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mTORC1 Prevents Epithelial Damage During Inflammation and Inhibits Colitis-Associated Colorectal Cancer Development

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Abstract

Epithelial cells lining the intestinal mucosa constitute a selective-semipermeable barrier acting as first line of defense in the organism. The number of those cells remains constant during physiological conditions, but disruption of epithelial cell homeostasis has been observed in several pathologies. During colitis, epithelial cell proliferation decreases and cell death augments. The mechanism responsible for these changes remains unknown. Here, we show that the pro-inflammatory cytokine IFNγ contributes to the inhibition of epithelial cell proliferation in intestinal epithelial cells (IECs) by inducing the activation of mTORC1. Activation of mTORC1 in response to IFNγ was detected in IECs present along the crypt axis and in colonic macrophages. mTORC1 inhibition enhances cell proliferation, increases DNA damage in IEC. In macrophages, mTORC1 inhibition strongly reduces the expression of pro-inflammatory markers. As a consequence, mTORC1 inhibition exacerbated disease activity, increased mucosal damage, enhanced ulceration, augmented cell infiltration, decreased survival and stimulated tumor formation in a model of colorectal cancer CRC associated to colitis. Thus, our findings suggest that mTORC1 signaling downstream of IFNγ prevents epithelial DNA damage and cancer development during colitis.

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Introduction

Intestinal epithelial cells (IEC) form a selective barrier that separates luminal contents from the underlying tissue, thus maintaining a functional barrier requires the number of IEC to remain constant. Therefore, a perfect balance between proliferating and dying cells is essential for maintaining epithelial homeostasis and proper function of the intestinal epithelium. Disruption of IEC homeostasis actively contributes to development and establishment of several pathologies, including inflammatory bowel diseases (IBD) and colorectal cancer [1].

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During colitis, numerous signaling pathways linked to the development and progression of colorectal cancer have been also associated with the inhibition of cell proliferation [2–4]. Therefore, understanding the specific mechanism controlling proliferation and IEC death in the colon is an important step in the identification of the machinery implicated in the development of several colonic pathologies.

Signaling networks, downstream of several molecules including cytokine receptors, provide a unique mechanism to integrate internal and external stimuli aimed to coordinate cellular responses. Phosphatidylinositol-3-kinase (PI3K) is a lipid kinase that regulates several biological processes including proliferation, survival and growth and is activated by cytokine receptors [5]. Its major function is mediated by the activation of the protein kinase B, also known as Akt [6]. Akt in turns activates several other effectors including the protein kinase complex mTORC1, a key regulator for metabolism and cell growth. mTORC1 is constituted by mTOR, Raptor, and mLST8, and the inhibitors of the complex PRAS40 and DEPTOR. mTORC1 main effects are controlled through rapamycin-sensitive phosphorylation of its substrates, S6 kinase 1 (S6 K1) and eIF4E-binding protein 1 (4E-BP1). mTORC1 activation is also important for inhibiting autophagy and increasing of protein and lipid synthesis, thus mTORC1 is necessary for preventing futile cycles of synthesis and degradation of cellular components [7,8]. mTORC1 has been implicated in the development of several pathological processes in the gut, including colorectal cancer development [9] and colitis, however the mechanism underlying those processes remain poorly understood.

Here, using *in vivo* and *in vitro* models of inflammation we demonstrated that IFNγ, a pro-inflammatory Th1cytokine that is upregulated in the colonic mucosa of colitic patients [10–12], negatively regulates IEC proliferation by inducing inactivation of Wnt/β-catenin signaling through mTORC1 [2,13–15]. Here we sought to analyze the role of mTORC1, a downstream target of Akt in the regulation of this process. We demonstrated that IFNγ induces activation of mTORC1 in IECs located along the crypt axis but also in immune cells (*e.g.* macrophages) present at the colonic mucosa. Interestingly, we observed that pharmacological inhibition of mTORC1 increased the number of proliferating IECs in the mucosa of colitic mice but this process resulted in enhanced epithelial damage and ulceration at the epithelium. Thus, mTORC1 inactivation augmented disease activity and mortality in a colitis model induced by the administration of DSS. Furthermore, mTORC1 inhibition triggered β-catenin dependent DNA damage in IECs and therefore stimulates CRC development associated to colitis. Such effects occurred because after mTORC1 inhibition macrophages failed to polarize to M1-phenotype and therefore an anti-inflammatory environment is created in the colon. Thus our findings strongly suggest that inhibition of mTORC1 during colitis could play important role in the development of colorectal cancer. However, they also demonstrated in the colonic mucosa the activation of mTORC1 by IFNγ plays a protective role not only in the maintenance of the epithelial integrity but also in the induction of IEC DNA damage.

Materials and Methods

Animals, Cell Culture and Crypt Isolation

Six- to eight-week-old (22–25 g of weight) C57BL/6 J or Lgr5-EGFP-IRES-creERT2 (β-catenin reporter mice; The Jackson Laboratories, Bar Harbor, ME) male mice were kept in standard day/night cycles conditions with free access to food and water. All experiments were approved by the Internal Committee for Care and Use of Laboratory Animals (CICUAL). The human IEC lines SW480 and RKO were obtained from ATCC (Manassas, VA) and cultured according to the provided protocols. Crypt isolations were performed as previously reported [14].

Antibodies and Reagents

2Antibodies against GAPDH sc-322 (Santa Cruz Biotechnology, Santa Cruz, CA), pSTAT1 Tyr701 #9167, pS6 #15967, pAkt Ser473 #4060, Akt1 #2967, pGSK3β #5558, pH2AX (γ-H2AX) mAb #9718, pp53 #9286, CHK1 # 2348 and pBRCA1 #9009 were obtained from Cell Signaling Technology (Danvers, MA). F4/80 Monoclonal Antibody (BM8) coupled to Biotin (eBioscience, Waltham, MA) was used according to manufacturer's instructions. p-Histone H3, ab32107 was obtained from Abcam (Cambridge, MA). Alexa conjugated antibodies were obtained from Thermo Fisher Scientific (Waltham, MA). AffiniPure goat and rabbit anti-horseradish peroxidase (HRP) were obtained from Jackson Immunoresearch (San Diego, CA) and 4′,6-Diamidino-2-phenylindole (DAPI) SC-3598 from Santa Cruz Biotechnology (Santa Cruz, CA). Recombinant mouse IFNγ (Pepro-Tech, Rocky Hill, NJ) was dissolved in 0.002% mouse serum albumin (MSA; Sigma-Aldrich, St. Louis, MO) and used at 2.5 μg/kg of weight. LiCl (Sigma-Aldrich, St Louis, MO) was used at 4 mg/mice and 4 μM *in vitro*. Rapamycin used as previously reported by us [14] Doxorubicin was obtained from Pfizer and used at 3.45 μM. AZD8055 and XAV939 were used as previously reported by us [16,17]. Dextran sulfate sodium YD318041799 (Carbosynth, San Diego, CA) was dissolved at 3% in drinking water.

Cytokine Treatment

Mice were randomly divided into groups. IFNγ was dissolved in PBS/MSA (0.02%) and administered intraperitoneally; control mice were injected with carrier alone. Two hours after cytokine administration mice were euthanized and colons processed for immunofluorescence and Western blot.

In Vivo Administration of Inhibitors and DSS-Induced Colitis Model

LiCl (4 mg/mice) was dissolved in PBS and Rapamycin (2 mg/kg) and AZD8055 (7.5 mg/kg) were dissolved in DMSO. The inhibitors were injected last 3 days of DSS treatment. Control group received vehicle alone. DSS-induced colitis model was previously described [18]. After treatment mice were euthanized and colons processed for immunofluorescence and Western blot analysis.

Proliferation Analysis and TOP-FOP Reporter Activity

Proliferation was assessed *in vivo* by intraperitoneal injection of 100 μg 5-ethynyl-2-deoxyuridine (EdU, Invitrogen). EdU incorporation was determined with a Click-iT EdU Cell Proliferation Kit (Invitrogen), according to the supplier's instructions. β-Catenin TCF/LEF reporter expression and *in vitro* cell proliferation assay. TCF reporter construct activity was measured using Dual Luciferase Reporter system (Promega, Madison WI) according to the manufacturer's instructions.

Immunofluorescence, Histology and Western Blot

Immunofluorescence, immunohistochemistry and Western blot of colonic mucosa were performed as previously described by us [14].

Statistical Analysis

All statistical analysis were performed in Prism 6.0 (GraphPad Software). Shapiro–Wilk, one-way ANOVA, two-tailed T, Dunnett,