NLRP6 protects *IL10*−/− mice from colitis by limiting colonization of *Akkermansia* muciniphila

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Summary

Dysfunction in host immune responses and pathologic alterations in the gut microbiota, referred to as dysbiosis, can both contribute to the development of inflammatory bowel disease (IBD). However, it remains unclear how specific changes in host immunity or the microbiota cause disease. We previously demonstrated that the loss of the innate immune receptor NLRP6 in mice resulted in impaired production of IL18 and increased susceptibility to epithelial-induced injury. Here, we show that NLRP6 is important for suppressing the development of spontaneous colitis in the *IL10*−/− mice model of IBD and that NLRP6-deficiency results in the enrichment of *Akkermansia muciniphila. A. muciniphila* was sufficient for promoting intestinal inflammation in both specific-pathogen free and germfree *IL10*−/− mice. Our results demonstrate that *A. muciniphila* can act as a pathobiont to promote colitis in a genetically-susceptible host and that NLRP6 is a key regulator of its abundance.

In Brief

NLRP6 is important for maintaining intestinal homeostasis. Seregin et. al. demonstrate that NLRP6 limits the colonization of mucolytic *A. muciniphila*, which is sufficient to induce colitis in specific pathogen free and germfree *IL10*−/− mice. Resistance to *A. muciniphila* colonization by NLRP6 is mediated by IL18.

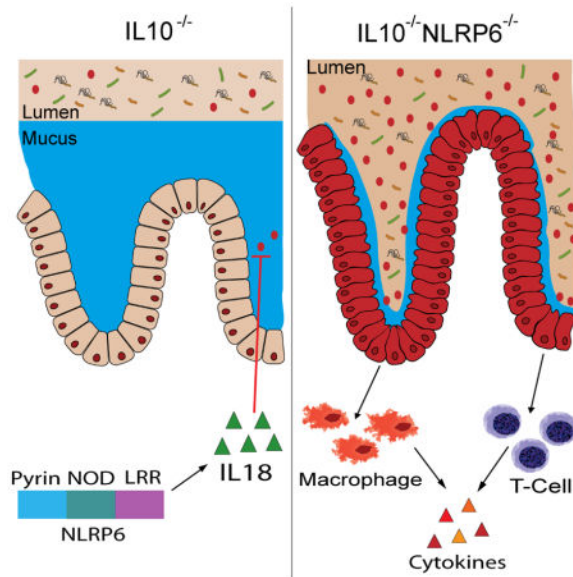[#]Lead contact: Grace Y. Chen (gchenry@umich.edu).

Introduction

Inflammatory bowel disease (IBD) is a significant health problem that afflicts approximately 0.5% of the general population in the Western world (Kaplan, 2015). The pathogenesis of IBD is not fully understood; however, host genetic factors, environmental exposures, and aberrant changes in the composition of the microbiota have all been implicated (Zhang and Li, 2014). In particular, the microbiome of IBD patients has been demonstrated to be of lower diversity than that of healthy controls (Joossens et al., 2011) with increased abundance of certain bacterial populations, such as *Enterobacteriaceae,* and increased mucosal adherence of bacteria (Johansson et al., 2008; Knights et al., 2014). In addition, defects in the immune system such as in the Nod-like receptor (NLR) family of pattern recognition receptors that are involved in the sensing of microbial and damage signals have been associated with IBD in humans and in mouse models of colitis (Rubino et al., 2012). In addition to their influence on inflammatory responses, the loss of function of these receptors has also resulted in changes in the composition of the microbiota, which in turn, may affect colitis susceptibility (Couturier-Maillard et al., 2013; Hirota et al., 2011).

NLRP6 is a member of the NLR family and has been presumed to function as part of an inflammasome based on its ability to interact with the adaptor protein ASC in overexpression assays and on the decreased levels of caspase-1 activation observed in the colon tissue of $Nlrp6^{-/-}$ mice (Grenier et al., 2002; Levy et al., 2015). Caspase-1 activation results in the cleavage of pro-IL1β and pro-IL18 to their mature forms (Netea et al., 2015), and consistently, NLRP6-deficient mice have impaired production of IL18 (Chen et al., 2011; Elinav et al., 2011; Levy et al., 2015). Furthermore, NLRP6 has been shown to protect mice against chemically-induced epithelial injury with dextran sulfate sodium (DSS) (Chen et al., 2011; Elinav et al., 2011; Normand et al., 2011; Seregin et al., 2016). The mechanisms involved in NLRP6-mediated protection remain to be fully elucidated, but have been related to a role in promoting the production of mature IL18, which is important for epithelial repair

and in regulating the composition of the microbiota (Chen et al., 2011; Elinav et al., 2011; Levy et al., 2015). More specifically, metabolites regulated by a "healthy" microbiome have been shown to signal through NLRP6 to modulate the production of IL18 and production of antimicrobial peptides (AMPs) that are important for epithelial barrier function, resistance to DSS-induced injury, and prevention of dysbiosis (Levy et al., 2015). NLRP6 deficiency was also previously demonstrated to be associated with an increased abundance of potentially colitogenic bacteria, namely, *Prevotella* (Elinav et al., 2011), although it has been unclear whether the enrichment of these bacterial populations are truly NLRP6-dependent and capable of precipitating or potentiating colitis.

Studies to date that identify a role for NLRP6 function in the pathogenesis of IBD have relied on the widely-used DSS model of colitis, and is an appropriate model to examine host responses during epithelial injury and repair (Brown et al., 2007), but may not necessarily encompass all aspects of IBD pathogenesis. Here, we identify a critical role for NLRP6 in protecting mice against the development of colitis using the IL10 knockout (KO) model. IL10 is an anti-inflammatory cytokine and polymorphisms in both IL10 and IL10R have been associated with IBD (Kaser et al., 2010). $IL10^{-/-}$ mice develop chronic enterocolitis that is T cell dependent and characterized by upregulated pathologic Th1 responses (Davidson et al., 1996). The development of spontaneous colitis, however, is highly dependent on the composition of the gut microbiota since the severity of disease can differ between mouse facilities (Keubler et al., 2015), and germfree (GF) $IL10^{-/-}$ mice do not develop inflammation (Sellon et al., 1998). Thus, $IL10^{-/-}$ mice have been considered an alternative, and perhaps preferred model, to the DSS-induced epithelial injury and colitis model for studying IBD. We demonstrate that specific pathogen free (SPF) $IL10^{-/-}Nlrp6^{-/-}$ mice develop spontaneous colitis with increased inflammation that does not occur in $IL10^{-/-}$ mice maintained at our facility. Moreover, $IL10^{-/-}Nlrp6^{-/-}$ mice harbored an altered microbiota with increased colonization of the mucin-degrader, *Akkermansia muciniphila*, that is not easily transferred to co-housed $IL10^{-/-}$ mice, resulting in lack of transmission of colitis to co-housed $IL10^{-/-}$ mice. Conventionalization of GF $Nlrp6^{-/-}$ also resulted in increased abundance of *A. muciniphila* compared to that in conventionized GF wildtype (WT) mice. Enforced colonization of *A. muciniphila* in SPF $IL10^{-/-}$ mice and more importantly, monocolonization of GF $IL10^{-/-}$ mice with *A. muciniphila* were sufficient to induce spontaneous colitis. Our results demonstrate that NLRP6 specifically regulates the colonization of *A. muciniphila* and that *A. muciniphila* can act as a pathobiont to promote colitis in genetically-susceptible mice. Thus, our study highlights the ability for NLRP6 to maintain intestinal homeostasis by limiting the colonization of specific colitogenic bacteria.

Results

Lack of functional NLRP6 makes $IL10^{-/-}$ mice prone to spontaneous colitis

We and others have previously shown that NLRP6 deficiency in mice is associated with protection against DSS-induced epithelial injury (Chen et al., 2011; Couturier-Maillard et al., 2013; Elinav et al., 2011; Seregin et al., 2016). DSS is toxic to the colonic epithelium resulting in epithelial apoptosis and mucosal ulceration that is subsequently followed by bacterial translocation and a commensal-driven inflammatory response (Kiesler et al., 2015).

The DSS colitis model, therefore, has often been considered an injury model rather than a model that truly reflects IBD pathogenesis. To further delineate the role of NLRP6 in modulating intestinal inflammatory responses, we generated mice doubly deficient in NLRP6 and IL10. IL10$^{-/-}$ B6 mice at our facility are resistant to the development of colitis (Nagalingam et al., 2013)(Figure S1A). However, by 8 weeks of age, *IL10$^{-/-}$Nlrp6$^{-/-}$* mice developed spontaneous colitis (Figure 1A). More specifically, there was increased epithelial hyperplasia and transmural inflammation resulting in higher histological scores than WT, *Nlrp6$^{-/-}$*, or *IL10$^{-/-}$* mice (Figure 1A). Flow cytometric analysis confirmed increased immune cell infiltration, in particular CD3$^+$ T cells and CD11b$^+$Ly6c^{hi} inflammatory monocytes, into the colon lamina propria of *IL10$^{-/-}$Nlrp6$^{-/-}$* mice (Figure S1). Moreover, severe rectal prolapse reflective of underlying inflammation occurred predominantly in *IL10$^{-/-}$Nlrp6$^{-/-}$* mice, typically developing at 2–4 months of age (Figure 1B). Consistent with the increased levels of inflammation at 8 weeks of age, *IL10$^{-/-}$Nlrp6$^{-/-}$* had increases in spleen weight and size and higher levels of fecal lipocalin (Lcn-2), a surrogate marker for intestinal damage and inflammation (Sherwood, 2012), as compared to that of *IL10$^{-/-}$* mice, which did not differ significantly from either age- and sex-matched WT and *Nlrp6$^{-/-}$* mice, (Figures 1C and 1D). The increased Lcn-2 levels and histological scores observed with *IL10$^{-/-}$Nlrp6$^{-/-}$* mice were also associated with increased bacterial translocation to the mesenteric lymph nodes (MLNs) (Figure 1E) and increased proinflammatory cytokine production in the colonic tissue (Figure 1F). Thus, NLRP6 is critically important for suppressing colitis in *IL-10$^{-/-}$* mice.

Cohousing of *IL10$^{-/-}$* with *IL10$^{-/-}$Nlrp6$^{-/-}$* mice does not result in transfer of colitis susceptibility

Since the development of colitis in *IL10$^{-/-}$* mice is, in part, facility-dependent likely reflecting differences in microbiota composition, we cohoused *IL10$^{-/-}$* mice with *IL10$^{-/-}$Nlrp6$^{-/-}$* mice at 4 weeks of age and then monitored them for the development of colitis until 3 months of age. Interestingly, despite cohousing, *IL10$^{-/-}$* mice did not develop significant colitis. Histological scores and fecal levels of Lcn-2 were similar to that of *IL10$^{-/-}$* that were not cohoused, in contrast to *IL10$^{-/-}$Nlrp6$^{-/-}$* mice (Figures 2A and 2C). More importantly, *IL10$^{-/-}$Nlrp6$^{-/-}$* mice developed significant colitis with higher histological scores, increased spleen weight, and Lcn-2 levels than cohoused *IL10$^{-/-}$* mice (Figures 2A–C). Finally, levels of IFNγ and IL1β production in the colons of *IL10$^{-/-}$Nlrp6$^{-/-}$* also remained significantly elevated compared to cohoused *IL10$^{-/-}$* mice (Figure 2D). We also evaluated *IL10$^{-/-}$* and *IL10$^{-/-}$Nlrp6$^{-/-}$* littermates, and observed similar increases in spleen weight and inflammation in *IL10$^{-/-}$Nlrp6$^{-/-}$* mice compared to that in *IL10$^{-/-}$* mice (Figure S2A–D).

Given the dependence on the composition of the gut microbiota for the development of colitis in *IL10$^{-/-}$* mice, we next determined if there were differences in bacterial populations between cohoused and non-cohoused *IL10$^{-/-}$* and *IL10$^{-/-}$Nlrp6$^{-/-}$* mice by performing 16S rRNA sequencing of bacterial DNA extracted from the stool of these mice. Despite cohousing, there remained significant differences in overall community structure between *IL10$^{-/-}$* and *IL10$^{-/-}$Nlrp6$^{-/-}$* mice (Figure S2E–F). As observed in IBD patients, the microbiome of *IL10$^{-/-}$Nlrp6$^{-/-}$* mice was also reduced in richness and α-diversity (Figure

3A) (Willing et al., 2010). We used the linear discriminant analysis (LDA) effect size (LEfSe) (Segata et al., 2011) method to identify operational taxonomic units (OTUs) that were the most differentially abundant between $IL10^{-/-}$ and $IL10^{-/-}Nlrp6^{-/-}$ mice. These OTUs, which represent bacterial sequences that are at least 97% identical to each other, also reflected bacterial populations that did not effectively transfer between mice genotypes. Relative to either non-cohoused or cohoused mice, *Bacteroides* (OTU63357), *Prevotella* (OTU63571), *Mucispirillum* (OTU66), *Akkermansia* (OTU2), and *Helicobacter* (OTU7) were more highly abundant in $IL10^{-/-}Nlrp6^{-/-}$ mice in contrast to $IL10^{-/-}$ mice (Figures 3B and 3C). There were also several bacterial populations that were significantly more abundant in $IL10^{-/-}$ mice compared to $IL10^{-/-}Nlrp6^{-/-}$ mice, although these were not differentially abundant in cohoused mice (Figures 3B and 3C). As the gut microbiome is typically maternally transmitted (Stappenbeck and Virgin, 2016) to further rule out the possibility that microbiome differences between $IL10^{-/-}$ and $IL10^{-/-}Nlrp6^{-/-}$ were due to independent breeding of these colonies, we performed similar analyses using $IL10^{-/-}$ and $IL10^{-/-}Nlrp6^{-/-}$ littermates. Based on average θ_{yc} distances, the community structures of $IL10^{-/-}$ and $IL10^{-/-}Nlrp6^{-/-}$ littermates were significantly different (Figure S2G–H). LEfSe analysis between $IL10^{-/-}$ and $IL10^{-/-}Nlrp6^{-/-}$ littermates revealed that *Prevotella* (OTU63571) and *Akkermansia* (OTU2), but not *Helicobacter* (OTU7) were more abundant in $IL10^{-/-}Nlrp6^{-/-}$ mice (Figure S2I). Altogether, these results strongly suggest that $IL10^{-/-}Nlrp6^{-/-}$ mice harbor multiple bacterial populations that are differentially abundant compared to $IL10^{-/-}$ mice despite cohousing or use of littermates, providing a possible explanation for colitis development in $IL10^{-/-}Nlrp6^{-/-}$ mice.

NLRP6 regulates the colonization of *Akkermansia muciniphila*

The above results suggest that NLRP6 deficiency in $IL10^{-/-}$ mice is associated with an altered microbiome that cannot be completely transferred to co-housed mice. However, as only $IL10^{-/-}Nlrp6^{-/-}$ mice develop significant colitis, it is possible that the observed differences in microbial composition are secondary to inflammation. To determine more directly whether NLRP6 regulates the composition of the gut microbiome, we generated germfree (GF) $Nlrp6^{-/-}$ mice and compared the colonization of these mice with GF WT mice conventionalized with the microbiome of WT SPF mice. Stool samples were collected from recolonized GF WT (gWT) and $Nlrp6^{-/-}$ (g$Nlrp6^{-/-}$) mice over a two-week period and microbial communities that established in these mice were analyzed based on 16S rRNA sequences. Both groups of mice received the same donor bedding and fecal material resulting in similar community structures shortly after conventionalization (day 3) (Figure S3A,B). However, after two weeks, the microbiome that established in g$Nlrp6^{-/-}$ mice exhibited less α-diversity and richness compared to that of gWT mice (Figures 4A and 4B). β-Diversity analysis using θ_{yc} also demonstrated that the microbial community structure of g$Nlrp6^{-/-}$ mice was distinct from that of gWT mice (Figure 4C). Consistently, significant differences in the relative abundances of several OTUs were found between gWT and g$Nlrp6^{-/-}$ mice. Based on LEfSe analysis, *Akkermansia* was the most differentially abundant OTU with significantly increased levels in g$Nlrp6^{-/-}$ mice (Figure 4D). The majority of these differentially abundant bacterial populations did not overlap with the OTUs found to be significantly different between cohoused or non-cohoused $IL10^{-/-}$ and $IL10^{-/-}Nlrp6^{-/-}$ mice (Figure 3B and 3C). We then examined the number of OTUs that were

differentially abundant between gWT versus g*Nlrp6*$^{-/-}$ mice, non-cohoused *IL10*$^{-/-}$ versus *IL10*$^{-/-}$*Nlrp6*$^{-/-}$ mice, and cohoused *IL10*$^{-/-}$ versus *IL10*$^{-/-}$*Nlrp6*$^{-/-}$ mice that were associated with an LDA score of 2 or higher by LEfSe analysis (Figure 4E). Only two OTUs were significantly different in relative abundance between all three pairwise comparisons, that being OTU97, which belongs to an unclassified bacterial phylum, and OTU2, *Akkermansia*. Based on the 16S rRNA sequence, OTU2 was identified to be the mucin-degrader *Akkermansia muciniphila* (Figure 4E). Quantitative PCR with primers specific to *A. muciniphila* confirmed the increased relative abundance of this bacterium in g*Nlrp6*$^{-/-}$ compared to gWT as well as in cohoused and non-cohoused *IL10*$^{-/-}$*Nlrp6*$^{-/-}$ mice compared to *IL10*$^{-/-}$ mice (Figures 4F, G and S3F). The increased relative abundance of *A. muciniphila* associated with NLRP6 deficiency was unlikely to be secondary to inflammation since fecal lipocalin, calprotectin, and cytokine levels were not significantly elevated or different between gWT and g*Nlrp6*$^{-/-}$ mice and neither mice developed frank colitis (Figure S3C–E and data not shown). Furthermore, increased levels of *Akkermansia* were also observed in younger 5 week old *IL10*$^{-/-}$*Nlrp6*$^{-/-}$ mice which had low levels of lipocalin compared to that of older mice (Figure S4A,B). Altogether, these results strongly suggest that NLRP6 regulates the composition of the gut microbiota and, in particular, the relative abundance of *A. muciniphila.*

IL18 modulates abundance of *A. muciniphila*

We and others have previously demonstrated that NLRP6 deficiency is associated with impairment in IL18 production (Chen et al., 2011; Elinav et al., 2011; Levy et al., 2015; Seregin et al., 2016). Consistently, we observed reduced IL18 levels in plasma and colons of *IL10*$^{-/-}$*Nlrp6*$^{-/-}$ mice as compared to IL10$^{-/-}$ mice, although this was not associated with reduced levels of caspase-1, caspase-8, or caspase-11 activation (Figure S4C–F), suggesting a different mechanism for the cleavage of pro-IL-18 in this context, which has been previously reported (Netea et al., 2015). In fact, in adult colitic *IL10*$^{-/-}$*Nlrp6*$^{-/-}$ mice, there are increased levels of caspase-1 activation compared to that in *IL-10*$^{-/-}$ mice consistent with increased inflammation and IL-1β production (Figure S4F). To determine if IL18 limits the relative abundance of *A. muciniphila*, we measured levels of *A. muciniphila* colonization in *IL18*$^{-/-}$ and *IL-18R*$^{-/-}$ mice. We observed significantly increased levels of *A. muciniphila* in *IL18*$^{-/-}$, but not *IL1β*$^{-/-}$ or *IL1R*$^{-/-}$ mice (Figure 5A). We next determined whether restoration of IL18 could reduce *A. muciniphila* colonization in SPF *Nlrp6*$^{-/-}$ mice by administering recombinant IL18 (rIL18) or rIL1β over three consecutive days and monitoring the relative abundance of *A. muciniphila* by qPCR. Within a few days after the last rIL18 injection, there was a dramatic reduction in *A. muciniphila* colonization to levels similar to that in SPF WT mice, which did not occur with rIL1β (Figures 5B and S4G). These results indicate that one mechanism by which NLRP6 regulates the colonization of *A. muciniphila* is through the production of IL18.

Increased colonization of *A muciniphila* is sufficient to trigger significant colitis in *IL10*$^{-/-}$ mice

Previous studies suggested that *Nlrp6*$^{-/-}$ mice have increased levels of *Prevotella* and TM7 compared to WT mice (Elinav et al., 2011), although it was not clear whether these observations were made in littermates and NLRP6-dependent. In our gWT and g*Nlrp6*$^{-/-}$

mice recolonized with the microbiota of SPF WT donors, which contained both *Prevotella* and TM7, the relative abundance of *Prevotella* and TM7, as determined by qPCR, was not significantly different (Figure S3G).

We next determined whether the enrichment of *A. muciniphila* that occurred in the absence of NLRP6 signaling contributed to the development of colitis in the context of IL10 deficiency in mice. To address this, we isolated a murine *A. muciniphila* strain and orally gavaged *A. muciniphila* into SPF *IL10$^{-/-}$* mice weekly over 7 weeks to maintain relatively stable colonization levels (~3%) during this period (Figure S5A). As a control for specificity, we gavaged with the same frequency and dose a separate cohort of SPF *IL10$^{-/-}$* mice with *Bacteroides acidifaciens* (Figure S5B) since its relative abundance was similar to that of *A. muciniphila* (~1–10%), but not significantly different between conventionalized GF WT and *Nlrp6$^{-/-}$* mice (Figure S3H). SPF *IL10$^{-/-}$* mice gavaged with *A. muciniphila* lost weight compared to mice gavaged with *B. acidifaciens* (Figure 6A). In addition, *A. muciniphila*-gavaged *IL10$^{-/-}$* mice developed significant colitis based on histological scoring compared to either control *IL10$^{-/-}$* mice or *IL10$^{-/-}$* mice gavaged with *B. acidifaciens* (Figure 6B). Consistently, *A. muciniphila*-gavaged *IL10$^{-/-}$* mice exhibited splenomegaly, increased colon weight/length ratios, dramatically elevated fecal Lcn-2 levels and increased bacterial translocation to the MLNs (Figures 6C–F). Importantly, there was significantly increased production of multiple pro-inflammatory mediators compared to either *IL10$^{-/-}$* mice gavaged with *B. acidifaciens* or *IL10$^{-/-}$* control mice (Figure 6G).

In murine colitis models and in IBD patients, there is penetration of the mucus layer that normally separates bacteria from the colonic mucosa, which may contribute to the development of colitis (Johansson et al., 2014). *A. muciniphila* is a mucin-degrader and, therefore, one possible mechanism by which *A. muciniphila* contributes to colitis development in *IL10$^{-/-}$* mice is by reducing the thickness of the mucus, thereby predisposing mice to bacterial penetration into the intestinal mucosa to induce inflammation. Consistently, greater bacterial translocation to the MLNs was observed in *IL10$^{-/-}$Nlrp6$^{-/-}$* and in *A. muciniphila*-gavaged *IL10$^{-/-}$* mice compared to that in *IL-10$^{-/-}$* mice and *B. acidifaciens*-gavaged *IL-10$^{-/-}$* mice, respectively (Figures 1E and 6F). In addition, *IL10$^{-/-}$Nlrp6$^{-/-}$* and *IL10$^{-/-}$* mice gavaged with *A. muciniphila* had a reduced mucus layer compared to *IL10$^{-/-}$* and *IL10$^{-/-}$* mice gavaged with *B. acidifaciens* based on Alcian blue staining of Methacarn-fixed colon sections (Figures S5C–E). We also observed a thinner mucus layer in *Nlrp6$^{-/-}$* and *IL18$^{-/-}$* mice that have elevated levels *A. muciniphila* (Figure S5E). These differences, however, were not due to a reduction in the number of goblet cells (Figure S5F) or in reduced expression of MUC2, a major component of mucus (Figure S5G). Furthermore, fluorescent *in-situ* hybridization (FISH) revealed the presence of *A. muciniphila* in the inner mucus layer in close proximity to the colon epithelium of *IL10$^{-/-}$* mice gavaged with *A. muciniphila* (Figure S5H). Altogether, these results support the colitogenic properties of *A. muciniphila*, which can promote colitis in SPF *IL10$^{-/-}$* mice when a threshold of colonization has been crossed.

It remained unclear if *A. muciniphila* promotes colitis directly or induces inflammatory responses indirectly by affecting the colonization of other potential colitogenic bacterial populations. We, therefore, monocolonized GF *IL10$^{-/-}$* mice with either *A. muciniphila* or

B. acidifaciens. Both bacterial strains efficiently colonized GF *IL10$^{-/-}$* mice after a single oral gavage and maintained high levels of colonization for the duration of the experiment (Figure 7A). Similar to what was observed with SPF *IL10$^{-/-}$* mice gavaged with *A. muciniphila,* GF *IL10$^{-/-}$* mice colonized with *A. muciniphila* were unable to gain weight unlike GF *IL10$^{-/-}$* mice colonized with *B. acidifaciens* (Figure 7B). *A. muciniphila* colonization also resulted in significantly increased histological scores and fecal Lcn-2 levels, which was associated with increased bacterial translocation consistent with its mucin-degrading properties (Figure 7D–E). Although GF *IL10$^{-/-}$* colonized with *B. acidifaciens* resulted in the upregulation of some proinflammatory cytokines as compared to GF *IL10$^{-/-}$* mice controls, *A. muciniphila*-monocolonized GF *IL10$^{-/-}$* mice induced them to a significantly greater extent and also upregulated additional cytokines including IL-6 and IL12p40 (Figure 7F).

Consistent with the colitogenic properties of *A. muciniphila*, stimulation of bone marrow-derived macrophages (BMDMs) isolated from *IL10$^{-/-}$* mice with heat-killed *A. muciniphila* extracts resulted in significantly increased production of IL-6 and IL-12p40 compared to that with *B. acidifaciens* extracts (Figures S6–7). *A. muciniphila* culture supernatants also upregulated cytokines to a greater extent than *B. acidifaciens*, but to a lesser degree than bacterial extracts (Figure S6A), The immunostimulatory activity of *A. muciniphila* extracts was not affected by either proteinase K or DNase/RNase treatment suggesting that the major immunostimulatory molecule from *A. muciniphila* is not a protein or nucleic acid (Figure S6A). Furthermore, the major immunostimulatory activity contained in *A. muciniphila* extracts required MyD88 and TLR4 signaling (Figure S6B), and therefore, likely to be lipopolysaccharide (LPS). To confirm, we purified the outer membrane (OM) as well as LPS from *A. muciniphila* and *B. acidifaciens* and demonstrated that both the OM and LPS derived from *A. muciniphila* upregulated cytokine production in *IL10$^{-/-}$* BMDMs to a greater extent than that from *B. acidifaciens* (Figure S6C–D). Moreover, although unstimulated CD11b^{+}Ly6G^{-} colonic myeloid cells isolated from the LP of *IL10$^{-/-}$* mice have generally high basal levels of inflammatory cytokines, stimulation with LPS purified from *A. muciniphila* resulted in significantly higher cytokine responses than that from *B. acidifaciens* (Figure S7B). Altogether, these results strongly suggest that *A. muciniphila* that normally colonizes the mouse gut can act as a pathobiont to promote colitis in a genetically-susceptible host.

Discussion

IBD is a devastating chronic disease that remains without a cure. Studies using mouse models of colitis have demonstrated that the pathogenesis of IBD is multifactorial and involves a complex interplay between host genetics, environment factors, including the gut microbiome, and the immune system. The current study further highlights the contributions of all three. We have demonstrated that NLRP6 protects mice deficient in IL10 from the development of colitis. NLRP6 was previously shown to be important for resistance against epithelial damage and subsequent inflammation in a chemically-induced epithelial injury model using DSS. In the DSS model, the onset of inflammation in NLRP6-deficient mice was associated with reduced production of IL18. IL18 deficiency, in turn, has been suggested to impair epithelial restitution after DSS-induced injury, which is consistent with

IL18 being upstream of MyD88 signaling that is important for commensal-induced reparative immune responses (Rakoff-Nahoum et al., 2004; Salcedo et al., 2010). In the current study, using an alternative model of IBD that is not dependent on epithelial injury and repair, we demonstrate that NLRP6 protects against the development of colitis in $IL10^{-/-}$ mice by limiting the colonization of colitogenic *A. muciniphila*.

Previous studies have shown that NLRP6 deficiency is associated with expanded colonization of *Prevotella* and TM7 and antibiotic treatment of $Nlrp6^{-/-}$ mice reduced its relative abundance and ameliorated colitis (Elinav et al., 2011). Cohousing $Nlrp6^{-/-}$ with WT mice resulted in transfer of bacteria and transmission of susceptibility to DSS-induced colitis (Elinav et al., 2011). These studies were the first indication that NLRP6 can regulate the composition of the gut microbiota to resist DSS-induced colitis. However, these studies only established an association between *Prevotella* colonization and NLRP6 deficiency, and it remained unclear whether any differences in microbiome composition were due to colony-dependent, but NLRP6-independent differences. We, therefore, generated GF $Nlrp6^{-/-}$ mice and compared the resulting microbiome composition to that of GF WT mice after conventionalization. Our results demonstrated that despite colonization with the same donor microbiota, the microbiomes that established in gWT and g$Nlrp6^{-/-}$ were distinct, suggesting that NLRP6 does indeed regulate the composition of the gut microbiota. Interestingly, there were no differences in the relative abundance of *Prevotella* or TM7 as previously reported even though the input donor microbiota harbored both bacterial populations, suggesting that NLRP6 does not regulate the abundances of these bacteria (Elinav et al., 2011). A significant increase in members of the family *Porphyromonadaceae* was also reported to be in enriched in $Asc^{-/-}$ mice (Henao-Mejia et al., 2012), and this increase was less clearly related to NLRP6 and colitis susceptibility. In the current study, we see different OTUs belonging to the family of *Porphyromonodaceae* that are either increased or decreased in abundance in our conventionalized germfree $Nlrp6^{-/-}$ mice compared to WT, and none of them were among the most differentially abundant bacterial populations in IL-10 versus IL-10/NLRP6 DKO mice. Thus, it is not clear the relative importance of different *Porphyromonodaceae* strains on colitis. Importantly, we found an expansion of *A. muciniphila* in g$Nlrp6^{-/-}$ mice. *A. muciniphila* was also significantly more abundant in $IL10^{-/-}Nlrp6^{-/-}$ mice compared to $IL10^{-/-}$ mice. Cohousing $IL10^{-/-}Nlrp6^{-/-}$ with $IL10^{-/-}$ mice over several weeks did not result in transfer of *A. muciniphila* or colitis-susceptibility to $IL10^{-/-}$ mice, indicating that lack of disease transmissibility with cohoused mice does not necessarily rule out microbiome contributions.

A. muciniphila is a Gram-negative, strictly anaerobic bacterium belonging to the *Verrucomicrobia* phylum. It is capable of degrading mucin and is also very abundant in the human gut (Derrien et al., 2004). Our study shows that repeated oral gavage of *A. muciniphila* resulted in increased colitis in SPF $IL10^{-/-}$ mice and, furthermore, monocolonization of GF $IL10^{-/-}$ mice resulted in increased inflammation as evidenced by histological evaluation, elevated lipocalin levels and induction of proinflammatory mediators, effectively fulfilling Koch's postulates. A potentially negative role for *A. muciniphila* within the intestine is also supported by studies demonstrating its increased abundance in mice that develop higher tumor burdens in a model of colitis-associated tumorigenesis (Baxter et al., 2014). The presence of *A. muciniphila* also exacerbated

Salmonella-induced colitis and is positively correlated with ulcerative colitis patients with active pouchitis and IBD patients that are treatment-resistant (Ganesh et al., 2013; White et al., 2009; Zella et al., 2011). Other small clinical studies of IBD patients, however, have shown contradictory results (Png et al., 2010; Rajilic-Stojanovic et al., 2013), and therefore studies of larger scale will clearly be needed to confirm the clinical relevance of *A. muciniphila* in IBD.

The mechanism by which *A. muciniphila* promotes colitis remains to be fully elucidated. Given its mucin-degrading properties, we hypothesized that *A. muciniphila* degrades the mucus layer, thereby allowing greater microbial access to the intestinal mucosa and facilitating commensal-driven inflammation in the setting of IL-10 deficiency. Indeed, we observed a thinner mucus layer in SPF *IL10$^{-/-}$* mice orally administered *A. muciniphila* associated with increased total bacterial translocation and the induction of inflammation likely driven by bacteria not limited to *A. muciniphila* in the setting of IL10 deficiency. How other bacterial populations can contribute to colitis in SPF *IL10$^{-/-}$* mice and whether the increased abundance of *A. muciniphila* contributes to the pathogenicity of other bacteria will need to be further investigated. However, our data suggests that *A. muciniphila* can itself be colitogenic since it is sufficient to cause inflammation in monocolonized GF *IL10$^{-/-}$* mice. As compared to *B. acidifaciens*, which was incapable of inducing colitis in GF or SPF *IL10$^{-/-}$* mice, *A. muciniphila* induced significantly higher levels of pro-inflammatory cytokines in the colon, indicating its colitogenic potential. Consistently, *A. muciniphila* extracts can induce robust cytokine production by *IL10$^{-/-}$* BMDMs in contrast to lower levels of cytokine production induced by *B. acidifaciens*. It was previously shown that colitis in *IL-10$^{-/-}$* mice is largely driven by colonic macrophages via MyD88 signaling likely triggered by intestinal bacteria (Hoshi et al., 2012). Our data suggests that *A. muciniphila*, and in particular, its LPS, can induce significantly higher levels of cytokine production in *IL-10$^{-/-}$* BMDMs and in *IL-10$^{-/-}$* CD11b$^+$Ly6G$^-$ colonic myeloid cells within the lamina propria than *B. acidifaciens*. The combination of *A. muciniphila*'s mucin-degrading ability and its highly immunstimulatory LPS activity likely contributes to the development of colitis in GF *IL-10$^{-/-}$* mice that does not occur with *B. acidifaciens*. It is important to note that the colitis in GF *IL-10$^{-/-}$* mice monoassociated with *A. muciniphila* is not as severe as that observed in SPF *IL-10$^{-/-}$* mice gavaged with *A. muciniphila* and suggests that the level of colitogenicity of *A. muciniphila* may be context-dependent. Identifying other bacterial populations that may interact and synergize with *A. muciniphila* to promote colitis in *IL-10$^{-/-}$* mice would be an important future endeavor. Indeed, it has been previously demonstrated that monoassociation of GF *IL-10$^{-/-}$* mice with different bacterial populations resulted in varying degrees of colitis (Kim et al., 2005).

Our data indicate that NLRP6 limits the colonization of *A. muciniphila* in an IL18-dependent manner. Both *IL18$^{-/-}$* and *IL18R$^{-/-}$* mice have increased *A. muciniphila* and furthermore, administration of rIL18 reduced the relative abundance of *A. muciniphila* in *Nlrp6$^{-/-}$* mice. Both *IL18$^{-/-}$* and *IL18R$^{-/-}$* mice also experience increased severity of DSS-induced colitis (Takagi et al., 2003). In addition, our results are consistent with previously published reports of microbiome changes observed in mice deficient in the AIM2 inflammasome. Similar to *Nlrp6$^{-/-}$* mice, *AIM2$^{-/-}$* mice are susceptible to DSS-induced colitis, have impaired IL18 production, and also have increased colonization of *A.*

muciniphila (Hu et al., 2015; Man et al., 2015). In addition, it was recently demonstrated that conventionalized GF *Nlrp6*$^{-/-}$ mice had a microbiome that was different from that of recolonized GF WT mice although it was not determined if recolonized GF *Nlrp6*$^{-/-}$ mice were enriched for any colitogenic bacteria (Levy et al., 2015). Administration of rIL18 also caused additional changes in the microbiome of *Nlpr6*$^{-/-}$ mice that did not occur in WT mice (Levy et al., 2015). Whether NLRP6 regulates the composition of other disease-inducing or protective bacteria through IL18 warrants further investigation. *Nlrp6*$^{-/-}$ mice also had reduced levels of AMP production that were IL18-dependent (Levy et al., 2015). Although AMPs may have selective bactericidal activity (Hooper et al., 2003), it is unclear whether this is the primary mechanism by while NLRP6 regulates *A. muciniphila* abundance. Our study also does not rule out the possibility that NLRP6 affects *A. muciniphila* colonization indirectly such as by influencing the abundance of other bacterial populations.

In summary, our data provide critical insights into how NLRP6 maintains intestinal homeostasis and establish a role for NLRP6 in limiting the colonization of IBD-inducing bacteria, in particular, the mucolytic *A. muciniphila*, which can promote colitis in both SPF and GF *IL10*$^{-/-}$ mice. Resistance to *A. muciniphila* accumulation by NLRP6 signaling is mediated at least in part by IL18. Our findings have significant clinical implications as they illustrate the ability of innate immune and cytokine signaling to affect colonization levels of bacteria that in the right context can have direct impact on colitis susceptibility. As the ligand for NLRP6 remains unknown, future studies to identify upstream regulators and the mechanism by which downstream effectors modulate the gut microbiome and IL18 production would be important for the development of strategies to maintain intestinal health and prevent IBD. The current work provides the necessary framework for these studies.

Experimental Procedures

Animals

SPF WT, *Nlrp6*$^{-/-}$, *IL10*$^{-/-}$, *IL18*$^{-/-}$, *IL18r1*$^{-/-}$, *IL1β*$^{-/-}$, and *IL1R*$^{-/-}$ mice (all B6 background) were bred in-house at the University of Michigan animal facility. *Nlrp6*$^{-/-}$*IL10*$^{-/-}$ mice were generated by crossing *Nlrp6*$^{-/-}$ females with *IL10*$^{-/-}$ males and then intercrossing F1 heterozygotes. Adult male or female 8–14 week-old mice were used except in cohousing experiments in which 4-week-old SPF *IL10*$^{-/-}$ and *IL10*$^{-/-}$ *Nlrp6*$^{-/-}$ mice were cohoused at 1:1 ratio for up to 8 weeks. GF WT, *IL10*$^{-/-}$ and *Nlrp6*$^{-/-}$ mice were housed and bred at the GF mouse facility, and sterility was regularly verified by aerobic and anaerobic cultures, Gram stains and qPCR. GF mice were conventionalized using bedding/feces pooled from several cages of SPF WT mice. gWT or g*Nlrp6*$^{-/-}$ mice were housed in 2–3 separate cages each to control for potential cage effects. Animal studies were conducted under protocols approved by the University of Michigan Committee on the Use and Care of Animals.

Treatment of mice with rIL18

Nlrp6$^{-/-}$ mice were administered 1 μg of sterilely filtered rIL18 (MBL) i.p. in 200 μl PBS for 3 consecutive days.

Assessment of colon inflammation

Colons were flushed free of feces, opened longitudinally, and jelly-rolled for formalin fixation and paraffin embedding. Histological assessment of H&E sections was performed in a blinded fashion by a pathologist (KAE) using a previously described scoring system (Seregin et al., 2016).

Reverse transcription and qPCR

Total RNA was isolated from colon tissue using the Nucleospin RNA kit (Machery-Nagel). cDNA synthesis was performed using iScript (Bio-Rad), and cDNA was used for qPCR using the SYBR Green Master Mix (Applied Biosystems) on the ABI 7900HT (Applied Biosystems). Bacterial DNA was isolated from fecal samples using the MoBio PowerSoil DNA Isolation Kit. Relative abundance of bacterial populations in stool samples was quantified by qPCR and normalized to the universal 16S rRNA gene EUB primers. Primer sequences are shown in Supplementary table I.

ELISA

Lcn-2 levels were measured from the supernatants of feces homogenized in PBS at 100 mg/ml and diluted to a range of 1:500 to 1:5000 using the Lcn-2/NGAL ELISA kit (R&D Systems).

Oral gavage of bacteria

A. muciniphila and *B. acidifaciens* were isolated from WT C57BL/6 mice. Specifically, cecal contents from mice were collected under strictly anaerobic conditions immediately after euthanasia, homogenized in anaerobic modified chopped-meat carbohydrate broth (CMCB) (Hehemann et al., 2012), and plated as serial dilutions onto either brain heart infusion (BHI) agar with 10% horse serum and gentamicin ($200 \ \mu g \ ml^{-1}$) or agar-solidified CMCB with gentamicin. Plates were cultured at 37° C anaerobically (85% N_2, 10% H_2, 5% CO_2) in a Coy anaerobic chamber and several hundred colonies representing various morphologies were picked into 96-well plates containing liquid CMCB with gentamicin. Growth kinetics at 600nm were monitored for three days using a plate handling device connected to an absorbance reader (Biotek, Winooski, VT) as previously described (Martens et al., 2011) and the resulting growth profiles used to distinguish culture wells that were likely to contain different organisms based on unique profiles. Sequencing of V4 of 16S rRNA was used to confirm identity of bacterial species. Prior to gavage, bacterial strains were grown anaerobically at 37° C overnight in CMCB. *IL-10$^{-/-}$* mice were gavaged with *A. muciniphila* or *B. acidifaciens* at a dose 2×10^8 CFU/mouse in 200 μl media.

Isolation of Bacterial DNA and 16S rRNA Sequences Analyses

DNA was isolated from fecal samples and processed as previously described and further detailed in supplemental methods (Kozich et al., 2013; Schloss et al., 2009). FASTQ sequence data can be obtained from the Sequence Read Archive at NCBI (Accession number SRP076233).

Bacterial translocation

Total DNA was extracted from MLNs using the MoBio PowerSoil DNA Isolation Kit and bacterial load quantified by qPCR using the universal 16S rRNA gene primers (EUB) in 20 ng DNA.

Statistical analysis

Statistically significant differences were determined using two-way ANOVA with a Bonferroni post-hoc test (time x genotype, p<0.05) (e.g., weight changes or fecal Lcn-2 time courses), by one-way ANOVA with a Student-Newman-Keuls post-hoc test (p value < 0.05) (e.g., qPCR analysis), or by 2-tailed Student's unpaired t test when only 2 groups are compared (e.g., fecal Lcn-2 levels). Kruskal–Wallis one-way analysis of variance non-parametric test was used for non-continuous variables (histology scoring). Differences in bacterial community structure were analyzed using AMOVA in mothur (Excoffier et al., 1992). Data are shown as mean ± SEM. Statistical analyses were performed using GraphPad Prism6 and mothur software.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- NLRP6 deficiency promotes colitis in IL10$^{-/-}$ mice.

- NLRP6 controls colonization of *A. muciniphila* in an IL18-dependent manner.

- *Akkermansia muciniphila* is sufficient to induce colitis in germfree *IL10*$^{-/-}$ mice.
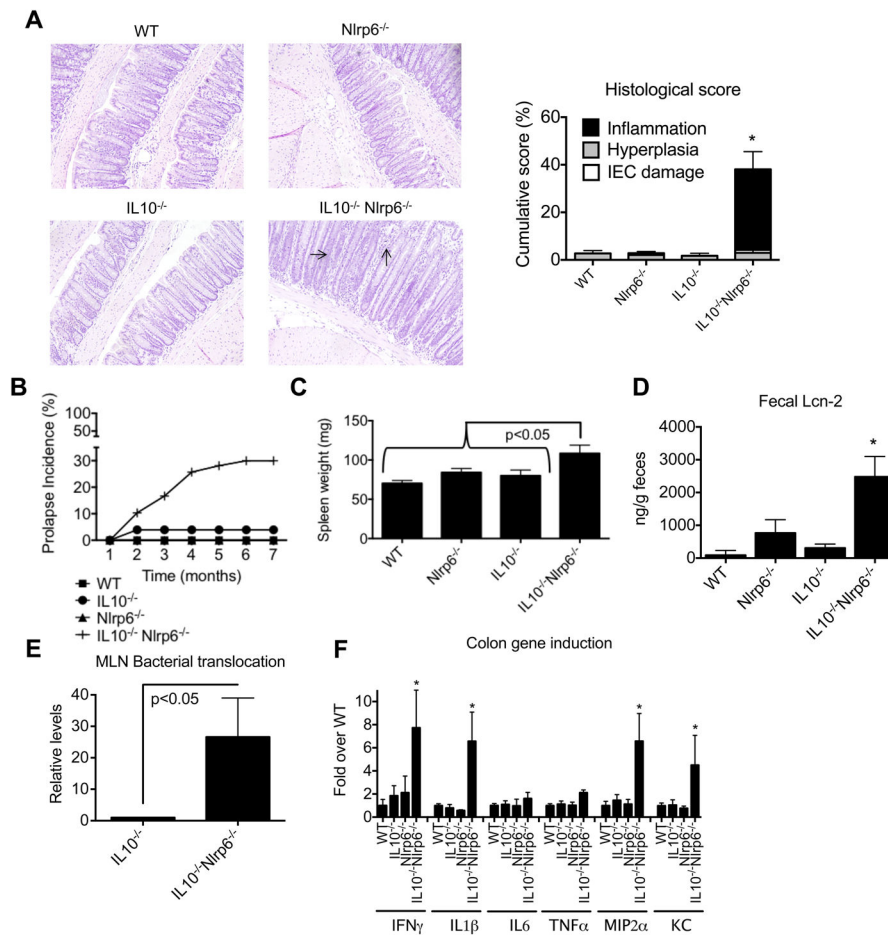
Figure 1. IL10$^{-/-}$Nlrp6$^{-/-}$ mice develop significant spontaneous inflammation in the colon
(**A**) Representative photographs and histological scores of colon sections from 8-week old
WT, *IL10$^{-/-}$, Nlrp6$^{-/-}$* and *IL10$^{-/-}$Nlrp6$^{-/-}$* mice. Inflammation and epithelial hyperplasia
are indicated by arrows. Original magnification = 200x. (**B**) Incidence of rectal prolapse in
WT (n=116), *Nlrp6$^{-/-}$*(n=89), *IL10$^{-/-}$* (n=51), and *IL10$^{-/-}$Nlrp6$^{-/-}$* (n=58). (**C**) Spleen
weights, (**D**) fecal lipocalin-2 levels, (**E**) relative levels of total bacteria/MLN in 14 week old
IL10$^{-/-}$ or *IL10$^{-/-}$Nlrp6$^{-/-}$* mice (n=7 mice/group), and (**F**) mRNA expression of pro-
inflammatory mediators relative to β-actin in the colons of WT (n=13), *IL10$^{-/-}$* (n=11)*,
Nlrp6$^{-/-}$* (n=12), and *IL10$^{-/-}$Nlrp6$^{-/-}$* (n=14) mice. Data are representative of three
independent experiments. * - p<0.05, as compared to all other genotypes. See also Figure
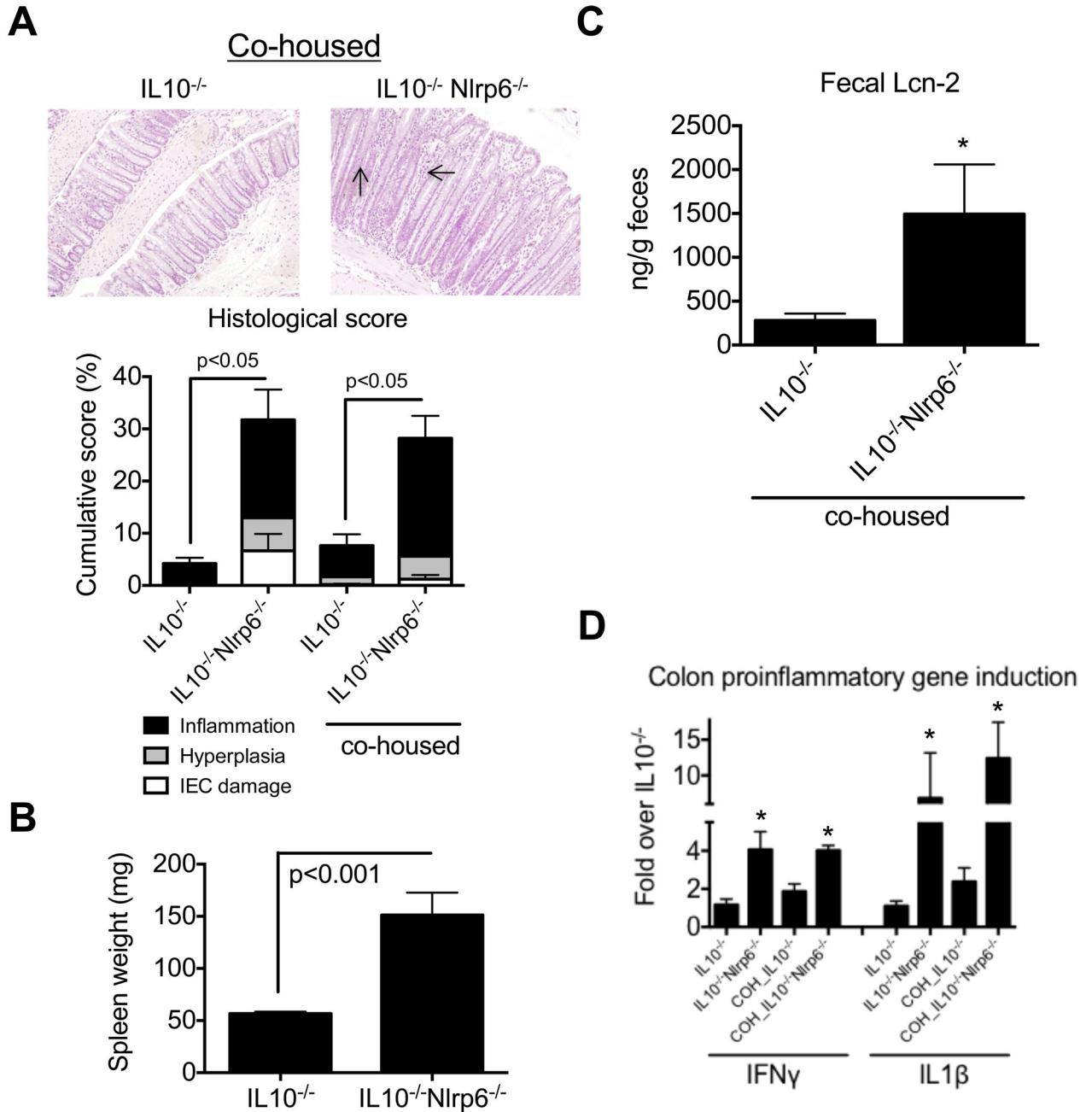S1.

A

Co-housed

IL10$^{-/-}$ IL10$^{-/-}$ Nlrp6$^{-/-}$

Histological score

- ■ Inflammation
- ▨ Hyperplasia
- □ IEC damage

co-housed

B

C

Fecal Lcn-2

co-housed

D

Colon proinflammatory gene induction

Figure 2. Increased colitis susceptibility in *IL10$^{-/-}$Nlrp6$^{-/-}$* mice does not transfer to *IL10$^{-/-}$* mice upon cohousing

(**A**) Representative micrographs and histological scoring of 12 week old *IL10$^{-/-}$* (n=6) and *IL10$^{-/-}$Nlrp6$^{-/-}$* (n=8) mice after 2 months of cohousing. Arrows point to Inflammation and hyperplasia. Original magnification = 200x. (**B**) Spleen weights and (**C**) fecal lipocalin-2 levels from cohoused *IL10$^{-/-}$* and *IL10$^{-/-}$Nlrp6$^{-/-}$* mice. (**D**) Relative mRNA expression of pro-inflammatory mediators in the colon of cohoused (COH) or non-cohoused *IL10$^{-/-}$* and *IL10$^{-/-}$Nlrp6$^{-/-}$* mice (n=8). Data are representative of two independent experiments. * - $p<0.05$ as compared to *IL10$^{-/-}$* mice. See also Figure S2.
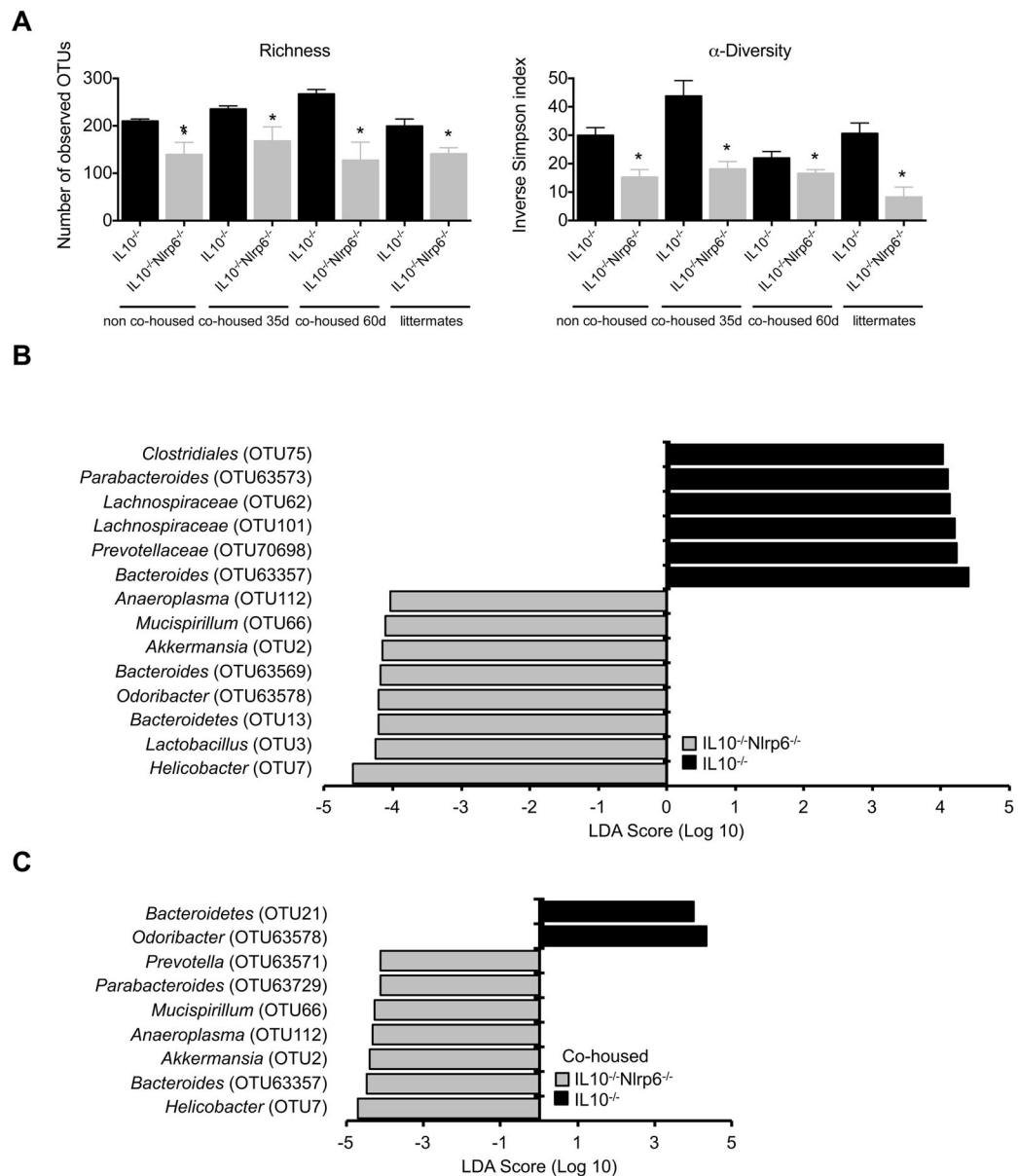
Figure 3. Inflammatory phenotype of *IL10−/−Nlrp6−/−* mice correlates with altered microbiome not corrected by cohousing

(**A**) Inverse Simpson's α-diversity index and observed community richness as measured by number of operational taxonomic units (OTUs), are shown for non-cohoused, cohoused (at 35 and 60 days), and littermate *IL10−/−* and *IL10−/−Nlrp6−/−* mice. LEfSe analysis shows bacteria that were most differentially abundant between (**B**) non-cohoused or (**C**) cohoused *IL10−/−* and *IL10−/−Nlrp6−/−* mice and indicate the effect size of differentially abundant OTUs in the colon. Data are representative of two independent experiments, n=8 for non-cohoused groups, n=6 and n=8 for cohoused *IL10−/−* and *IL10−/−Nlrp6−/−* mice respectively. * - p<0.05 as compared to *IL10−/−* group. See also Figure S2.
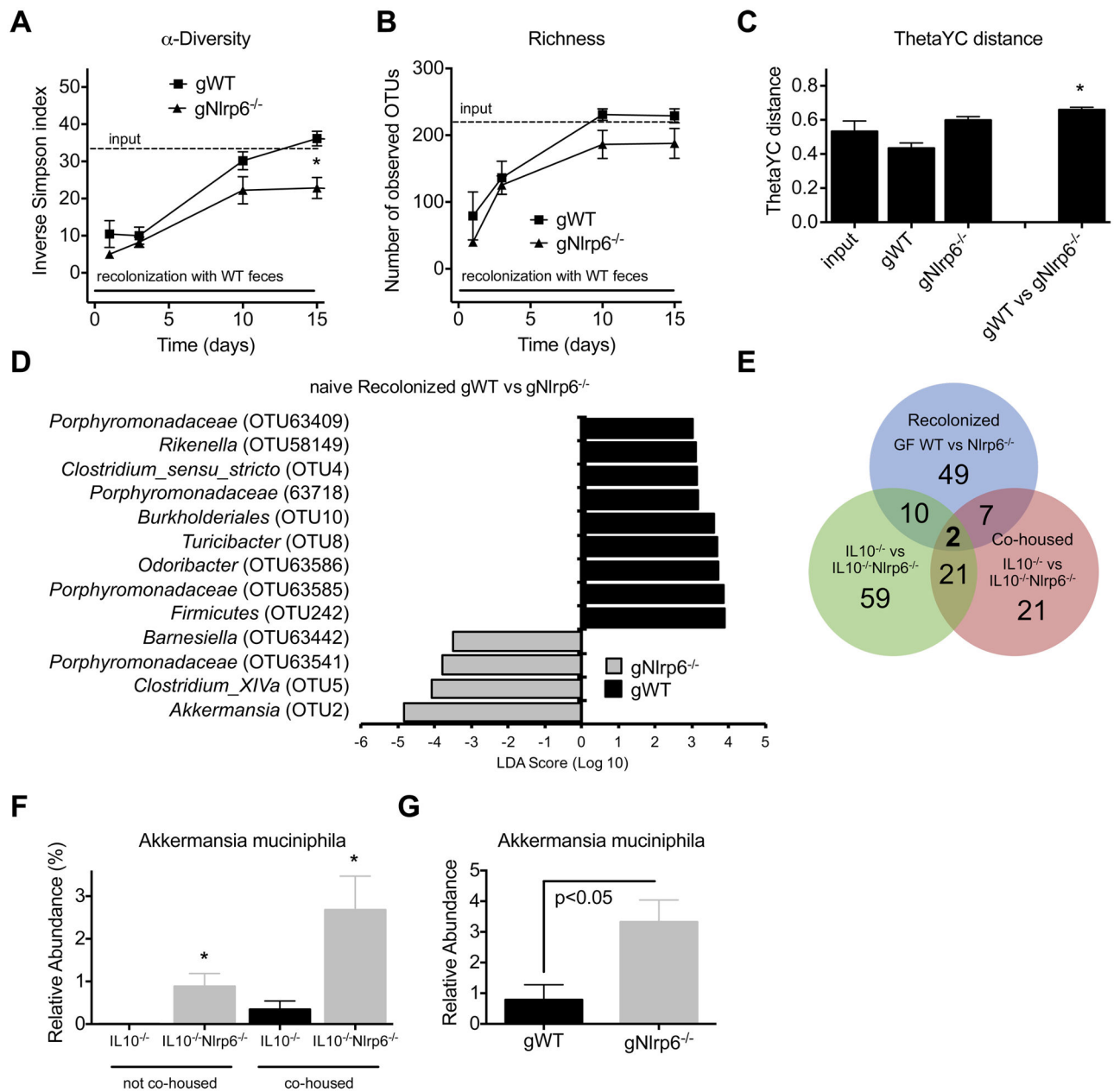
Figure 4. Conventionalized GF *Nlrp6*$^{-/-}$ mice have altered microbiota associated with reduced α-diversity and richness and dramatically increased *A. muciniphila* colonization

(**A**) Time course for inverse Simpson's α-diversity index and (B) observed community richness are shown for *gWT* and *gNlrp6*$^{-/-}$ mice. (C) Average θ$_{yc}$ distance within or between groups of *gWT* and *gNlrp6*$^{-/-}$ mice. (D) LEfSe results show most differentially abundant OTUs. (E) Venn diagram illustrating the number of bacterial OTUs that were differentially abundant (LDA score > 2) between the indicated mice. (**F**) Relative abundance of *A. muciniphila* in the colons of 12-week old non-cohoused or cohoused *IL10*$^{-/-}$ and *IL10*$^{-/-}$*Nlrp6*$^{-/-}$ mice normalized to total bacteria. (**G**) Relative abundance of *A. muciniphila* in colons of *gWT* and *gNlrp6*$^{-/-}$ mice (day 15 after conventionalization); n=8

for non-cohoused groups, n=6 and n=8 for cohoused $IL10^{-/-}$ and $IL10^{-/-}Nlrp6^{-/-}$ mice respectively; n=5, n=8 for gWT and $gNlrp6^{-/-}$ mice respectively. * - p<0.05 as compared to corresponding gWT group (A–C) or to $IL10^{-/-}$ group (F). See also Figure S3.
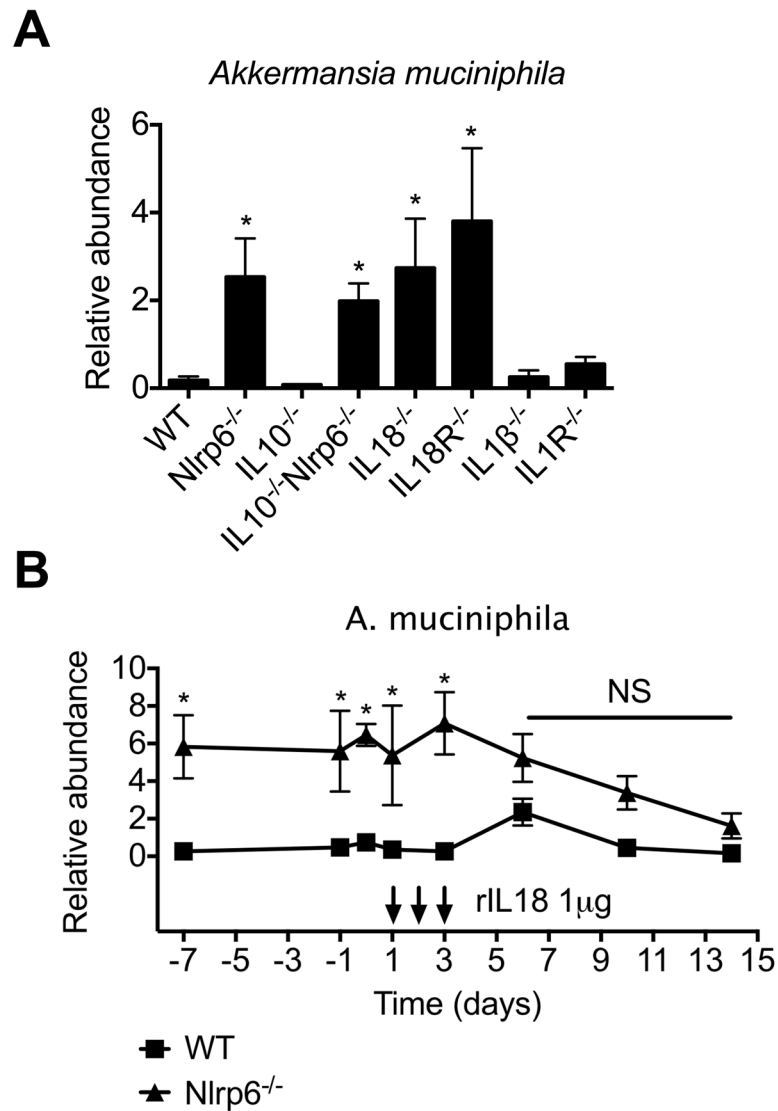
A

Figure 5. rIL18 is sufficient to reduce *A. muciniphila* colonization in *Nlrp6*$^{-/-}$ mice
(**A**) Relative abundance of *A. muciniphila* from fecal samples collected from adult WT, *IL18*$^{-/-}$, *IL18R*$^{-/-}$ (n=8 mice/genotype), *IL1β*$^{-/-}$, *IL1R*$^{-/-}$, *IL10*$^{-/-}$, *Nlrp6*$^{-/-}$ and *IL10*$^{-/-}$*Nlrp6*$^{-/-}$ mice (n=5 mice/genotype) and (**B**) *Nlrp6*$^{-/-}$ mice before and after treatment with rIL-18 (treatment days indicated by arrows). Data are representative of two independent experiments. * - $p<0.05$ compared to WT. See also Figure S4.
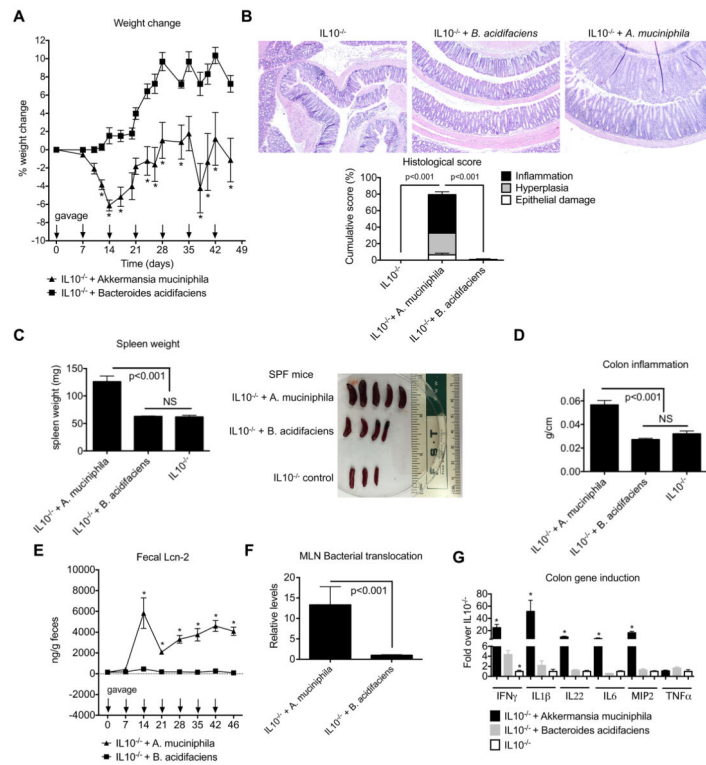
Figure 6. *A. muciniphila* gavaged into SPF *IL10*$^{-/-}$ mice triggers colitis
SPF *IL10*$^{-/-}$ mice were left untreated or gavaged weekly with 2×10^8 CFU/ml of *A. muciniphila* or *B. acidifaciens*. **(A)** Percent weight change of age- and sex-matched groups of mice. **(B)** Representative micrographs and histological scores of colon sections from *IL10*$^{-/-}$ + no gavage, *IL10*$^{-/-}$ + *A. muciniphila* or *IL10*$^{-/-}$ + *B. acidifaciens* mice (200x). **(C)** Spleen weights (left) and size (right), **(D)** colon inflammation index (weight/length), **(E)** fecal lipocalin-2 levels as measured by ELISA, **(F)** levels of total bacteria/MLN and **(G)** relative mRNA expression of various pro-inflammatory mediators in the colons from *IL10*$^{-/-}$ + no gavage, *IL10*$^{-/-}$ + *A. muciniphila* or *IL10*$^{-/-}$ + *B. acidifaciens* mice as determined by qPCR with β-actin used as the housekeeping gene control. Data are representative of three independent experiments, n=11, n=13, n=12 for *IL10*$^{-/-}$ control, *IL10*$^{-/-}$ + *A. muciniphila* or *IL10*$^{-/-}$ + *B. acidifaciens* groups of mice respectively. * - p<0.05 as compared to *IL10*$^{-/-}$ + *B. acidifaciens* group. See also Figure S5.
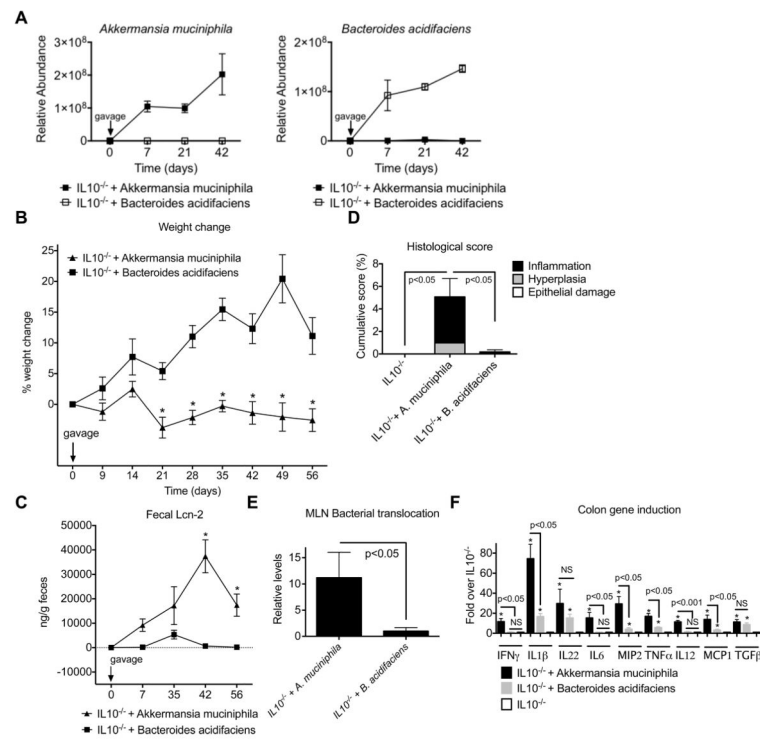
Figure 7. *A. muciniphila* is sufficient to trigger inflammation in GF *IL10*⁻/⁻ mice

(**A**) GF *IL10*⁻/⁻ mice were monocolonized with *A. muciniphila* or *B. acidifaciens* and specific colonization confirmed by qPCR. (**B**) Percent weight change, (**C**) fecal lipocalin-2 levels, (**D**) histological inflammatory scores, and (**E**) normalized levels of total bacteria/MLN in monoassociated GF *IL10*⁻/⁻ mice. (**F**) mRNA expression of various pro-inflammatory mediators relative to β-actin in the colons of the indicated mice. n=11 for GF *IL10*⁻/⁻ + *A. muciniphila* and *GF IL10*⁻/⁻ + *B. acidifaciens* groups, and n=6 for GF *IL10*⁻/⁻ mice. * - p<0.001 compared to GF *IL10*⁻/⁻ + *B. acidifaciens* (B, C) or to GF *IL10*⁻/⁻ (F).