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Graphical abstract

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Gut epithelial TSC1/mTOR controls RIPK3-dependent necroptosis in intestinal inflammation and cancer

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Abstract

While the Western-diet and dysbiosis are the most prominent environmental factors associated with inflammatory bowel diseases (IBDs), the corresponding host factors and cellular mechanisms remain poorly defined. Here we report that the TSC1-mTOR pathway in the gut epithelium represents a metabolic and innate immune checkpoint for intestinal dysfunction and inflammation. mTOR hyperactivation triggered by the Western-diet or Tsc1-ablation led to epithelium necroptosis, barrier disruption, and predisposition to DSS (dextran sulfate sodium)-induced colitis and inflammation-associated colon cancer. Mechanistically, our results uncovered a critical role for TSC1-mTOR in restraining the expression and activation of RIPK3 in the gut epithelium through Trim11-mediated ubiquitination and autophagy-dependent degradation. Notably, microbiota-depletion by antibiotics or gnotobiotics attenuated RIPK3 expression and activation, thereby alleviating epithelial necroptosis and colitis driven by mTOR hyperactivation. mTOR primarily impinged on RIPK3 to potentiate TNF- and microbial PAMP-induced necroptosis, and hyperactive mTOR and aberrant necroptosis were intertwined in human IBDs. Together, our data reveal a previously unsuspected link between the Western-diet, microbiota and necroptosis, and identify the mTOR-RIPK3-necroptosis axis as a driving force for intestinal inflammation and cancer.
**Introduction**

Inflammatory bowel disease (IBD) composed of Crohn’s disease (CD) and ulcerative colitis (UC) are chronic and relapsing inflammatory disorders manifested in the gastrointestinal (GI) tract (1, 2). Despite a continuous increase in IBD incidence worldwide, the complex molecular and cellular mechanisms underlying IBD pathogenesis remain incompletely understood and the clinical treatments for IBDs are unsatisfactory (3-5). To date, metagenomic analysis on IBD patients has identified over 200 genetic risk loci involving a broad spectrum of physiological processes, namely innate immune recognition, adaptive immunity, autophagy and ER stress (2, 6). Consistent with the importance of maintaining “microbial-tolerance” in the gut, a diversity of innate receptor pathways, such as TLRs, NLRs and Dectins, have been linked to IBD pathogenesis (6). Moreover, the identification of *ATG16L1* and *IRGM* as IBD susceptible genes has led to the discovery of autophagy as an integral component of intestinal homeostasis and inflammation (7-9). Indeed, autophagy is involved in the secretion of anti-microbial peptides and resolution of ER stress in Paneth cells (7), as well as the control of inflammasome activation in enterocytes (8, 9). Nevertheless, genetic risk factors only account for a small fraction of IBD incidence (i.e., 20-40% for CD and 10% for UC) (3). Recently, environmental factors, such as the Western-diet enriched with protein/fat and microbiota dysregulation have been considered as major contributors to the ever-increasing IBD incidence (2-5). Hence, it is widely believed that the etiology of IBD involves the complex interplay between host factors, microbiota and dietary factors (2, 10). However, the underlying molecular and cellular components bridging these multi-layered interactions remain largely elusive.

The intestinal epithelium comprises functionally distinct cell types such as absorptive enterocytes, secretory goblet cells and Paneth cells, which together act as a physical barrier separating the microbiota in the lumen from the immune cells residing in the laminar propria (4, 5). Recently, the regulated necrotic cell death, namely necroptosis,
has been implicated in the regulation of intestinal homeostasis (11-13). A number of pro-inflammatory and pro-death signals, such as TNF, IFNs, and PAMPs, are capable of triggering necroptosis, especially when caspase-8 is inactivated (13, 14). The initiation of necroptosis involves the activation of protein kinases RIPK1 and RIPK3, which subsequently phosphorylate the executioner molecule MLKL to induce cell membrane rupture (13, 15-18). Often causing the release of a myriad of DAMPs, such as IL-1α, IL-1β and HMGB1, necroptosis has been regarded as a highly proinflammatory process that requires stringent control (13-15). Indeed, a number of negative regulators such as caspase-8, FADD, A20, CHIP, c-FLIP, MK2, TBK1 and TAK1 have been implicated in the control of necroptosis (19-27). Remarkably, genetic ablation of either Casp8 or Fadd in intestinal epithelial cells (IECs) is sufficient to induce necroptosis and spontaneous ileitis and/or colitis (19, 28). Moreover, aberrant RIPK3 activation and necrotic cell death have also been implicated in pediatric and adult IBDs (19, 29). While the aforementioned studies indicate a causative role for RIPK3 and necroptosis in IBD, a protective role for RIPK3 in colitis has also emerged (30, 31). However, in the latter scenario, the anti-inflammatory function of RIPK3 was restricted to the mononuclear phagocytes (MNPs), which contrasts with its pro-necroptotic function in the IECs (30-32). Therefore, the role of RIPK3 and necroptosis in IBD pathogenesis remains under debate (33, 34) and further studies are required to clarify its cell type-specific roles in intestinal homeostasis and inflammation.

mTOR plays crucial roles in cell growth/proliferation, development, immunity and tumorigenesis in response to a diversity of extracellular and intracellular signals, including glucose, amino acids, cytokines and PAMPs (35-38). Growth factors, cytokines and TLR ligands trigger mTOR activation primarily through the phosphorylation and inactivation of the repressor TSC1/TSC2 by a plethora of upstream protein kinases, such as PI3K/AKT and ERK (36, 37, 39). In contrast, nutrients such as amino acids induce mTOR activation mainly through the heteromeric GTPase Rags (32, 36). Once activated, mTOR (herein referred to as
mTORC1) functions through a myriad of downstream effectors to regulate diverse physiological processes, such as anabolism, autophagy, cell growth and proliferation (36, 37). Under cell growth and rapid proliferation, mTOR is required to promote the phosphorylation of translational regulator 4E-BP1, as well as ribosome protein S6, both of which are vital regulators of protein synthesis. Moreover, under nutrient-sufficient conditions, mTOR can contribute to autophagy inhibition through phosphorylating and inactivating the autophagy initiator ULK1 (40). Further, the emerging evidence has also begun to link mTOR to intestinal homeostasis and tumorigenesis (41-45). Genetic ablation of mTOR in IECs revealed a critical role for mTOR in the regulation of caloric restriction (41), Paneth/goblet cell development (42, 44), and proliferation of transformed epithelium (43). Given the widespread association between mTOR dysregulation, chronic inflammation and colon cancer, it becomes imperative to understand whether and how environmental factors might influence mTOR activation in the pathogenesis of IBD and colon cancer.

Breakdown of ingested nutrients, such as proteins, fatty acids, and carbohydrates in the GI tract, generates a variety of intermediary macromolecules, which can be further processed by microbiota (4, 10). Together, diet and microbiota constitute the most potent environmental factors profoundly shaping epithelium homeostasis and mucosal immunity (5, 46, 47). As such, the Western-diet and dysbiosis have been widely linked to IBD, albeit the corresponding host factors and cellular mechanisms are still poorly understood. Here we report that the epithelial TSC1-mTOR pathway is instrumental in the regulation of intestinal homeostasis, inflammation and tumorigenesis. Our results further reveal a novel role for mTOR in the regulation of RIPK3 expression and necroptosis in the gut epithelium, a process profoundly shaped by the Western-diet and microbiota. Overall, our findings support the emerging notion that epithelial necroptosis is a risk factor for intestinal inflammation and cancer, and provide new insight into the host response to dietary/microbial cues in the gut.
Results

The Western-diet promotes mTOR hyperactivation and necroptosis in IECs

The Western-diet is regarded as a major risk factor for IBD, yet how the Western-diet enriched with high-protein/high-fat influence intestinal homeostasis and inflammation remains poorly understood. To this end, we fed mice with the Western-diet containing either high-protein (60% protein) or high-fat (60%) for 2 weeks, followed by DSS (dextran sulfate sodium, M.W. 35-50kD) treatment to induce intestinal inflammation. Compared to the mice fed with a regular diet, high-protein- and high-fat-fed mice suffered severer colonic inflammation, as demonstrated by the pronounced induction of proinflammatory genes (such as Tnf, Il1b, Il6, Cxcl1, Ccl2, and Ccl5), extensive ulceration, and heightened clinic symptoms in the colon in response to DSS treatment (Supplementary Figure 1, A-C). The mice fed with a high-protein or high-fat diet also displayed a high degree of morbidity and mortality (Supplementary Figure 1D and Figure 1, A and B). Moreover, prominent TUNEL-positive epithelial cells were detected in the colonic mucosae fed with a high-protein or high-fat diet (Figure 1C and Supplementary Figure 1E), hence associating IEC death with the Western-diet.

Given the recently emerged role for IEC necroptosis in intestinal inflammation (19, 27, 28), we wondered whether the necroptotic RIPK3-MLKL pathway could be involved in response to the Western-diet. To test this scenario, we treated both wild-type and Mlkl-deficient mice with a high-protein diet for 2 weeks before DSS challenge. While high-protein diet substantially exacerbated IEC death in wild-type mice, it failed to enhance IEC death or proinflammatory response in Mlkl-deficient mice (Figure 1C and Supplementary Figure 1F), indicating a crucial role of MLKL in the Western-diet-triggered IEC death and colonic inflammation. By immunoblotting, we found that MLKL was markedly phosphorylated and activated in high-protein diet-treated IECs (Figure 1D). Similarly, RIPK3 phosphorylation was also highly induced in IECs pretreated with the high-protein diet (Figure 1D), hence correlating IEC necroptosis with the protein-enriched diet. Moreover, aberrant activation of
RIPK3/MLKL pathway was also associated with the IECs pretreated with a high-fat diet (Supplementary Figure 1G). These results hence reveal an unexpected link between the Western-diet and IEC necroptosis in the pathogenesis of colitis.

Given the crucial role of mTOR in nutrient-sensing, we sought to test the role of mTOR in Western-diet-triggered epithelial necroptosis. At first, we assessed the impact of the Western-diet on epithelial mTOR activity by immunoblotting and immunohistochemistry staining (IHC). Following DSS challenge, S6 and 4EBP-1/2, two well-known substrates of mTORC1, became greatly phosphorylated in the IECs pretreated with high-protein or high-fat diet, indicative of mTOR activation by the Western-diet (Figure 1D and Supplementary Figure 1G). Consistently, IHC revealed the presence of pronounced p-S6 in IECs treated with high-protein or high-fat diet as well (Figure 1E and Supplementary Figure 1H). Further, the Western-diet induced prominent phosphorylation of AKT and GSK3α/β, two upstream kinases controlling mTOR activation through the TSC1/TSC2 complexes (48-50) (Supplementary Figure 1I). Consistently, the phosphorylation of TSC2 was also more abundantly present in the Western-diet-treated IECs (Supplementary Figure 1I). These results revealed intestinal epithelial mTOR hyperactivation as a host response to the Western-diet.

Based on the above observations, we sought to investigate the association between epithelial mTOR and necroptosis in human IBD, which has been strongly linked to the Western-diet. We collected colon tissues from 14 patients with active Crohn’s disease and examined mTOR activation via immunohistochemistry staining. We also assessed IEC necroptosis in these samples by H&E and TUNEL staining. Histology analysis revealed the widespread presence of necrotic cells in the mucosa of IBD patients, especially in the inflamed area (Figure 1F). In contrast, epithelial cell death was barely detectable in the non-IBD controls (Figure 1F). Notably, the necrotic cells in the IBD mucosae displayed unique morphology and histology characteristics, such as disintegrated cell membrane, shrunken eosinophilic cytoplasm and pyknotic nuclei (Figure 1F), thus resembling necroptotic cells as previously described (19, 29).
TUNEL-staining corroborated these results by demonstrating that there were higher numbers of necrotic cells in IBD mucosa than non-IBD controls (Figure 1, G and H). By immunohistochemistry analysis, we found that the majority of IBD samples exhibited a higher degree of p-S6 staining than control individuals (Figure 1, G and H), and the abundances of p-S6 were well-correlated with the extent of necroptosis in IBD patients (Figure 1I). Together, these data indicate an interesting link between the Western-diet, mTOR activation and necroptosis in intestinal inflammation.

*Epithelial TSC1-mTOR regulates necroptosis and intestinal barrier function*

Next, we employed a genetic approach to mimic the Western diet-triggered mTOR hyperactivation, specifically in the IEC compartment, to interrogate the role of epithelial mTOR in necroptosis and inflammation. By crossing Tsc1 floxed mice onto Villin-cre transgenic strain, we generated Tsc1 floxed:Villin-cre (Tsc1IEC-KO) mouse strain in which the mTORC1 pathway was highly activated only in the IECs (Figure 2A). Unlike wild-type IECs, IECs isolated from Tsc1IEC-KO colonic crypts showed robust phosphorylation of RIPK3 and MLKL (Figure 2B), indicating the activation of RIPK3/MLKL pathway. Consistently, the TUNEL assay revealed prominent epithelial cell death without caspase-3 activation in the Tsc1IEC-KO colon (Supplementary Figure 2A). Hence, these data suggest that TSC1-mTOR may be involved in the modulation of necroptosis in the IECs, possibly in cooperation with the well-defined necroptosis-eliciting signals such as TNF or/and microbial-derived PAMPs (19, 28, 51).

To further test this notion, we bred Mlkl−/− mice (52) onto Tsc1IEC-KO strain, thereby generating Tsc1IEC-KO/Mlkl−/− mice which presumably are refractory to necroptosis. As expected, the ablation of Mlkl alleviated the profound epithelial cell death in Tsc1IEC-KO colon, rendering Tsc1IEC-KO/Mlkl−/− colon exhibiting a basal level of IEC death comparable to Mlkl−/− mice (Figure 2C). These data further support necroptosis as the major contributor to the aberrant IEC death in Tsc1IEC-KO mice. On the other hand, Ki-67 staining revealed mostly normal epithelial proliferation in the colon, but
slightly accelerated epithelial proliferation in the ileum of \(Tsc1^{IEC-KO}\) mice (Supplementary Figure 2B). The histology analysis revealed largely normal presence of enterocytes and goblet cells in the \(Tsc1^{IEC-KO}\) intestine (Supplementary Figure 2, C and D), except for fewer Paneth cells resided at the bottom of the ileum crypts (Supplementary Figure 2D). Additionally, the \(Tsc1^{IEC-KO}\) ileum also expressed fewer amounts of lysosome and defensin than wild-type ileum (Supplementary Figure 2E), further demonstrating a crucial role for TSC1-mTOR in the maintenance and functionality of Paneth cells.

As intestinal barrier dysfunction has been frequently linked to the Western-diet and aberrant epithelial cell death (53, 54), we went on to test whether mTOR hyperactivation might compromise the epithelium barrier function. Following oral gavage, we found that about 2-fold more FITC-dextran (M.W. 3-5 kD) was detected in the blood of \(Tsc1^{IEC-KO}\) mice, indicative of disrupted epithelial barrier function (Figure 2D). Strikingly, the intestinal barrier dysfunction associated with TSC1 IEC-deficiency was completely rescued upon the concomitant ablation of \(Mlkl\), thus suggesting a causative role for IEC necroptosis in intestinal barrier dysfunction (Figure 2D). Correspondingly, we found that a subset of pro-inflammatory genes, especially \(Il6, Il1b,\) and \(Cxcl1\), were upregulated in \(Tsc1^{IEC-KO}\) colon (Figure 2E). Moreover, a more significant number of immune cells, such as macrophages, neutrophils, and T cells, were infiltrated into the \(Tsc1^{IEC-KO}\) colon compared with wild type controls (Figure 2F). Hence, these results collectively demonstrate that epithelial mTOR hyperactivation can lead to IEC necroptosis and homeostasis disruption in the gut.

*Epithelial mTOR hyperactivation predisposes to severe colitis*

Corresponding to the aforementioned disrupted homeostasis, \(Tsc1^{IEC-KO}\) mice were highly susceptible to DSS-induced colitis. With a modest 2.5% of DSS challenge, \(Tsc1^{IEC-KO}\) mice exhibited extremely severe colitis, showing extensive ulceration,
marked epithelial erosion, and pronounced infiltration of leukocytes (Supplementary Figure 3, A and B). Consistently, Tsc1IEC-KO mice displayed much worse clinic symptoms and considerably shortened colons compared to the wild type controls (Supplementary Figure 3, C and D). Consequently, while marked morbidity and mortality were associated with Tsc1IEC-KO mice, all the wild-type mice survived from relatively mild colitis (Figure 3, A and B). Therefore, these results collectively demonstrate that mTOR hyperactivation in the IECs can contribute to susceptibility to colitis.

Following DSS challenge, the intestinal barrier of Tsc1IEC-KO mice was further disrupted, leading to the presence of about 5-fold more FITC-dextran in their bloodstream than wild-type mice (Figure 3C). Consistently, many pro-inflammatory genes, such as Tnf, Il1b, Il6, Cxcl1, Ccl2, and Ccl5, were highly expressed in the Tsc1IEC-KO colon following DSS treatment (Figure 3D), indicative of robust inflammatory response. Following DSS treatment, Tsc1IEC-KO IECs exhibited pronounced phosphorylation on RIPK3 and MLKL, along with elevated RIPK3 and MLKL expression as compared to the wild-type IECs (Figure 3E). However, the phosphorylation and expression levels of RIPK1 were comparable in wild-type and Tsc1IEC-KO IECs (Figure 3E). Moreover, while the genetic ablation of Mlkl abolished the expression and phosphorylation of MLKL, it had no impact on the activation of the upstream kinases RIPK1/RIPK3 in both wild-type and Tsc1IEC-KO IECs (Figure 3E). Following DSS challenge, the prominent IEC death associated with Tsc1IEC-KO colon was significantly alleviated upon the concomitant ablation of Mlkl, and the Tsc1IEC-KO/Mlkl−/− compound mice showed a similar extent of IEC death to Mlkl−/− mice (Figure 3F). Consistently, both Tsc1IEC-KO/Mlkl−/− and Mlkl−/− mice also had comparable colitis symptoms induced by DSS (Figure 3G), implicating an indispensable role for MLKL in driving the severe colitis in Tsc1IEC-KO mice. Together, these data strongly indicate that epithelial necroptosis executed by MLKL has a crucial role in promoting intestinal dysfunction and overt inflammation in Tsc1IEC-KO mice.
Given the above association of mTOR hyperactivation with epithelial necroptosis, we then rigorously interrogated the role of mTOR in IEC death and gut inflammation. At first, we attempted to alleviate mTOR activation in wild-type and Tsc1<sup>−/−</sup> IECs by intraperitoneally administering rapamycin. Following consecutive intraperitoneal injections of rapamycin for 5 days, TUNEL-positive epithelial cells were markedly reduced in Tsc1<sup>IEC-KO</sup> colon, but unaltered in wild-type colon (Figure 4A). Notably, rapamycin treatment largely reversed the aberrant IEC death in Tsc1<sup>IEC-KO</sup> colon, leading to restored homeostasis as observed in wild-type colon (Figure 4A). Upon 2.5% DSS challenge, rapamycin-pretreated Tsc1<sup>IEC-KO</sup> mice showed a notable reduction in colitis symptoms, including inflammation, histopathology, morbidity, and mortality, compared to vehicle-treated Tsc1<sup>IEC-KO</sup> mice (Supplementary Figure 4, A and B and Figure 4, B and C). On the contrary, the rapamycin pretreatment regimen employed here (rapamycin treatment for 3 days before DSS challenge, and 2 days after DSS challenge) failed to ameliorate colitis symptoms in wild-type mice (Supplementary Figure 4, A and B and Figure 4, B and C). Notably, more extended rapamycin treatment started after the DSS challenge has been associated with either reduced or enhanced colitis in WT mice (55, 56). Hence, it seems possible to ameliorate IEC death and colitis by tempering mTOR activity. Subsequently, we sought to alleviate mTOR activity in Tsc1<sup>IEC-KO</sup> IECs through a genetic approach. In an attempt to generate IEC-specific TSC1/mTOR-double knockout mice, we failed to obtain Tsc1<sup>IEC-KO</sup>/mTor<sup>IEC-het</sup>, Villin-cre mice due to their embryonic lethality. Nevertheless, we found that Tsc1<sup>IEC-KO</sup>/mTor<sup>IEC-het</sup>, Villin-cre mice (herein named as Tsc1<sup>IEC-KO</sup>/mTor<sup>IEC-het</sup>), with only one mTor allele deleted in the Tsc1<sup>IEC-KO</sup> IECs, showed considerable reduction in mTOR activity (Figure 4D). Strikingly, assessment of necroptotic signaling by immunoblotting revealed diminished expression and phosphorylation of RIPK3 in Tsc1<sup>IEC-KO</sup>/mTor<sup>IEC-het</sup> IECs (Figure 4D). Further, Following DSS challenge, Tsc1<sup>IEC-KO</sup>mTor<sup>IEC-Het</sup> mice suffered a lower degree of tissue damage (Supplementary Figure 4C) and survived much better than Tsc1<sup>IEC-KO</sup> mice (Figure 4E). Hence, these
data establish a central role for the TSC1-mTOR axis in the control of intestinal homeostasis and inflammation.

Besides dietary nutrients, the TSC1-mTOR pathway has also been implicated in the interaction with microbiota (57), another environmental factor strongly tied to IBD. Interestingly, microbial-derived PAMPs such as LPS, polyI:C, and flagellin were able to promote mTOR activation in the ex vivo cultured enteroid cells (Supplementary Figure 4D). To determine the influence of microbiota on epithelial mTOR and necroptosis in vivo, we implemented both germ-free and antibiotics-depletion models. Strikingly, while the tonic phosphorylation of RIPK3/MLKL was evidently observed in IECs isolated from mice reeled in SPF facility, RIPK3/MLKL activation became barely detectable in germ-free IECs (Figure 4F). It is noteworthy that the expression levels of RIPK3 and MLKL proteins also diminished in germ-free IECs (Figure 4F). Remarkably, the phosphorylation of 4EBP-1/2 was considerably lower in germ-free IECs, indicative of decreased mTOR activity (Figure 4F). Hence, these data suggest that the activation of both RIPK3/MLKL and mTOR in the IECs is contingent on the microbiota.

Next, we depleted microbiota through orally administering a cocktail of antibiotics in drinking water, and then dissected necroptotic signaling in 5 weeks. Notably, microbial depletion through antibiotics alleviated RIPK3 protein accumulation and RIPK3 phosphorylation in Tsc1IEC-KO IECs (Supplementary Figure 4E). Moreover, antibiotics treatment also dampened the phosphorylation of MLKL in IECs, further supporting a crucial role for the microbiota in the activation of necroptotic signaling (Supplementary Figure 4E). Following DSS treatment, antibiotics-treated Tsc1IEC-KO mice had very mild inflammation and tissue damage (Supplementary Figure 4, F and G), showing significantly improved clinic manifestation and morbidity as compared with the antibiotics-untreated Tsc1IEC-KO mice (Figure 4G and Supplementary Figure 4H). Antibiotics treatment also alleviated DSS-induced colitis in wild-type mice (Figure 4G and Supplementary Figure 4, F-H). Hence, microbiota and mTOR may
form a liaison in regulating epithelial necroptosis and colitis.

**RIPK3 overexpression underlies mTOR-promoted necroptosis and colitis**

The studies shown above revealed that mTOR hyperactivation, associated with either the Western-diet (Figure 1D and Supplementary Figure 1G) or Tsc1- ablation (Figure 2B, Figure 3E and Figure 4D), was able to promote RIPK3 activation without influencing RIPK1, hence ruling out a possible role for mTOR in the upstream components of TNF- and TLR3-elicted necrotic signaling, such as TAK1, TBK1 or Trif. It is also noteworthy that in addition to its phosphorylation, the expression of RIPK3 was also substantially increased upon mTOR hyperactivation (Figure 1D, Figure 2B, Figure 3E, Figure 4D and Supplementary Figure 1G). Based on these observations, we reasoned that RIPK3 overexpression might underlie mTOR-promoted necroptosis and colitis. To rigorously test this hypothesis, we first validated the quality of purified IECs in this study. By comparing the expression of Epcam in purified IEC versus the whole colon tissue, we observed profound enrichment of the epithelium marker Epcam in purified colonic epithelial cells, thus validating our IEC purification approach (Figure 5A). Notably, the increase in RIPK3 expression was more robust in Tsc1IEC-KO IECs than in the whole colon tissue of Tsc1IEC-KO mice (Figure 5A), highlighting the IEC-specific RIPK3 accumulation in response to mTOR hyperactivation.

To definitively address whether RIPK3 overexpression per se contributes to IEC necroptosis, we fine-tuned RIPK3 expression in the IECs through a genetic approach. As RIPK3 can exert a protective role on colitis through a necroptosis-independent mechanism (30, 31), to avoid the confounding effect from RIPK3-deficiency, we took advantage of Ripk3-heterozygosity which does not exhibit notable phenotype on the wild-type background (30). Strikingly, ablating only one allele of Ripk3 gene in the Tsc1IEC-KO mice considerably alleviated epithelium RIPK3 expression, rendering Tsc1IEC-KO/Ripk3+/- IECs expressing similar levels of RIPK3 proteins to wild-type and
Ripk3\(^{+/−}\) IECs (Figure 5B). Consistent with attenuated RIPK3 expression, the phosphorylation and activation of RIPK3 (Figure 5B), as well as epithelial cell death, were markedly reduced in \(Tsc1^{IEC-KO/Ripk3^{+/−}}\) IECs as compared to \(Tsc1^{IEC-KO}\) IECs (Figure 5C). These results collectively implicate RIPK3 overexpression as the driving force for aberrant necroptosis in the \(Tsc1^{IEC-KO}\) IECs. In support of this notion, Ripk3-heterozygosity restored the intestinal barrier function in \(Tsc1^{IEC-KO/Ripk3^{+/−}}\) and \(Ripk3^{+/−}\) mice exhibited similar levels of FITC-dextran in their blood circulation (Figure 5D). Following DSS challenge, \(Tsc1^{IEC-KO/Ripk3^{+/−}}\) mice had attenuated inflammation/tissue damage in the colon (Supplementary Figure 5, A-C), as well as modest weight loss/morbidity compared to \(Tsc1^{IEC-KO}\) mice (Figure 5E). Interestingly, \(Ripk3^{+/−}\) mice showed similar colitis symptoms (Figure 5E and Supplementary Figure 5, A-C) and IEC death (Supplementary Figure 5D) to the wild-type mice. Overall, these data highlight the necessity for precision control on RIPK3 expression in the IECs.

**mTOR regulates ubiquitin-dependent RIPK3 degradation via autophagy**

Considering excessive RIPK3 can impose such a detrimental effect, we went on to investigate how mTOR impacts RIPK3 abundance. Although RIPK3 overexpression exhibited in \(Tsc1^{IEC-KO}\) IECs resembles what has been shown in \(Casp8^{IEC-KO}\) and \(Fadd^{IEC-KO}\) IECs (19, 28), unlike \(Casp8^{IEC-KO}\) and \(Fadd^{IEC-KO}\) IECs, \(Tsc1^{IEC-KO}\) IECs did not upregulate the expression of \(Ripk3\) mRNAs (Supplementary Figure 6A). Interestingly, RIPK3 accumulated in the wild-type organoid cells upon blocking autophagy flux with chloroquine (CQ) or bafilomycin A1 (BFA) (Figure 6A), suggesting the involvement of autophagy in RIPK3 degradation. In contrast, with the pronounced accumulation of autophagy adaptor p62 in the steady-state, the \(Tsc1^{IEC-KO}\) organoid cells seemed to have only minimal autophagy flux, which was refractory to further inhibition by chloroquine (Figure 6A). Similar to p62, RIPK3 proteins were highly enriched in the steady-state \(Tsc1^{IEC-KO}\) organoid cells, and unchanged with chloroquine or BFA treatment (Figure 6A). Notably,ULK1 phosphorylation on
Ser757, an event responsible for autophagy suppression by mTOR (40), was highly induced in \( Tsc1^{IEC-KO} \) IECs, in accordance with a lower level of LC3-I to LC3-II conversion in these cells (Figure 6B). These data further indicated a marked inhibition of autophagy flux by mTOR in \( Tsc1^{IEC-KO} \) IECs. To extend these observations, we applied EBSS medium, which does not contain amino acids to induce autophagy through amino acid starvation. Remarkably, amino acid starvation induced robust autophagy, as demonstrated by rapid p62 degradation in the wild-type cells (Figure 6C). Correspondingly, RIPK3 and MLKL, but not RIPK1, declined in wild-type cells following AA starvation (Figure 6C). Conversely, neither RIPK3 nor MLKL degraded in \( Tsc1 \)-deficient cells, which were refractory to autophagy induction by AA starvation (Figure 6C). Furthermore, we observed prominent RIPK3 polyubiquitination, including both K48- and K63-type polyubiquitination on RIPK3 in wild-type cells (Figure 6D). However, RIPK3 ubiquitination, especially the K48-type polyubiquitination, was considerably decreased in \( Tsc1 \)-deficient cells (Figure 6D). Consistent with the role of p62 in recognition of ubiquitinated cargo proteins for autophagy degradation, immunoprecipitation assay demonstrated that p62 was able to form complexes with RIPK3 and MLKL, but not RIPK1 (Supplementary Figure 6B). Hence, these results collectively suggest a crucial role for autophagy in the regulation of RIPK3 degradation.

Considering that \( Tsc1 \)-deficient cells had less ubiquitinated RIPK3 than wild-type cells (Figure 6D), we reasoned mTOR might act through an E3 ubiquitin ligase to regulate this process. To this end, we focused on TRIM family members, as many of them have been implicated in autophagy and innate immunity (58). By screening the family of TRIM proteins for their ability to mediate RIPK3 degradation (data not shown), we identified TRIM11 as a candidate E3 ligase for RIPK3 degradation (Figure 6E). Overexpression of wild-type but not the E3 ligase-inactive mutant (C56A) (59) of Trim11 diminished RIPK3 in HT-29 cells (Supplementary Figure 6C). Moreover, overexpression of mTOR or blockade of the autophagosome with either chloroquine or bafilomycin A1 abolished TRIM11-mediated RIPK3 degradation.
(Figure 6F and Supplementary Figure 6D). Further, overexpression of mTOR decreased Trim11-promoted auto-polyubiquitination (Supplementary Figure 6E). Collectively, these data strongly implicate the mTOR-Trim11 axis in the regulation of the autophagic degradation of RIPK3. Next, we conducted an in vivo ubiquitination assay to test whether TRIM11 functions as an E3 ligase for RIPK3. Indeed, TRIM11 formed complexes with RIPK3 (Supplementary Figure 6F) and catalyzed notable ubiquitination on RIPK3 proteins (Supplementary Figure 6G). Hence, Trim11 may act as an E3 ligase to regulate RIPK3 ubiquitination and degradation. To test this hypothesis, we generated Trim11−/− HT-29 cells by CRISPR-Cas9 technology (Figure 6G). Remarkably, both Trim11−/− cells generated by two independent sgRNAs demonstrated increased susceptibility to TNF-induced necroptosis in the presence of zVAD and Smac mimetic (TSZ), hence revealing a negative role for Trim11 in necroptosis (Figure 6G). Consistently, Trim11−/− cells demonstrated elevated phosphorylation and expression of RIPK3, which led to the hyper-activation of MLKL following the stimulation with TSZ (Supplementary Figure 6H). Next, we cultured WT and Trim11−/− cells with EBSS in combination with Vps34 inhibitor SAR405 (60), thereby assessing the involvement of autophagy in RIPK3 degradation in these cells. While AA starvation elicited similar levels of autophagy and p62 degradation in WT and Trim11−/− cells, AA starvation-induced RIPK3 degradation was considerably lower in the Trim11−/− cells (Figure 6H). Since Vps34 inhibitor SAR405 was able to block both p62 and RIPK3 degradation (Figure 6H), these results support the notion that TRIM11 may specifically regulate RIPK3 degradation through autophagy. Consistently, knockdown of Trim11 also led to elevated expression and phosphorylation of RIPK3 in wild-type MEFs after stimulation with TSZ (Supplementary Figure 6I). Conversely, knockdown of Trim11 expression did not have any detectable effect on RIPK3 expression or phosphorylation in the Tsc1−/− MEFs (Supplementary Figure 6I), likely attributable to an inhibitory role of mTOR in Trim11 function as shown above (Figure 6F). Collectively, these results suggest that TSC1-mTOR may impinge on both selective autophagy and Trim11 in controlling RIPK3 expression and activation in the IECs.
mTOR regulates microbial PAMP- and TNF-induced necroptosis

The above observations suggest that autophagy blockade by mTOR can lead to RIPK3 accumulation and susceptibility to necroptosis. Next, we sought to explore the cytokine/microbial signals likely responsible for eliciting gut epithelium necroptosis in vivo. Although TNF and polyI:C have been implicated in the induction of IEC necroptosis, whether TSC1-mTOR impinges on these signals remain unclear. To this end, we employed ex vivo cultured enteroid cells to comprehensively investigate what necroptotic signals are responsible for IEC necroptosis in vivo. Corroborating the role of mTOR in cell growth and proliferation, Tsc1IEC-KO enteroid cells grew faster and larger than their wild-type counterparts (Supplementary Figure 7A). By Western blotting, we observed marked elevation of RIPK3 and MLKL expression in Tsc1IEC-KO enteroids, which were abrogated by rapamycin treatment (Figure 7A). In contrast, other regulators of necroptosis, especially RIPK1, CHIP, FADD, and Caspase-8, were similarly expressed in Tsc1IEC-KO enteroids (Figure 7A). Consistently, p-RIPK3 was notably induced in the Tsc1IEC-KO enteroids contingent on mTOR activation (Figure 7A). Hence, the ex vivo enteroid culture reproduced well the corresponding phenotypes manifested by in vivo IECs as described above. In the presence of TLR3 ligand polyI:C or TNF alone, wild-type enteroids had negligible cell death; however, Tsc1IEC-KO enteroids demonstrated a significant extent of cell death to polyI:C alone (Figure 7B), implicating endogenous PAMPs as the possible trigger for necroptosis in Tsc1IEC-KO colon. With the presence of a broad-spectrum caspase inhibitor z-VAD, polyI:C, TNF, and IFN-β all triggered robust cell death in both types of enteroids; nevertheless, Tsc1IEC-KO enteroids showed more pronounced cell death which was blunted by Ripk3-heterozygosity (Figure 7C). In addition to polyI:C, TLR ligands LPS, flagellin, and MDP also triggered much severe cell death in Tsc1IEC-KO enteroid cells, as assessed by PI staining (Supplementary Figure 7B). Hence, Tsc1IEC-KO enteroids are highly susceptible to a variety of necroptosis-inducing signals, particularly polyI:C and TNF.
Next, we extended our study to MEF and HT-29, two cell lines widely used for necroptosis study. As previously reported (17, 18), TNF was able to induce necroptosis in both MEF and HT-29 cells in the presence of z-VAD and Smac mimetic. Notably, knockout or knockdown of Tsc1 considerably increased TNF-induced necroptosis in these cells (Figure 7D and Supplementary Figure 7C). Similarly, polyI:C stimulation also triggered robust necroptosis in Tsc1-knockout MEFs (Figure 7D). It is noteworthy that RIPK3 but not RIPK1 expression was also elevated in Tsc1-knockout cells, revealing a general role for TSC1-mTOR in the regulation of RIPK3 expression (Figure 7E). Consistently, Tsc1-deficiency also led to a substantial elevation in RIPK3 and MLKL phosphorylation, without significant influence on RIPK1 upon polyI:C or TNF stimulation (Figure 7, E and F). Surprisingly, Tsc1-deficiency did not have a significant impact on TNF-induced activation of NFκB and MAPKs, except for somewhat impairment on p38 activation (Figure 7G). Together, these results demonstrate that the TSC1-mTOR axis participates in the regulation of RIPK3 expression and activation in a variety of necroptosis-inducing signals, especially polyI:C and TNF.

The mTOR-RIPK3-necroptosis axis promotes chronic inflammation and cancer

Chronic inflammation, a hallmark for IBD, and a driving force for human cancers, especially colon cancers, has been intrinsically associated with necroptosis. In this regard, we went on to interrogate how epithelium mTOR-regulated necroptosis influences chronic inflammation and colon cancer. Repeated DSS challenge induces chronic inflammation in the colon, which can drastically promote tumor development in conjunction with carcinogen AOM (Azoxymethane). Although a standard regimen was previously used by us (61) to induce colitis-associated colon cancer, here we modified the DSS treatment strategy to accommodate the hyper-susceptibility of Tsc1IEC-KO mice (Supplementary Figure 8A). Despite being challenged with a less rigorous regimen (1.5%, 1.5% and 1% of DSS, respectively), Tsc1IEC-KO mice still
developed robust inflammation and suffered extended morbidity and mortality (Supplementary Figure 8B). While all the wild type mice survived to the end of the regimen, approximately 60% of Tsc1IEC-KO mice died of overt inflammation during treatment (Supplementary Figure 8B).

By analyzing the surviving mice, we noticed prominent colonic tumorigenesis in Tsc1IEC-KO mice, which had nearly 2-fold more tumors per colon (averaging 14 tumors/colon vs 5 tumors/colon) than wild-type mice (Figure 8A and Supplementary Figure 8C). Moreover, Tsc1IEC-KO mice also had 5-times more large tumors (15% vs 3%) than wild-type mice (Figure 8A). Besides, histological analysis revealed a high degree of dysplasia in the Tsc1IEC-KO tumors (Figure 8B), indicating accelerated tumor progression. Compared to the wild-type tumors, the Tsc1IEC-KO tumors showed much stronger p-S6 staining (Figure 8C), as well as abundant accumulation of RIPK3 proteins (Figure 8D). Regardless of genotypes, all the tumors showed much higher RIPK3 expression than the respective normal colon tissues (Figure 8, D and E). Consistent with elevated p-RIPK3 and p-MLKL in Tsc1IEC-KO tumors (Figure 8E), more necrotic cells were presented in Tsc1IEC-KO tumors than in wild-type tumors (Figure 8F). Moreover, Tsc1IEC-KO tumors showed a drastic increase in the induction of proinflammatory mediators Cox2, Mmp10, and Ereg, as well as cytokines Il1b, Il6, and Il22 (Supplementary Figure 8D). Together, these results revealed that colon cancer is associated with hyperactive mTOR and profound necroptosis.

Considering both tumor-suppressive and tumor-promoting roles have been reported on RIPK3 and necroptosis in various cancers (34, 62, 63), we employed Tsc1IEC-KO/Ripk3+/− mice to further assess the role of RIPK3 and necroptosis in colitis-associated colon cancer. As compared to Tsc1IEC-KO tumors, we observed a profound reduction in inflammatory cytokines/mediators in Tsc1IEC-KO/Ripk3+/− tumors (Figure 9A), suggesting a critical role for RIPK3 in driving the overt inflammation in Tsc1IEC-KO tumor. Correspondingly, Tsc1IEC-KO/Ripk3+/− mice exhibited decreased tumorigenesis, with fewer tumors per mouse than Tsc1IEC-KO mice (Figure 9B).
contrast, Ripk3+/− mice exhibited a slight increase in tumor number compared to wild-type mice (Figure 9B), corroborating with increased tumorigenesis in Ripk3+/− mice in previous reports (34, 63). It is important to note that while Tsc1 IEC-deficiency led to a substantial increase in colon cancer development in wild-type mice (∼2.52 fold), its tumor-promoting effect was considerably dampened in Ripk3+/− mice (∼1.77 fold) (Figure 9B). Besides, Tsc1IEC-KO/Ripk3+/− tumors exhibited a lower level of epithelial necroptosis than Tsc1IEC-KO tumors (Figure 9C), hence revealing a positive correlation between necroptosis and colonic tumorigenesis. Collectively, these data support a tumor-promoting role for epithelial RIPK3 and necroptosis in colitis-associated colon cancer.
Discussion

Here we propose that epithelial mTOR functions as a metabolic checkpoint for intestinal dysfunction and inflammation through the integration of dietary and microbial cues in the gut. Compelling evidence from this study firstly demonstrates that epithelial mTOR hyperactivation, an event frequently associated with the Western diet, dysbiosis and genetic alterations, is intimately linked to aberrant RIPK3-MLKL activation, overt IEC necroptosis, and barrier dysfunction. Further, our results also implicate the epithelial mTOR-RIPK3-necroptosis axis as a vicious circuit in the pathogenesis of IBD and cancer. Therefore, we believe that through a constant survey of environmental signals, epithelium mTOR might operate as a rheostat balancing autophagy and necroptosis, two processes critically involved in intestinal homeostasis and inflammation (Supplementary Figure 9).

Epithelium homeostasis rests on the dynamic equilibrium of cell proliferation and death, and aberrant cell death can lead to intestinal barrier disruption and inflammation. Along with apoptosis, necroptosis has emerged as a critical player in the regulation of intestinal homeostasis (13). While mTOR has been previously linked to intestinal epithelium growth and proliferation (42-44, 64), here we discovered an unexpected role for mTOR in epithelium necroptosis. Overall, studies from others (41, 44) and us strongly suggest that precision control of mTOR activity in the gut epithelium is crucial for intestinal homeostasis. In support of this notion, we found that mTOR hyperactivation triggered by the Western-diet or genetic ablation of Tsc1 was able to tilt the balance from pro-cell growth towards pro-cell death, thereby shifting intestinal homeostasis to barrier disruption and inflammation. Moreover, in human IBDs, aberrant mTOR activation and prominent necroptosis were intertwined and intimately associated with overt inflammation. Hence, our results identify mTOR as a metabolic hub linking environmental risk factors to epithelium disequilibrium, thereby providing a molecular explanation for the epidemiological association of the Western-diet with IBD.
While RIPK3 expression can be regulated via both transcriptional and post-transcriptional mechanisms, our results indicate that mTOR primarily regulates RIPK3 at the post-transcriptional level. It has been reported that a fraction of polyubiquitinated RIPK3 protein undergoes rapid degradation by the proteasome (65). Moreover, RIPK3 protein ubiquitinated by E3 ligase CHIP can also be degraded by lysosome (27). Here, our results suggest selective autophagy as a novel mechanism mediating RIPK3 degradation, thus adding another layer of regulation on RIPK3. Under amino acid-restricted conditions, autophagy can be induced to promote rapid RIPK3 degradation. Conversely, amino acid surplus or Tsc1-deficiency led to mTOR hyperactivation andULK1 phosphorylation, which resulted in autophagy shutdown and ensuing RIPK3 accumulation. Additionally, we also identified TRIM11 as a novel E3 ubiquitin ligase responsible for RIPK3 polyubiquitination and autophagy degradation. Although TRIM11 has been previously linked to the degradation of misfolded polyQ and AIM2 (59, 66), its role in necroptosis has not been implicated. Our data suggest that TRIM11 likely primarily assemble K48-type polyubiquitin chains on RIPK3, a marker could be recognized by autophagy adaptor p62 for autophagosome degradation. Interestingly, mTOR appears to be able to counteract TRIM11-mediated ubiquitination and degradation of RIPK3, although the precise mechanism remains to be elucidated. Hence, our study suggests that mTOR may prevent RIPK3 degradation through two interrelated mechanisms that converge on autophagy degradation of RIPK3 proteins.

In addition to rapamycin, we found that microbiota-depletion considerably ameliorated RIPK3 accumulation and activation in Tsc1-deficient IECs. These findings collectively suggest that epithelium TSC1-mTOR has a crucial role in responding to microbiota, whereby maintaining a delicate balance between autophagy and necroptosis in the gut. It is important to note that while MLKL expression is mostly regulated independent of RIPK3 (18, 52), we observed concomitant elevation of MLKL and RIPK3 in Tsc1-deficient as well as the Western diet-treated IECs. Given
RIPK3 and MLKL can also execute necroptosis-independent functions (13, 34, 52), the co-regulation of both together reinforces the notion that the TSC1-mTOR pathway has a designated role in necroptosis. Nevertheless, whether autophagy has a similar role in MLKL regulation remains unknown at present, but certainly warrants further investigation. Interestingly, a recent study reports that Atg16L1-deficient Paneth cells are hyper susceptible to TNF-triggered necroptosis (67), thus providing another paradigm for the intricate interplay of autophagy and necroptosis. Hence, we propose that autophagy may serve as a checkpoint for necroptosis in controlling intestinal homeostasis and inflammation.

Despite the intrinsic link of necroptosis to barrier disruption and inflammation, two hallmarks of IBD, the role of necroptosis in colitis has yet to be unequivocally established. While blockade of RIPK1 by Nec-1 led to attenuated DSS-induced colitis (68), thus suggesting a proinflammatory role for necroptosis, studies on Ripk3-knockout mice failed to support this conclusion (30, 33). Instead, a protective role executed by the CD11chi mononuclear phagocytes has been suggested for RIPK3 (30, 31). While RIPK3 tends to execute necroptosis-independent roles in the immune cell compartment, studies from others (19, 28) and us strongly suggest that RIPK3 is reserved predominantly for necroptosis in the IECs. Genetic ablation of either Tsc1, Casp8 or Fadd in IECs all led to RIPK3 accumulation, aberrant epithelium necrosis and exacerbated experimental or spontaneous colitis. While others (19, 28, 51, 69) have shown that pharmacological inhibition or genetic ablation of Ripk3 in Casp8IEC-KO and FaddIEC-KO mice alleviated epithelium death and colitis, our data demonstrate that genetic depletion of Ripk3 or Mlkl were able to ameliorate epithelium necrosis, restoring barrier function and repressing colitis in Tsc1IEC-KO mice. Collectively, these observations uncover a causative role for epithelium RIPK3 and MLKL in intestinal homeostasis and colitis. Additionally, we also validated a strong and widespread correlation between mTOR activation and epithelium necroptosis in human IBDs, hence providing novel insight into the widespread association of IBD with the Western diet. With all these pieces of evidence, we would
like to propose that in the gut epithelium, RIPK3 and necroptosis may form a bad liaison driving IBD pathogenesis, thus should be considered as therapeutic targets in the future.

The role of necroptosis in cancer appears complex, while a large number of studies implicate RIPK3 as a tumor-suppressor (62), a tumor-promoting role for RIPK3 has also emerged, especially in pancreatic cancer and intrahepatic cholangiocarcinoma (ICC) (70, 71). The fact that RIPK3 is shut down in most cancer cell lines has led to the popular view that RIPK3 and necroptosis are incompatible with cancer development (17, 62, 72, 73). Corroborating with this hypothesis, Ripk3-knockout mice showed increased tumorigenesis following AOM+DSS treatment (34, 63). Paradoxically, we and others (74) observed an increase, rather than a decrease of RIPK3 expression in colon tumors induced by AOM+DSS regimen or Apc\textsuperscript{min\textsuperscript{h}} heterozygosity. Moreover, the ablation of one allele of Ripk3 in Tsc1\textsuperscript{IEC-KO} mice led to decreased tumorigenesis and concomitant reduction in necroptosis and inflammation. Hence, it is plausible that RIPK3-promoted necroptosis might have contributed to the overt chronic inflammation in Tsc1\textsuperscript{IEC-KO} mice, thereby driving compensatory cell proliferation to accelerate tumor progression. Although more studies are certainly required to clarify the role of RIPK3 in tumorigenesis, our study nonetheless suggests that RIPK3 can be upregulated in a subset of cancers with mTOR dysregulation, thus should be considered as a therapeutic target. As a proof-of-principle, Smac mimetic has been shown to be effective in the induction of necroptosis and treatment of RIPK3\textsuperscript{high} colon cancers and AMLs (74, 75). Considering mTOR dysregulation is widely associated with human cancers, it will be of great interest to explore how to harness RIPK3 overexpression for cancer therapy in individuals with gain-of-function mTOR or loss-of-function Caspase-8.

In summary, our results present the TSC1-mTOR axis as a node of the complex immune/metabolic network in the gut epithelium, delicately balancing autophagy and necroptosis via integrating environmental cues. We also provide compelling evidence
suggesting that mTOR and necroptosis as underpinnings of the Western-diet-/dysbiosis-triggered intestinal dysfunction, inflammation, and cancer. Hence, we propose that RIPK3 and necroptosis should be considered as a potential target for the treatment of IBD and colon cancer.
Methods

Mice

Tsc1 floxed (76), mTor floxed (77) and Villin-cre transgenic mice (78) were purchased from Jackson Laboratories. Ripk3−/− and Mlkl−/− mice on C57BL/6 background were described previously (23, 52). Tsc1 floxed mice were bred onto Villin-cre mice to generate intestinal epithelium-specific Tsc1-knockout mice Tsc1floxed/Villincre (Tsc1IEC-KO). Tsc1IEC-KO mice were backcrossed to wild type C57BL/6 mice for 6 generations. In all experiments, littermates carrying loxp-flanked alleles but without cre recombinase were used as controls (Tsc1floxed). mTorfloxed, Ripk3−/− and Mlkl−/− mice were bred onto Tsc1IEC-KO mice at least three generations to generate compound mutant mice. For dietary experiments, mice were fed with a standard diet (D10012G, Research Diets) which contains 20% protein/16% fat, a high-protein diet comprising 60% protein/16% fat or high-fat diet (D12492, Research Diets) with 20% protein/60% fat. All the mice were bred and maintained in a specific pathogen-free animal facility at Institut Pasteur of Shanghai, except for the germ-free C57BL/6 mice, which were bred and maintained in GF conditions in vinyl isolators of the animal facility at the Third Military Medical University.

Human samples

Paraffin-embedded specimens of colonic mucosae were obtained from control individuals (n=14) or active Crohn’s disease patients (n=14) at the First Affiliated Hospital of Sun Yat-sen University. The diagnosis of Crohn’s disease was based on a standard combination of clinical, endoscopic and histological criteria.

Induction of colitis and clinical score

Age- and gender-matched 8 to 10-week-old littermates were challenged with DSS
(MP biomedicals, MW 36,000-50,000) for 5 days in drinking water to induce colitis. Unless mentioned otherwise, mice were treated with 2.5% DSS. All the mice were co-housed before and during colitis induction, and littermates were used for experiments whenever possible. After a 5-day DSS treatment, mice were given regular water till the end of the experiment. For rapamycin treatment, mice were i.p. injected with 5mg/kg rapamycin dissolved in PBS with 5% Tween 80 for 5 days (3 days before DSS treatment and 2 days after DSS treatment). DSS-induced clinical scores were determined by average scores of weight loss, bleeding, and stool condition as described previously (79). Briefly, for weight loss: no weight loss was scored 0, 1-5% weight loss was scored 1, 5-10% weight loss scored 2, 10-20% weight loss scored 3, and more than 20% weight loss scored 4; for bleeding: negative hemoccult was scored 0, positive hemoccult scored 2, gross bleeding scored 4; for stool: well-formed pellets were scored 0, pasty and semiformed stools scored 2, and liquid stools scored 4.

Statistics

The survival curve was analyzed by Log-rank (Mantel-Cox) test. The unpaired, 2-tailed Student’s t-test was used to compare two groups; One-way ANOVA was used for multiple groups. p < 0.05 is considered significant.

Study approval

The written patient consent was provided, and ethic approval for the use of human samples was granted by the Ethical Committee of the First Affiliated Hospital of Sun Yat-sen University before collecting human tissue samples. All the animal studies were conducted in compliance with the protocol approved by the Institutional Animal Care and Use Committee at Institut Pasteur of Shanghai.
Author contributions


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Figure 1. The Western-diet promotes mTOR hyperactivation and necroptosis in the IECs. (A, B) The C57BL/6 mice (n=9-12) fed with normal diet (20% protein, 16% fat), high-protein diet (60% protein) or high-fat diet (60% fat) for 2 w were treated with 3.5% DSS in drinking water for 5 d, and then switched on regular water. (C) IF staining of the colon sections from the WT and Mikt-/- mice pre-fed with normal or high-protein diet and then challenged with 2.5% DSS for 5 d (n=6). The Epcam/TUNEL double-positive cells denoted by arrows were counted over 5 high power fields (400×) per sample (n=30). Scale bars: 20 μm. (D) Immunoblots of the purified colonic epithelial lysates following 2.5% DSS treatment for 5 d on p-RIPK1 (Ser166), p-RIPK3 (Ser232), p-MLKL (Ser345), p-S6 (Ser235/236), or p-4EBP1 (Thr37/46). The numerical numbers under Western blot bands represent relative quantifications over Actin. (E) IHC staining of the colon sections fed with various protein diets for 2 w. Scale bars: 50 μm. (F, G) H&E and immunostaining of the control or IBDs’ mucosal sections. The black arrows denote the necrotic IECs with shrunken eosinophilic cytoplasm and pyknotic nuclei, and the white arrows indicate the TUNEL-positive IECs. Scale bars: 20 μm for H&E and TUNEL, 100 μm for p-S6. (H) Statistical quantification of the necrotic cell no. and p-S6 staining intensity by Image J (n=14). (I) Spearman correlations between necrotic cell no. and p-S6 intensity in control and IBDs’ mucosae (n=14). The data were pooled from 2 independent experiments (A, B) or representative of 3 independent experiments (C-G) and shown as means±SEM. ****p<0.0001; NS, not significant; by Log-rank test (A, B) or unpaired Student’s t test (C, H).
Figure 2. The Tsc1IEC-KO mice exhibit disrupted intestinal homeostasis. (A, B) Immunoblots of the purified colonic epithelial lysates from Tsc1fl/fl and Tsc1IEC-KO mice for p-RIPK1 (Ser166), p-RIPK3 (S232), p-MLK (S345), p-S6 (S235/236), or p-4EBP1 (Thr27/46). The numerical numbers under Western blot bands represent relative quantifications over Actin. (C) IF staining of the colon sections (n=8) from various genotypes. The Epcam/TUNEL double-positive cells were counted over 5 high power fields (400×) per sample (n=30). Scale bars: 20 μm. (D) The mice (n=6-8) fasted for 4 h were gavaged with FITC-dextran and bled in 4 h. (E) qPCR analysis of inflammatory cytokines and chemokines mRNAs (normalized to beta-actin) in the steady-state colons (n=7). (F) IF staining and quantification of various immune cells in the steady-state colons (n=8). Scale bars: F4/80 and Ly6G, 20 μm; CD3, 50 μm. The data were pooled from 2 independent experiments (D, E) or representative of 3 independent experiments (A-C, F) and shown as mean±SEM. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001; NS, not significant; by unpaired Student’s t test (E, F) or One-way ANOVA (C, D).
Figure 3. Aberrant epithelial necroptosis contributes to the heightened colitis in Tsc1EC-KO mice. The Tsc1f/f and Tsc1EC-KO mice were treated with 2.5% DSS for 5 d and then with regular water. (A, B) The body weight (A) and survival (B) were documented on the treated mice (n=16-18) daily. (C) The leakage of FITC-dextran from the GI to the bloodstream was measured in the DSS-treated mice for 3 d (n=6). (D) qPCR analysis of proinflammatory gene expression (normalized to beta-actin) in the colons treated with DSS for 5 d (n=6). (E) Immunoblots of colonic epithelial lysates from the mice treated with DSS for 5 d (n=3). p-RIPK1 (Ser166), p-RIPK3 (S232), and p-MLKL (S345) antibodies were used. The numerical numbers under Western blot bands represent relative quantifications over Actin. (F) IF staining on colon sections from various genotypes of mice treated with DSS for 5 d (n=6). The double-positive cells were counted over 5 high power fields (400x) per sample (n=30). Scale bars: 20 μm. (G) The body-weight of the mice treated with DSS for 5 d (n=7-8). The data were pooled from 3 independent experiments (A, B, G) or representative of 3 independent experiments (C, D, E, F) and shown as mean±SEM. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001; NS, not significant; by Log-rank test (B), unpaired Student’s t test (C, D, G) or One-way ANOVA (F).
Figure 4. The microbiota-mTOR axis regulates epithelial necrosis and colitis. (A) The mice were intraperitoneally injected with vehicle or rapamycin (5mg/kg, for 5 d), and the colon sections (n=5) were stained by IF and the double-positive cells were quantified over 30 images. Scale bars: 20 μm. (B, C) 3 d after rapamycin treatment, the mice were treated with 2.5% DSS for 5 d in the presence of rapamycin for only 2 d, and the body-weight (B) and survival (C) were recorded daily. (D) Immunoblots of the colonic epithelial lysates from the indicated mice (n=2). (E) The survival curve of the mice treated with 2.5% DSS for 5 d (n=12). (F) Immunoblots of the colonic epithelial cell lysates from B6 mice reeled in germ-free or SPF facility (n=24). p-RIPK1 (Ser166), p-RIPK3 (S232), p-MLKL (S345), p-S6 (S235/236), and p-4EBP1 (Thr27/63) were used. The numerical numbers under Western blot bands represent relative quantifications over Actin. (G) The mice untreated or pretreated with antibiotics (ABX) for 5 w were challenged with 2.5% DSS for 5 d to induce colitis, and the body-weights were recorded daily (n=5). The data were pooled from 3 independent experiments (B, C, E) or representative of 3 independent experiments (A, D, F, G) and shown as mean±SEM. ***p<0.001, ****p<0.0001; NS, not significant; by Log-rank test (C, E), unpaired Student’s t test (B, G), or One-way ANOVA (A).
Figure 5. RIPK3 accumulation correlates with severe IEC necroptosis and colitis. (A, B) Immunoblot of the protein lysates from the whole colon tissues or purified IECs (p-RIPK3 (S232), p-MLKL (S345), p-S6 (S235/236)). The numerical numbers under Western blot bands represent relative quantifications over Actin. (C) IF staining and quantification of the double-positive cells in the colon sections (n=6). Scale bars: 20 μm. (D) The intestinal permeability assay on Tsc1IEC-KO/Ripk3+/− and Tsc1IEC-KO/Ripk3+/− mice (n=5). (E) The mice were treated with 2.5% DSS for 5 d, and the body-weights were recorded daily (n=9–12). The data were pooled from 3 independent experiments (E) or representative of 3 independent experiments (A–D) and shown as means±SEM. **p<0.01, ****p<0.0001; NS, not significant; by unpaired Student's t test.
Figure 6. Autophagy and TRIM11 collaborate in RIPK3 degradation. (A) Immunoblots of protein lysates from the ex vivo organoids treated with 20 μM chloroquine (CQ) or 0.2 μM bafilomycin A1 (BFA) for 18 h. (B, C) Immunoblots of the cell lysates from WT and Tsc1EC-KO IECs (B) or WT and Tsc1EC KO MEFs treated with amino acid-free EBSS medium for various times as indicated (p-ULK1 Ser757) (C). (D) The cell lysates from MEFs were immunoprecipitated with control or anti-RIPK3 antibodies, and then immunoblotted with respective antibodies. (E, F) HEK293T cells were transfected with various combinations of plasmids for 36 h. (G) WT and TRIM11-KO HT-29 cells confirmed by immunoblotting were treated with 10 ng/ml hTNF (T), 1 μM BV-6 (S) and 20 μM zVAD (Z) for 24 h, and cell cytotoxicity was measured by LDH release (n=3 biological replicates). (H) The WT and TRIM11-KO HT-29 cells were treated with EBSS medium w/o or w/ 10 μM SAR405 for 24 h. The cell lysates were immunoblotted and quantified by Image J. The numerical numbers under Western blot bands represent relative quantifications over Actin (B, C). Tubulin (A) or GAPDH (E, F, H). The data were representative of 3 independent experiments and shown as mean±SEM. **p<0.01, ***p<0.0001, by unpaired Student’s t test.
Figure 7. mTOR regulates the microbial PAMP- and TNF-induced necroptosis. (A) Immunoblots of protein lysates of the ex vivo organoids treated with 10 ng/ml rapamycin for 48 h. (B, C) Organoid viability was quantified by cellular ATP levels after treatment with 10ng/ml mTNF or 10 μg/ml poly(I:C) alone (B), or in combinations with 10 nM BV-6 (S) or 20 μM zVAD (Z) for 24 h (n=4). (D) The WT and Tsc1−/− MEFs were treated with 10 ng/ml mTNF (T), 100 nM BV-6 (S) and 20 μM zVAD (Z), w/o w/o 10 μM Nec-1 for 6 h; or treated with 50 μg/ml poly(I:C) (P) and 20μM zVAD (Z), w/o w/o 5μM GSK872 for 16-20 h. Cell cytotoxicity was measured by LDH release (n=3). (E-G) The MEFs were treated with 10 ng/ml mTNF (T), 100 nM BV-6 (S) and 20 μM zVAD (Z) or 50 μg/ml poly(I:C) (P) and 20μM zVAD (Z) for various times as indicated. p-RIPK1 (Ser186), p-RIPK3 (S232), p-MLKL (S345), p-S6 (S235/236), p-IkBα (Ser32), p-IKKα/β (Ser176/180), p-p38 (Thr180/Tyr182), and p-Jnk (Thr183/Tyr185) were used. The numerical numbers under Western blot bands represent relative quantifications over Actin. The data were representative of 3 independent experiments and shown as mean±SEM. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, by unpaired Student’s t test.
Figure 8. mTOR regulates RIPK3 overexpression and necroptosis in colitis-associated cancers. (A) The tumor number and volume were assessed in Tsc1f/f and Tsc1IEC-KO mice challenged with the CAC regimen for 80 d (n=9-16). (B-D) H&E and IHC staining of colon tumors from the CAC-challenged mice. Scale bars: 100 μm for H&E and p-S6, 50 μm for Ki-67 and RIPK3. (E) Immunoblots of protein lysates from the colon tumors (T) or the adjacent normal colon tissues (N) of the CAC-challenged mice (n=3-4). p-RIPK1 (Ser166), p-RIPK3 (S232), p-MLKL (S345), and p-S6 (S235/236) were used. The numerical numbers under Western blot bands represent relative quantifications over Actin. (F) IF staining and quantification of the Epcam/TUNEL double-positive cells in the colon tumor sections of the CAC-challenged mice (n=5). Scale bars: 20 μm. The data were pooled from 2 independent experiments (A) or representative of 2 independent experiments (B-F) and shown as mean±SEM. **p<0.01, ***p<0.001, ****p<0.0001, by unpaired Student’s t test.
Figure 9. RIPK3 promotes inflammation and necroptosis in Tsc1EC-KO colon cancers. (A) qPCR analysis of proinflammatory gene expression (normalized to beta-actin) in the colon tumors (T) and normal colon tissues (N) following the CAC regimen (n=6). (B) The tumor numbers formed in various genotypes of mice upon CAC challenge (n=7-13). (C) IF staining and quantification of the double-positive cells in the colon tumor sections (n=25). Scale bars: 20 μm. The data were pooled from 2 independent experiments (B) or representative of 2 independent experiments (A, C) and shown as mean±SEM. *p<0.05, **p<0.01, ****p<0.0001; by One-way ANOVA.