The anti-inflammatory IFITM genes ameliorate colitis and partially protect from tumorigenesis by changing immunity and microbiota

Zoya Alteber, Adi Sharbi-Yunger, Meirav Pevsner-Fischer, Dan Blat, Lior Roitman, Esther Tzehoval, Eran Elinav & Lea Eisenbach

Department of Immunology, The Weizmann Institute of Science, Rehovot 76100, Israel

Keywords
Inflammatory bowel disease, T helper 17 cells, T helper 1 cells, immune disorders, tumor immunology, microbiome.

Correspondence
Lea Eisenbach, Department of Immunology, Weizmann Institute of Science, Rehovot 76100, Israel.
E-mail: lea.eisenbach@weizmann.ac.il

Received 21 March 2017; Revised 18 October and 4 December 2017; Accepted 6 December 2017

doi: 10.1111/imcb.12000

Immunology & Cell Biology 2018; 1–14

Abstract

Inflammation plays pivotal roles in different stages of tumor development. Screening for predisposing genetic abnormalities and understanding the roles these genes play in the crosstalk between immune and cancer cells will provide new targets for cancer therapy and prevention. The interferon inducible transmembrane (IFITM) genes are involved in pathogenesis of the gastrointestinal tract. We aimed at delineating the role of IFITM3 in colonic epithelial homeostasis, inflammation and colitis-associated tumorigenesis using IFITM3-deficient mice. Chemical induction of colitis in IFITM3-deficient mice results in significantly increased clinical signs of inflammation and induction of invasive tumorigenesis. Bone marrow transplantation showed that cells of the hematopoietic system are responsible for colitis deterioration. In these mice, impaired cytokine expression skewed inflammatory response toward pathogenic Th17 with reduced expression of the anti-inflammatory cytokine IL10 during the recovery phase. Intriguingly, mice lacking the entire IFITM locus developed spontaneous chronic colitis from the age of 14 weeks. Sequencing the 16S rRNA of naïve mice lacking IFITM3 gene, or the entire locus containing five IFITM genes, revealed these mice had significant bacterial differences from their wild-type littermates. Our novel results provide strong evidence for the essential role of IFITM genes in ameliorating colitis and colitis-associated tumorigenesis.

INTRODUCTION

Chronic inflammatory disorders of the gastrointestinal tract are commonly called inflammatory bowel diseases (IBD). The main clinical forms of IBD are Crohn’s disease (CD) and ulcerative colitis (UC). Patients with IBD are at increased risk to develop colorectal cancer (CRC) and IBD is among the top three high-risk conditions for CRC. Three main factors were shown to affect the pathogenesis of IBD: genetic background, the host immune system and gut microbiota. Normal intestinal homeostasis is based on the dynamic interactions between intestinal epithelial cells, intestinal microbes and local immune cells. These interactions are also essential for mounting protective immunity to pathogens. Defects in epithelial barrier function, innate immune recognition or immune regulatory circuits may accelerate the aberrant expression of pathological inflammatory responses in IBD.

The Interferon inducible transmembrane proteins (IFITM) have emerged as potent viral inhibitors of a broad range of RNA viruses. However, the exact mechanism underlying their viral restriction action remains uncertain. In addition, these genes are involved in multiple molecular and cellular processes such as fetal development, cell cycle control, cellular adhesion and invasion. Recently, it was shown that mice lacking a locus containing five IFITM genes (IFITMdel) suffer from
IFITM genes prevent colitis and tumorigenesis
Z Alteber et al.

age-related metabolic dysfunction with hyposensitivity to insulin, obesity and microglia morphologic abnormalities.5 Several findings associate IFITM genes with pathogenesis of the gastro-intestinal tract. IFITM2 and IFITM3 genes are shown to be overexpressed in colon cancer both in human and mouse.6-8 The antiproliferative activity of IFITM genes has been associated with their tumor-suppressor function.4 Yang and coworkers showed that knockdown of IFITM1 gene promotes cancer growth in vivo.9 Moreover we have demonstrated that IFITM2 inhibits oncogenic transformation in vitro.10 In addition, due to their altered expression in transformed tissues, IFITM genes are overexpressed in colitis-associated colon cancer and in severely inflamed mucosa of UC and CD patients.11,12 Furthermore, polymorphism in the IFITM3 and IFITM1 genes was found to be associated with susceptibility to UC.13,14

These evidences led us to hypothesize that IFITM genes may be involved in colon inflammation and colitis-associated tumorigenesis, predisposing dysfunctional allele carriers to these diseases. To test this assumption and to delineate the role of IFITM genes in inflammation and colitis-related tumor formation we use IFITM3-deficient and IFITMdel mouse strains.

In this work we describe, for the first time, that IFITM3 deficiency results in deterioration of dextran sodium sulfate (DSS)-induced colitis, whereas deletion of the IFITM locus predisposes mice to develop spontaneous colitis. IFITM3-deficient mice are prone to develop intense Th17 inflammatory response with increased infiltration of macrophages and effector T cells to the colon lamina propria. Moreover, both IFITM3-deficient and IFITMdel mice have substantial alterations in intestinal microbiota with notable increased abundance of Prevotella and Mucispirillum genera. We also show that, IFITM3-deficient mice demonstrate increased susceptibility to azoxymethane (AOM)/DSS-mediated colitis-associated tumorigenesis and develop invasive adenocarcinomas with high frequencies of proliferating cells.

RESULTS

Increased susceptibility to colitis in IFITM3- and IFITMdel- deficient mice

To study whether IFITM3 affects colitis, we induced acute and chronic colitis in IFITM3−/− mice, and in their IFITM3+/− and WT littermates. To induce an acute colitis model, mice were treated with 2% DSS dissolved in their drinking water for 7 days.15 The DSS model has been shown to recapitulate many of the clinical observations, associated with UC in humans, by affecting the integrity of the mucosal epithelial barrier.16 As shown in Figure 1a, IFITM3 relative expression was induced substantially in WT animals due to DSS administration. This elevation increased with the course of the disease. Interestingly, while in WT the fold of induction of IFITM3 expression was nearly 30, it was not significantly upregulated in IFITM3+/− colons (threelfold). The expression of IFITM1, another IFITM family member, was not altered with colitis progression (Figure 1b). Therefore, the induction is specific to IFITM3.

Mice were weighed daily and examined endoscopically to assess the severity of the colitis on days 8 and 22 from the onset of the treatment. IFITM-deficient (IFITM3−/− and IFITM3+/−) mice lost significantly more weight (Figure 1c) and displayed substantially more severe clinical signs of inflammation than their WT littermates (Figure 1d and e, Supplementary figure 1A). Although the DSS dose that we have selected is not lethal, a slightly higher mortality rate was observed in IFITM3−/− mice (data not shown), indicative of the severity of the disease. In agreement with these findings, H&E staining of histologic sections taken from the colons after 7 days of 2% DSS treatment showed dramatic leukocyte recruitment and crypt loss in the IFITM3-deficient mice compared with WT control mice (Figure 1f). IBDs are considered chronic conditions and therefore we examined IFITM3 role also in chronic inflammation. Mice were subjected to three cycles of 2% DSS treatment separated by a 2-week administration of regular water.15 In this chronic model, we again encountered increased clinical signs of inflammation in IFITM3-deficient mice as was confirmed by endoscopic evaluation (Supplementary figure 1B, C). Altogether, these data indicate that IFITM3 gene attenuated both acute and chronic colitis. It significantly influenced the initial response to epithelial damage and recovery. IFITM3 deficiency resulted in prolonged and severe inflammation.

Due to high homology between the IFITM family members, there may be a functional compensation for IFITM3 deletion by other family members. To test this possibility we examined IFITMdel mice, harboring a deletion of entire Ifitm cluster encompassing the complete family locus.17 Strikingly, these mice developed spontaneous chronic colitis from the age of 14 weeks (Figure 1g). These data indicate that other family members may have partially compensated for IFITM3 deletion. Moreover, the occurrence of colitogenic signs in mice that were not exposed to any external challenge emphasizes the anti-inflammatory activity of IFITM genes.
Figure 1. IFITM3 gene attenuates inflammation during acute DSS-induced colitis. (a) Relative IFITM3 mRNA expression WT and IFITM3+/– mice on indicated days following 2% DSS-coltitis induction. (b) Relative IFITM1 mRNA expression in WT mice on indicated days following 2% DSS-coltitis induction. (c) Weight loss relative to initial weight in indicated groups following DSS challenge. (d) Endoscopic colitis grades assessed on days 8 and 22 after DSS initiation. (e) Representative images taken during colonoscopy of mice on day 8. (f) Representative hematoxylin and eosin staining of colons on days 3 and 8 following colitis induction of indicated groups. (g) Endoscopic colitis grades of naive IFITMdel (WT, +/+ and −/− mice). For data presented in a, b, n = 5–8. Data presented in c–f are representative of five individual experiments, n = 7–30. For data presented in g, n = 9–12. *P < 0.05, **P < 0.01, ***P < 0.001. DSS, dextran sodium sulfate; IFITM, interferon inducible transmembrane; DSS, dextran sodium sulfate.
Expansion of myeloid intestinal cells in colons of inflamed IFITM3−/− mice

IFITM proteins are expressed ubiquitously in humans. Even in the absence of IFN stimulation, primary tissues and cell lines express basal levels of IFITM proteins. To test whether the proinflammatory response, detected in IFITM3-deficient mice, was caused by the hematopoietic system or the epithelial compartments, we generated BM chimeras. After exposure of mice to 2% DSS, KO→WT chimeras, similarly to KO→KO controls, exhibited significantly strengthened features of colitis as assessed by colonoscopy at day 8 after the onset of DSS treatment (Figure 2a). This indicated that in IFITM3-deficient mice, cells of the hematopoietic arm were responsible for colitis deterioration.

The mammalian gastrointestinal tract is colonized by trillions of microorganisms, mostly commensal bacteria. The symbiotic relationship between the microbiota and the host is tightly regulated by the immune system. While constantly exposed to dietary and environmental antigens, the host immunocytes regulate the composition of microbiota and protect against invading pathogens. Intestinal mononuclear phagocytes, DCs and macrophages, are important mediators of gut homeostasis and its breakdown.

To further elucidate how IFITM3 attenuated colon inflammation, we studied DC and macrophage populations in colons of naïve and inflamed mice. Induction of colitis resulted in macrophage expansion with the most substantial elevation in IFITM3−/− and +/− mice (Figure 2b). CD11b+ and CD11b− DCs were also elevated during the course of inflammation; however, there were no

Figure 2. The hematopoietic arm is responsible for colitis deterioration in IFITM3-deficient mice. (a) Summary of endoscopic colitis grades assessed on day 8 after DSS initiation for KO→WT, WT→KO BM chimeras, as well as WT→WT and KO→KO controls. (b) Frequencies of F4/80+Ly6ChighCD64+ lamina propria macrophages out of CD45+MHCIIhigh cells. (c) Percentages of GFP expressing lamina propria macrophages. (d, e) Frequencies of lamina propria-isolated CD4+ and CD8+ T cells (respectively) and their activation state, as defined by the expression of CD44 and CD62L. (f) Representative dot plots for IFNy and IL17 intracellular cytokine staining of lamina propria-isolated CD4+ T cells stimulated with PMA, ionomycin and brefeldin A. (g) Summary of the data presented in f. All data present samples collected from WT and IFITM3−/− mice on indicated days following DSS administration. Data presented in a, summaries two individual experiments, n = 5–9 for each group. Data presented in b–g are summaries of two individual experiments, n = 3–5 in each. **P < 0.01, ***P < 0.001. DSS, dextran sodium sulfate; IFITM, interferon inducible transmembrane.
IFITM3 deficiency skewed the immune response toward Th17

In addition to the innate immune system, cells of the adaptive immunity also mediate homeostatic and protective responses in the intestine. Among intestinal lymphocytes, CD4+ T cells represent a major population responsible for managing these responses. However, when aberrant CD4+ T cell accumulation occurs, the outcome may result in development of chronic inflammation. In IFITM3-deficient mice, a significant expansion of CD4+ T cells was detected as colitis progressed. Moreover, these mice presented higher frequencies of effector (CD62L−CD44+) CD4+ T cells (Figure 2d). Similarly to CD4+ T cells, we detected an elevation in population of CD8+ T cells in IFITM3−/− mice. We observed a trend for increased CD8+ effector T cells proportions in IFITM3-deficient groups (Figure 2e), indicating potential CTL activity at the site of inflammation.

Next, we analyzed the populations of IFN-γ, IL-4, IL-17, and TNFα secreting CD4+ and CD8+ T cells. Intriguingly, we detected that while wild-type mice developed (IFNγ+ and STAT-3-activating) Th1 responses, in IFITM3−/− mice the pathogenic (IL17+) Th17-dominated pathway was evoked (Figure 2f and g). We also showed that the Th2 response was not involved in our model, as no significant expression of IL4 by CD4+ T cells was detected in all groups (data not shown).

To determine whether altered T cells cytokine secretion in IFITM-deficient mice is also reflected in characteristic cytokine responses, we investigated the expression pattern of Th17-associated cytokines. The development of Th17 cells in mice requires signaling via the TCR in combination with signaling from TGFβ, IL1β and a STAT-3-activating cytokine, such as IL6. We further examined the expression of these cytokines, in the different groups with the progression of the colitis. RNA was isolated from intestines on different days of DSS-induced inflammation and cytokine expression by quantitative RT-PCR was measured. As shown in Figure 3a, TGFβ was specifically upregulated in IFITM3−/− mice, but not in the WT group. IL1β and IL6 were upregulated in all groups along the progression of the colitis; however, the elevation in cytokines levels, from day 0 to 8, was significant only in IFITM3 depleted animals (Figure 3b and c). TNFα, another proinflammatory cytokine was also upregulated substantially in IFITM3-deficient mice (Figure 3d). Remarkably, while WT mice significantly increased the expression of the anti-inflammatory IL10 cytokine, IFITM3+/− and −/− mice maintained low levels of its expression (Figure 3e). Moreover, IFITM3-deficient mice expressed relatively increased levels of IL27p28 cytokine at colitis onset (day 0) compared to WT. These expression levels were reduced in IFITM3-deficient mice with colitis progression (day 8), whereas WT mice showed the opposite trend (Figure 3f).

Overall, these findings suggest that while in WT mice, the induction of intestinal inflammation drove the expression of Th1 cytokines, in IFITM3-deficient mice altered cytokine profile promoted the development of pathogenic Th17 response.

The microbiota mediates exacerbation in disease severity in IFITM3−/− mice

Intestinal microbiota plays an essential role in shaping host immune responses in steady-state and disease. However, shifts in microbiota composition can lead to dysbiosis and result in severe intestinal disease. Germ-free mice were documented to harbor a deficient immune response, particularly in impaired generation and maturation of gut-associated lymphoid tissues. Specifically, microbiota may promote the development of Th17 cells and prompt DCs and macrophages to secrete pro-inflammatory cytokines upon pathogenic insult. Since IFITM3-deficient mice developed severe inflammation upon DSS administration with high percentages of macrophages and IL17-secreting T cells, we hypothesized that microbiota may be directly involved in this phenomenon. The IFITM3 protein was shown to restrict several pathogenic viruses that enter host cell by fusing with its membranes or late endosome or lysosome. There is a possibility that this inhibitory activity is not restricted only to viral, but also to bacterial or fungal pathogens. An additional explanation suggests an indirect microflora-mediated effect that may occur as a result of altered virus inhibition in IFITM3-deficient colons, in line with studies that describe synergistic interactions between bacteria and viruses in disease.

To test this hypothesis, mice were treated with a mixture of antibiotics (Abx) (vancomycin, neomycin, metronidazole and ampicillin) or left untreated prior to DSS administration, as described in the Methods. Ten days following the onset of DSS application colitis was assessed. As shown in Figure 4a and b, treatment with antibiotics resulted in amelioration of colitis in both Abx-treated WT and IFITM3−/− mice, compared to control mice. However, only in IFITM3−/− mice this...
amplification reached statistical significance. This indicates possible alterations in intestinal microbiota of IFITM3−/− mice, affecting their immune responses to challenges such as breakdown of the epithelial barrier.

IFITM genes mediate distinct microbial composition

We next examined the fecal microbiota composition of the different groups by sequencing of the 16S ribosomal RNA gene. We collected fecal samples from IFITM3 and IFITMdel (WT, +/+ or −/−) mice before treatment (naïve), 5 days post-DSS treatment (IFITM3+/− only) and 21 days-post antibiotics treatment (WT and IFITM3+/−). DNA was prepared and each sample was barcoded separately.

We acquired an average of ~44 800 reads per sample and obtained 1597 operational taxonomic units (OTUs). Then multivariable analysis of the fecal microbiota was performed. IFITM3+/− and −/− mice showed similar microbiome configuration clustered separately from WT group (Figure 4c and d). Likewise, IFITMdel+/− group clustered separately from IFITMdel−/− and WT mice (Figure 4e and f). More than 100 OTUs were significantly altered in abundance (false discovery rate corrected P < 0.05 for each OTU) between the different genotypes in each strain (data not shown). Moreover, naïve +/+ and −/− mice, from IFITMdel strain showed statistically significant increased species richness in their stool compared to their WT littermates (Figure 4g and h). Naïve IFITM3+/− and −/− mice sowed similar trend, but did not reach statistical significance (Figure 4i and j).

Taxon-based analysis was performed separately for IFITM3 and IFITMdel naïve mice. It revealed that for IFITM3 strain, 18 families and 15 genera were significantly changed in abundance between the WT, +/+ and −/− littermates (Supplementary figure 3A, Figure 5a). When compared to WT mice, 11 genera were significantly increased (Prevotella, Dorea, Odoribacter, Parabacteroides, Turicibacter, uncultured Erysipelotrichaceae, uncultured Coriobacteriaceae, Anaeroplasma, AF12, Anaerofustis, Christensenella), and 3 genera were significantly reduced (Bacteroides, Desulfovibrionaceae, Enterococcus) in the IFITM3−/− littermates. For IFITMdel strain 17 families and genera were changed in abundance between the littermates (Supplementary figure 3B, Figure 5b).

When compared to WT mice, 13 genera were significantly increased (Mucispirillum, Prevotella, Anaeroplasma, Ruminococcus, Odoribacter, Anaerostipes, Desulfovibrionaceae, Clostridiomycetes, Coprobacillus, Christensenella, Anaerofustis, uncultured Erysipelotrichaceae and Desulfovibrionaceae) and 4 genera were significantly reduced (Bacteroides, Parabacteroides, Bilophila, Staphylococcus) in the IFITMdel−/− littermates.

To evaluate the effect of antibiotics or of DSS treatment on the bacterial composition, we plotted P-value as a function of fold of change. The horizontal axis indicates the fold representation (defined as the log2 of the ratio of the percentage of genera present in IFITM3+/− mice treated with antibiotics or with DSS vs. naïve IFITM3−/− mice). The left side of the axis indicates taxa whose representation is greater in naïve mice; the right denotes
taxa whose representation is greater in treated mice. The origin represents equivalent recovery of taxa in both groups. The vertical axis represents the minus log10 of the P-value calculated for each taxon as defined by G-test. This representation allows identification of specific taxa that have significantly changed. As expected, treatment with antibiotics resulted in a substantial reduction in bacterial diversity, and survival of strains resistant for this treatment. Moreover, treatment with DSS resulted in diversity reduction as well (data not shown).

IFITM3 reduces tumorigenesis in colitis-associated colon cancer model

Colorectal cancer (CRC) may develop sporadically or as a complication of inflammatory bowel disease (IBD).25 Thus, we next sought to assess the role of IFITM3 gene in colitis-driven tumorigenesis. Azoxy methane (AOM), a chemical agent that has been most successfully used in rodent models of CRC, initiates cancer by DNA alkylation.26 Combined administration of AOM and

Figure 4. Deterioration of colitis in IFITM3−/− mice is mediated by the microbiota. (a) Mice were administered drinking water containing ampicillin, vancomycin, neomycin and metronidazole (Abx) 3 weeks prior to treatment with 2% DSS (for another 7 days). Summary of endoscopic colitis grades assessed on day 10 following DSS administration is presented. (b) Weight loss relative to initial weight following DSS challenge in mice shown in a. (c) Principal coordinates analysis (PCoA) of unweighted UniFrac distances based on 16S rRNA analysis collected from feces of naïve IFITM3 WT, +/- or +/- mice. (d) One-way ANOVA with Bonferroni post-test statistical analysis of unweighted unifrac distances presented in panel c. Significance was achieved between WT and IFITM3−/− mice. (e) PCoA of unweighted UniFrac distances based on 16S rRNA analysis collected from feces of naïve IFITMdel WT, +/- or +/- mice. (f) One-way ANOVA with Bonferroni post-test of unweighted unifrac distances presented in panel e. Significance was achieved only between WT and IFITMdel−/− mice. Bacterial richness is presented by number of observed species (g) and i) or by Shannon index (h) and j) for naïve IFITMdel mice (g and h), and for naïve IFITM3 mice (i and j), at the depth of 8312 and 7856 reads for each strain, respectively. In c and e, each symbol represents one mouse. DSS, dextran sodium sulfate; IFITM, interferon inducible transmembrane.
repeated cycles of DSS treatment (AOM/DSS), mimic human inflammation-derived CRC.\textsuperscript{27,28} We induced CRC by administration of 7.5 mg kg\textsuperscript{1} AOM combined with two subsequent DSS cycles, separated by 14 days of regular water. Similarly to the data obtained from the chronic colitis model, we detected a significant weight reduction (data not shown) and increased clinical signs of inflammation (Figure 6a) 54 days after the onset of the experiment. Tumors induced by AOM/DSS treatment, were expected to be detectable by 10th week from the onset of the experiment.\textsuperscript{28} However, in IFITM\textsuperscript{+/-} and \textsuperscript{-/-} groups we were able to detect polyps by means of endoscopy already by day 19, and tumors by day 38. Depletion of IFITM3 resulted in increased tumorigenesis as was shown by a significantly elevated tumor scoring on days 38 and 54 (Figure 6b and c). In line with the notion that AOM-induced tumors often lack mucosal invasiveness,\textsuperscript{29} we did not detected tumor invasion in WT mice. However, in IFITM3-deficient mice we found multiple foci of invasive adenocarcinomas (Figure 6d).

Tumor scoring assembles number of tumors and their size. Thus, to assess how IFITM3 gene affects tumorigenicity, we examined the amount of tumors sized below and above 25% of the luminal diameter. As shown in Figure 6e, IFITM3 gene strongly affects tumor incidence, as we detected an increase in the amount of tumors sized below 25% of the luminal diameter, whereas no difference was observed in tumors sized above 25%. These data demonstrate that the IFITM3 gene functions to attenuate inflammation and colitis-derived tumorigenesis, by reducing tumor formation.

DSS induces damage to the epithelial barrier, and upon recovery a wound healing process that includes increased crypt epithelial cell proliferation occurs. IFITM2, an IFITM3 homolog, was found to induce apoptosis.\textsuperscript{10} Moreover, IFITM1, another IFITM family member, was

### Figure 5
Dominant indicator phylotypes for IFITM3 and IFITMdel strains. Taxon based analysis at the genus level among WT, +/– and –/– genotypes of IFITM3 (a) and IFITMdel (b) strains. All the presented taxa are statistically significant in the change in abundance, as was determined by one-way ANOVA followed by Kruskal–Wallis Test. *unclassified genus of the designated family. Each column represents a sample from a single mouse as described in Figure 4. IFITM, interferon inducible transmembrane.
Figure 6. IFITM3-deficient mice are highly susceptible to colitis-associated CRC. Chronic colitis-associated tumorigenesis was induced by injection with 7.5 mg kg\(^{-1}\) azoxymethane and two subsequent cycles of 2% and 1.5% of DSS separated by 2-week administration of regular water. (a) Endoscopic colitis grades assessed on day 54 after CRC initiation. (b) Endoscopic tumor grades assessed on indicated days after CRC initiation. (c) Representative images taken during colonoscopy of mice at day 54. (d) Representative histopathologic sections of colon adenocarcinomas (upper panel, bars: 500 μm) and invasive tumor foci (lower panel, arrows, bars: 100 μm). (e) Number of tumors above and below 25% of luminal diameter on day 54. (f) Quantitative analysis of Ki67-positive cells per total nuclei in colons of mice 36 days after CRC initiation as was quantified using imaging analysis software (ImageJ). (g) Immunofluorescence labeling for Ki67 (red) in colons 36 days after CRC initiation. Cells nuclei were stained with Dapi (blue) (x20 magnification, a merged picture is shown). Data presented in a–e are summaries of three individual experiments, \(n = 16-20\) for each group. For data presented in f, g \(n = 4-6\) for each group in two independent experiments. *\(p < 0.05\), **\(p < 0.01\), ***\(p < 0.001\). CRC, colorectal cancer; DSS, dextran sodium sulfate; IFITM, interferon inducible transmembrane.
shown to mediate the antiproliferative actions of IFNγ. We detected increased tumorigenesis in IFITM3+/− and −/− mice following treatment with DSS/AOM. To test whether this increased susceptibility to tumor initiation stems from increased proliferation, we assessed Ki67 incorporation by immunohistochemistry. Indeed, IFITM3-deficient mice showed increased Ki67 staining 36 day following AOM/DSS initiation (Figure 6f and g).

DISCUSSION

In this study, we demonstrate that IFITM genes are essential for maintaining intestinal homeostasis. Specifically, IFITM3 gene is shown to play critical role in ameliorating experimental colitis and preventing inflammation-associated tumorigenesis.

Surprisingly, the susceptibility to acute and chronic DSS-induced colitis as well as to AOM/DSS carcinogenesis was shared by both IFITM3+/− and −/− genotypes. This phenomenon is further supported by reduced IFITM3 expression by IFITM3+/− mice upon DSS administration, as opposed to WT mice. Although IFITM3 heterozygous mice carry one wild-type allele, it seems that this allele is non-functional or untranslated. Epigenetic modifications known to play essential roles in intestinal immunity and mucosal homeostasis. Genomic imprinting is an epigenetic event that occurs during embryogenesis and results in genes that are expressed from only one parental allele. Remarkably, above 50 percent of imprinted genes in mouse, are situated on chromosome 7 and clustered into five domains. One of these imprinted domains is located in high proximity to IFITM locus on the chromosome. Although it has never been suggested before, it is possible that IFITM3 gene undergo this form of monoallelic expression. This might be the reason that heterozygous mice show very similar phenotype to knockout mice, yet more experiments are required to further demonstrate this. Intriguingly, unlike IFITM3+/− mice that share the phenotype with IFITM3−/− mice, IFITMdel+/− mice develop milder spontaneous colitis relatively to IFITMdel−/− animals indicating other IFITM genes do not undergo allelic exclusion.

Next, BM transplantation experiments showed that hematopoietic cells are mainly responsible for colitis deterioration in IFITM3-deficient mice. IFITM1, IFITM2 and IFITM3 are expressed by a wide variety of immune cells, including bone marrow-derived DCs, macrophages, and mucosal mast cells. IFITM6 expression was specifically induced in BM-derived and splenic macrophages of tumor-bearing mice. In another recently published study it was shown that microglia and BM-derived macrophages extracted from IFITMdel mice exhibited impaired morphology and intensively infiltrated choroid plexus upon Poly I:C treatment. Based upon these data we studied the expression of these cells in the intestine of mice during DSS-colitis. Induction of colitis resulted in macrophage expansion in the colons of all the mice, with the most substantial accumulation in IFITM3-deficient mice. In addition, an increase in GFP+ macrophages and DCs in IFITM3−/− mice was detected with colitis progression, suggesting specific induction of the IFITM3 promoter. This may indicate that these cells play a role in deterioration of inflammation in IFITM3-deficient mice.

Intestinal CD4+ T cells are essential mediators of immune homeostasis. DCs generate primary T-cell responses and mediate immune tolerance to prevent inflammation in response to gut microflora. However, in the IBD-associated immune response, CD4+ T cells accumulate and mediate tissue injury. Following DSS administration, IFITM3−/− mice displayed a substantial increase in the expansion of effector CD4+ and CD8+ T cells, with the progression of the disease. Moreover, IFITM3 might be involved directly or indirectly in the development of Th1 responses, as CD4+ T cells from IFITM3−/− mice, secreted mainly IL17. Interleukin-17 (IL17) is a proinflammatory cytokine that mediates the pathogenesis of several inflammatory disorders. Th17 cells are considered as the major source for IL17 production. Combined signaling of TGFβ, IL1β and IL6 was shown to induce the retinoid-related orphan receptor-α (RORα) and RORγt transcription factors, which are required for the development of Th17 responses. On the contrary, IL27p28 cytokine was shown to reduce inflammation by suppressing differentiation of Th17 and enhancing the development of IL10 producing anti-inflammatory T cells. The anti-inflammatory cytokine IL10 is essential for maintaining intestinal homeostasis and tolerance toward commensal flora. Depletion of IL10 or its receptor leads to severe colitogenic phenotype in mice and humans. Induction of colitis by DSS resulted in substantial elevation in expression of pro-inflammatory cytokines in IFITM3-deficient animals. Intriguingly, in IFITM3+/− and −/− mice the expression of the anti-inflammatory IL10 and IL27p28 cytokines was unaffected and downregulated, respectively. However, WT mice induced the expression of these cytokines, with the progression of the disease, indicative of induction of recurrence phase. Impaired IL10 and IL27 production may cause the development of the more severe manifestations of colitis, as was shown in IFITM3-deficient animals. It is likely that in these mice, insufficient expression of IL27 subsequently reduces the expression of IL10, which skews the inflammatory response towards Th17, and results in colitis deterioration.

Microbiota consisting of bacteria, fungi, parasites and viruses, inhabit mucosal surfaces of the gastrointestinal tract. It is essential for host defense against pathogens.
and inflammatory disorders as well as for digestion of nutrients and production of vitamins.

Intestinal microbiota co-evolved with the host in a symbiotic relationship. Hence, it promotes tolerance of macrophages and DCs towards the commensal flora. However, upon homeostasis breakdown, these cells are triggered by the microbiota to secrete pro-inflammatory cytokines and elicit immune response.\(^1\) The development of Th17 responses were shown to depend on the microbiota as germ-free or antibiotic-treated mice have significantly reduced Th17 intestinal population.\(^2\) In particular, segmented filamentous bacteria (SFB) was shown to promote Th17 generation in mice.\(^3\) In agreement with these evidences and due to the notion that IFITM3 is a potent viral inhibitor, we have investigated the contribution of microbiota to the tendency of IFITM3-deficient mice to develop colitis. Antibiotic administration induces dramatic and persistent perturbations to the intestinal microbiota and affects immune defense against pathogens. As intestinal microbial populations are symbiotic, treatment with antibiotics specific for one class of bacteria may indirectly deplete antibiotic-resistant bacteria.\(^4\) We have adapted a combined regime of antibiotics, that depletes intestinal microbiota and affects several aspects of immune response.\(^5\) This treatment resulted in amelioration of colitis in IFITM3\(^{−/−}\) mice compared to IFITM3\(^{+/−}\) control mice. This hints to potential alterations in intestinal microbiota of IFITM3\(^{−/−}\) mice, affecting their immune response to challenges such as breakdown of the epithelial barrier. Further experiments such as fecal microbiota transplantation into germ-free mice are needed to verify this hypothesis.

To further analyze the intestinal microbial diversion in IFITM3 and IFITMdel mice, we performed in-depth 16S rRNA sequencing. We identified significant perturbations in the variety of commensal microflora in IFITM3-deficient (\(^{+/−}\) and \(^{−/−}\)) mice even without a challenge. Similarly, mice deficient of the entire IFITM locus (IFITMdel\(^{−/−}\)) exhibited substantially altered microbial composition and density, when compared to their heterozygous and WT littermates. These alterations may cause compromised homeostasis and potentiate their carriers to severe and chronic inflammation upon epithelial or mucosal insult. Remarkably, bacterial communities of IFITM3\(^{+/−}\) and \(^{−/−}\) mice clustered together, separately from microbiota of WT littermates, in correlation with their susceptibility to develop more severe DSS-colitis. Likewise, IFITMdel\(^{−/−}\) mice that carry substantially diverged bacterial component, develop spontaneous colitis at greater severity than IFITMdel\(^{+/−}\) or WT littermates.

We found differences in the representation of the genus *Prevotella* within the *Prevotellaceae* family, showing significant increase in abundance in IFITM3-deficient and IFITMdel\(^{−/−}\) mice. Expended representation of *Prevotellaceae* was already described in murine colitis models and in IBD patients.\(^6\) Enzymes from *Prevotella* were found to be involved in mucin oligosaccharide degradation.\(^7\) The genus *Mucispirillum* within *Deferribacteraceae* family, another putative mucin degrader.\(^8\) was found in significantly greater amounts in IFITMdel\(^{−/−}\) mice. Mucus layer covers the intestinal surface and protects the underlying epithelium against pathogenic agents. Under steady state conditions mucus is constantly degraded and replenished. Yet once this natural procedure disturbed, increased sensitivity to spontaneous or chemically induced colitis occurs.\(^9\) A reduction in mucus layer thickness that caused increased exposure of the microbes to the gut immune system was demonstrated in IBD patients.\(^10\) Hence, we may speculate that increased prevalence of *Prevotella* and *Mucispirillum* may affect mucus layer integrity and increase sensitivity to DSS-colitis or induce spontaneous colitis. Increased *Ruminococcus gnavus* was found in fecal microbiota of CD patients, indicating on a role of this bacteria in the onset or the perpetuation of IBD.\(^11\) We also have noted a significant induction in *Ruminococcaceae* family, and the genus *Ruminococcus*, in stools of IFITMdel\(^{−/−}\) mice. On the contrary, *Bacteroides*, that is abundant in mucosa of UC patients,\(^12\) was decreased in feces from IFITM3-deficient and IFITMdel\(^{−/−}\) mice. Overall, these findings suggest a potential dysbiosis that may affect the epithelium and the immune cells within intestine, and result eventually in colitogenic phenotype of IFITM3- and IFITMdel-deficient mice. However, further extensive study, and additional experiments are required to determine a causative association between microbiota and colitis predisposition in our model.

Inflammation may contribute to carcinogenesis through several mechanisms, among them are genotoxicity, aberrant tissue repair, proliferation and survival signaling.\(^13\) Intriguingly, microbial organisms are implicated in about 20% of human malignancies, and pathogens and commensal pathobions have recently been recognized as being involved in inflammatory processes that promote tumor growth.\(^14\) Inflammatory bowel diseases are associated with a high risk of colon carcinogenesis. As IFITM genes are overexpressed in colon tumors, and IFITM-deficient mice suffer from susceptibility to DSS-induced colitis and microbiota perturbations, we examined whether these mice are also prone to inflammation-induced carcinogenesis. Subjecting IFITM3-depleted animals to AOM/DSS resulted in increased tumorigenesis. Tumors from IFITM3\(^{+/−}\) and \(^{−/−}\) mice invaded into the mucosa and differentiated to adenocarcinomas. IFITM3 gene was shown to mainly affect tumor incidence rather than tumor progression. Moreover, absence of IFITM3 increases epithelial cell proliferation following AOM/DSS treatment. Greater epithelial growth may stem from exposure to altered...
microbial composition, elevated expression and secretion of proinflammatory cytokines and impaired immune response with intensive infiltration of effector T cells and macrophages.

In summary, we have shown that IFITM genes participate in gene-microbiota–immune response interactions, which mediate intestinal homeostasis. Further studies of the precise biological mechanisms for IFITMs actions, will provide additional knowledge concerning inflammation and inflammation-associated carcinogenesis processes.

METHODS

Animals

IFITM3 knockout mice were generated by insertion of EGFP to exon 1 of the IFITM3 gene, abolishing its expression, as described previously. IFITMdel mice were created by a deletion of a 120-kb region on Chr7, harboring the entire Ifitm family locus but no other known gene. Mice were a generous gift of The European Mouse Mutant Archive (EMMA). For all experiments age- and sex-matched, generous gift of The European Mouse Mutant Archive (EMMA). For all experiments age- and sex-matched, generous gift of The European Mouse Mutant Archive (EMMA). For all experiments age- and sex-matched, generous gift of The European Mouse Mutant Archive (EMMA). For all experiments age- and sex-matched.

Induction of colitis and colitis-associated cancer

Acute colitis was induced by administration of 2% Dextran sodium sulfate (DSS) (MP Biomedicals, molecular mass 36 000–50 000 Da) in the drinking water for 7 days, followed by regular water. For chronic colitis induction, mice were given three successive cycles of 2% of DSS dissolved in their drinking water, separated by 14 days administration of regular water. Colitis-associated cancer was induced by intraperitoneal injection of AOM (Sigma-Aldrich, Rehovot, Israel) at a dose of 7.5 mg kg⁻¹ body weight. On the same day (day 1) mice received 2% DSS in the drinking water for 7 days, followed by 14 days of regular water. Subsequently, mice received 1.5% DSS for 6 days in the second cycle.

Endoscopic clinical scoring

Colonoscopy was performed in a blinded fashion for colitis and tumor monitoring, using a high resolution mouse video endoscopic system. It consists of a miniature endoscope (scope 1.9 mm outer diameter), a xenon light source, a triple chip camera and an air pump to achieve regulated inflation of the mouse bowel (Karl Storz, Tuttlingen, Germany). The endoscopic procedure was viewed on a color monitor and digitally recorded. The colonoscopy allowed for real-time evaluation of 3–4 cm of colon from the anal verge to the splenic flexure. Colitis scoring was based on stool consistence, vascular pattern, granularity of mucosal surface, transparency of the colon and appearance of fibrin (0–3 points for each). Colitis score is a summation of individual scores. Tumor grading was calculated according to tumor size relative to the circumference of the colon as established previously.

Generation of bone marrow chimeras

For bone marrow chimera (BM) generation, recipient mice (host) were lethally irradiated with two doses of 450 and 500 rads, with 3 h interval between the two irradiations. A day later, mice were intravenously injected with 2 × 10⁶ BM cells/0.2 mL PBS, isolated from donor’s femurs and tibiae. BM recipients were maintained on antibiotics (Ciprofloxacin 20 μg mL⁻¹, Bayer) for 3 weeks and were allowed to rest for another 3 weeks before use.

Intestinal cell isolation and flow cytometric analysis

The method for isolation of hematopoietic cells from the intestine was modifed from protocol established previously. Briefly, the colon was isolated, washed with PBS and cut longitudinally to 5 mm pieces. Colon pieces were incubated with HBSS (without Ca²⁺ and Mg²⁺) containing 5% FBS, 2 mmol L⁻¹ EDTA and 0.15 mg mL⁻¹ (1 mmol L⁻¹) DTT (Sigma-Aldrich) at 37°C shaking at 250 rpm. The supernatant contains epithelial cells and intra-epithelial lymphocytes. The remaining colon was digested in PBS¹/₄ containing 5% FBS, 1 mg mL⁻¹ Collagenase VIII (Sigma-Aldrich), and 0.1 mg mL⁻¹ DNase I (Roche) at 37°C shaking at 250 rpm. The digested cell suspension was then washed with PBS and passed sequentially through 100 and 40 μm cell strainers to collect lamina propria lymphocytes. For T cells analysis, a mix of intra-epithelial lymphocytes and lamina propria lymphocytes was further subjected to separation by Percoll (GE Healthcare) density gradient and further stained with appropriate antibodies: CD4, CD8, CD3, CD44, CD62L (eBioscience). For intracellular staining cells (1 × 10⁶) were incubated for 6 h at 37°C, 5% CO₂ in lymphocyte medium supplemented with 50 ng mL⁻¹ of phorbol 12-myristate 13-acetate (Sigma-Aldrich) and 750 ng mL⁻¹ ionomycin (Sigma-Aldrich). Brefeldin A (GolgiPlug, 1 μg mL⁻¹, BD) was added for the last 4 h. Then, cells were harvested, suspended in staining buffer, and blocked with anti-CD16/32 (eBioscience). Anti-CD4 or CD8 antibodies (eBioscience) were added, followed by washing and fixation with 4% paraformaldehyde for 20 min at 4°C. Furthermore, cells were permeabilized in PBS containing 5% FCS, 0.1% saponin (Sigma-Aldrich) and 0.1% sodium azide for 15 min at 4°C and incubated with anti-IFN-γ, IL17 or IL4 (eBioscience) for 30 min. For analysis of myeloid cells, lamina propria lymphocytes fraction was further stained with following antibodies: CD45, CD45.2, Ly6C, CD11c, CD11b, IAb, CD64 (all from BioLegend), CD103 (M290) (BD Bioscience) and F4/80 (CLeA-3-1) (Serotec). All samples were acquired and electronically compensated on LSR II (BD) and exported for analysis with FlowJo (Tree Star).
Quantitative real-time PCR

Total RNA was extracted from colon tissue using RNeasy mini kit (QIAGEN). cDNA was synthesized from 1 µg of total RNA using SuperScript® II Reverse Transcriptase (Invitrogen). Relative gene expression was detected by performing quantitative Real-Time PCR with Platinum SYBR Green (Invitrogen) on an ABI 7300 instrument (Applied Biosystems). Quantification of the PCR signals of each sample was performed by comparing the cycle threshold values (Ct), in duplicates, of the gene of interest with the Ct values of the TBP housekeeping gene. Primers sequences are listed in Supplementary table 1.

Histological examination and immunohistochemistry

Colons were fixed in 4% PFA and embedded in paraffin. Sections of 10 mm were stained with hematoxylin and eosin (H&E) using standard procedures. For immunofluorescence staining, cryosections of 10 mm were fixed in ice-cold acetone for 10 min, blocked in PBST containing 5% normal horse serum for 1 hr at room temperature and incubated overnight with primary antibodies. Next, sections were incubated with labeled secondary antibodies, and finally incubated with Hoechst (Molecular Probes) to visualize nuclei. Primary antibodies were anti-Ki67 (5 µg mL⁻¹, Abcam, Cambridge, UK) and anti-IFITM3 (10 µg mL⁻¹, R&D systems, Minneapolis, MN, USA).

Combined antibiotics regime

Mice were administered drinking water containing ampicillin (1 g L⁻¹, Sigma-Aldrich), vancomycin (500 mg L⁻¹, Mylan, Paris, France), neomycin sulfate (1 g L⁻¹, Sigma-Aldrich) and metronidazole (1 g L⁻¹, Sigma-Aldrich) 3 weeks prior to administration of 2% DSS. After administration of antibiotics for 3 weeks, mice rested 3 days prior to beginning of the DSS regime. Control mice received drinking water without antibiotics.

Microbiota analysis by 16S rRNA gene sequencing

Feces were collected from mice and DNA was isolated using PowerSoil DNA Isolation Kit (Mo Bio Laboratories, Solana Beech, CA, USA). For 16S amplicon pyrosequencing, PCR amplification was performed spanning the V3/4 region using the primers 515F/806R of the 16S rRNA gene and subsequently sequenced using 500 bp paired-end sequencing (Illumina MiSeq, San Diego, CA, USA). Reads were then processed using the QIIME (quantitative insights into microbial ecology) analysis pipeline with USEARCH against the Greengenes database as described elsewhere.46,50

Statistical analysis

Data are presented as the mean ± s.e.m. Analysis of variance was performed by one-way ANOVA followed by Tukey-Kramer HSD for multiple comparison tests for both individual experiments and composite data. Statistical significance for single data points were assessed by the Student’s two-tailed t-test. Survival curves were generated using the product limit method of Kaplan and Meier, and comparisons were made using the log-rank test. All analyses were performed using Prism 7 (GraphPad Software). In all cases, a P < 0.05 was considered statistically significant.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

REFERENCES