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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The manuscript contains 55.342 characters (including spaces).

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 grove 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. *Lcn2*$^{-/-}$ / *Il10*$^{-/-}$ animals exhibited a markedly altered microbial community structure and expansion of certain species particularly *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 *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 *Lcn2*$^{-/-}$ / *Il10*$^{-/-}$ mice translated histologically into adenomatous polyps with low−grade dysplasia (Figure 2A). In *Lcn2*$^{-/-}$/ *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 *Lcn2*$^{-/-}$ / *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 *Lcn2*$^{-/-}$/ *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 *Lcn2*$^{-/-}$/*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 *Lcn2*$^{-/-}$/*Il10*$^{-/-}$ and *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 *Il10*$^{-/-}$ and particularly in *Lcn2*$^{-/-}$/*Il10*$^{-/-}$ mice (Figure 5A) and abrogated tumor formation (Figure S4A).

In line with a critical role of the microbiota, newborn *Il10*$^{-/-}$ mice were cross−fostered (CF) at birth with *Lcn2*$^{-/-}$/*Il10*$^{-/-}$ mothers (Figure 5B) and developed more severe colitis than non−CF littermates (Figure 5C). Conversely, *Lcn2*$^{-/-}$/*Il10*$^{-/-}$ pups CF with *Il10*$^{-/-}$ mothers demonstrated milder colitis than their non−CF littermates (Figure 5C). Notably, *Il10*$^{-/-}$ mice CF with *Lcn2*$^{-/-}$/*Il10*$^{-/-}$ mothers had comparable tumor counts and densities to non−CF *Lcn2*$^{-/-}$/*Il10*$^{-/-}$ mice (Figures 5D to 5F). In contrast, *Lcn2*$^{-/-}$/*Il10*$^{-/-}$ animals CF with *Il10*$^{-/-}$ 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, *Il10*$^{-/-}$ and *Lcn2*$^{-/-}$/*Il10*$^{-/-}$ mice adopted a comparable microbiota when raised by the same mother, which was reflected mainly on 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 *Il10*$^{-/-}$ and *Lcn2*$^{-/-}$ / *Il10*$^{-/-}$ mice age and gender−matched and in a 1−to−1−ratio (Figure S4B). As expected, genotype−specifically housed *Il10*$^{-/-}$ and

Lcn2$^{-/-}$/*Il10*$^{-/-}$ littermates either did or did not develop excessive mucosal inflammation and polyps (Figure S4C to S4E). In contrast, *Il10*$^{-/-}$ mice co-housed with *Lcn2*$^{-/-}$/*Il10*$^{-/-}$ animals developed more severe colitis (Figure S3C). With respect to tumor formation, co-housed *Il10*$^{-/-}$ and *Lcn2*$^{-/-}$/*Il10*$^{-/-}$ animals developed comparable tumor numbers, yet significantly higher than observed in genotype-specifically housed *Il10*$^{-/-}$ and significantly lower than in genotype-specifically housed *Lcn2*$^{-/-}$/*Il10*$^{-/-}$ littermates (Figure S4D). Differences in tumor numbers were not reflected in tumor areas which were comparable to genotype-specifically housed *Lcn2*$^{-/-}$/*Il10*$^{-/-}$ littermates (Figure S4E). 16S sequencing data from co-housed animals revealed that co-housed *Il10*$^{-/-}$ mice acquired a microbiota highly similar to that of their *Lcn2*$^{-/-}$/*Il10*$^{-/-}$ "cagemates" (Adonis testing showed no significant differences between *Lcn2*$^{-/-}$/*Il10*$^{-/-}$ single-housed and any of the co-housed *Il10*$^{-/-}$ and *Lcn2*$^{-/-}$/*Il10*$^{-/-}$ animals) (Figures S4F and S4G).

To test for a potential impact of coprophagy-delivered *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 *Il10*$^{-/-}$ mice did not result in a relevant increase in stool Lcn2 in *Lcn2*$^{-/-}$/*Il10*$^{-/-}$ mice relativizing 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 *Il10*$^{-/-}$ and Lcn2$^{-/-}$/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 3×10^5 CFU 5-ethynyl-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 3×10^5 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 × 10^5 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 peformed 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: 5.7×10^{-3} to 3.5×10^{-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

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) (Flo et al., 2004). Mice were intercrossed to generate *Wt*, *Lcn2*$^{-/-}$, *Il10*$^{-/-}$, and *Lcn2*$^{-/-}$ / *Il10*$^{-/-}$ and moreover *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. 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 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 studies upon oral application of Alistipes finegoldii or putredinis and Robinsoniella peoriensis

Alistipes finegoldii (46020T) and Alistipes putredinis (45780T) were obtained from the CCUG (Culture Collection, University of Göteborg, Sweden) and propergated 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 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 QIAshreedder 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 ANOVA 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

Conceptualization, A.R.M., R.R.G. and H.T.; Methodology, A.R.M. and R.R.G.; Investigation, A.R.M., R.R.G., V.K., A.P., J.W. and J.S.; Formal Analysis, J.W., S.R. and H.H.; Writing – Original Draft, A.R.M. and R.R.G.; Writing – Review & Editing, A.R.M., R.R.G., T.E.A., S.L.K., J.W., J.F.B., A.K., H.T.; Funding Acquisition, H.T., A.K. and A.R.M.; Resources, P.L.M., A.S., D.O-H., Supervision, H.T.

ACKNOWLEDGEMENTS

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).

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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.

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.

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 *Il6*$^{-/-}$/*Lcn2*$^{-/-}$/*Il10*$^{-/-}$ triple knockout mice (pink squares). Each datapoint represents an individual animal.

(C) Representative H&E−stained colon sections of indicated genotypes (scale bars, 250µm for *Tko*, and 100µm for the remaining genotypes).

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

(D and E) Comparison of tumor numbers and areas between *Il10*$^{-/-}$ (light blue triangles), *Il6*$^{-/-}$ / *Il10*$^{-/-}$ (blue triangles), *Dko* (dark blue squares), and *Il6*$^{-/-}$ / *Lcn2*$^{-/-}$ / *Il10*$^{-/-}$ 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 *Lcn2*$^{-/-}$ / *Il10*$^{-/-}$ (*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), *Lcn2*$^{-/-}$ (dark grey), *Il10*$^{-/-}$ (light blue), and *Lcn2*$^{-/-}$ / *Il10*$^{-/-}$ (*Dko*, dark blue). Indicated significances refer to *Il10*$^{-/-}$ 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.

*, 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*$^{-/-}$, *Il10*$^{-/-}$, and *Lcn2*$^{-/-}$ / *Il10*$^{-/-}$ (*Dko*) animals. Each data point represents an individual animal.

(B) Experimental outline of cross−foster experiments.

(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.

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*$^{-/-}$ / *Il10*$^{-/-}$ 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*$^{-/-}$/*Il10*$^{-/-}$ 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*$^{-/-}$ and *Dko* mice cross−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^{3+}), 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*$^{-/-}$ and *Lcn2*$^{-/-}$/*Il10*$^{-/-}$ (*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).

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 3×10^5 *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*$^{-/-}$ 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.