Lcn2^−/− / Il10^−/− mice (Figures 3A to 3E), and IL-6 governs intestinal tumorigenesis via STAT3 (Grivennikov et al., 2009). In this sense, we noted increased STAT3 phosphorylation in the right– compared to the left–sided colon (Figure 7D). A reciprocal picture was seen in Il10^−/− animals (Figure 7E). Noteworthy, comparing the 16S data from Lcn2^−/− / Il10^−/− double– and Il6^−/− / Lcn2^−/− / Il10^−/− triple–deficient mice revealed a roughly similar microbiome (Figure S6A). In other words, Il6^−/− / Lcn2^−/− / Il10^−/− mice were (partly) protected from the development of inflammation and right–sided tumors (see Figure 3A to Figure 3F) despite the presence of Alistipes (Figure S6B)
further corroborating an important link between the right–sided tumor location, *Alistipes*, and the IL-6–STAT3 axis.

In search for additional relevant tumor–promoting pathways, we finally conducted whole-genome expression analyses of right– versus left–sided intestinal tissue samples (Figure S6C). Remarkably, gene ontology (GO) analysis confirmed the relevance of the “positive regulation of tyrosine phosphorylation of STAT3 protein” in the right– compared to the left–sided colon (Figure 7F). Other GO terms significantly overrepresented included “epithelial cell morphogenesis”, “somatic stem cell division” and “retinoic metabolic process” (Figure 7F). Ingenuity Pathway Analysis (IPA) identified “Cancer” as the most significantly regulated topic (p value range: $5.7 \times 10^{-3}$ to $3.5 \times 10^{-8}$) of which a large proportion of differentially regulated genes were part of to the cancer categories “Adenoma”, “Intestinal adenoma”, “Colorectal adenoma”, “Invasion of Cancer cells”, and “Growth of Tumor” (yellow dots, Figure S6D). Together, data derived from whole genome expression analyses were in support of the clinical phenotype and gave a hint to additional relevant pathways.
DISCUSSION

Herein we report that Lcn2 protects from colonic inflammation and intestinal carcinogenesis due to modulation of the intestinal microbiota in the IL-10 knockout model of colitis. Il10\(^{-/-}\) mice are widely used to study genetic and environmental cues during colitis as genetic variation in the human IL-10 receptor has been linked to IBD (Glocker et al., 2009). Il10\(^{-/-}\) colitis is perpetuated by a detrimental immune response against colonizing bacteria and Il10\(^{-/-}\) mice do not develop colitis under germ–free conditions (Sellon et al., 1998). Monoassociation of germ–free Il10\(^{-/-}\) mice instigates intestinal inflammation with individual kinetics depending on the commensal species that in turn is dependent on TLR signaling through the adaptor protein MyD88 (Kim et al., 2005; Rakoff-Nahoum et al., 2006). Notably, in our setting Lcn2–deficiency was associated with a remarkably early onset of intestinal inflammation in Il10\(^{-/-}\) mice that was evident in only 4–week–old mice and peaked at 12 weeks (Figures S2C to S2F and Figure 1A to 1F). Cross–fostering experiments, which are set up immediately after birth and which induce a permanent microbiota shift shaped by the nursing mother (Daft et al., 2015), strongly suggested that the microbiota was the determinant factor irrespectively of the genotype in our model. Unlike cross–fostering, in co–housing, in which mice with an established microbiota are put together at weaning, the disease–causing microbiota and the disease phenotype were transferred predominantly to Il10\(^{-/-}\) animals. This is line with previously published data and may reflect the higher complexity of the Lcn2\(^{-/-}\)/Il10\(^{-/-}\) microbiota which renders Lcn2\(^{-/-}\)/Il10\(^{-/-}\) mice more colonization resistant than lower–complexity Il10\(^{-/-}\) mice (Elinav et al., 2011; Stecher et al., 2010).

Unlike other anti–microbial peptides (AMP) such as α–defensins or lysozyme, which are constitutively expressed and stored as precursors in Paneth cell secretory granules,
Lcn2 is strongly up-regulated in response to bacterial pathogens (Behnsen et al., 2014; Chakraborty et al., 2012). This phenomenon is not restricted to the intestinal mucosa, but is observed at various bacterially stressed epithelial borders including the nasal and airway mucosa, and the skin (Berger et al., 2006; Nelson et al., 2005; Sorensen et al., 2003). In contrast to RegIIIγ, a secreted anti-bacterial lectin also strongly induced in a MyD88-dependent manner (Vaishnava et al., 2011), Lcn2 was dispensable for maintaining an interlaced area separating the microbiota from the intestinal epithelium in the steady state. However, in the context of chronic intestinal inflammation Lcn2-deficiency resulted in disruption of the spatial segregation, widespread direct contact of bacteria with the intestinal epithelium and excessive innate and adaptive immune activation.

Signals derived from the microbiota emplace mucosal defense strategies including the induction of AMPs to contain commensals within the intestine, a process disrupted during infection and chronic inflammation (Bevins and Salzman, 2011). This has often been reported to result in a reduction of species diversity or an overgrowth of Proteobacteria as seen in IBD (Manichanh et al., 2012). However, in IBD it remains a matter of debate whether dysbiosis triggers or is a consequence of inflammation (Sartor, 2008). Accordingly, species diversity was markedly reduced in Il10−/− mice, which produce high concentrations of Lcn2 in the intestine derived from both, IECs and infiltrating mono- and polymorphonuclear cells (Figures S2B and S5B to S5D). We arguably observed a compensatory expression of various AMPs when Lcn2 was concomitantly deficient (Figures S5E to S5H). Although species diversity was not diminished in Lcn2−/−/ Il10−/− mice, we noted markedly altered microbial signatures (increased bacterial load and variability in alpha- and beta-diversity) suggesting a dysregulation of intestinal community structure commonly referred to as dysbiosis.
Lcn2’s important role as an anti-microbial defense molecule becomes evident in mouse models of Gram-negative bacterial sepsis. Thereby, its protective role against infection is linked to its ability to withhold iron from bacteria. This function is based on the binding of iron–loaded bacterial siderophores such as enterobactin by Lcn2, and thus withholding iron as an essential nutrient and pathogenicity factor from bacteria (Berger et al., 2006; Flo et al., 2004; Nairz et al., 2009; Raffatellu et al., 2009). Accordingly, Lcn2 deficiency may increase iron availability for certain intestinal bacteria and can cause a growth advantage of facultative pathogenic bacteria over commensals based on their ability to bind and re–ingest intestinal iron via siderophores (Deriu et al., 2013). This mechanism is relevant in our model of chronic colonic inflammation in Il10−/− mice where the subsequent deletion of Lcn2 resulted in striking alterations of the gut microbiota characterized by the expansion of bacterial populations with pathogenic properties.

The altered microbiota was critical for inflammation and spontaneous tumorigenesis in Lcn2−/− / Il10−/− mice. Dysbiosis may promote the rise and expansion of particular bacterial populations with pathogenic properties, sometimes referred as pathobionts (Chassaing et al., 2011). We identified three OTUs specifically overrepresented in Lcn2−/− / Il10−/− mice, namely two from unclassified Lachnospiraceae and one from Alistipes spp. Recently, we and others identified Alistipes spp. as one of the top ten most abundant genera associated with human colorectal carcinoma (Feng et al., 2015). In line with this, we now provide experimental evidence that Alistipes, which thrives in the inflamed colon during Lcn2–deficiency, potently induced inflammation and tumorigenesis in Il10−/− mice. The molecular mechanisms by which Alistipes modulates the propensity for tumor formation and the specific growth in the right–sided colon remain so far unclear but we provide evidence for an impact of Alistipes spp. on the IL-6–STAT3 pathway thereby being a driving force for chronic intestinal inflammation.
Previous studies demonstrated site-specific tumor formation in the cecum which depends on the presence of a specific microbiota (Bongers et al., 2014). Notably, *Alistipes* accumulated in the right-sided colon after gavage which could be a consequence of optimal growth conditions or the anaerobic milieu. Interestingly, tumorigenesis is dependent on colonic inflammation as demonstrated by the requirement of IL-6 signaling and only minor effects on small intestinal adenoma formation in the *Apc*\(^{min/+}\) model (Reilly et al., 2013). Myeloid and epithelial NF–κB and STAT3 activation and pro-inflammatory cytokines such as TNF-α, IL-6, and IL-22 promote IEC hyperplasia and tumorigenesis (Greten et al., 2004; Grivennikov et al., 2009; Huber et al., 2012). Early in the disease course, IL-6 and IL-22 were strongly activated in our model as was phosphorylated STAT3, which was also more pronounced in the right-sided colon. Correspondingly, *Il6\(^{-/-}\) / Lcn2\(^{-/-}\) / Il10\(^{-/-}\) triple-deficient mice exhibited fewer and smaller adenomas despite the presence of *Alistipes*, demonstrating that IL-6–STAT3 signaling, probably induced by *Alistipes*, critically drives tumor formation in our model.

In conclusion, our data indicate that Lcn2 in the inflamed intestine is critically important to control bacterial community and particularly *Alistipes* which perpetuates disease and promotes tumorigenesis in an IL-6–dependent manner. Our work provides another example of the intricate interplay between the host and the microbiota during intestinal inflammation and highlights the importance of Lcn2 in keeping dysbiosis and intestinal inflammation at bay.
MATERIALS AND METHODS

A detailed description of materials and methods used in this paper can be found in the Supplemental Information.

Animal experiments

$\text{Il10}^{-/-}$ mice and $\text{Il6}^{-/-}$ mice, both on a C57BL/6J background, were purchased from Jackson Laboratories. $\text{Lcn2}^{-/-}$ mice were kindly provided by S. Akira (University of Osaka, Osaka, Japan) (Flo et al., 2004). Mice were intercrossed to generate $\text{Wt}$, $\text{Lcn2}^{+/-}$, $\text{Il10}^{-/-}$, and $\text{Lcn2}^{-/-}/\text{Il10}^{-/-}$ and moreover $\text{Il6}^{-/-}$, double–deficient ($\text{Il6}^{-/-}/\text{Lcn2}^{+/-}$, $\text{Il6}^{-/-}/\text{Il10}^{-/-}$), or triple–deficient ($\text{Il6}^{-/-}/\text{Lcn2}^{-/-}/\text{Il10}^{-/-}$) litters. All mice were bred and maintained under specific pathogen–free conditions. All experiments were approved by the Austrian Ministry of Science and Research (66011/75/5/3b/2012 to A.R.M.) and supervised by the local veterinarian authorities.

Cross–fostering

Newborn litters from $\text{Il10}^{-/-}$ and $\text{Lcn2}^{-/-}/\text{Il10}^{-/-}$ mice were split and swapped at a 1–to–1 ratio between respective mothers within 24 hours of birth (outlined in Figure 5B). Accordingly, each mother raised 50% of her own and 50% of cross–fostered pups. After 3 weeks, the offspring was weaned gender–specifically and in a 1–to–1 ratio according to genotype. Mice were analyzed at 12 weeks of age.

Antibiotic treatment

4–week–old mice were treated with a combination of ciprofloxacin and metronidazole prepared with Kool-Aid in sterile drinking water. Antibiotics were changed three times per week for in total 8 weeks (see Figure 5A). Mice were analyzed at 12 weeks of age.
Association and tracking studies upon oral application of *Alistipes finegoldii*

Alistipes finegoldii (46020T) and Alistipes putredinis (45780T) were obtained from the CCUG (Culture Collection, University of Göteborg, Sweden) and propagated anaerobically on Columbia agar. For in vivo experiments animals were gavaged with bacteria or vector control. Mice were evaluated after three days, one week or eight weeks for inflammation, tumor development or colonization by FISH. In other experiments EdU–labeled *Alistipes* were visualized by Click chemistry and analyzed by flow cytometry or confocal microscopy.

**In vivo barrier function experiments**

12–week–old mice were gavaged with FITC dextran. Serum fluorescence was measured on a PHERAstar plus microplate reader (BMG Labtech, Ortenberg, Germany).

**Colon explant cultures**

10 mm pieces of colonic tissue were weighed and cultured in RPMI1640 and cytokine release was assayed by multiplexed bead-based assays or ELISA.

**Gene Expression Analysis**

Colonic tissues scrapings were preserved in RNAlater solution and total RNA was extracted using QIAshredder and RNeasy Mini columns (Qiagen). Quantitative real–time PCR was performed on a Mx3000P instrument with SYBR-green chemistry. Primer sequences are shown in Table S4.
Whole-Genome Expression Analysis and Bioinformatics

RNA from proximal and distal colonic tissue samples was analyzed on an Affymetrix GeneTitan platform using Affymetrix® Mouse Gene 2.0 ST whole transcript arrays at IMGM (Munich, Germany). Bioinformatics is described in detail in the Supplementary Experimental Procedures.

Bacterial FISH

For FISH analyses colonic tissue pieces were mounted in modified Carnoy’s fixative and processed as described (Swidsinski et al., 2007). Briefly, sections were hybridized with respective fluorophore-labelled probes, visualized by fluorescence microscopy, and scored as described previously (Swidsinski et al., 2007).

Isolation of bacterial DNA and 16S rRNA sequences analyses

Bacterial DNA was extracted using a FastDNA SPIN Kit (MP Biomedicals) and a Precellys®24 homogenizer (Peqlab). The V1-V2 region of the bacterial 16S rRNA gene was amplified with 27F-338R primers together with adapter sequences and multiplex identifiers, and processed on an Illumina MiSeq sequencer. Detailed bioinformatics is given in the Supplementary Experimental Procedures.

Statistical analysis

All results are expressed as means ± standard error. Normally-distributed data groups were analyzed by one-way ANOVA followed by post-hoc Tukey. Non-normally distributed data were analyzed by Kruskal–Wallis test followed by Dunn’s multiple comparisons test where appropriate (GraphPad Prism 6.0).
SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, four figures, and four tables and can be found with this article online.

AUTHOR CONTRIBUTION


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We thank Professor Akira for kindly providing Lcn2–deficient animals, Teresa Fritz for valuable input and helpful discussions regarding animal experiments, Ines Brosch, Barbara Enrich, and Sabine Geiger–Schredelseker for outstanding support in histological concerns and technological assistance, Tim Raine for outstanding assistance in flow cytometry, Hartmut Glossmann and Hans-Günther Knaus for generously providing lab space, Hermann Dietrich and his team of the central animal laboratory facility for the excellent care of our mice. This work was supported by the Christian–Doppler–Research Foundation (to HT), the Austrian Science Fund project grant P21530-B18 (to AK), the Tyrolian Science Fund 0404/1812 (to TEA) and the Tyrolean science fund project UNI-0404/1480 (to ARM).
REFERENCES


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FIGURE LEGENDS

Figure 1: Absence of Lcn2 results in colitis aggravation and spontaneous tumor formation in IL-10–deficient mice

(A and B) Histologic severity scores of 12-week—old wildtype (Wt) (light grey circles, n=9), Lcn2−/− (dark grey triangles, n=10), Il10−/− (light blue triangles, n=10), and Lcn2−/−/ Il10−/− double knockout (Dko) mice (dark blue squares, n=13), along with representative H&E-stained sections of colons (Scale bars, 400µm).

(C and D) Mean colon lengths in mm (c), and macroscopic photographs (d) of colons and spleens of mice from the indicated genotypes.

(E and F) Analysis of mRNA expressions in colonic mucosal scrapings (e), and protein release from colonic explant cultures of the indicated cytokines (f) of Wt, Lcn2−/−, Il10−/−, and Dko mice (n = 6 to 8 per group, 2 independent experiments).

(G) Immunohistochemical analysis of infiltrating leukocytes. Number of cells per crypt axis immuno–positive for the indicated surface marker in Wt, Lcn2−/−, Il10−/−, and Dko mice. N=5, 5 crypts per mouse.

(H) Bone marrow chimeras were generated using both Il10−/− and Dko mice as host and bone marrow donor. Histologic colitis severity was analyzed 8 weeks after bone marrow transplantation to assess the effect of hematopoietic versus non-hematopoietic Lcn2 on colitis severity in Il10−/−(bm) → Il10−/− (n=11), Dko (bm) → Il10−/− (n=11), Il10−/− (bm) → Dko (n=8), and Dko (bm) → Dko (n=11) mice.

See also Figure S1 and Figure S2. All data are given as means. Error bars indicate the s.e.m. of samples within a group. *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001; one–way ANOVA (post–hoc Tukey’s) or Kruskal–Wallis test (post–hoc Dunn’s).
Figure 2: Lcn2−deficiency eventuates in spontaneous right−sided tumor formation in IL−10−deficient mice.

(A) Stereomicroscopic, macroscopic pictures of representative ceca of Il10−/− and Lcn2−/−/Il10−/− (Dko) mice along with respective H&E stainings. Scale bars, 1200µm.

(B and C) Wt (n=6), Lcn2−/− (n=6), Il10−/− (n=6), and Dko (n=8) animals were examined stereomicroscopically and histologically for the presence of adenomatous polyps. Tumor incidence and tumor areas (tumor area/total cecum area) are shown.

(D and E) Western blot analyses of phosphorylated and total STAT3 from colonic epithelial scrapings and corresponding densitometrical analyses of indicated the genotypes.

(F and G) Immunohistochemistry for phosphorylated STAT3 in the indicated experimental groups. Phosphorylated STAT3 positive nuclei along the crypt axis were assessed separately for epithelial and adjacent inflammatory cells. STAT3−positive cells were normalized to total crypt cells (n=5 to 6 per group, at least 4 crypts per section).

(H and I) Immunohistochemistry for BrdU on colonic tissue sections of wildtype (Wt), Lcn2−/−, Il10−/−, and Dko animals 2 hours after injection of BrdU (n=6 per group, scale bar, 50µm). BrdU−positive cells (brown nuclei) along the crypt axis (at least 6 per section) were counted and normalized to total crypt cells (BrdU+ plus BrdU− (blue)). Left bars outline 2 hours, right bars 24 hours proliferation.

See also Figure S3.

Figure 3: IL−6 drives inflammation and right−sided tumor formation in Lcn2−/−/Il10−/− mice

(A and B) Histologic severity scores and mucosa thicknesses compared between Wt, Lcn2−/−, Il10−/−, and Lcn2−/−/Il10−/− (Dko) mice with or without additional IL−6−deletion.
Tko indicates \( \text{Il}6^{-/-} / \text{Lcn}2^{-/-} / \text{Il}10^{-/-} \) triple knockout mice (pink squares). Each datapoint represents an individual animal.

(C) Representative H&E-stained colon sections of indicated genotypes (scale bars, 250\( \mu \)m for Tko, and 100\( \mu \)m for the remaining genotypes).

(C) Representative macroscopic captures of ceca of indicated genotypes.

(D and E) Comparison of tumor numbers and areas between \( \text{Il}10^{-/-} \) (light blue triangles), \( \text{Il}6^{-/-} / \text{Il}10^{-/-} \) (blue triangles), Dko (dark blue squares), and \( \text{Il}6^{-/-} / \text{Lcn}2^{-/-} / \text{Il}10^{-/-} \) triple-deficient (Tko, pink squares) animals.

All data are given as means. Error bars indicate the SEM of samples within a group. *, \( P < 0.05 \); **, \( P < 0.01 \); ***, \( P < 0.001 \) by one-way ANOVA (post-hoc Tukey’s) or Kruskal–Wallis test (post-hoc Dunn’s).

Figure 4: Lcn2/IL-10 double-deficient mice exhibit structural mucosal damage, barrier leakage, and an altered microbial ecology

(A) Representative picture captures of fluorescence in situ hybridizations on Carnoy-fixed colonic tissue sections using a FITC-labeled pan-bacterial EUB338 probe (yellow to green, lower panel). Nuclei were counterstained with DAPI (blue, top panel). Genotypes are indicated. The epithelium (E) was adjusted to the upper and the feces (F) containing bacteria to the bottom part of the image. The sterile mucus layer (M) highlighted by two dashed lines was lost in \( \text{Lcn}2^{-/-} / \text{Il}10^{-/-} \) (Dko) animals. The white arrows point at leukocytes infiltrating the feces. Original magnification 200×.

(B) Slides were scored for the indicated features as described in detail in the Supplementary Experimental Procedures. Data are presented as percent involvement according to the respective genotype. Wildtype (Wt, light grey), \( \text{Lcn}2^{-/-} \) (dark grey),
II10−/− (light blue), and Lcn2−/−/II10−/− (Dko, dark blue). Indicated significances refer to II10−/− versus Dko mice. n=6 per group, data are expressed as means.

(C and D) Quantitative analysis of the total copies of bacterial 16S rDNA in mesenteric lymph nodes (input material 20ng, normalized to host 18S rDNA, n=6 per group), and serum fluorescence intensity 4 hours after gavage of FITC-dextran in indicated mice (n=6 per group). Data are given as means. Error bars indicate the SEM.

(E) Abundances of major phyla and major genera of bacterial communities in the indicated cecal DNA samples collected at 12 weeks. An asterisk indicates significant differences between all indicated groups by ANOVA (FDR corrected). Additional statistical details are given in Table S1.

(F and G) Species–evenness and –richness as indicated by Shannon and Chao1 indices are outlined. Data are shown as boxplots with values as median (horizontal line), 75% confidence interval (box), and minimum/maximum values (whiskers), n=4 per group.

See also Table S1. *, P < 0.05; **, P < 0.01; ***, P < 0.001 by one–way ANOVA (post–hoc Tukey’s) or Kruskal–Wallis test (post–hoc Dunn’s).

Figure 5: Colitis and tumors are responsive to antibiotics and transmissible to cross–fostered animals

(A) Mice were treated with broad-spectrum antibiotics or water. The treatment plan is indicated below the abscissa. Histologic severity scores of mice with (filled symbols) or without (open symbols) antibiotic treatment at 12 weeks are demonstrated for wildtype (Wt), Lcn2−/−, II10−/−, and Lcn2−/−/II10−/− (Dko) animals. Each data point represents an individual animal.

(B) Experimental outline of cross–foster experiments.
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**(C)** Histologic severity scores of *Il10*−/− pups cross-fostered (CF) with *Dko* [*Il10* (*Dko*), n=9] and *Dko* pups CF with *Il10*−/− mothers [*Dko* (*Il10*), n=6] together with respective littermate controls [*Il10* (*Il10*), n=6; and *Dko* (*Dko*), n=7]. Data represent 3 independent experiments.

**(D)** Representative macroscopic photographs of ceca of the indicated groups.

**(E and F)** Tumor numbers and areas were determined at week 12 for *Il10* (*Il10*), light blue triangles, *Il10* (*Dko*), turquoise triangles, *Dko* (*Il10*), green squares, n=6, and *Dko* (*Dko*), dark blue squares, mice.

**(G and H)** Abundances of major phyla and major genera of bacterial communities in indicated cecal DNA samples of cross-fostered mice. An asterisk indicates significant differences between all indicated groups by ANOVA (FDR corrected). Additional statistical information is given in Table S2.

**(I and J)** Species evenness and species richness as demonstrated by Shannon and Chao1 indices are outlined. Data are illustrated as boxplots with values as median (horizontal line), 75% confidence interval (box), and minimum/maximum values (whiskers), n=3−4 per group.

**(K and L)** Ordination plots of bacterial communities, including different genotypes and housing conditions, based on Bray-Curtis dissimilarities and weighted Unifrac index. There was a significant relationship between geographic distance and community dissimilarities for both plots (*, adonis test, P < 0.001). Legend: (single) means genotype-specifically, single-housed animals, the other conditions are outlined above.

See also Figure S4 and Table S2. Data are given as means. Error bars indicate the SEM of samples within a group. *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001; by one-way ANOVA (post-hoc Tukey’s) or Kruskal–Wallis test (post-hoc Dunn’s).
**Figure 6:** *Alistipes* species are overrepresented in *Lcn2<sup>−/−</sup> / *Il10<sup>−/−</sup>* animals and capable of evoking colitis and right-sided colonic tumors when orally transmitted.

(A) 16S data were ordinated according to community compositions (redundancy analysis, RDA) and overlayed by a ‘bioplot’ function to indicate genotype or housing specific genera. At the genus level significantly enriched genera in *Lcn2<sup>−/−</sup> / *Il10<sup>−/−</sup>* mice were indicated with red arrows, and further investigated at species level (OTUs). Legend: (single) means genotype-specifically, single-housed animals; the genotype put in brackets indicates the genotype of the respective foster mother.

(B) Relative abundances of OTU808 (*Alistipes*) in single-housed *Dko*, and *Il10<sup>−/−</sup>* and *Dko* mice crossed-fostered with *Dko* mothers versus all other genotypes are indicated.

(C) Effect of increasing concentrations of recombinant Lcn2 on the growth of *Alistipes putredinis* (45780T) and *Alistipes finegoldii* (46020T) on Columbia blood agar plates. Lysozyme was used as a positive control. n=5 per group, each experiment was performed at least three times independently.

(D) *Alistipes finegoldii* was cultured in thioglycollate broth for five days and supernatants were tested for the presence of siderophores using a colorimetric SideroTec assay. Medium only served as a negative control (n=5).

(E) *Alistipes finegoldii* were grown with or without the indicated concentrations of deferoxamine (DFO), iron (III) ammonium sulfate (Fe<sup>3+</sup>), iron-free enterobactin (ENT), or iron-laden enterobactin (Fe–ENT) again with or without 5µM recombinant siderophore-free Lcn2. Numbers of CFU were evaluated on Columbia blood agar plates (n=5, 2 independent experiments).

(F) Fecal EdU–positive *Alistipes finegoldii* were quantified by flow cytometry in *Il10<sup>−/−</sup>* and *Lcn2<sup>−/−</sup> / *Il10<sup>−/−</sup>* (*Dko*) mice 24, 48, and 72 hours after oral gavage (n=5 per group).
(G and H) 4-week-old Wt, Lcn2−/− and Il10−/− mice were gavaged with vector control or Alistipes finegoldii and histology was scored after 7 days (2 independent experiments). Representative H&E stainings are shown and bottom–to–top distances (µm) are indicated at the y-axis.

(I and J) 4-week-old Wt, Lcn2−/− and Il10−/− mice were gavaged with vector control or Alistipes finegoldii (46020T) once per week over 8 weeks and histology was scored (i, 2 independent experiments). (j) Representative macroscopic photographs of the ceca of 12-week-old Il10−/− mice with or without Alistipes.

(K and L) Tumor numbers and tumor areas were quantified by stereomicroscopy and histologic assessment (2 independent experiments).

See also Figure S5. Kruskal–Wallis test (post–hoc Dunn’s). Data represent mean and error bars indicate the SEM. *, P < 0.05; **, P < 0.01; ***, P < 0.001.

Figure 7: Site–preferential emergence of tumors in Dko mice is associated with a distinct colonization pattern of Alistipes, local differences in STAT3 activation, and differential expression of genes involved in tumorigenesis.

(A) Representative H&E–stained sections of colonic "swiss-rolls" of Il10−/− and Dko mice. Proximal and distal ends are indicated. Original magnification 3.5×.

(B) Per segment analysis of histologic severity of the indicated colonic sections was scored in Il10−/− and Dko animals (N=5–6 per group). **, P < 0.01. Student’s t-test and one–way ANOVA.

(C) Il10−/− mice (n=5) were gavaged with 3×10⁵ Alistipes finegoldii and their abundance was determined by FISH in the indicated colon segments three days later. The confocal image represents the cecum of a Carnoy-fixed tissue section. Nuclei of the epithelium (E) are shown in blue (DAPI), the mucus layer (M) is emphasized by two dashed lines,
the cecal feces (C) shows all bacteria in green (EUB338-FITC) with interspersed *Alistipes* as depicted in yellow to red (*Alistipes*-Cy5, indicated by white arrows). Original magnification 400×. *, P < 0.05; **, P < 0.01. One−way ANOVA, post−hoc Tukey’s test.

(D and E) Phosphorylated and total STAT3 of right−sided (cecum and proximal colon) and distal colonic scrapings of *Il10<sup>−/−</sup>* and *Dko* mice were determined by Western blot and quantified by densitometry. Student’s t-test.

(E) Whole genome expression analysis of paired proximal (right−sided) versus distal colonic tissue samples identified 327 significantly differentially regulated genes which were imported to ClueGO to obtain a functional network of differentially regulated biological processes. Each node represents a significantly affected gene ontology term. Nodes are connected and grouped shared genes (kappa score>0.3). The node size represents the enriched p−value corrected by the Benjamini−Hochberg method.

See also Figure S6.
SUPPLEMENTARY INFORMATION

Lipocalin 2 protects from colonic inflammation and tumorigenesis through its microbiota modulating properties

Short title: Lcn2 in intestinal inflammation

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II. SUPPLEMENTARY FIGURES

Figure S1 (related to Figure 1)

(A) Comparative picture captures of hind legs and perianal regions of WT, Lcn2−/−, IL10−/−, and Lcn2−/−/IL10−/− double knockout (Dko) mice.

(B) Representative H&E-stained longitudinal colon section of a Dko animal. Besides other features such as hyperplasia and mixed-cell infiltrate, crypt-abscesses (black arrows) and crypt dropouts (white dashed line) are indicated.

(C) Representative overview of H&E- and indicated immunohistochemical stainings of paraffin-embedded tissue sections of 12-week-old Wt, Lcn2−/−, IL10−/−, and Lcn2−/−/IL10−/− double knockout (Dko) mice. Bars: (H&E) 500 μm; Original magnification IHC 300x.
(A) Verification of BM chimeric mice (combinations indicated below the abscissa) was carried out 4 weeks after transplantation. Mice were found to be fully chimerized, as assessed by specific ELISAs for Lcn2 and IL-10 in freshly isolated, LPS-stimulated peripheral blood mononuclear cells. LPS-stimulated wildtype peripheral mononuclear cells served as a positive control for the IL-10 ELISA.

(B) Chimerism was further evaluated on paraffin-embedded colonic tissue sections of 12-week-old animals. Representative picture captures of the indicated combinations showing either positivity or negativity in intestinal epithelial cells and/or inflammatory cells.

(C and D) Comparison of histology scores of (c) 4- and (d) 8-week-old animals.

(E and F) Relative mRNA expression (x-fold) for IL-1β, IL-6, IFNγ, IL-17A, and IL-22 in colonic tissue samples. N=5-6 per group. 2 independent experiments.
Data are given as means. Error bars indicate the SEM. *P < 0.05, **P < 0.01, ***P < 0.001 Kruskal–Wallis ANOVA (Dunn’s).

Figure S3 (related to Figure 2)

(A) Example of malignant transformation from an adenomatous polyp into an invasive adenocarcinoma, breaking through the muscularis mucosae. The carcinoma is highlighted by a black dashed line.

(B) Western blot analyses of phosphorylated and total C/EBP-β and NF-κB from colonic epithelial scrapings of the indicated genotypes. GAPDH served as a loading control.
(D and E) Western blots of phosphorylated and total C/EBP-β and NF-κB were analysed by densitometry (relative light units) using ImageJ normalized to GAPDH (n=4 per group).

**Figure S4** (related to Figure 5)

Colitis and right−sided tumors are transmissible by co−housing

(A) Macroscopic photographs of ceca of 12-week-old Il10+/− and Dko mice after 8-weeks of antibiotic treatment. Tumor numbers and areas in respective animals are shown. N=6 per group, 2 independent experiments.

(B) Experimental outline of co−housing experiments.
Histologic severity scores of 12–week–old $\text{IL10}^{+/+}$ (IL10) co–housed with $\text{Lcn2}^{+/+} / \text{IL10}^{+/+}$ (Dko) animals and respective genotype–specifically housed littermates are shown. IL10 (IL10), light blue triangles, n=10. IL10 (Dko), turquoise triangles, n=10. Dko (IL10), green squares, n=8. Dko (Dko), dark blue squares, n=6.

Right–sided tumors are transmissible from double–deficient (Dko) to co–housed $\text{IL10}^{+/+}$ mice, which demonstrate higher tumor incidences and extents. Co–housed Dko animals had lower tumor numbers yet a comparable tumor area compared to their genotype–specifically housed littermates. Tumor areas are outlined as per cent of overall cecum surface. IL10 (IL10), light blue triangles, n=10. IL10 (Dko), turquoise triangles. Dko (IL10), green squares. Dko (Dko), dark blue squares. Each symbol indicates an individual animal.

Ordination plots of bacterial communities, including either single–housed or co–housed $\text{IL10}^{+/+}$ or $\text{Lcn2}^{+/+} / \text{IL10}^{+/+}$ (Dko) animals based on Bray–Curtis dissimilarities and weighted Unifrac index. Adonis testing showed no significant differences between $\text{Lcn2}^{+/+} / \text{IL10}^{+/+}$ single–housed and any of the co–housed $\text{IL10}^{+/+}$ and $\text{Lcn2}^{+/+} / \text{IL10}^{+/+}$ animals. IL10 (IL10), light blue triangles, n=10. IL10 (Dko), turquoise triangles, n=10. Dko (IL10), green squares, n=8. Dko (Dko), dark blue squares.

Fecal Lcn2 protein levels in cecal stool samples of co–housed or genotype–specifically–housed $\text{IL10}^{+/+}$ and Dko mice were determined by specific ELISA (n=6).

Ordination plots of bacterial communities, including either 4–week–old or 12–week–old single–housed $\text{IL10}^{+/+}$ or $\text{Lcn2}^{+/+} / \text{IL10}^{+/+}$ (Dko) animals based on Bray–Curtis dissimilarities and weighted Unifrac index, showed a clear segregation already at 4 weeks. 4–week–old IL10 single–housed (4W), purple triangles; 4–week–old Dko single–housed (Dko 4W), azure squares; 12–week–old IL10 single–housed (IL10 12W), light blue triangles; 12–week–old Dko single–housed (Dko 12W), dark blue squares.

Mean and SEM are indicated. *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001 by one–way ANOVA, post–hoc Tukey’s test.
Moschen, Gerner et al.  Lcn2 in intestinal inflammation

Figure S5 (related to Figure 6)
(A) Quantitative PCR analysis of the total copies of Alistipes spp. 16S rDNA in the cecum of the indicated genotypes (n=6 per group).

(B) Immunoblot analysis of epithelial Lcn2 in 8-week-old Wt and Il10<sup>-/-</sup> mice. N=3, one representative experiment out of 2 experiments is shown.

(C and D) Epithelial Lcn2 mRNA expression of 8-week-old Wt and Il10<sup>-/-</sup> mice together with the corresponding fecal Lcn2 protein concentrations measured by ELISA (N=6).

(E–H) mRNA expressions of the indicated antimicrobial peptides (Defa1, Defensin alpha 1; Defensin beta 1; Camp, Cathelicidin antimicrobial peptide; Reg3g, Regenerating islet-derived 3 gamma) in colonic epithelial scrapings. Data were normalized to β-glucuronidase and hypoxanthine–guanosine phosphoribosyl transferase (n=6 per genotype). *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001 by one-way ANOVA (post-hoc: Tukey’s).

(I) Fecal pellets were prepared for flow cytometry and EdU–positive Alistipes finegoldii were quantified. Outlined is the gating strategy for unstained (baseline), Dko and Il10<sup>-/-</sup> mice after 24 hours. Percentages of singlets and EdU–positive cells are indicated.

(J–N) EdU-positive Alistipes (red) were stained on Carnoy–fixed tissue section of Il10<sup>-/-</sup> and Dko animals. Nuclei were counterstained with DAPI (blue). A 200 x 200 μm square was acquired by confocal microscopy. Numbers of EdU–positive Alistipes were quantified within 150μm (K) and within 75μm (L) from the epithelial margin (dashed line). The number of EdU–positive Alistipes in contact with the epithelium was counted (M), and the mucus area in μm<sup>2</sup> (N) was determined with ImageJ. Student’s t–test.

Figure S6 (related to Figure 7)
(A) Ordination plots of the bacterial communities used for Figure 5K and 5L including \( \text{Il6}^{−/−} / \text{Lcn2}^{−/−} / \text{Il10}^{−/−} \) triple–deficient (Tko) animals highlighted in pink circles. Tko animals show a microbiota comparable to Dko animals. The other genotypes are indicated in the legend.

(B) Relative abundances of OTU808 (Alistipes) in Dko and Tko versus all other single–housed animals, genotypes are indicated.

(C) Whole genome expression analysis was performed on paired proximal (right-sided) versus distal colonic tissue samples of Dko animals (n=5) using Affymetrix Mouse Gene 2.0 ST arrays. The heatmap of gene expression levels (Z-scores) indicates 32 genes with increased (>3–fold) and 21 genes with decreased (<3–fold) expression in the proximal versus the distal colon (false discovery rate [FDR] Q < 0.1). Red indicates increased and blue decreased expression.

(D) Ingenuity Pathway Analysis (IPA) of 327 significantly differentially regulated genes (196 with increased (>1.5–fold) and 131 genes decreased (<1.5–fold) expression) identified “Cancer” as the most significantly regulated term in the “Top Diseases and Bio Functions” category including 166 involved transcripts (p value range: \( 5.7 \times 10^{-3} \) to \( 3.5 \times 10^{-8} \)). The volcano plot indicates all genes (grey), significantly up– (red) or down– (blue) regulated genes, and 41 significantly regulated genes from the cancer categories “Adenoma”, “Intestinal adenoma”, “Colorectal adenoma”, “Invasion of Cancer cells”, and “Growth of Tumor” (yellow).
## I. SUPPLEMENTARY TABLES

### Table S1. Statistical analysis of fecal microbial community structures.

<table>
<thead>
<tr>
<th></th>
<th>Average abundances</th>
<th>Anova p among all groups</th>
<th>Anova p between IL10⁻/⁻ and Dko (FDR corrected)</th>
<th>Phyla</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacteroidetes</td>
<td>59.41%</td>
<td>1.28E-02</td>
<td>0.133</td>
<td>0.133</td>
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<tr>
<td>Firmicutes</td>
<td>37.34%</td>
<td>8.58E-03</td>
<td>0.142</td>
<td>0.142</td>
</tr>
<tr>
<td>uc_Porphyromonadaceae</td>
<td>18.09%</td>
<td>1.83E-06</td>
<td>8.53E-06</td>
<td>8.02E-02</td>
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<tr>
<td>Bacteroides</td>
<td>16.74%</td>
<td>1.68E-04</td>
<td>5.88E-04</td>
<td>9.91E-04</td>
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<td>uc_Lachnospiraceae</td>
<td>15.81%</td>
<td>1.60E-01</td>
<td>2.04E-01</td>
<td>9.33E-02</td>
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<tr>
<td>Clostridium_XVIII</td>
<td>8.57%</td>
<td>3.55E-04</td>
<td>8.27E-04</td>
<td>1.25E-01</td>
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<tr>
<td>uc_Cytophagaceae</td>
<td>6.75%</td>
<td>6.62E-04</td>
<td>1.32E-03</td>
<td>1.01E-01</td>
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<td>uc_Erysipelotrichaceae</td>
<td>5.99%</td>
<td>5.77E-02</td>
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<tr>
<td>Alistipes</td>
<td>5.20%</td>
<td>7.12E-01</td>
<td>7.12E-01</td>
<td>3.73E-01</td>
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<tr>
<td>uc_Rikenellaceae</td>
<td>3.65%</td>
<td>1.53E-06</td>
<td>8.53E-06</td>
<td>4.40E-05</td>
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<tr>
<td>uc_Prevotellaceae</td>
<td>2.81%</td>
<td>6.61E-01</td>
<td>7.12E-01</td>
<td>7.04E-01</td>
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<td>Lactobacillus</td>
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<td>9.13E-02</td>
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<tr>
<td>Parabacteroides</td>
<td>1.97%</td>
<td>2.82E-03</td>
<td>4.94E-03</td>
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<td>Parasutterella</td>
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<tr>
<td>uc_Marinilabiaceae</td>
<td>1.61%</td>
<td>1.05E-06</td>
<td>8.53E-06</td>
<td>4.17E-02</td>
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</table>

Differences between major taxa among all groups (Wt, Lcn2⁻/⁻, IL10⁻/⁻, Dko) as well as a post-hoc analysis of differences between IL10⁻/⁻ and Dko mouse microbiomes are shown. Major taxa were defined as phyla and genera with average abundance ≥1% in the whole dataset. ANOVA was performed to test the differences among groups showing significant p values before and after correction for multiple testing. Furthermore, ANOVA results between IL10⁻/⁻ and Dko mice before and after multiple testing corrections are shown. Significant values are indicated by bold print. Bacterial abundances are log transformed to achieve a normal distribution.
Major taxa are defined as phyla and genera with average abundance ≥1% in the whole dataset. ANOVA was performed to test for differences between mice fostered by mothers of different genotypes (IL10<sup>−/−</sup> and Dko) as well as to test between the two genotypes of mice (IL10<sup>−/−</sup> and DKO) fostered by each kind of mother. P-values are shown before and after corrections for multiple testing. The data show that the microbiomes are overall separated by the genotype of the nursing mother instead of genotype of the fostered mice. Bacterial abundances are log transformed to achieve a normal distribution.
Table S3. List of antibodies used.

<table>
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<tr>
<th>Antigen</th>
<th>Label</th>
<th>Isotype</th>
<th>Clone/Catalogue No</th>
<th>Source</th>
<th>Application</th>
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<tbody>
<tr>
<td>CD3</td>
<td>Rabbit IgG</td>
<td>A 0452</td>
<td>Dako</td>
<td></td>
<td>IHC</td>
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<tr>
<td>CD45</td>
<td>Rat IgG2b</td>
<td>30-F11</td>
<td>Biolegend</td>
<td></td>
<td>IHC</td>
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<td>F4/80</td>
<td>Rat IgG2b</td>
<td>Cl:A3-1</td>
<td>AbDSerotec</td>
<td></td>
<td>IHC</td>
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<tr>
<td>CD45R/B220</td>
<td>Rat IgG2a</td>
<td>RA3-6B2</td>
<td>BDPharmingen</td>
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<td>IHC</td>
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<tr>
<td>NGAL/Lcn2</td>
<td>Goat IgG</td>
<td>AF3508</td>
<td>R&amp;D Systems</td>
<td></td>
<td>WB</td>
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<tr>
<td>Stat3</td>
<td>Rabbit IgG</td>
<td>4904</td>
<td>Cell Signaling</td>
<td></td>
<td>WB</td>
</tr>
<tr>
<td>pStat3</td>
<td>Rabbit IgG</td>
<td>9145</td>
<td>Cell Signaling</td>
<td></td>
<td>WB, IHC</td>
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<tr>
<td>C/EBPβ (LAP)</td>
<td>Rabbit IgG</td>
<td>sc-150</td>
<td>Santa Cruz</td>
<td></td>
<td>WB</td>
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<tr>
<td>pC/EBPβ (LAP)</td>
<td>Rabbit IgG</td>
<td>3084</td>
<td>Cell Signaling</td>
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<td>WB</td>
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<td>NF-κB p65</td>
<td>Rabbit IgG</td>
<td>4764</td>
<td>Cell Signaling</td>
<td></td>
<td>WB</td>
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<tr>
<td>pNF-κB p65</td>
<td>Rabbit IgG</td>
<td>3033</td>
<td>Cell Signaling</td>
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<td>Lcn2</td>
<td>Rat IgG</td>
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<td>Enzo</td>
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<td>IHC</td>
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<td>GAPDH</td>
<td>Rabbit IgG</td>
<td>2118</td>
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<td>WB</td>
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<tr>
<td>anti goat</td>
<td>HRP</td>
<td>Goat IgG</td>
<td>P 0449</td>
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<td>anti rabbit</td>
<td>HRP</td>
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<td>IHC</td>
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<td>anti rat</td>
<td>HRP</td>
<td>Rat IgG</td>
<td>MP-7444</td>
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<td>IHC</td>
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HRP, horseradish peroxidase.

Table S4. Primer pairs used for SYBRgreen and TaqMan quantitative real-time PCR.

<table>
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<tr>
<th>Gene Symbol (Accession No)</th>
<th>Forward Primer (5′→3′)</th>
<th>TaqMan Probe (FAM-5′→3′-BHQ1 (1))</th>
<th>Reverse Primer (5′→3′)</th>
</tr>
</thead>
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<tr>
<td>Mm IL-1b (NM_008361)</td>
<td>GCAACTTTTCCTGAACCCAAC</td>
<td>ATCTTTGGGGGCCTCAACT</td>
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<td>Mm IL-6 (NM_03116)</td>
<td>TGTTCCTCTGGAAAATCCTGGA</td>
<td>ATGAGAAAGAAGTTGTCGCTG</td>
<td>AAGTGTCATTGTTTCTCATCA</td>
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<tr>
<td>Mm IFNγ (NM_008337)</td>
<td>AGGCCAAAAGAAGTGCATTCC</td>
<td>TTGGCAACCTGAGTCACACAA</td>
<td>TGAATGCTGGCCCTGG</td>
</tr>
<tr>
<td>Mm IL-17A (NM_010552)</td>
<td>TTTAATCTCCCTGGGCAA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mm IL-22 (NM_016971)</td>
<td>TGTCGGGCTCATCAGGGAGA</td>
<td>ACGAGCGATGCTCATCC</td>
<td></td>
</tr>
<tr>
<td>Mm defensin, alpha 1 (NM_010031)</td>
<td>AAGAGACTAAACTGAGGACGAC</td>
<td>GACACGCAGGCGGCTGA</td>
<td></td>
</tr>
<tr>
<td>Mm defensin, beta 1 (NM_007843)</td>
<td>TCTGGTGTAGATATGTTTTCTT</td>
<td>TGTCTTGCTCAAGACTTGTA</td>
<td></td>
</tr>
<tr>
<td>Mm Camp* (NM_009291)</td>
<td>GCTGATTCCTGCATACACTGTA</td>
<td>GGCCAGCCGGGAAGTTTCTT</td>
<td></td>
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<tr>
<td>Mm Reg3g** (NM_011260)</td>
<td>CCGTCGCTTATGCTCTATTTG</td>
<td>GACAGACACAAAGATGTCCTG</td>
<td></td>
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<tr>
<td>Alistipes f. (Roager et al., 2014)</td>
<td>GTACTAATCCCACAATACCTCAG</td>
<td>CTATACCAAGCCATGCCCCCATCTT</td>
<td></td>
</tr>
<tr>
<td>16S (ribosomal DNA)</td>
<td>ACTCTACGGAGGCCAGCAGT</td>
<td>GTATTACC GGCTGGC</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: Mm, Mus musculus; (1) BHQ1, black hole quencher; *Camp, cathelicidin antimicrobial peptide; **Reg3g, regenerating islet-derived 3 gamma
III. SUPPLEMENTARY EXPERIMENTAL PROCEDURES

Animal husbandry

Il10+/− mice and Il6−/− mice, both on a C57BL/6J background, were purchased from Jackson Laboratories. Lcn2−/− mice were kindly provided by S. Akira (University of Osaka, Osaka, Japan), and backcrossed to a C57BL/6J background for at least 10 generations (Flo et al., 2004). In order to generate double-deficient animals, we intercrossed Lcn2−/− and Il10+/− animals to receive Lcn2−/+ / Il10+/− animals. The entire cohort consisting of Wt, Lcn2+/−, Il10+/−, and Lcn2−/+ / Il10−/− was generated by intercrossing heterozygous litters for at least 5 generations. In other experiments breeding pairs were set up to achieve single–deficient Il6−/−, double–deficient (Il6−/−/Lcn2−/+ , Il6−/−/Il10−/−) or triple–deficient (Il6−/−/Lcn2−/+ / Il10−/−) litters. All mice were bred and maintained under specific pathogen-free conditions, under a strict 12 hours light–dark cycle, with ad libitum access to standard chow and water at the central laboratory animal facility of the Medical University of Innsbruck. Mice were screened for the presence of Helicobacter spp. and tested negative for Helicobacter bilis, Helicobacter muridarum, Helicobacter typhlonius, and positive for Helicobacter rodentium. qPCR for Helicobacter hepaticus was negative. Further, mice tested serologically negative for murine norovirus. All experiments were approved by the Austrian Ministry of Science and Research (66011/75/5/3b/2012 to A.R.M.) and supervised by the local veterinarian authorities.

Cross–fostering

Newborn litters from Il10−/− and Lcn2−/− / Il10−/− mice were split and swapped at a 1–to–1 ratio between respective mothers within 24 hours of birth (outlined in Figure 5B). Accordingly, each mother raised 50% of her own and 50% of fostered pups. After 3 weeks, the offspring was weaned gender–specifically and in a 1–to–1 ratio according to genotype. Mice were analyzed at 12 weeks of age.

Co–housing

4–week–old, age– and gender–matched Il10−/− and Lcn2−/− / Il10−/− mice were weaned at a 1–to–1 ratio into new cages and co–housed for 8 weeks (see Figure S2B). No more than 4 mice per cage were co–housed. Mice were analyzed at 12 weeks of age.

Antibiotic treatment

4–week–old mice were treated with a combination of ciprofloxacin (0.2 g/l, Sigma Aldrich) and metronidazole (1 g/l Sigma Aldrich) prepared with Kool-Aid (2 g/l, Kraft Foods Group, Northfield, IL) in sterile drinking water. Antibiotics were changed three times per week for in total 8 weeks (see Figure 5B). Mice were analyzed at 12 weeks of age.

Association and tracking studies upon oral application of Alistipes finegoldii

Alistipes finegoldii (46020T) and Alistipes putredinis (45780T) were obtained from the CCUG (Culture Collection, University of Göteborg, Sweden). All strains were propagated on Columbia agar with 5% sheep blood under strictly anaerobic conditions. using GENbag or GENbox jar in combination with GENbox anaer atmospheric generators and anaerobic indicators (bioMérieux, Lyon, France) for 48 hours. For in vitro experiments, Alistipes finegoldii were grown anaerobically in thiglycollate medium (TG, Fluka). All working steps were performed in an anaerobic Glove Box Type 90400. Alistipes were exposed to increasing concentrations of siderophore–free recombinant murine Lcn2 (R&D Systems, Minneapolis, MN) and then plated on Columbia agar. Lysozyme (Sigma–Aldrich) served as a positive control. In other experiments, bacteria were grown in the presence or absence of defereroxamine mesylate (DFO), iron(III) ammonium sulfate (both Sigma–Aldrich), iron–free enterobactin (ENT, iron free) or iron–laden Enterobactin (Fe-ENT, both EMC Microcollections) at the indicated concentrations and combined with or without recombinant Lcn2 prior to plating on Columbia agar. For in vivo experiments, 6–week–old Wt, Lcn2−−, and Il10−− mice were gavaged once with 3 × 10⁷ colony–forming units (CFU) of Alistipes finegoldii in 200µl PBS. Control mice received PBS only. Mice were sacrificed after 1 week and histology was assessed. In other experiments, 8–week–old Il10−− mice were gavaged with 3 × 10⁷ CFU of Alistipes and sacrificed after 3 days. Intestines were removed and ceca, proximal, middle, and distal colons processed per segment before proceeding to FISH analysis. To study tumor development, 4–week-old Wt, Lcn2−−, and Il10−− mice were inoculated with 3 × 10⁵ CFU of Alistipes finegoldii once a week for a total of 8 weeks. Inflammation and development of polyps were evaluated macroscopically and confirmed by histological examination. For Alistipes tracking experiments, bacteria were grown for 48 hours in the presence of 5-ethynyl-2′-deoxyuridine (EdU, Thermo Fisher Scientific). Cells were spun down and adjusted in PBS. 6–week–old animals were gavaged with EdU–labeled cells. Stool pellets were collected the baseline and on 3 consecutive days starting after 24 hours. Mice were sacrificed after 72 hours and ceca and proximal colons were preserved in Carnoy’s fixative. Ceca and colon were processed and stained with a Chick-iT® EdU Alexa Fluor® 594 Imaging Kit (Thermo Fisher) according to manufacturer’s instructions for Alistipes detection. 200µm × 200µm images were acquired on a Zeiss Axioobserver.
Z1 in combination with a LSM700 confocal laser scanning system. Number of Alistipes were counted separately for a 150µm and a 75µm area from the epithelial edge. Furthermore Alistipes in direct contact with the epithelium were determined and the mucus area in µm² was measured in ImageJ.

**Flow cytometry**

EdU-positive Alistipes in fecal pellets were quantified by flow cytometry. Briefly, 100mg of stool was processed using PreCellys CK14 tubes containing staining buffer (1% BSA in PBS) in combination with a Precellys®24 homogenizer (both Peqlab, Erlangen, Germany). Samples were then centrifuged at 0.8×g for 15 min at 4°C and 200µl of supernatants passed through 20µm cell strainers prior to pelleting cells at 8000×g for 5 min. Pellets were processed using a Click-iT® EdU Flow Cytometry Assay Kit AF647 (Thermo Fisher) according to manufacturer’s instructions and acquired on a BD FACSVerse equipped with a volumetric flow sensor (Becton Dickinson).

**Detection of siderophores**

For detection of siderophores, thioglycollate medium with or without Alistipes finegoldii was incubated anaerobically for 72 hours, centrifuged and supernatants stored at -80°C. Presence of siderophores was assayed using a SideroTec Assay™ (Emergen Bio, Maynooth, Ireland) according to the manufacturer’s instructions.

**Bone marrow chimeras**

Four to five-week-old Il10−/− and Lcn2−/−/Il10−/− mice were irradiated with 10 Gray using a linear accelerator with an iodium-192 radioactive source. After 4–6 hours mice were reconstituted intravenously with 2 × 10⁶ bone marrow cells derived from Il10−/− or Lcn2−/−/Il10−/− mice. Post irradiation, mice received 10mg/L neomycin sulfate prophylactically via the drinking water, ad libitum, for 2 weeks. Chimeric mice (Il10−/− with Il10−/− or Lcn2−/−/Il10−/− bone marrow, or Lcn2−/−/Il10−/− with Il10−/− or Lcn2−/−/Il10−/− bone marrow, respectively) were analyzed 8 weeks thereafter for severity of colitis. For the verification of chimerism, peripheral blood leukocytes were enriched for monocytes (by attachment) and stimulated with 100ng LPS. Supernatants were assayed for the presence of Lcn2 and IL-10 by ELISA (Figure S2A). Colonic chimerism was further verified by immunohistochemistry for Lcn2 on paraffin-embedded tissue sections from 12-week-old mice (Figure S2B).

**In vivo barrier function experiments**

12-week-old Wt, Lcn2−/−, Il10−/−, and Lcn2−/−/Il10−/− mice were gavaged with 0.6 mg/g body weight of an 80 mg/mL solution of FITC dextran (Sigma Aldrich, St. Louis, MO). Serum was prepared 4 hours later. A standard curve was prepared using serial dilutions of dextran in mouse serum. Fluorescence emission was measured on a PHERAstar plus microplate reader (BMG Labtech, Ortenberg, Germany) at an excitation of 485 nm and an emission of 521 nm.

**Histological procedures and scoring**

Colon, including cecum and rectum, were removed, flushed with cold PBS and immediately fixed in 10% buffered formalin. Fixed tissues were further transferred into an automatic tissue processor (Leica, Wetzlar, Germany). After sectioning all slides were stained with hematoxylin and eosin (H&E). All slides were scanned on a Panoramic Scan digital slide scanner and investigated using Panoramic Viewer software (both 3DHistech, Budapest, Hungary) in a blinded fashion. Inflammatory activity was assessed using a score previously described by Adolph et al with minor modifications as detailed below (Adolph et al., 2013). Each of four histological criteria (polymorphonuclear infiltrate, mononuclear infiltrate, epithelial injury and epithelial hyperplasia) was determined as absent (0), mild (1), moderate (2), or severe (3). Each parameter was assigned an extent factor reflecting its overall involvement ranging from 1 (< 10%), 2 (10–25%), 3 (25–50%), and 4 (>50%). Dysplasia assessment followed architectural (crypt branching, complex glandular crowding, intraluminal papillary tufting) and cytological (loss of cell polarity) features.

**Immunohistochemistry, BrdU incorporation**

Four-micrometer sections were deparaffinised, rehydrated and antigens were retrieved by pressure cooking in antigen unmasking solution (Vector Laboratories, Burlingame, CA). Endogenous peroxidase activity was quenched with 3% hydrogen peroxide. Non-specific protein binding sites were blocked using 2.5% normal horse serum. Primary antibodies used for immunohistochemistry are listed in Table S3. Slides were incubated with respective primary antibodies at 4°C overnight. Targets were visualized using an ImmPRESS HRP-conjugated anti–rabbit, anti–rat, or anti–goat polymeric system (Vector Laboratories, Burlingame, CA) together with 3–amino–9–ethylcarbazole (AEC) as a chromogen. Slides were counterstained with hematoxylin QS (Vector Labortories, Burlingame, CA) and mounted aequously.

For proliferation assays, animals were injected with 2.5 mg bromodeoxyuridine (BrdU, Becton Dickinson, Franklin Lakes, NJ) in 500 µL PBS. Colons were harvested after 2 or 24 hours. BrdU-positive nuclei were identified using a BrdU detection kit (Becton Dickinson). Data are expressed as BrdU-positive nuclei per crypt axes normalized to total nuclei per crypt.

**Colon explant cultures**
After cleansing with ice-cold PBS, a 10 mm piece of colon was opened longitudinally, weighed, and cultured in RPMI1640 supplemented with FBS, penicillin, streptomycin and amphotericin B. Supernatants were harvested after 12 hours, centrifuged, aliquoted, and stored at −80°C until further analyses.

Measurement of cytokines with ELISA and multiplex assays

Concentrations of cytokines in culture supernatants were assayed using commercially available ELISA kits (BD Pharmingen). Multiplex assays were performed on a Bio–Plex 200 system using Bio–Plex–Pro Reagents (Bio–Rad, Hercules, CA). For the measurement of fecal Lcn2, 100mg of stool samples from genotype–specifically or co-housed animals was re-suspended in PBS using a Precellys homogenizer. Suspensions were centrifuged at 10,000 × g for 10 minutes and supernatants were analysed for Lcn2 using a Mouse Lipocalin-2/NGAL Quantikine ELISA Kit (R&D Systems). All assays were performed according to manufacturer’s instructions.

Western Blot

Cells or mucosa scrapings were lysed using M-PER mammalian protein extraction reagent supplemented with HALT proteinase and phosphatase inhibitor cocktails (Thermo Fisher Scientific Inc., Rockford, IL). Protein concentrations were determined by Bradford protein assay with BSA standards (Bio-Rad). Lysates were separated under reducing conditions on bis-acrylamide gels using a mini-PROTEAN3 electrophoresis system (Bio-Rad). Proteins were blotted onto Hybond-P PVDF membranes (GE Healthcare Bio-Sciences, Pittsburgh, PA). All the following steps including (i) blocking with 5% dry milk in TPBS, (ii) incubation with primary antibodies (listed in Table S3), (iii) applying anti-rabbit secondary antibodies (Dako, Glostrup, Denmark), and (iv) washing steps were carried out on a SNAP i.d. protein detection system (Merck Millipore, Billerica, MA). Bands were visualized using a chemiluminescent substrate and Amersham Hyperfilms (GE Healthcare Bio-Sciences).

Gene Expression Analysis

Tissues were preserved in RNAlater solution (Ambion, Austin, TX). After homogenization in RLT buffer, lysates were applied to QIAshredder on RNAeasy columns according to the manufacturer’s instructions (Qiagen, Hilden, Germany). One microgram of total RNA was converted to cDNA using a M-MLV kit in combination with random hexamer primers. Real-time PCR was performed using gene-specific primers in combination with SYBR-green chemistry on a Mx3000P instrument. Primer sequences are shown in Table S4. PCR conditions were 95°C for 2 minutes followed by 40 cycles of 95°C for 30 seconds, 60°C for 30 seconds and 72°C for 30 seconds. Data were analysed on Stratagene’s MxPro software using the standard curve method. Glucuronidase beta and hypoxanthin phosphoribosyltransferase served as internal controls. Primer sequences are demonstrated in Table S4.

Whole-Genome Expression Analysis and Bioinformatics

RNA was isolated from proximal (cecum and proximal colon) and distal tissue samples that were harvested from formalin-fixed, paraffin-embedded tissue sections of Lcn2−/−Il10−/− mice using an RNeasy FFPE Kit (Qiagen). RNA integrity number (RIN) as measured by an Agilent 2100 bioanalyzer was ≥ 8 for all samples. Whole genome expression analysis was performed on an Affymetrix GeneTitan platform using Affymetrix® Mouse Gene 2.0 ST whole transcript arrays at IMGM (Munich, Germany). Microarray data were analyzed within “ using Bioconductor packages as described previously (Hackl et al., 2010). The package “oligo” and the robust multiarray average (RMA) method were used for preprocessing, quantile normalization, and probe summarization. Resulting intensities were log2–transformed and differentially expressed genes were identified using the moderated t–test (package “limma”). As distal and proximal samples originated from the same Lcn2−/−Il10−/− colon, a paired analysis was performed. P-values were adjusted for multiple testing based on the false discovery rate according to the Benjamini-Hochberg procedure. Probesets (genes) were filtered for mRNA Refseq annotation according to the MoGene 2.0 ST array annotation provided from Affymetrix and were considered significantly differentially if they showed an adjusted p-value (FDR) < 0.1 and a >1.5-fold change. Heatmaps for gene expression levels (Z-score) were generated using Genesis. Enriched gene ontology terms, pathways, biological and disease processes were identified using the Database for Annotation, Visualization and Integrated Discovery (DAVID), ClueGO, and Ingenuity pathway analysis (IPA).

Bacterial FISH

Stool–containing colonic tissue samples were fixed in modified Carnoy’s fixative (Swidsinski et al., 2007). Tissue sections were deparaffinised and hybridized with a pan-bacterial Eub338 5′–FITC–GCT GCC TCC CGT AGG AGT probe, and species–specific A.fine447 5′–Cy3– GGC TCC TAC ACG TAA AAG CGT, or R.peor982 5′–Cy3– ACG TTA CTG TCC GGT CAA GAG probes. After washing, slides were counterstained and coveredslipped using ProLong® Gold Antifade Reagent including 4′,6–diamidino–2–phenylindole (DAPI, Life Technologies, Grand Island, NY). Samples were viewed and imaged on a Nikon Eclipse up-right science microscope with epifluorescence or on a Zeiss Axiobserver Z1 in combination with a LSM700 confocal laser scanning system. Slides were scored as described previously (Swidsinski et al., 2007). Briefly, three 1000× picture captures for each FISH probe were taken from the lumen, the outer mucus layer, and the inner mucus layer in close proximity to the epithelium. Leukocytes (in DAPI) were counted in a 50µm2 microscopic field representative for the region of interest. Bacteria were counted separately for all three regions. Bacterial numbers were based on the approximation, that a 10µl solution containing...
10^7 bacteria per ml is equal to 40 bacteria per microscopic field at a magnification of 1000× (Amann et al., 1995). Where single bacterial cells were morphologically not definable, represented by a confluent, homogenous “fluorescence band”, the concentration was assigned a value of > 10^12 per ml. If single bacterial cells were not distinguishable within the bacterial mass but areas with “empty gaps” were present, the bacterial concentration was assigned a value of > 10^11 per mL. In all other cases single bacteria were counted individually. Mucus integrity was evaluated on a transversal section of the colon and the proportion of altered or damaged mucus structure was quantified as percent of the investigated area. Crypt invasion, as defined by the presence of > 5 bacteria localized within a colonic crypt, was scored as present or absent. Bacteria in direct contact with the underlying epithelium i.e. without segregation by an intact mucus layer were denoted as “attaching bacteria”. All slides were examined by two independent investigators. Only slides with clear and crisp hybridization signals were included into enumeration. Mean values and standard deviations were calculated for bacteria concentrations and measurements of layer (e.g., mucus or interlaced) thickness.

Isolation of bacterial DNA and 16S rRNA sequences analyses
Stool was collected from the cecum and stored at −80°C short-term. Microbial DNA was extracted using a FastDNA™ 2 mL SPIN Kit (MP Biomedicals, Santa Ana, CA) in combination with a Precellys®24 homogenizer (Peqlab, Erlangen, Germany). The V1-V2 region of the bacterial 16S rRNA gene was amplified with primers 27F and 338R, linked with double index (8nt on each of the primers), and then processed on Illumina MiSeq sequencer with MiSeq Kit V3 (300bp paired-end). Sequencing outputs were performed as previously described (Geirnaert et al., 2015). In summary, sequences were first merged with FLASH software (Magoc and Salzberg, 2011), and then filtered according to quality. Chimera removal was carried out using Uchime (Edgar et al., 2011), against recommended databases. A subset of 10,000 sequences per sample each was used for the following analysis. The correction for the input material was based on stool weight for DNA extraction (100mg/ml), amount of input material for PCR amplification (20ng/PCR), and the downsampling to 10,000 reads per sample. Clustering of sequences was performed using Uclust (Edgar, 2010), and a 97% level OTU table was created with Perl scripts. Classification of the sequences was performed using RDP classifier (Wang et al., 2007), for each read, a 80% confident threshold was used and reads with <80% confidence were named as “unclassified” and suffixed with the corresponding family. FastTree was used to create a maximum-likelihood tree (Price et al., 2010). Phylogenetic diversity and weighted Unifrac distances were calculated using implementations in Mothur. Bacterial community comparisons were carried out using the "Vegan" R package. Bray-Curtis dissimilarity, Jaccard dissimilarity, unweighted and weighted Unifrac distances (Lozupone and Knight, 2005) were analyzed using constrained principle component analysis (Anderson, 2003), and analysis of dissimilarity "adonis" in R to test effect genotype and co-housing effects.

Statistical analysis
All results are expressed as means ± standard error. All data were assessed for normality by Kolmogorov-Smirnov or Shapiro-Wilk test. For normally-distributed data differences between groups were determined using one-way ANOVA followed by post-hoc Tukey or Bonferroni corrected for multiple comparison. Accordingly, non-normally distributed data were analyzed by Kruskal–Wallis non-parametric ANOVA and differences between groups calculated by Dunn’s test corrected for multiple comparison. All statistical tests were performed with SPSS statistics 20.0 (IBM, SPSS Inc., NY), and independently confirmed by GraphPad Prism version 6.0 (GraphPad Software, La Jolla, CA).
IV. SUPPLEMENTARY REFERENCES


