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Host and microbiota interactions are critical for development of murine Crohn's-like ileitis

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Abstract

Deregulation of host-microbiota interactions in the gut is a pivotal characteristic of Crohn's disease. It remains unclear, however, whether commensals and/or the dysbiotic microbiota associated with pathology in humans are causally involved in Crohn's pathogenesis. Here we show that Crohn's-like ileitis in *Tnf*^{ARE+} mice is microbiota-dependent. Germ-free *Tnf*^{ARE+} mice are disease-free and the microbiota and its innate recognition through Myd88 is indispensable for TNF overexpression and disease initiation in this model. The epithelium of diseased mice shows no major defects in mucus barrier and paracellular permeability. However, *Tnf*^{ARE+} ileitis associates with reduction of lysozyme-expressing Paneth cells, mediated by adaptive immune effectors. Furthermore, we show that established but not early ileitis in *Tnf*^{ARE+} mice involves defective expression of antimicrobials and dysbiosis, characterized by *Firmicutes* expansion, including epithelial-attaching Segmented Filamentous Bacteria, and decreased abundance of *Bacteroidetes*. Microbiota modulation by antibiotics treatment at an early disease stage rescues ileitis. Our results suggest that the indigenous microbiota is sufficient to drive TNF overexpression

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and Crohn's ileitis in the genetically susceptible *Tnf ARE/+* hosts, whereas dysbiosis in this model results from disease-associated alterations including loss of lysozyme-expressing Paneth cells.

Keywords

Crohn's disease; ileitis; TNF; microbiota; germ-free; Paneth cell

Introduction

A mutually beneficial relationship has been evolutionarily shaped in the mammalian gut between the host and the complex communities of commensal microorganisms, collectively called as the “microbiota”. Misbalanced host-microbiota interactions are considered to underlie the pathogenesis of Inflammatory Bowel Diseases (IBD), chronic inflammatory disorders of the intestine with two major forms, Crohn's disease and ulcerative colitis.¹ Crohn's disease is manifested primarily in the terminal ileum, a part of the intestinal tract which provides a unique microenvironment for the growth of microbial communities. Contrary to the large bowel, the ileum is characterized by the formation of a single-layered and porous mucus barrier which is penetrant to bacteria.² Antimicrobial peptides secreted by Paneth cells localized at the basal part of the crypts and an extensive network of lymphoid tissues, including Peyer's patches and isolated lymphoid follicles, provide antimicrobial clearance and immunosurveillance.^{3, 4} Furthermore, the composition of mucosa-associated microbial populations in the ileum significantly differs from that of the colon both in healthy humans and in IBD patients,⁵ whereas a series of studies has established that Crohn's disease is associated with dysbiosis.⁶ A recent relevant study showed that treatment-naïve pediatric Crohn's disease associates with enrichment of *Enterobacteriaceae* and depletion of Clades IV and XIVa of *Clostridia* in the mucosa of the terminal ileum.⁷ Apart from these microbial alterations associated with disease, genome-wide association studies (GWAS) have indicated several microbial recognition and host defense genes as associated with both Crohn's disease and ulcerative colitis.⁸ For example, risk alleles in *NOD2* and *ATG16L1* genes lead in an additive manner to an abnormal Paneth cell phenotype in Crohn's disease patients,⁹ indicative of a possible implication of defective microbial clearance in pathogenesis. Although recent progress in Crohn's disease genetics and microbiome analysis strengthens the concept that misbalanced host-microbiota interactions underlie pathogenesis and also establish the development of a dysbiotic microbiota as a key feature associated with pathology, the causalities in abnormal host-microbiota interactions with regard to Crohn's disease pathogenesis remain unknown.

Mouse models of IBD are essential for the understanding of mechanisms implicating host-microbiota interactions in human disease; however, relevant studies have been restricted in colitis models so far.¹⁰ *Tnf ARE/+* mice chronically overproduce TNF and spontaneously develop a fully penetrant Crohn's-like IBD pathology localized specifically in the terminal ileum and occasionally also in the proximal colon.¹¹ *Tnf ARE/+* mice display typical histopathological features of human Crohn's disease such as massive infiltration of acute and chronic effector cells in the lamina propria (LP), villi blunting and development of transmural inflammation.¹¹ Local overexpression of TNF in the gut specifically by intestinal

epithelial cells (IECs) is sufficient to induce full-blown Crohn's-like IBD pathology in this model.¹² Thus, the *Tnf*^{ARE} model is an ideal tool for the study of intestinal epithelial responses to luminal bacteria and vice versa, in a disease context sharing many common characteristics with human Crohn's disease.

Here, we study host-microbiota interactions in murine modeled Crohn's disease and demonstrate that pathology in *Tnf*^{ARE/+} mice depends on the presence of the commensal microbiota which is required for TNF overexpression in the intestine. We also evidence epithelial alterations occurring in established but not in early disease in *Tnf*^{ARE/+} mice and show that a dysbiotic microbiota is shaped in diseased mice. Our results suggest that commensals are sufficient to initiate Crohn's disease pathogenesis in the presence of proinflammatory genetic alterations whereas an overall dysbiotic microbiota with significant alterations at the level of phyla develops in the inflamed mucosa secondarily to disease and is not required for disease initiation.

Results

Crohn's-like IBD pathology and disease-initiating TNF overexpression in the intestine of *Tnf*^{ARE/+} mice depend on the presence of bacteria

To examine the role of the intestinal microbiota in Crohn's disease pathogenesis we generated germ-free *Tnf*^{ARE/+} mice and compared disease development to *Tnf*^{ARE/+} mice reared under Specific Pathogen Free (SPF) conditions. Strikingly, germ-free *Tnf*^{ARE/+} mice were completely protected from IBD pathology showing no signs of inflammatory infiltration or villi blunting in the terminal ileum as evidenced by histological analysis at the age of 15 weeks, an age when *Tnf*^{ARE/+} mice show fully developed IBD pathology.¹³ In sharp contrast to germ-free *Tnf*^{ARE/+} mice, Crohn's-like pathology was readily evidenced in the ileum of 11-week-old control *Tnf*^{ARE/+} mice originating from the same mouse colony and raised under identical but non-sterile, specific-pathogen-free (SPF) housing conditions (Figure 1a, b). These results demonstrate that the development of Crohn's-like IBD pathology in *Tnf*^{ARE/+} mice is microbiota-dependent. The complete microbial dependence of intestinal pathology in *Tnf*^{ARE/+} mice suggests a possible implication of the microbiota in the very early events driving pathogenesis in this model, which are related to TNF overproduction. Relevantly, we have previously established that IECs are a major local source of early TNF overexpression in *Tnf*^{ARE/+} mice and that mice engineered to overexpress TNF specifically by IECs develop full Crohn's-like IBD pathogenesis in the ileum.¹² We examined the necessity of the intestinal microbiota in ileal TNF overexpression, by performing RT-PCR analysis in the ileum showing significantly higher TNF expression levels in the ileum of SPF compared to germ-free *Tnf*^{ARE/+} mice (Figure 1c). These results show that microbial signals are necessary for the induction of aberrant TNF expression in the intestine, a condition sufficient for disease initiation in this model. Since innate recognition of the microbiota by several TLRs (Toll-like receptors) initiates signaling cascades through the Myd88 adaptor molecule and activates cytokine production, including TNF,¹⁴ we hypothesized that Myd88-mediated innate signaling may play a central role in IBD pathogenesis in the *Tnf*^{ARE} model. Indeed, *Tnf*^{ARE/+Myd88^{-/-} mice examined at the age of 16 weeks, a stage when fully-developed disease is observed,¹³ showed a significantly}

attenuated IBD pathology compared to *Tnf*^{ARE/+} littermate controls, characterized by less inflammatory infiltration in the ileal mucosa and the submucosa and showing no signs of transmural inflammation, as it was the case for *Tnf*^{ARE/+} controls (Figure 2a, b). To examine whether Myd88 is required for TNF overexpression in *Tnf*^{ARE/+} mice, we isolated peritoneal macrophages from *Tnf*^{ARE/+}, *Tnf*^{ARE/+}*Myd88*^{-/-}, *Myd88*^{-/-} and WT control mice and measured TNF secretion in response to LPS stimulation. Macrophages isolated from *Tnf*^{ARE/+}*Myd88*^{-/-} mice showed defective LPS-induced TNF secretion compared to *Tnf*^{ARE/+} macrophages and reached levels similar to that of WT macrophages (Figure 2c). This suggests that innate response to LPS through Myd88 drives TNF overexpression and subsequently mediates IBD pathogenesis in *Tnf*^{ARE/+} mice. These results establish that TLR/Myd88-mediated innate recognition is required for TNF-driven IBD pathology development, in contrast to the homeostatic role established for TLR/Myd88 signaling in epithelial-injury induced colitis,¹⁵ and also indicate the presence of additional, Myd88-independent, innate pathways mediating TNF-driven murine Crohn's-like pathogenesis.

The inflamed ileum of *Tnf*^{ARE/+} mice is characterized by epithelial alterations involving loss of lysozyme-expressing Paneth cells and defective antimicrobial responses

High resolution endoscopy performed in the terminal ileum of diseased *Tnf*^{ARE/+} mice showed intestinal villi with increased diameter and an irregular shape, an observation consistent with the increased inflammatory infiltration in the LP in this model (Figure 3a). The intestinal epithelium of diseased *Tnf*^{ARE/+} mice is also characterized by accumulation of $\alpha E^+CD8\alpha\beta^+$ effector intraepithelial T-lymphocytes (Figure 3b). We have previously shown that these cells home to the ileum in a $\alpha E\beta 7$ integrin-dependent manner where they predominate over the resident non-conventional TCR $\gamma\delta$ and TCR $\alpha\beta CD8\alpha\alpha$ T-lymphocytes and express interferon (IFN)- γ and TNF.¹⁶ To examine the possible effects of cytokine-expressing immune effectors on intestinal epithelial functions we characterized barrier properties, epithelial cell type populations and gene expression in the intestinal epithelium of diseased *Tnf*^{ARE/+} mice. TNF and IFN- γ have been suggested to promote a leaky paracellular transport of solutes across the intestinal epithelium.¹⁷ We examined the possible presence of gross defects in paracellular epithelial barrier permeability in the terminal ileum of diseased *Tnf*^{ARE/+} mice by measuring blood to lumen ⁵¹Cr-EDTA clearance. No difference was found between *Tnf*^{ARE/+} mice and WT littermates in the paracellular permeability for this ~400 kD large molecule in the ileum (Figure 3c). In addition, no significant difference was found in the lumen to blood permeability of orally-administered 20kDa FITC-dextran (Figure 3d). Although malfunctions in the paracellular epithelial barrier in *Tnf*^{ARE/+} mice cannot be generally excluded based on these experiments, these argue against the presence of major defects permitting the passage of >20kDa luminal molecules. At the level of transcellular ion transport and ion channel function, we have previously reported defects in bicarbonate secretion and fluid absorption in the ileum of *Tnf*^{ARE/+} mice which may affect luminal pH and gut microbiota composition in these mice.¹⁸ The reduced fluid absorption in the ileum is compensated in the colon of *Tnf*^{ARE/+} mice,¹⁸ thus no diarrhea is observed (Figure 3e). Quantification of Goblet cells per villus by Periodic acid-Schiff staining (PAS) showed no difference between diseased *Tnf*^{ARE/+} and WT control mice (Figure 3f). Furthermore, mucin 2 secretion by Goblet cells was also found similar between *Tnf*^{ARE/+} mice and WT controls (Figure 3g). These results suggest that no

major defects occur in the formation of the mucus barrier in the inflamed ileum of *Tnf*^{ARE/+} mice. In contrast, Paneth cell quantification by immunostaining using anti-lysozyme antibodies revealed significantly reduced lysozyme-expressing Paneth cell numbers per crypt in the ileum of *Tnf*^{ARE/+} mice at the age of 8 and 16 weeks, an intermediate and a full-blown disease stage respectively (Figure 4a). 16 week-old *Tnf*^{ARE/+} mice show significantly reduced Paneth cells compared to 8 week-old *Tnf*^{ARE/+} mice, indicative of an association between pathology stage and Paneth cell numbers. In addition, 8-week-old *Tnf*^{ARE/+} mice showed significantly reduced Paneth cell numbers in crypts surrounded by extensive submucosal inflammation as compared to crypts located in areas where inflammation is less extensive or restricted in the mucosa (Figure 4b). These results show that disease progression in *Tnf*^{ARE/+} mice is associated with Paneth cell loss as assessed by immunostaining against lysozyme. To examine whether adaptive immune system effectors are required for this effect we examined Paneth cell numbers in alymphoid *Tnf*^{ARE/+Rag1^{-/-} mice. *Tnf*^{ARE/+Rag1^{-/-} mice show strongly attenuated pathology but also persistent infiltration of neutrophils in the LP and villi blunting.¹³ Quantification of Paneth cells by immunostaining against lysozyme in 16 week-old *Tnf*^{ARE/+Rag1^{-/-} mice showed significantly attenuated Paneth cell loss in their ileal crypts when compared to *Tnf*^{ARE/+} mice, to values approximating those of WT animals (Supplementary Figure 1a, c). In agreement with the previously described infiltration of neutrophils in the latter,¹³ lysozyme-producing inflammatory cells were observed in the LP of both *Tnf*^{ARE/+} and *Tnf*^{ARE/+Rag1^{-/-} mice (Supplementary Figure 1a). Thus, adaptive immune system effectors are essential for Paneth cell loss in *Tnf*^{ARE/+} mice whereas innate inflammatory infiltrates appear not to be sufficient to mediate Paneth cell loss. Furthermore, lysozyme immunostainings in 16 week-old *Tnf*^{ARE/+Ifng^{-/-} mice, which also display an attenuated pathology,¹³ showed a significantly attenuated Paneth cell loss compared to *Tnf*^{ARE/+} littermate controls (Supplementary Figure 1b, c). These results indicate a role of IFN- γ -secreting adaptive immune effector cells in Paneth cell reduction in *Tnf*^{ARE/+} mice. Whether this is a direct effect of IFN- γ via its receptor on Paneth cells needs further investigation. Paneth cell-specific targeting of IFN- γ receptor may clarify this issue.}}}}}

To examine the functional consequences of Paneth cell loss in the epithelium of *Tnf*^{ARE/+} mice we tested antimicrobial responses since these cells are a major source of antimicrobial peptides in the small intestine. RT-PCR analysis performed in the ileum of 8 week-old *Tnf*^{ARE/+} mice and littermate WT controls for a panel of antimicrobial genes showed significantly decreased expression levels for the alpha defensin genes (*Defa*) *Defa21* and *Defa29*, the complement receptor type 2 (*CR2*) and the lysozyme-encoding gene *Lyzc1* in *Tnf*^{ARE/+} mice (Figure 4c, d). These results suggest that IBD pathology in *Tnf*^{ARE/+} mice is associated with diminished antimicrobial gene expression, in agreement with the loss of Paneth cells observed in these mice. To compare the above findings with inflammation levels, we measured gene expression levels of *Tnf*, the major driver of inflammation and pathogenesis in *Tnf*^{ARE/+} mice. In agreement with our previous results on an early local *Tnf* overexpression before disease onset in this model,¹² *Tnf*^{ARE/+} mice showed increased *Tnf* expression in the ileum compared to their WT littermates at 4, 8 and 18 weeks of age (Figure 4e).

Dysbiosis, increased abundance of Segmented Filamentous Bacteria and increased epithelial attachment of bacteria in the terminal ileum of diseased *Tnf*^{ARE/+} mice

Antimicrobial peptides can affect the composition of the intestinal microbiota.¹⁹ To determine if alterations in the expression of antimicrobial peptides and/or bicarbonate secretion defects observed in *Tnf*^{ARE/+} mice altered the composition of the microbiome, we analyzed the mucosa-associated microbiome in the distal ileum of *Tnf*^{ARE/+} and co-housed WT control littermates by 16S rDNA sequencing.²⁰ Before disease onset, at 4 weeks of age, no significant difference in alpha diversity, beta diversity, or the relative abundance of the different phyla was found between WT and *Tnf*^{ARE/+} mice (Figure 5). After 8 weeks of age, a progressive increase in the relative abundance of *Firmicutes* was observed with a concomitant significant decrease in *Bacteroidetes* at 18 weeks of age. This progression was accompanied by a significant decrease in alpha diversity (species richness) and significant separation of WT mice from *Tnf*^{ARE/+} mice by weighted UniFrac analysis (Figure 5). These results indicate that the composition of the microbiome in the ileum of diseased *Tnf*^{ARE/+} mice and co-housed WT control littermates is markedly different. Detailed analysis of the 16S rDNA sequencing data revealed the presence of 139 operational taxonomic units (OTUs) present across the different samples after filtering (average read fraction $92 \pm 7\%$). At 4 and 8 weeks of age none of these OTUs was significantly different between co-housed *Tnf*^{ARE/+} mice and WT littermate controls (Figure 6, Supplementary Figure 2). At 18 weeks of age a significant dysbiosis was observed between co-housed *Tnf*^{ARE/+} mice and WT littermate controls. Out of the 139 OTUs detected, 23 were significantly different between WT and *Tnf*^{ARE/+} mice. In *Tnf*^{ARE/+} mice 20 out of 21 OTUs that were decreased in abundance were of the phyla *Bacteroidetes*. Two OTUs (greengenes id: 376862, 425767) were significantly increased in *Tnf*^{ARE/+} mice compared to co-housed WT control-littermates (Figure 6, Supplementary Figure 2). These two OTUs both belonged to the phylum *Firmicutes* and the genus *Candidatus* Arthromitus or *Candidatus* Savagella,²¹ informally known as Segmented Filamentous Bacteria (SFB). These results show that at 18 weeks of age, compared to co-housed WT littermate controls, *Tnf*^{ARE/+} mice have an increased relative abundance of SFB in the terminal ileum.

The dysbiosis associated with loss of Paneth cells and defects in antimicrobial genes expression observed in diseased *Tnf*^{ARE/+} mice, may affect not only the composition but also the number of mucosa-associated bacteria. We performed FISH analysis using a eubacterial probe on terminal ileal sections obtained from *Tnf*^{ARE/+} and WT littermate controls. We observed an increased number of bacteria in direct contact with IECs in *Tnf*^{ARE/+} mice compared to WT controls (Figure 7a, b). To confirm these results we quantified the number of bacteria by pan-bacterial 16S rDNA qPCR analysis. While the levels of bacterial 16S rDNA in the stool were not significantly different between WT and *Tnf*^{ARE/+} mice (Figure 7c), an increased number of mucosa-associated bacteria was detected in *Tnf*^{ARE/+} compared to WT animals (Figure 7d). Similar results were obtained using SFB specific primers²² i.e. *Tnf*^{ARE/+} mice showed an increase in the levels of mucosa-associated SFB (Figure 7e). These results suggest that in diseased *Tnf*^{ARE/+} mice an increased number of bacteria, likely SFB, are in close contact with IECs.

Modulation of the microbiota by antibiotics treatment reduces ileitis in *Tnf^{ARE/+}* mice

To determine if we can affect Crohn's-like disease progression by modulating the microbiota at an early disease stage, we treated *Tnf^{ARE/+}* mice with water or with 0.5 g/L vancomycin in the drinking water. Antibiotic treatment was initiated at 6 weeks of age, an early disease stage when the first signs of pathology are observed, and maintained until analysis at 20 weeks of age, when full-blown disease is expected (Figure 8a). Histopathological analysis showed severe Crohn's-like disease in *Tnf^{ARE/+}* mice treated with water (n = 5). In contrast, none of the *Tnf^{ARE/+}* mice treated with antibiotics (n = 5) showed signs of a Crohn's-like disease (Figure 8b-d). These results establish the critical role of the microbiota in the progression of Crohn's-like IBD and also highlight the therapeutic potential of microbiota modulation by antibiotics when administered at the early stages of chronic intestinal inflammation.

Discussion

The results presented in this study collectively provide a mechanistic interpretation of the complex host-microbiota dialogue in Crohn's disease pathogenesis modeled in *Tnf^{ARE/+}* mice. First, we show that Crohn's-like IBD pathology in the *Tnf^{ARE}* model is microbiota-dependent, originating from TNF overexpression in the intestine which is driven by a normal, indigenous microbiota. Second, we show that Crohn's-like IBD pathology in *Tnf^{ARE/+}* mice is associated with dampened antimicrobial responses and reduction of Paneth cells, mediated by adaptive immune system effectors. We further establish that secondarily to disease these conditions shape a dysbiotic gut microbiota in *Tnf^{ARE/+}* mice which is not sufficient to transfer disease to co-housed WT mice but also involves increased abundance of epithelial-adhering SFB which may further promote disease.

Concerning our first conclusion that Crohn's-like IBD pathogenesis in *Tnf^{ARE/+}* mice is microbiota-dependent, studies performed in other animal models of IBD, mainly in colitis models, have been controversial so far. Thus, an essential role in colitis pathogenesis has been shown for the microbiota in *IL-10^{-/-}* mice and in HLA-B27 transgenic rats which were completely rescued from colitis under germ-free conditions.^{23, 24} In contrast, germ-free conditions attenuated but did not prevent spontaneous pathology in the *IL-2^{-/-}* mouse model of colitis.²⁵ The role of the microbiota in murine ileitis was examined so far only in the SAMP1/YitFc model in which germ-free conditions led to an attenuated phenotype but did not prevent pathogenesis.²⁶ Consistently with the fact that adaptive immune effectors are a primary cause of disease in this model bearing transferable pathogenic activity,²⁷ the persistence of the SAMP1/YitFc ileal pathology under germ-free conditions has been attributed to defects in immunoregulation.²⁶ These studies indicate the presence of microbiota-independent mechanisms sufficient for IBD pathogenesis, particularly when adaptive immune responses are deregulated. In contrast, the microbiota is indispensable for the development of ileitis in the *Tnf^{ARE}* model where innate and adaptive immune responses form a continuum. Although aberrant adaptive immune responses are an important component of the fully-developed IBD pathology in *Tnf^{ARE/+}* mice,¹⁶ ileal pathology is observed in *Tnf^{ARE/+}Rag1^{-/-}* mice, involving infiltration of neutrophils and villi blunting.^{11, 13} Thus, germ-free studies in mouse models of IBD collectively suggest that the

microbiota is indispensable for pathogenesis when deregulated inflammatory mechanisms predominate. By contrast, when aberrant adaptive immune responses are the major driver of disease, the microbiota appears to be dispensable for pathology initiation.

Our second conclusion is that Crohn's-like pathogenesis in *Tnf*^{ARE/+} mice relies on the commensal microbiota whereas global changes in microbial communities, referred to as dysbiosis, are secondary to disease. This conclusion stems from our findings that significant alterations in gut microbiota composition at the level of phyla and at the level of the individual OTUs detected are observed only in late disease in *Tnf*^{ARE/+} mice and thus appear not to be essential for the initiation of IBD pathogenesis. By contrast, the commensal microbiota is highly potent in inducing local TNF expression in the intestine of WT mice and TNF overexpression in the intestine of *Tnf*^{ARE/+} mice at an early time-point, before disease onset. An important question raised by these findings is whether TNF transcriptional activation in the gut depends on specific bacterial taxa within the indigenous microbiota. Bacteria within commensals which are highly potent in inducing TNF transcriptional activation would function as pathobionts in the context of TNF-dependent ileitis in *Tnf*^{ARE/+} mice,²⁸ in a similar manner to the role played by *Prevotellaceae* in colitis susceptibility in *Nlrp6*^{-/-} mice for example.²⁹ The identification of such pathobionts could provide alternatives for localized targeting of TNF secretion in the gut. Thus, commensals or specific pathobionts within commensals, and not necessarily an overall dysbiotic microbiota, may be highly relevant to the early events initiating Crohn's disease, particularly in the presence of genetic variants which, either directly or indirectly, promote pro-inflammatory activation and local TNF expression in the gut.

Our present results however do not exclude a possible disease-exacerbating function of the dysbiotic microbiota developed in diseased *Tnf*^{ARE/+} mice. In the *T-bet*^{-/-}*RAG2*^{-/-} mouse model of colitis the dysbiotic microbiota has been shown to be colitogenic and, interestingly, this activity is transferable even to WT mice.³⁰ Although the dysbiotic microbiota of *Tnf*^{ARE/+} mice is not sufficient to transfer pathology in co-housed WT mice, the findings in the *T-bet*^{-/-}*RAG2*^{-/-} model indicate that the inflamed intestine may privilege the development of particularly harmful bacterial strains which may also bear full pathogenic potential. The increased abundance of SFB and their epithelial attachment in the inflamed ileum of *Tnf*^{ARE/+} mice may contribute to disease perpetuation in this model. SFB have been shown to both induce Th17 cells in the LP and tightly adhere to the surface of epithelial cells in the terminal ileum of mice bearing Th17 cells.³¹ We have previously shown that *Tnf*^{ARE/+} mice display increased Th17 responses in the ileal LP compared to WT controls.¹⁶ It is likely that the increased SFB abundance and their epithelial attachment in the ileum, reported here, underlies the increased Th17 responses in *Tnf*^{ARE/+} mice.

The dysbiosis and the increased bacterial attachment on the epithelium observed in *Tnf*^{ARE/+} mice secondarily to pathogenesis can be largely explained by the loss of Paneth cells and the associated disturbances in the expression of antimicrobial peptide genes. Paneth cells play a key role in intestinal antimicrobial surveillance, host defense and homeostasis and the involvement of Paneth cell dysfunction in IBD pathophysiology is under intense investigation, in light of GWAS results indicating abnormalities in Paneth cell function as risk factors for disease.³ Paneth cells in Crohn's disease patients have been

shown to strongly overexpress TNF, a fact indicating that these cells have the potential to introduce inflammatory signals in the intestinal mucosa under some conditions.³² Reduced Paneth cell alpha defensins are observed in the ileum of Crohn's disease patients, though not in association with inflammation grade.³³ In addition, in agreement with early reports,³⁴ patients with active Crohn's disease have recently been shown to display reduced numbers of Paneth cells in their crypts, a defect suggested to result from increased necroptotic Paneth cell death.³⁵ Whether loss of lysozyme⁺ cells in *Tnf ARE/+* mice reflects Paneth cell death or a dysfunction of alive Paneth cells needs to be addressed in future studies. It is likely that loss of lysozyme⁺ Paneth cells in *Tnf ARE/+* mice is caused by IFN- γ and TNF, both overproduced in their inflamed ileum. TNF has been shown to trigger Paneth cell necroptosis in mice bearing epithelial-specific caspase-8 ablation,³⁵ whereas acute exogenous TNF administration in normal mice leads to Paneth cell dysfunction and loss of cellular integrity.³⁶ In addition, IFN- γ produced during mucosal responses against the protozoan parasite *Toxoplasma gondii* leads to reduction of Paneth cells.³⁷ IFN- γ is also a trigger for Paneth cell degranulation, epithelial extrusion and death, in organoids and *in vivo* upon anti-CD3 antibody injection.³⁸ However, the physiological context of murine ileitis is markedly different from the above models; thus, the requirement of TNF and IFN- γ for Paneth cell loss should be directly examined by Paneth cell-specific ablation of the respective receptors in *Tnf ARE/+* mice.

A study published while our paper was under peer-review,³⁹ showed that *Tnf ARE* ileitis is microbiota-dependent and associated with dysbiosis; results that are in agreement with our results. Schaubeck et al. also concluded that *Tnf ARE* ileitis is transmissible by disease-associated but not healthy microbiota. This conclusion was based on the fact that germ-free *Tnf ARE/+* mice developed ileitis upon caecal content transfer from diseased but not from non-responder (healthy) *Tnf ARE/+* mice present in their colony; transfer from WT mice was not tested. The authors did not explain, however, why diseased *Tnf ARE/+* mice did not transmit ileitis to the co-housed non-responders of the same litter, a fact that argues against a transmissible phenotype. Therefore, the evidence presented in the work by Schaubeck et al. was not firmly conclusive as to whether ileitis is transmissible in the *Tnf ARE* model by disease-associated but not healthy microbiota and that intestinal pathology is secondary to dysbiosis.

Our study establishes a mouse model of Crohn's disease in which the indigenous intestinal microbiota is causatively involved in pathogenesis under genetic conditions favoring TNF overexpression. In this model, dysbiosis is a result and not a cause of intestinal pathology. Future studies may clarify whether in humans the indigenous microbiota has the potential to play a similar pathogenic role in the presence of genetic conditions which favor inflammatory activation in the gut or affect the antimicrobial defenses that normally maintain commensals at check.

Methods

Mice, design of in vivo experiments and study approval

Mice were bred and maintained on a C57BL/6/J genetic background in the animal facilities of BSRC "Alexander Fleming" (for Figures 2, 3, 4a-b) and the Icahn School of Medicine at

Mount Sinai (for Figures 4c-e, 5-8) under specific pathogen-free conditions. As an exception, *Tnf*^{ARE+/Ifng^{-/-} mice were maintained on a mixed C57BL/6J×129Sv genetic background in the animal facilities of BSRC “Alexander Fleming”. *Tnf*^{ARE/+} mice,¹¹ *Myd88^{-/-}* mice,⁴⁰ *Rag1^{-/-}* mice⁴¹ and *Ifng^{-/-}* mice⁴² have been previously described as indicated. For all in vivo experiments littermate and sex-matched mice were used. Mice were randomly assigned to experimental groups on a first-come basis. No mice were excluded from analyses performed. *Tnf*^{ARE/+} mice were analyzed before disease onset (4 weeks) and/or at an intermediate (8 weeks), established (12 weeks) or full-blown (16 weeks) disease stage, based on the previously described disease course for this model in the animal facilities of BSRC “Alexander Fleming”.¹³ A similar *Tnf*^{ARE/+} disease course was observed in the animal facilities of Icahn School of Medicine at Mount Sinai as shown in Supplementary Figure 3.}

All mice were used in accordance with the guidance of the Institutional Animal Care and Use Committee of BSRC “Alexander Fleming” and the guidelines of the Animal Care and Use Committee of Icahn School of Medicine at Mount Sinai.

Generation of germ-free *Tnf*^{ARE/+} mice

Germ-free mice were generated and raised at the Instituto Gulbenkian de Ciência, Oeiras, Portugal. C57BL6J *Tnf*^{ARE/+} and WT mice were transferred to germ-free conditions upon rederivation by caesarean section, as described in the relevant EMMA protocol (http://strains.emmanet.org/protocols/GermFree_0902.pdf). Briefly, 20 days post-coitum donor females were euthanized and their uteri transferred to a sterile chamber through a reservoir filled with 1% Virkon S. Pups were extracted and adopted by C3H/HeN Germ-Free foster mothers. The microbiological status of the isolator and the animals were monitored 3 weeks after transfer and every 3 weeks onward. Germ-free and SPF control *Tnf*^{ARE/+} mice were raised and maintained under strictly identical conditions, except the microbiological status. These include food pellets, drinking water, humidity and temperature.

Histopathological analysis of *Tnf*^{ARE/+} mice

Histological analysis of *Tnf*^{ARE/+} mice crossed with *Myd88^{-/-}*, *Rag1^{-/-}* and *Ifng^{-/-}* mice and of germ-free *Tnf*^{ARE/+} mice was performed in the terminal ileum as described previously.¹² The proximal and distal colon was also examined. Briefly, the tissue was embedded in paraffin and stained with H&E. Semi-quantitative assessment of intestinal pathology was performed in a blinded fashion. The following scoring system was used: 0= normal, 1= villi blunting and mucosal inflammation, 2= villi blunting, extensive mucosal inflammation and submucosal inflammation, 3= extensive submucosal inflammation, 4= transmural inflammation.

Pan-bacterial PCR amplification

Total genomic DNA was isolated from tissue using the DNeasy Blood & Tissue Kit (Qiagen). Quantitative PCR analysis and SFB primer sequences are described elsewhere.⁴³ Relative quantity was calculated by the Ct method and normalized by the presence of mouse ubiquitin.

Biome analysis

DNA was obtained from distal ileum (2 cm) using the DNeasy Blood and Tissue Kit (QIAGEN). Bacterial 16S rRNA genes were amplified using the primers as described.⁴⁴ Sequencing was performed with an Illumina MiSeq sequencer for sequencing as previously described.⁴⁴ Detailed methods for DNA Extraction, 16S rDNA Amplification, Multiplex sequencing and Analysis of 16S rDNA Sequences are provided in the Supplementary Methods.

Antibiotic treatment

Mice were treated for the indicated time with 0.5 g/L vancomycin (Western Medical Supply) ad libitum in the drinking water for the indicated time.

Statistical analyses

For graphs and statistical analyses indicated we used GraphPad Prism, SigmaPlot or R statistical environment. Sampling distributions were tested for normality with a threshold of $\alpha=0.05$ (GraphPad Prism). For $P<0.05$ in the normality test, differences in means were tested for statistical significance with two-tailed Mann-Whitney U test (Wilcoxon rank-sum test). For $P>0.05$ in the normality test, F test or Bartlett's test were used for homoscedasticity. For similar variances (F test, $P>0.05$) unpaired two-tailed Student's t-test was applied, for unequal variances (F test, $P<0.05$) unpaired two-tailed Welch's t-test was applied. P -values <0.05 were considered as statistically significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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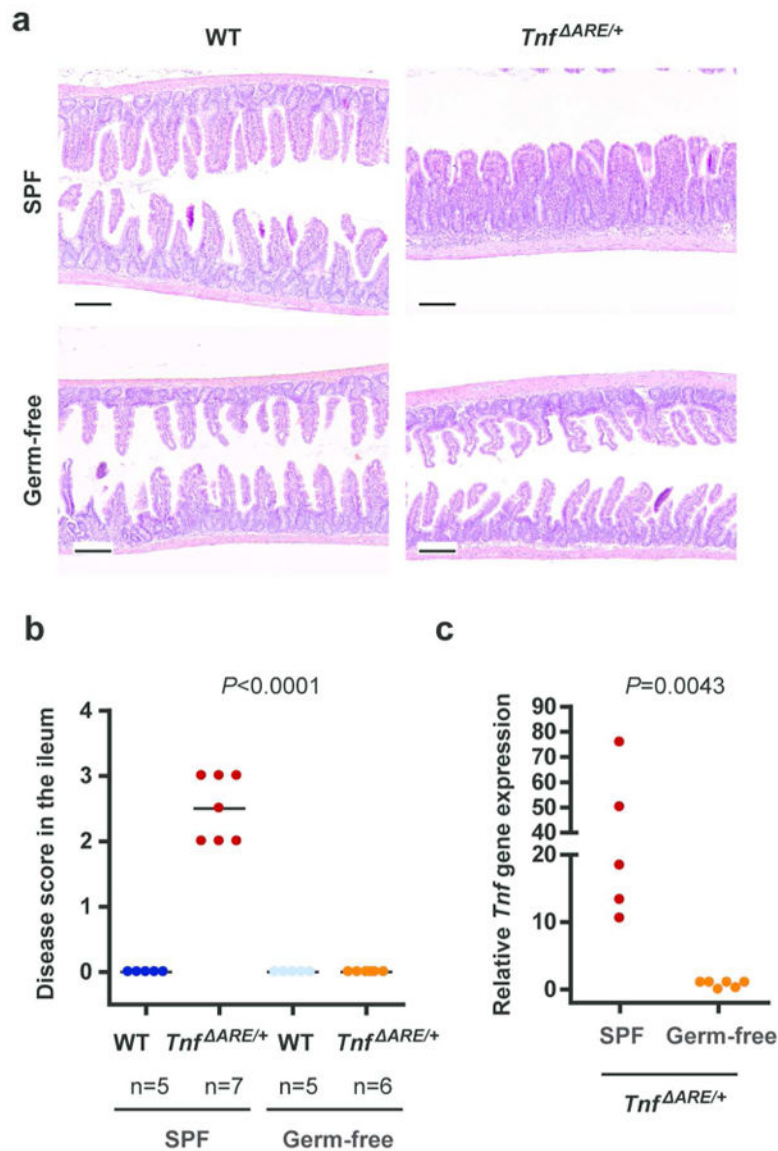


Figure 1. Germ-free *Tnf*^{ARE/+} mice are completely rescued from TNF overexpression in the intestine and Crohn's-like IBD pathology

(a) Histological analysis was performed in the terminal ileum of $n = 6$ *Tnf*^{ARE/+} mice raised under germ-free conditions at the age of 15 weeks. $N = 7$ *Tnf*^{ARE/+} mice raised under Specific Pathogen Free (SPF) conditions (11 weeks) were used as controls. Representative H&E-stained sections are shown. (Scale bars: 100 μm).

(b) Histopathological analysis performed in the terminal ileum of germ-free and SPF control *Tnf*^{ARE/+} mice examined at the age of 15 and 11 weeks respectively. Statistical significance was calculated by Kruskal-Wallis one-way analysis of variance.

(c) Quantification of TNF gene expression in whole tissue biopsies from the terminal ileum of *Tnf*^{ARE/+} mice reared under germ-free and SPF conditions, examined at the age of 15 and 11 weeks respectively. Statistical significance was calculated with two-tailed Mann-Whitney test.

All data shown represent mean ± SEM.

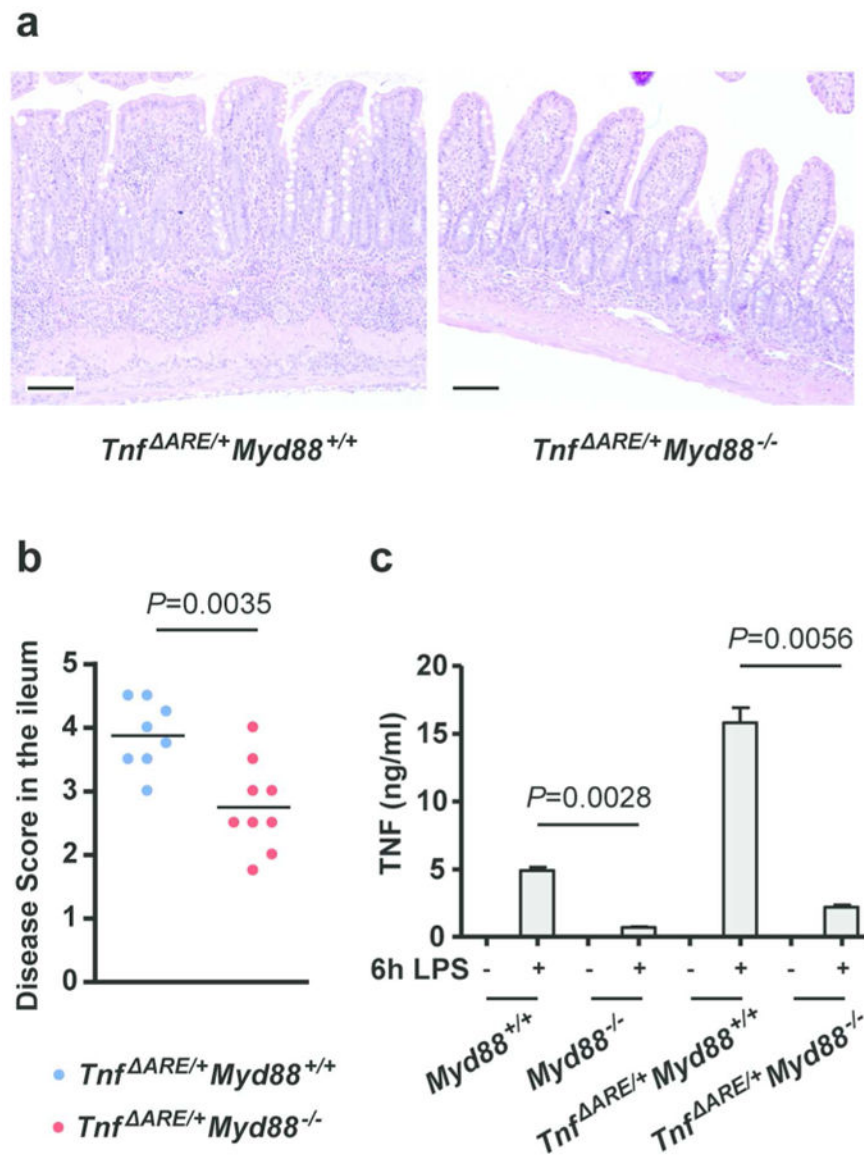


Figure 2. Myd88 mediates TNF overexpression and IBD pathogenesis in $Tnf^{ARE/+}$ mice

(a) Crohn's-like pathology is attenuated in the terminal ileum of 16-week-old $Tnf^{ARE/+} Myd88^{-/-}$ mice as compared to $Tnf^{ARE/+}$ littermate controls. Representative H&E-stained sections are shown. (Scale bars: 50 μm).

(b) Histopathological analysis performed in the terminal ileum of $Tnf^{ARE/+} Myd88^{-/-}$ and $Tnf^{ARE/+}$ control mice at the age of 16 weeks. Statistical significance was calculated with Mann-Whitney test. (** $P < 0.01$)

(c) Thioglycollate-elicited peritoneal macrophages (see SI Methods) were isolated from 16-week-old $Tnf^{ARE/+}$, $Tnf^{ARE/+} Myd88^{-/-}$, $Myd88^{-/-}$ and WT control mice (n = 3 mice per genotype) and stimulated with LPS for 6 hours. TNF secretion was measured by ELISA. Macrophages isolated from $Tnf^{ARE/+} Myd88^{-/-}$ mice show defective LPS-induced TNF secretion. Statistical significance was calculated with unpaired Welch t test. (** $P < 0.01$) All data shown represent mean \pm SEM.

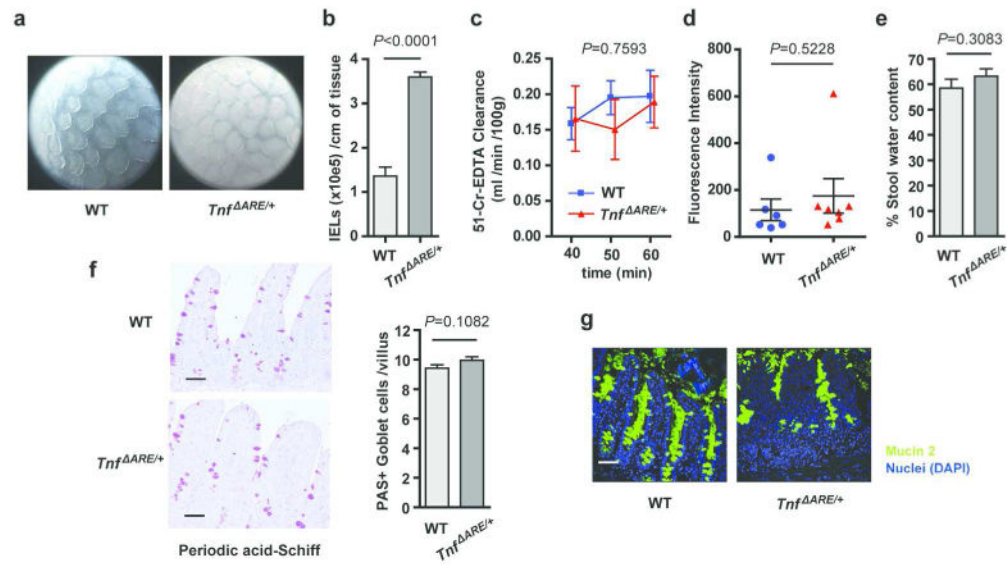


Figure 3. Epithelial barrier function in the ileum of diseased *Tnf*^{ARE/+} mice

(a) Endoscopic pictures from the terminal ileum of 12 week-old *Tnf*^{ARE/+} mice and WT controls. Representative of n = 2 independent experiments.

(b) Quantification of intraepithelial lymphocytes (IELs) isolated from the ileum of 12 week-old *Tnf*^{ARE/+} mice and WT controls (n=5 each). Unpaired two-tailed Student *t*-test (***) *P* < 0.001)

(c) Epithelial paracellular permeability, as measured by blood to lumen ⁵¹Cr-EDTA clearance in the terminal ileum of 12 week-old *Tnf*^{ARE/+} mice (n=4) and WT controls (n=4). No significant difference (ns) was found between the two groups by Two-way ANOVA.

(d) Epithelial barrier permeability of 12 week-old *Tnf*^{ARE/+} mice (n=7) and WT controls (n=6), as measured by lumen to blood passage of orally-delivered 20kDa FITC-dextran.

(e) Stool water content of *Tnf*^{ARE/+} mice (n=24) and WT littermates (n=18) at the age of 16 weeks. No significant (ns) differences were found by unpaired two-tailed Student *t*-test.

(f-g) Goblet cell numbers and mucus secretion show no major defects in the inflamed ileum of 12-week-old *Tnf*^{ARE/+} mice compared to littermate WT controls (n=3 per group) as shown by Periodic acid-Schiff (PAS)-staining (e) and mucin 2 immunostaining (f). Unpaired two-tailed Student *t*-test. (Scale bars: 50 μm).

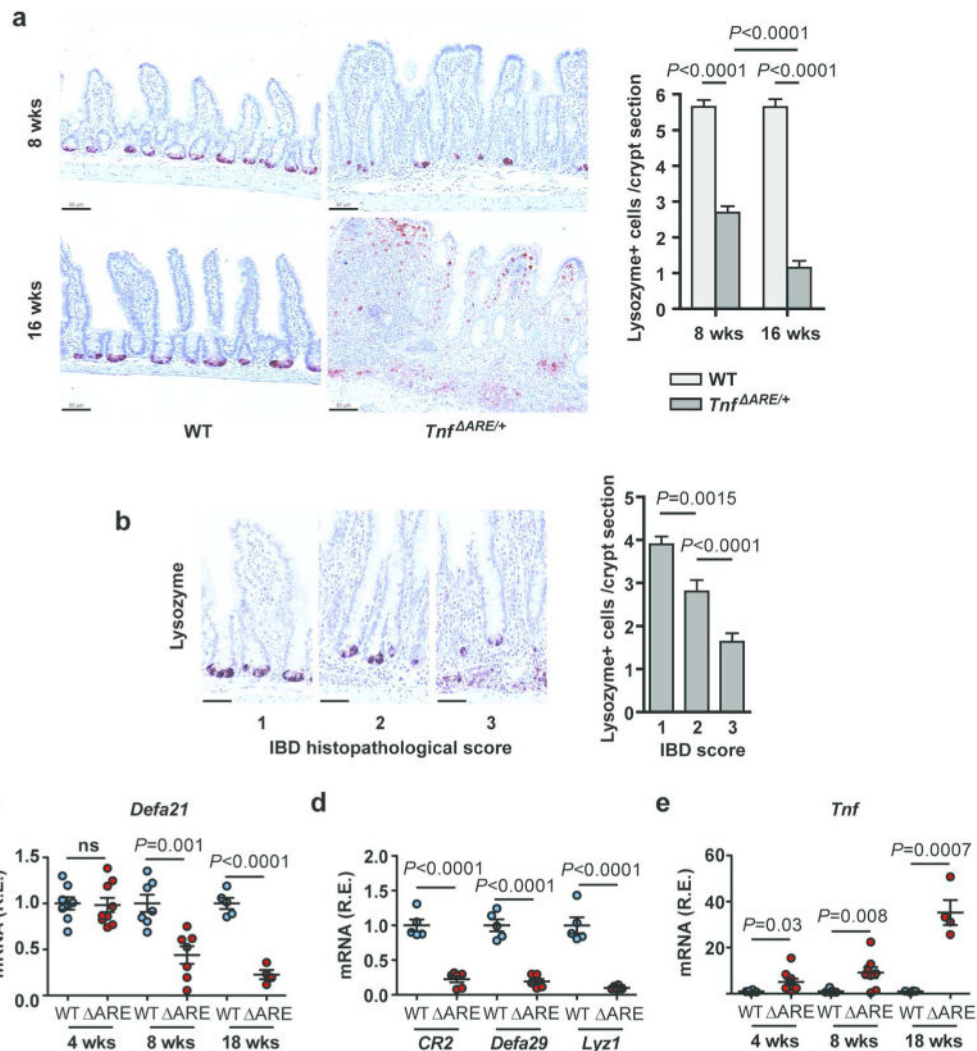


Figure 4. Loss of Paneth cells and altered expression of antimicrobials in the ileum of diseased *Tnf^{ARE/+}* mice

(a) Immunohistochemistry for Lysozyme for the quantification of Paneth cells per crypt in the terminal ileum of *Tnf^{ARE/+}* mice at the age of 8 and 16 weeks and littermate WT controls (n=3/group). Two-tailed Mann-Whitney test (***) $P < 0.001$.

(b) Paneth cell numbers are significantly reduced as the local IBD histopathological score increases in the crypts of 8 week-old *Tnf^{ARE/+}* mice (n=3). A total of n=108 well-oriented crypts were examined. Indicative lysozyme immunostaining pictures are shown for each disease stage. (Scale bars: 33 μ m). Statistical significance was calculated by two-tailed Mann-Whitney test (** $P < 0.01$).

(c) Relative mRNA expression of *Defa21* in the last 2 cm of the terminal ileum of WT and *Tnf^{ARE/+}* mice at different ages (n= 4-8/group). Two-tailed Student *t*-test.

(d) Relative expression of antimicrobials *Cr2*, *Defa29* and *Lyz1* in whole tissue extracts from the last 2 cm of the ileum of WT and *Tnf^{ARE/+}* mice at 18 wks (n= 6/group). Two-tailed Student *t*-test.

(e) Relative expression of *Tnf* in whole tissue extracts from the last 2 cm of the ileum of WT and *Tnf*^{ARE/+} mice at different ages (n= 4-8/group). Two-tailed Student *t*-test. R.E., Relative Expression. Each dot represents an animal. All data shown represent mean ± SEM.

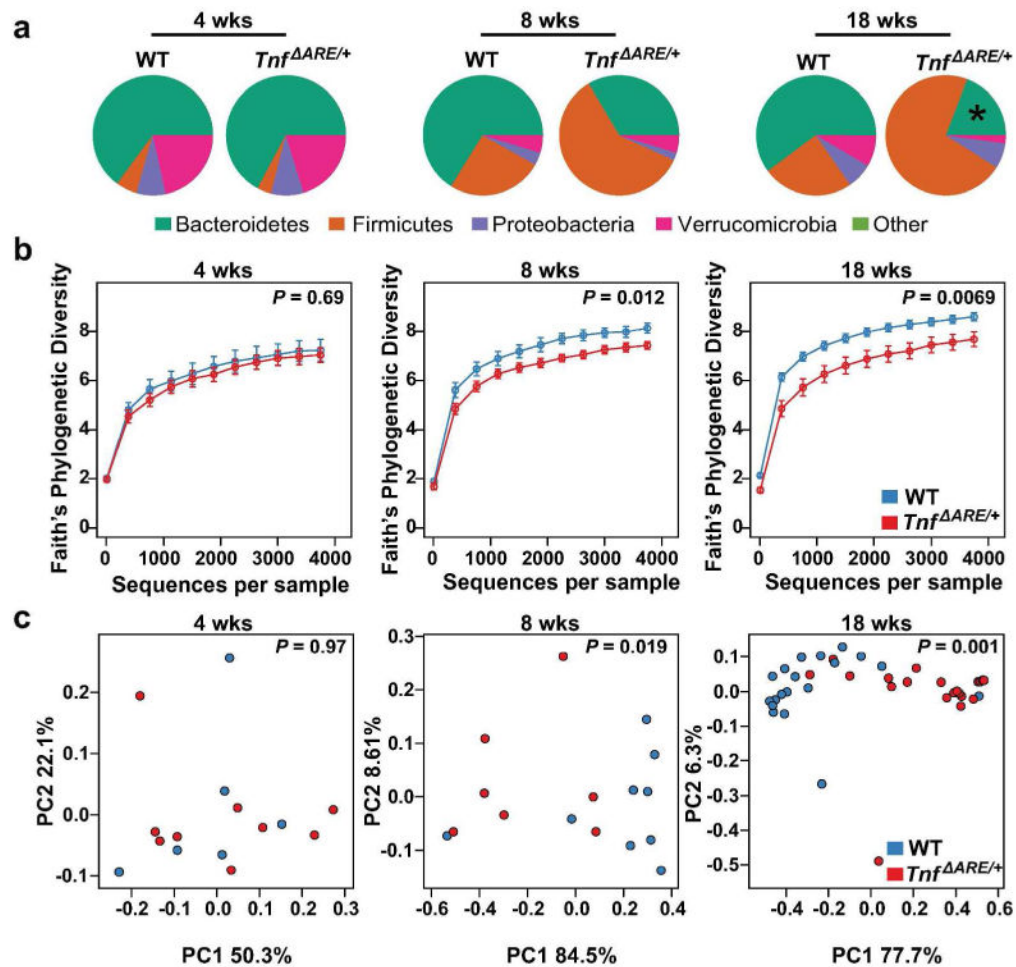


Figure 5. Biome analysis of *Tnf^{ARE/+}* mice

(a) Relative abundance of phyla present in the terminal ileum of WT and *Tnf^{ARE/+}* mice at 4 (W, n = 6; T, n = 9), 8 (W, n = 9; T, n = 7), and 18 (W, n = 19; T, n = 20) weeks of age. Data shown represent the most abundant phyla, while low abundant and unclassified OTUs were grouped in 'Other', * represents a significant ($q < 0.05$) change in abundance between age-matched WT and *Tnf^{ARE/+}* mice.

(b) Alpha diversity in *Tnf^{ARE/+}* mice at 4, 8 and 18 weeks of age. Statistical significance was calculated with Mann-Whitney test.

(c) Weighted Unifrac distances between *Tnf^{ARE/+}* and co-housed WT litter-mate controls at 4, 8 and 18 wks of age. Statistical significance was calculated with Adonis test.

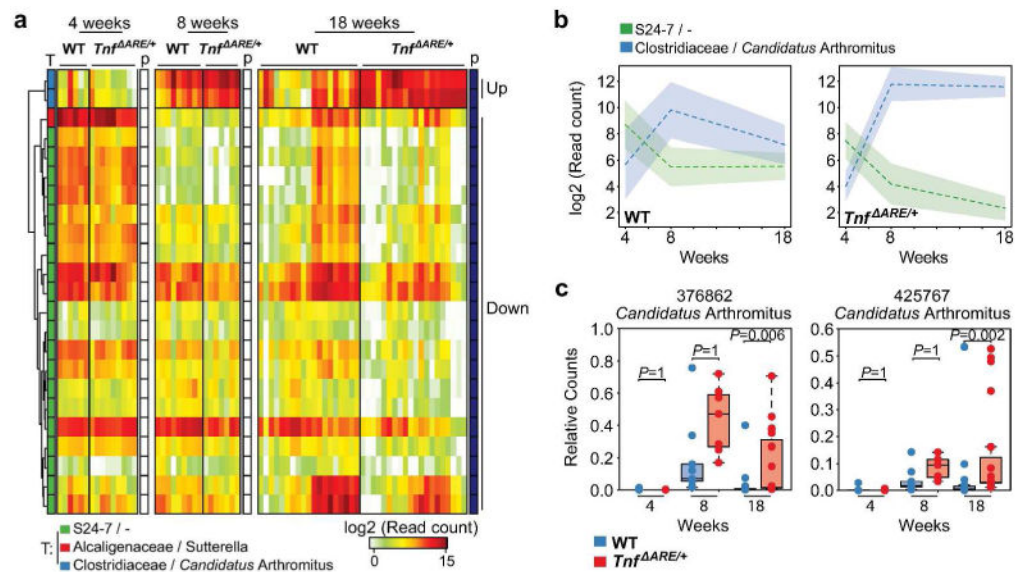


Figure 6. Increased abundance of *Candidatus Arthromitus* in *Tnf*^{ARE/+} mice

(a) In total 139 OTUs were detected across all samples. Pearson hierarchical clustering of the abundance profiles of the 23 OTUs that were significantly altered in the terminal ileum of *Tnf*^{ARE/+} mice compared to co-housed WT littermate controls at 4, 8 and 18 weeks. 4 weeks: WT (n = 6), *Tnf*^{ARE/+} (n = 9); 8 weeks: WT (n = 9), *Tnf*^{ARE/+} (n = 7); 18 weeks: WT (n = 19), *Tnf*^{ARE/+} (n = 20). Two OTUs, 376862 and 425767, were significantly enriched at 18 weeks; both represent the genus *Candidatus Arthromitus*. Each column represents a different mouse. Left colored boxes indicate the family/genus taxonomy classification (T). Right colored boxes indicate if the comparison (p) between WT and *Tnf*^{ARE/+} shows significant difference (blue, $P < 0.05$). (b) LOESS regression of the median read counts of the S24-7/- and Clostridiaceae/*Candidatus Arthromitus* OTUs shown in (a). Shaded region represents standard error. (c) Boxplots showing the relative abundance of the two OTUs (376862, 425767) enriched in *Tnf*^{ARE/+} mice at 18 weeks of age.

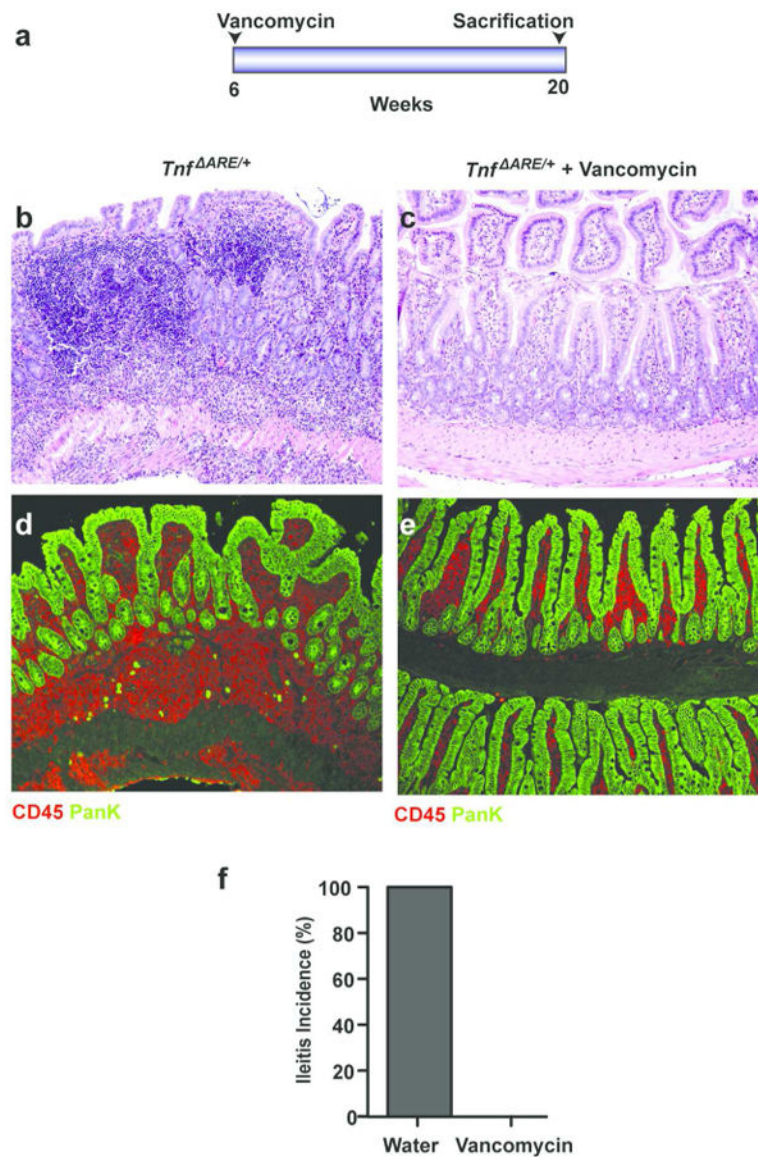


Figure 7. Increased number of mucosa-associated bacteria in the terminal ileum of *Tnf*^{ARE/+} mice

(a-b) Visualization of bacterial localization in the distal ileum of WT and *Tnf*^{ARE/+} mice at 18 wks. Transversal sections of the distal ileum were hybridized with a eubacterial 16S probe (red) and counterstained with Pan-keratin antibody to visualize the intestinal epithelial cells (green) and DAPI for nuclear staining (blue). Notice the increased attachment of bacteria to the epithelium of *Tnf*^{ARE/+} mice when compared to WT animals. Shown are representative images (a) and quantification of 5 mice per group (b).

(c-d) Bacterial content in the stool (c) and in the distal ileum mucosa (d) of WT (n = 4) and *Tnf*^{ARE/+} mice (n = 5) at 18 wks of age determined by pan-bacterial 16S qPCR amplification relative to host ubiquitin.

(e) Relative expression of mucosa-associated SFB in the terminal ileum of WT and *Tnf*^{ARE/+} mice at 18 weeks of age.

R.E., Relative Expression. Each dot presents an animal. Data are expressed as mean \pm SEM. Statistical significance was calculated with two-tailed Student *t*-test. Scale bars = 50 μ m.

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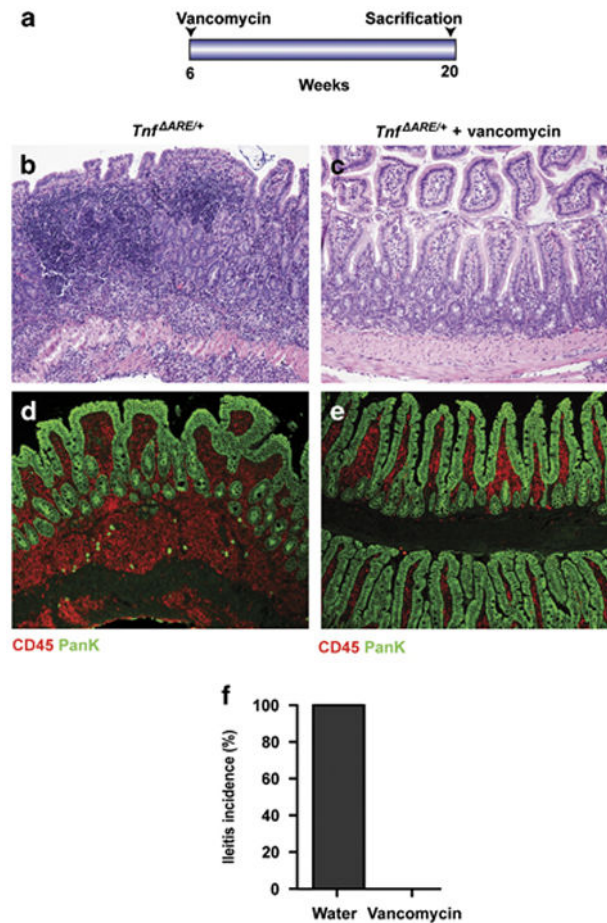


Figure 8. Antibiotics treatment rescues ileitis in *Tnf*^{ARE/+} mice

(a) Diagram of antibiotic treatment. *Tnf*^{ARE/+} mice at 6 weeks of age were treated with water (n=5) or vancomycin (n=5). Animals were sacrificed at 20 weeks of age.

Inflammatory infiltrates in the ileum of *Tnf*^{ARE/+} mice were completely abrogated in animals treated with vancomycin (c) when compared to water treated controls (b). All mice (n = 5 per group) were analyzed histologically and representative H&E-stained sections are shown.

(d-e) Immunostaining of CD45 and Pan-keratin in the terminal ileum of *Tnf*^{ARE/+} mice treated with water (n = 5) (d) or vancomycin (n = 5) (e), sacrificed at 20 weeks of age.

(f) Ileitis incidence in 20-week-old *Tnf*^{ARE/+} mice treated with water (n = 5) or vancomycin (n = 5).