7-2023

Studies of the Role of mTORC1 in Modulating Intestinal Epithelial Barrier Function

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STUDIES ON THE ROLE OF mTORC1 IN MODULATING INTESTINAL EPITHELIAL BARRIER FUNCTION

by

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A THESIS

Presented to the Faculty of

The Graduate College at the University of Nebraska

In Partial Fulfillment of Requirements

For the Degree of Master of Science

Major: Nutrition and Health Sciences

Under the Supervision of Professor Regis Moreau

Lincoln, Nebraska

July 2023

STUDIES ON THE ROLE OF mTORC1 IN MODULATING INTESTINAL EPITHELIAL BARRIER FUNCTION

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University of Nebraska, 2023.

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Mechanistic target of rapamycin complex 1 (mTORC1) signaling impacts intestinal inflammation by modulating the production of cytokines in both intestinal epithelial cells and macrophages. Recent evidence revealed that THP-1-derived macrophages with elevated mTORC1 activity (THP-1shTSC2 macrophages), exhibited increased proinflammatory cytokine production when stimulated with lipopolysaccharide (LPS) compared to counterpart macrophages with repressed or baseline mTORC1 activity. Furthermore, it has been shown that mTORC1 governs intestinal barrier function as evidenced by elevated transepithelial electrical resistance (TEER) and para/transcellular permeability in Raptor-depleted Caco-2 cells (cells with repressed mTORC1 activity) compared to Caco-2 cells with elevated or baseline mTORC1 activity. The present study investigated the barrier function characteristics of Caco-2 cell monolayers upon exposure to secretagogues produced by activated THP-1-derived macrophages. Conditioned media from LPS-treated (LP) or LPS+curcumin-treated (LC) THP-1shTSC2 macrophages were applied basolaterally to Raptor-depleted Caco-2 cells (R Caco-2 cells) and Caco-2 cells with baseline mTORC1 activity (S Caco-2 cells) grown and differentiated in Transwells. TEER and paracellular transport of FITC-dextran 4 kDa were measured to assess the integrity of the epithelial barrier. Results showed that upon LP exposure, the initially elevated TEER of R Caco-2 cells collapsed and exhibited increased permeability to FITC-dextran 4 kDa

(P<0.0001) while S Caco-2 cells experienced a temporary decrease in TEER and mild leakiness to FITC-dextran followed by the recovery of their pre-treatment TEER. These results suggested that inflammatory factors secreted by macrophages in LP disrupted epithelial Caco-2 cell transport, particularly when mTORC1 activity was downregulated. LP dose-dependently disrupted the TEER of R Caco-2 cells (P<0.001), whereas LC alleviated the disruption. The study identified TNF-α as one of the macrophage-derived secretagogues causing TEER disruption since TNF-α blocking antibody effectively prevented the decrease of R Caco-2 cells TEER caused by basolaterally supplied recombinant TNF-α or LP (P<0.001). Additionally, results showed that the p38 MAPK, JNK, and IKKβ signaling pathways were involved in the collapse of TEER since specific pharmacological inhibitors provided protection (P<0.001). In conclusion, these studies provided new insights into how mTORC1, p38 MAPK, JNK, and IKKβ signaling pathways impact intestinal epithelial barrier function in Caco-2 cells upon macrophage and TNF-α challenges and inferred potential therapeutic targets for enhancing intestinal barrier integrity.

DEDICATION

To the Almighty God, the creator of the vast cosmos, the giver and sustainer of lives.

ACKNOWLEDGEMENTS

I would like to express my profound gratitude to Dr. Regis Moreau for extending to me the invaluable opportunity to become a part of his esteemed research lab. Ever since I joined his lab, his unwavering support, exceptional mentorship, and consistent availability to provide timely advice, invaluable insights, and uplifting encouragement have been instrumental in propelling me forward throughout this program. I am sincerely grateful for his guidance, which has greatly contributed to my scientific growth.

I would also like to extend my heartfelt appreciation to my esteemed committee members, Dr. Sathish Kumar Natarajan, and Dr. Lee Jaekwon. Their dedication of time, valuable suggestions, and unwavering guidance have played a vital role in enhancing the quality of my work.

Furthermore, I would like to express my gratitude to Dr. Mary Ann Johnson, the Chair of the Department, whose guidance and leadership have been invaluable in fostering a conducive academic environment. I would also like to acknowledge the consistent support provided by the NHS administrative staff, namely Amy Brown, Morgan Harris, and Melinda Nolan.

Lastly, I cannot emphasize enough my gratitude towards the most significant individuals in my life, my beloved family members, whose unwavering support, prayers, and words of encouragement have been a constant source of motivation and inspiration throughout this journey. I acknowledge that this accomplishment would not have been possible without their unwavering support.

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LIST OF ABBREVIATIONS

Akt: AKT serine/threonine kinase 1, Protein kinase B (PKB)

AMPK: AMP-activated protein kinase

ATP: Adenosine-triphosphate

BCA: Bicinchoninic acid

BSA: Bovine serum albumin

CM: Conditioned media

CKI: Cocktail of inhibitors

CREB: cAMP response element binding protein

CUR: curcumin

DMSO: dimethyl sulfoxide

DSS: Dextran sulfate sodium

DTT: dithiothreitol

EMEM: Eagle's minimum essential media

ERK: Extracellular signal regulated kinase

FBS: Fetal bovine serum

FITC: Fluorescein isothiocynate

HBSS: Hanks Balanced Salt Solution

HRP: Horseradish peroxidase

IBD: Inflammatory Bowel Disease

IL: Interleukin

IkBα: Inhibitor of kappa B

IKKα: inhibitor of nuclear factor kappa B kinase subunit alpha

IKK: IkBα kinase

IRS: Insulin receptor substrate

JNK: c-Jun N-terminal kinases

LPS: Lipopolysaccharide

LP: LPS treated THP-1shTSC2 macrophages

LC: LPS+CUR treated THP-1shTSC2 macrophages

mAb: monoclonal antibody

MAPK: mitogen activated protein kinase

MEK: Mitogen activated protein/extracellular signal regulated kinase

mTORC1: mechanistic target of rapamycin complex 1

mTORC2: mechanistic target of rapamycin complex 2

NFkB: Nuclear factor kappa B

PAMP: Pathogen associated molecular pattern

PBS: phosphate buffered saline

PI3K: phosphoinositide 3 kinase

PRAS40: proline-rich Akt substrate 40

p70S6K: p70 ribosomal protein S6 kinase B1

Raptor: regulatory-associated protein of mTOR

Ras: GTPase Ras proteins

Rheb: Ras homolog enriched in brain

RPMI medium: Roswell Park Memorial Institute 1640 medium

RSK: ribosomal S6 kinase

S6: 40S ribosomal protein S6

TLR: Toll-like receptor

TNFα: Tumor necrosis factor alpha

TRADD: Tumor necrosis factor receptor type 1-associated DEATH domain

TRAF2: TNFαR associated factor 2

TSC1/2: Tuberous sclerosis complex 1/2

ZO-1/2; Zonula occludens-1/2

4EBP1, eukaryotic translation initiation factor 4E binding protein

CHAPTER 1

LITERATURE REVIEW

1. INTRODUCTION

The gastrointestinal (GI) tract performs various functions such as digestion, absorption, excretion, and protection, and is composed of several organs including the mouth, stomach, small intestine, large intestine, and anus. The small intestine is made up of the duodenum, jejunum, and ileum, while the large intestine is further divided into the cecum, colon, and rectum. The intestinal epithelium of the small intestine is composed of various distinct cell types, each serving specific functions to maintain the overall balance and stability of the intestinal environment [1].

The intestinal epithelium is constantly exposed to various forms of physical, chemical, and biological insult or injury, thus for the continuity of its function, a renewal process occurs every 4-5 days [1]. At the base of the crypts, there exist intestinal epithelial stem cells (ISC) that undergo continuous division, generating all the different types of epithelial cells that populate the intestine [2, 3]. Paneth cells, found in the crypts, and Goblet cells, located in the villi, play crucial roles in maintaining the intestinal epithelium. They secrete mucin and antimicrobial substances, which combine to form a protective coat at the luminal side of the epithelium [3-5]. Enterocytes, which constitute 80% of the small intestine cells, are primarily situated along the villi and actively facilitate the absorption of nutrients from the lumen to the lamina propria. Enteroendocrine cells, also present in the villi, contribute to digestion through the regulated secretion of hormones [3-5].

The regulation of ISC function is tightly controlled by several signaling pathways, including the Wnt, Notch, and Hippo pathways. These pathways play significant roles in determining the fate of ISCs, including their proliferation, differentiation, and migration up the villi. Together, these signaling pathways maintain the integrity of the intestinal epithelium, ensuring efficient nutrient absorption and providing a barrier against harmful toxins and pathogens [5, 6].

The intestinal epithelium is composed of columnar epithelial cells arranged in a monolayer that are tightly connected by multiprotein complexes known as tight junctions (TJs) [7]. These TJs seal the paracellular space between adjacent epithelial cells, thereby regulating the movement of water, electrolytes, dietary substances, microorganisms, and other external agents into the underlying lamina propria, where immune cells are located [8, 9]. Rather than functioning as a rigid physical barrier, the intestinal epithelium acts as a finely tuned gate that selectively allows the passage of substances, thereby facilitating the development of the intestinal immune system and immune tolerance [10].

Several signaling pathways, such as Wnt and Notch pathways, have been reported to regulate TJs in the intestine. The Wnt pathway activation has been shown to enhance the expression of the TJ protein claudin-4, which is critical for controlling the paracellular movement of luminal antigens, thereby maintaining the health of the gut and overall wellbeing [11]. Additionally, the mechanistic target of rapamycin complex 1 (mTORC1), a central regulator of cell growth and metabolism, has been reported to be critical for intestinal barrier function by stimulating ISCs to divide and migrate from the crypts [12]. This process is promoted by the activation of mTORC1 via various stimuli such as growth factors, amino acids, and even commensal microbiota. Since ISCs play a

crucial role in intestinal barrier repair and renewal, which includes the formation of new TJs, therefore, the regulation of ISCs function by mTORC1 may be important for the maintenance of intestinal barrier function [13-16].

Intestinal epithelium homeostasis is linked to the integrity of TJs; however, certain physiological and pathological conditions can disrupt or modulate the paracellular and/or transcellular pathways responsible for the epithelium selective permeability. Disruption can lead to the production and/or entry of inflammatory mediators resulting in chronic gut inflammation which can further disrupt TJ proteins and increase permeability [17, 18]. Disruption of the intestinal epithelial barrier is widely recognized as a significant characteristic of mucosal inflammation. Numerous studies have focused on this phenomenon, primarily in chronic autoimmune conditions such as inflammatory bowel disease, which includes Crohn's disease (CD) and ulcerative colitis (UC).

Furthermore, impaired integrity of the gut barrier is common in various extraintestinal, and systemic disorders characterized by inflammation [17-19]. These conditions include alcoholic liver disease, non-alcoholic fatty liver and steatohepatitis, liver cirrhosis, severe acute pancreatitis (SAP), primary biliary cholangitis (PBC), type 1 and type 2 diabetes, as well as depression. [17-21].

It is important to recognize that an increased permeability of the gut barrier can coexist with other dysfunction of the intestinal host defense, which may involve malfunctioning Paneth cells leading to impaired secretion of antimicrobial peptides as well as abnormal mucin production by Goblet cells [22-23]. When these cellular and secretory processes of gut epithelial defense are simultaneously impaired, excessive exposure to gut microbes can occur, which in turn triggers an immune response and the

release of various inflammatory mediators within the intestinal mucosa [24-25]. Inflammatory mediators such as tumor necrosis factor α (TNF-α), interleukins and interferon γ (IFN-γ) play a role in accelerating the disruption of TJs and increasing the permeability of the gut barrier thereby exacerbating mucosal inflammation [26-29].

Therefore, understanding the molecular regulation of intestinal barrier function, particularly in an inflammatory milieu, will improve barrier function and contribute to identifying therapeutic targets for preventing or treating associated disease conditions.

2. INTESTINAL EPITHELIAL BARRIER FUNCTION

The intestinal epithelium, found within the body, comprises the largest mucosal surface. In adult humans, it spans an impressive surface area of approximately 30 square meters (m^2) [30]. The organization of the intestinal epithelium is specifically designed to optimize surface area and enhance its absorptive capacity [31, 32]. This is achieved through various structural features, including folds at the organ level, tubular invaginations known as crypts, projections called villi at the multicellular level, and subcellular apical membrane projections called microvilli or brush border [30, 32]. Together, these architectural elements afford high functional efficiency to the intestinal epithelium.

The intestinal epithelium serves as a protective barrier separating the external environment (the gut lumen) from the internal body space (the serosa) (Fig. 1). At the tissue level, the barrier function is established by the secretion of mucins and antimicrobial proteins from Goblet and Paneth cells, which help prevent bacteria and viruses from accessing the epithelial surface. Goblet cells also engage the immune system by

transporting bacterial antigens across the intestinal epithelium towards immune cells beneath it, thus aiding in defense against pathogens [33-35].

At the cellular level, the epithelium physical barrier is achieved through the formation of a cohesive and polarized monolayer of cells, whose cell membranes are interconnected and linked to the basement membranes through protein complexes,. Within this cell monolayer, intercellular junctions are classified into three functional groups: TJs, adherens junction, and desmosomes [36, 37]. While TJs and adherens junctions have been recognized as crucial for regulating the permeability of the gut barrier, the precise function of desmosomes is still not fully understood [38, 39].

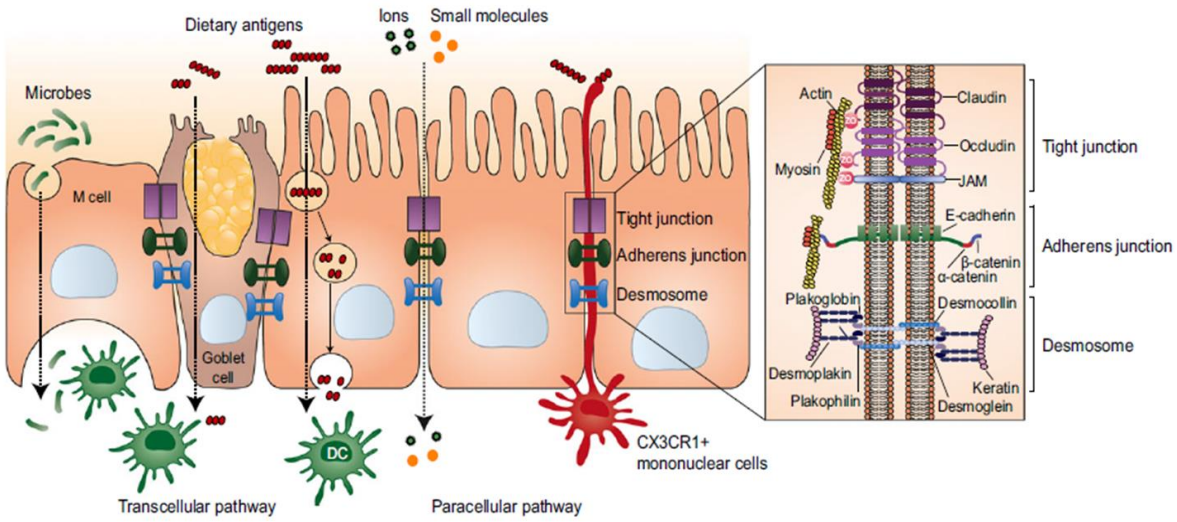


Fig. 1. Transcellular and paracellular routes. Intestinal permeability is regulated by paracellular and transcellular pathways. In the transcellular pathway, larger molecules cross the apical and basolateral membranes of epithelial cells via passive transport, passive diffusion facilitated by efflux pumps, active transport, or endocytosis. Goblet cells can also deliver luminal antigens to immune cells via Goblet cell-associated antigen passages. The paracellular pathway facilitates the transport of small molecules, ions, and solutes between vicinal epithelial cells. TJs located near the apical surface primarily regulate paracellular transport. (Source: Galipeau & Verdu [37])

2.1 Intestinal electrical resistance

The tight junction barrier plays a crucial role in regulating the absorption of nutrients and drugs administered orally, which makes the intestinal epithelium a central focus of studies examining the transport of substances across it [40]. To evaluate the integrity of the intestinal epithelial barrier *in vitro*, a commonly employed non-invasive technique called transepithelial electrical resistance (TEER) is utilized. TEER serves as a reliable readout of TJs function and epithelium permeability. Moreso, it serves as a valuable tool for evaluating the nature and extent of both the transcellular and paracellular pathways. TEER provides real-time insights into physiological and pathological conditions, thus offering valuable information when studying cellular processes *in vitro*. [40]. To conduct TEER measurements, two electrodes are positioned on opposite sides of the epithelial cell layer, and a minute electrical current is transmitted between them. By quantifying the voltage drop across the layer, it becomes possible to calculate the resistance through the utilization of Ohm's law (Resistance = Voltage / Current) [40, 41]

TEER values are typically expressed in units of ohms per square centimeter (Ω·cm2). Elevated TEER correspond to a more robust and intact epithelial barrier, indicating tighter junctions between cells [40, 41]. Conversely, lower TEER values indicate a compromised barrier function. Changes in TEER inform on modifications in the integrity of the epithelial layer, such as heightened permeability or damage due to inflammation, infection, or other pathological conditions [40, 41].

TEER measurements find common application in cell culture models aimed at simulating intestinal, renal, and vascular epithelia. These measurements are employed to evaluate the barrier function of cells and investigate the impact of various factors, including drugs, toxins, and disease states, on epithelial integrity.

Numerous factors can influence the electrical resistance of the intestinal epithelium, including inflammation, infections, stress, and specific diseases. These factors can affect tight junction proteins and the overall structure of the intestinal epithelium, leading to alterations in electrical resistance and the barrier function it represents [41]

2.2 Tight junctions

Tight junctions (TJs) represent the most apical intercellular junctions and play a crucial role in maintaining the barrier integrity and epithelial polarity (Fig. 1). TJs form a circular protein seal around each cell, and effectively restrict the diffusion of ions and prevent the translocation of luminal antigens, such as organisms and toxins, to the lamina propria [42]. The multiprotein complexes that constituting TJs involve four classes of transmembrane proteins: occludin, claudins, junctional adhesion molecules (JAM), and tricellulin [43]. These transmembrane elements found in TJs establish adhesive connections with their counterparts on the adjacent cell membrane. On the cytoplasmic side of the membrane, these components interact with various scaffolding proteins, including the well-known members of the 'zonula occludens' (ZO) protein family [44, 45].

Occludin is a pivotal protein involved in the dynamic assembly and disassembly of TJs. The precise location of occludin within the cell membrane is controlled by phosphorylation of specific residues, namely serine (Ser), threonine (Thr), and tyrosine (Tyr). When occludin undergoes extensive phosphorylation, it primarily locates within TJs, whereas when it is dephosphorylated, it relocates to the cytoplasm. This occludin's phosphorylation significantly impacts the stability of TJs and regulates the passage of molecules between cells (paracellular transport) [46, 47].

Claudins, on the other hand, are the main TJs determining barrier function. Claudins effectively regulate the movement of ions through the paracellular space. Like occludin, they are subject of posttranslational phosphorylation of specific serine and threonine residues [48]. Claudins form channels that possess biophysical properties of ion channels, selectively permitting the passage of selected ions [48]. To date, only a limited number of claudins have been characterized as pore-forming proteins. Among them, claudin-2, claudin-10b, and claudin-15 function as cation pores, while claudin-10a and claudin-17 serve as anion pores [48-52]. The barrier forming claudins include claudin-1, 3, 4, 5, 6, 8, 9, 11, 12, 14, 17, 18, and 19 [53-58]. However, it should be noted that certain claudins are known to form pores only in the presence of specific interactions with other claudins. For example, the combination of claudin-16 and claudin-19 has been observed to function as a cation pore, whereas claudin-4 and claudin-8 act as anion pores [59, 60]. The claudin composition of TJs varies significantly among different segments of the intestines, dictated by their distinct physiological functions. The permeability of ions with different sizes and charges is also determined by specific amino acids present in the extracellular loops of claudins. Consequently, the diffusion of substances through the paracellular pathway varies depending on the specific types of claudins expressed [39-41]. Furthermore, several lines of evidence have uncovered the significant contribution of various other claudins in influencing the barrier characteristics of epithelial cells by impacting their electrical resistance. Certain claudins have been shown to enhance resistance [61, 62], while others have been found to diminish it [63]. For instance, claudin-4 (CLD 4) has been shown to increase resistance in a dose-dependent manner by selectively discriminating against Na^+ [64-67]. Christina et al. [64] reported that claudins establish the charge selectivity of tight

junctions through the electrostatic effects of their extracellular amino acids that carry charges and through this theory, they discovered that when a positively charged residue at position 46 in the first domain of CLD4 was mutated to a negatively charged residue, the discrimination against Na+ ions was reduced and there was increased paracellular conductance. Therefore, modifying the charge at specific sites on claudins can impact paracellular transport. Furthermore, studies using MDCK cell lines have demonstrated that claudins 8, 14, and 11 decrease paracellular conductance and exhibit selectivity against cations [65-68].

Another group of proteins involved in TJ regulation is the Junctional Adhesion Molecules (JAMs), which belong to the immune globulin subfamily. JAM proteins are expressed in various cell types, including epithelial and endothelial cells, as well as leukocytes and platelets. Within epithelial TJs, JAM-A, JAM-C, CAR, ESAM, and JAM4 are present and interact laterally with other proteins at intercellular contacts to facilitate the proper assembly and functioning of polarized TJs. JAMs play a vital role in regulating intestinal permeability and inflammation [69].

Tricellulin is a specialized protein located at intercellular contacts between three adjacent cells, where it plays a crucial role in maintaining and forming the epithelial barrier. It creates a seal that prevents the passage of macromolecules while minimally affecting ion permeability [70-71].

The transmembrane TJ proteins, including claudins, occludin, and JAMs, are connected to the cytoskeletal actomyosin fibers by members of the Zonula Occludens (ZO) protein family, namely ZO-1, ZO-2, and ZO-3. These ZO proteins play multifaceted roles in cellular permeability, adhesion regulation, TJ formation, stabilization, and signal

transmission from intercellular junctions to the inner cell, thereby influencing processes like cell migration [72].

Collectively, these various proteins – occludin, claudins, JAMs, tricellulin, and ZO proteins – intricately orchestrate the formation, stability, and functioning of tight junctions. Their phosphorylation and interactions finely tune the permeability characteristics of these junctions, which are crucial for maintaining tissue integrity and homeostasis.

2.3 Mechanisms of intestinal epithelial barrier permeability

Molecules can be transported from the intestinal lumen to the lamina propria through two well-defined mechanisms (Fig. 1). The first is paracellular diffusion, which involves the movement of molecules through the TJs located between adjacent intestinal epithelial cells (IECs). These tight junctions act as barriers, controlling the passage of substances and maintaining the integrity of the intestinal barrier. The second mechanism is transcellular transport, where molecules traverse through the intestinal epithelial cells themselves. This process involves the molecules being taken up at the apical membrane of the IECs, passing through the cytoplasm, and then being released into the underlying lamina propria at the basolateral membrane (Fig. 1)

Both paracellular diffusion and transcellular transport play crucial roles in the absorption of nutrients, ions, and other essential molecules from the intestinal lumen into the bloodstream. These mechanisms are carefully regulated to ensure that harmful substances are not allowed to pass through the intestinal barrier, maintaining the overall health and function of the digestive system.

2.3.1 Paracellular transport pathway

Basically, the tight junction allows for the passage of solutes and water through two distinct paracellular pathways which are (a) the pore pathway and (b) the leak pathway. These pathways can be differentiated based on their selective permeability to different sizes and charges, as well as their capacity for transport. The pore pathway is a pathway characterized by high conductivity, and it exhibits selectivity based on charges and size, demonstrating an upper limit of approximately 6 to 8 Å in diameter [73]. This means that only ions or molecules within this size range can pass through this pathway. Moreover, the pore pathway shows preference for specific charges, allowing the passage of certain charged particles while restricting others [74].

In contrast, the leak pathway, which has lower conductivity, is not as well-defined in terms of its upper size limit. However, it is estimated to have an upper limit of around 100 Å in diameter. Unlike the pore pathway, the leak pathway lacks selectivity for charges, allowing the nonselective passage of ions and molecules regardless of their charge [75]. This leak pathway serves as a less regulated pathway for the movement of larger substances, enabling their traversal across the cellular membrane [75].

(a) The pore pathway

Irrespective of whether they exhibit cation-selectivity or anion-selectivity, all studied claudin channels are found to be size-selective, permitting paracellular passage exclusively for molecules with a diameter approximately between 6 to 8 Å [73, 76]. These channels are instrumental in defining the pore pathway and are exemplified by claudin-2 and claudin-15, both forming cation-selective channels that facilitate the paracellular movement of small cations, like Na+ ions, as well as water [77]. Notably,

recent findings demonstrated that the knockout of claudins 2 and 15 in mice resulted in significant defects in paracellular Na+ flux, along with impaired cellular sodium-coupled nutrient transport [56]. Paracellular Na+ efflux via claudin-2 and claudin-15 channels plays a pivotal role in transcellular nutrient transport. Additionally, a study by Van Itallie et al. [76] revealed that increased flux of 7-Å diameter polyethylene glycol was correlated with heightened ion conductance (i.e., reduced TEER). Further analysis of tight junction protein expression unveiled that MDCK II, the line exhibiting greater polyethylene glycol flux, expressed claudin-2, whereas the less permeable MDCK C7 line did not. The introduction of claudin-2 expression into MDCK C7 cells resulted in reduced TEER and enhanced paracellular flux of 7-Å diameter polyethylene glycol, while larger polyethylene glycols were unaffected. Consequently, Van Itallie et al. [76] concluded that claudin-2 expression leads to an increased number of small tight junction pores. Weber et al. [77] treated T84 intestinal epithelial cell monolayers with interleukin-13 (IL-13) and discovered that this treatment enhanced paracellular cation permeability but had no impact on the flux of FITC 4-kDa dextran. Further investigation revealed that IL-13 selectively induced claudin-2 expression, and blockade of claudin-2 up-regulation via siRNA prevented IL-13-induced conductance increases. Therefore, the activation of the pore pathway, specifically through the upregulation of claudin-2 expression, is a selective effect induced by IL-13. [77].

Moreover, apart from IL-13, extensive in vivo and in vitro studies have identified several other factors that contribute to the upregulation of claudin-2 in intestinal epithelial cells. These factors include IL-1, IL-6, IL-13, IL-22, and tumor necrosis factor-α (TNF-α) [78]. Moreso, several studies have demonstrated that the expression of CLDN2 is

upregulated through the activation of phosphatidylinositol-3-kinase (PI3K) signaling in response to treatment with TNF-α [78-81]. Consequently, intestinal epithelial claudin-2 has become a focal point of extensive investigation, with many studies proposing the development of targeted strategies to modulate the tight junction channels associated with the pore pathway. More so, Takigawa et al. [82] reported that treating epithelial cell monolayers with an anti-CLDN2 monoclonal antibody (mAb) targeting a specific extracellular loop of CLDN2 can enhance barrier integrity. Their study demonstrated that the anti-CLDN2 mAb effectively mitigated the reduction in tight junction (TJ) integrity caused by TNF-α. Furthermore, when cells were co-treated with both anti-TNF-a mAb and anti-CLDN2 mAb, the attenuating effects on TJ integrity showed an additive response [82]. Overall, targeting CLDN2 and its interactions with specific TJ proteins, holds the potential to modulate claudin channels and regulate the permeability of the pore pathway with therapeutic potential of maintaining and restoring barrier function in conditions characterized by compromised TJ integrity.

(b) The leak pathway

In contrast to the pore pathway, which only allows the passage of small molecules up to 8-Å in diameter, the leak pathway permits the movement of larger molecules, including lactulose, mannitol, and FITC-4 kDa dextran, across tight junctions. The leak pathway has a more extensive capacity and can accommodate molecules with diameters of up to 100-Å, without being selective based on charge [70]. Although the exact molecular structure of the leak pathway remains unidentified, it is known to be influenced by various proteins, such as occludin [71, 72], tricellulin [73], ZO-1 [74], and perijunctional actomyosin [71-75].

Various lines of evidence suggest that the activation of intestinal epithelial MLCK plays a role in accelerating immune-mediated inflammatory bowel disease (IBD), while the genetic deletion of intestinal epithelial MLCK reduces disease severity [81, 82]. Interestingly, claudin-2 upregulation, typically observed in experimental immune-mediated IBD, is diminished in mice lacking intestinal epithelial MLCK, but this effect can be restored by introducing constitutively active MLCK into intestinal epithelial cells [82]. This indicates a connection between the leak pathway and the pore pathway in disease development.

Previous studies have also shown that occludin overexpression enhances the barrier function of MDCK monolayers, and studies on both MDCK and Caco-2 occludin knockdown lines demonstrated increased paracellular permeability to macromolecules up to approximately 100 Å in diameter [70, 77]. Besides occludin, ZO-1 has also been reported to contribute to increased leak pathway permeability. This is linked to the TNF-α-mediated interaction between ZO-1 and occludin, with previous studies revealing the importance of the coiled-coil occludin/ELL domain within the cytoplasmic C-terminal occludin tail, which depends on the K433 residue forming part of the occludin binding surface for ZO-1 [70, 77]. The interaction with ZO-1 may play a central role in MLCK-dependent regulation of the leak pathway, as ZO-1 directly binds to F-actin, unlike occludin [79]. Consistently, the actin binding region of ZO-1 is essential for in vitro barrier regulation by MLCK, and ZO-1 knockdown increases leak pathway permeability of epithelial monolayers [74].

In addition to occludin and ZO-1, tricellulin has also been implicated in the regulation of the leak pathway [70, 71]. This is supported by findings that tricellulin overexpression

reduces paracellular macromolecular flux and morphological analyses demonstrating a distinctive tight junction structure at tricellular contacts, which is disrupted in tricellulin knockout mice [70]. Tricellulin's contribution to increased leak pathway permeability may be related to occludin endocytosis since the loss of occludin leads to the expansion of tricellulin distribution, including bicellular tight junction regions [70, 71]. Overall, the actin cytoskeleton, ZO-1, occludin, and tricellulin are all implicated in the regulation of the leak pathway.

2.3.2 Transcellular transport pathway

The process of transcellular transport involves the movement of substances across epithelial cells through various mechanisms such as active transport and endocytosis-transcytosis-exocytosis [83, 84]. Within the intestinal lumen, substances can be taken up into the apical (luminal) side of the epithelial cells through processes like active transport, facilitated diffusion, or secondary active transport. An example of this is the uptake of glucose, which is facilitated by the sodium-glucose co-transporter 1 (SGLT1). SGLT1 utilizes the energy derived from the sodium gradient to transport glucose against its concentration gradient, allowing it to enter the epithelial cells [27, 36]

Once inside the epithelial cell, the absorbed substances need to be transported across the cytoplasm to reach the basolateral side of the cell. This transport across the cytoplasm ensures that the substances can be effectively transferred from the apical to the basolateral membrane of the epithelial cells [84]. Additionally, under normal physiological conditions, there is evidence that molecules with a molecular weight (MW) greater than 600 Da, such as food antigens and peptides, are sampled by the epithelial cells through endocytosis at the apical membrane. These molecules are then transcytosed

towards the lamina propria. During transcytosis, full-length peptides or proteins are partially degraded in acidic and lysosomal compartments. Eventually, they are released in the form of either amino acids (total degradation) or breakdown products (partial degradation) at the basolateral pole of enterocytes [84].

However, during pathological conditions like inflammatory bowel disease (IBD), there can be an increased permeability of the intestine, leading to a higher rate of macromolecule penetration. This increased permeability is often associated with an augmented transcytosis of luminal materials [83, 84]. Previous studies have indicated that the enhanced endosomal uptake of antigens in these conditions is mediated by TNFα which has been linked to increased transcellular transport [83, 84].

Overall, in the context of intestinal inflammation, there is a simultaneous increase in both paracellular and transcellular transport pathways. The increased transport activity adversely impacts the local immune response. Consequently, a detrimental cycle is formed, wherein luminal antigens gain access to the lamina propria, where they interact with immune cells. This interaction triggers the secretion of factors that enhance intestinal permeability. These factors, in turn, contribute to the progressive deterioration of the intestinal epithelial barrier function, thus exacerbating the condition [83-85].

3. MECHANISTIC TARGET OF RAPAMYCIN COMPLEX 1 (mTORC1) PATHWAY AND ITS COMPONENTS

The mechanistic target of rapamycin (mTOR) is a well-conserved threonine/serine kinase found in two distinct protein complexes: mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). mTORC1 comprises five essential protein components: mTOR (the catalytic subunit of the complex), regulatory-associated protein

of mTOR (Raptor), mammalian lethal with Sec13 protein 8 (mLST8), proline-rich Akt substrate 40 kDa (PRAS40), and DEP-domain-containing mTOR-interacting protein (Deptor) [86]. Raptor plays a vital role in the formation of mTORC1, facilitating the recruitment and binding of mTOR substrates such as p70 S6K1 and 4E-BP1 [87] (Fig. 1). Meanwhile, mLST8 contributes to the stability of the mTOR-Raptor association by interacting with the kinase domain of mTOR, thus exerting a positive influence on mTOR activation [86-88]. On the other hand, PRAS40 and Deptor interact with mTORC1 and serve as inhibitors of its activity. Interestingly, upon mTORC1 activation, both PRAS40 and Deptor undergo degradation [86-88].

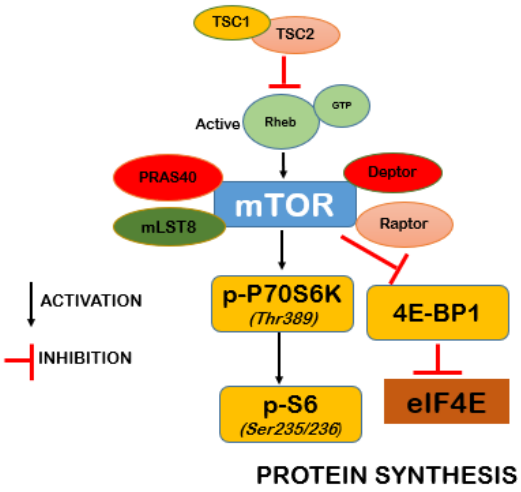


Fig. 2. mTORC1 pathway leading to protein synthesis. The inactivation of the TSC1/2 complex enables GTP-bound Rheb to bind to and activate mTORC1, leading to a series of phosphorylation events. This, in turn, drives the process of protein synthesis by promoting the phosphorylation of p70S6K, subsequent phosphorylation of S6, and inhibitory phosphorylation of 4EBP1.

Furthermore, the activity of mTORC1, a key signaling complex involved in various cellular processes, is tightly regulated by growth factors. These growth factors exert their influence on mTORC1 through two primary pathways: the PI3K/Akt pathway and the Ras/MAPK pathway. Both pathways converge on the tuberous sclerosis heterodimeric complex (TSC1/2), which serves as an upstream inhibitor of mTORC1 signaling. The TSC1/2 complex is responsible for inhibiting the GTPase function of Rheb, a critical regulator of mTORC1 activity [89-91]

Genetic evidence has established that Rheb acts downstream of TSC1/2. Consequently, the elimination or inactivation of either TSC1 or TSC2 leads to an increase in the fractional GTP charge of Rheb, resulting in the constitutive activation of mTORC1 [92]. Additionally, the activation of mTORC1 is facilitated by the inhibitory phosphorylation of the TSC1/2 complex by Erk and Akt. This phosphorylation event stimulates the activation of p70S6K, which in turn phosphorylates mTOR (Ser2448) in a positive feedback loop. Akt also phosphorylates PRAS40 (Thr246), an accessory partner and inhibitor of mTORC1. In its non-phosphorylated form, PRAS40 negatively regulates mTORC1 by competing with 4EBP1 and p70S6K for binding with mTORC1. However, phosphorylation of PRAS40 by mTOR and Akt leads to the dissociation of p-PRAS40 from the complex, allowing 4EBP1 and p70S6K to bind [93, 94].

4. ACTIVATION OF mTORC1 IN INTESTINAL EPITHELIAL CELLS AND MACROPHAGES

Recent evidence suggests that mTORC1 plays a crucial role in regulating intestinal barrier function [95, 96]. Activation of mTORC1 stimulates intestinal stem cells (ISCs) to undergo division and migration from the crypts, which is essential for

barrier repair and renewal. Various stimuli, including growth factors, amino acids, and even commensal microbiota, can activate mTORC1 and promote this process [95-98]. Given the critical role of ISCs in maintaining the integrity of the intestinal barrier, the regulation of their function through mTORC1 activation is crucial. However, previous studies have reported that sustained activation of intestinal mTORC1 resulting from repeated insults or injuries to the mucosa can be detrimental [99]. Uncontrolled mTORC1 activation has been associated with abnormal cell dedifferentiation, exacerbation of the gut inflammatory response, and the development of tumors [99]. Therefore, while mTORC1 activation is essential for the normal functioning and maintenance of intestinal stem cells (ISCs) and the integrity of the intestinal barrier, its unregulated and persistent activation can have harmful consequences.

Moreover, mTORC1 not only regulates the immune response in the intestinal epithelium but also modulates innate immune responses in the intestine by influencing macrophages [100]. When intestinal epithelial cells experience damage or injury, they release damage-associated molecular patterns (DAMPs), such as ATP or formylated peptides. These DAMPs attract wound-associated macrophages (WAMs) from the periphery to the injured epithelium, challenging mucosal immune tolerance [100-102]. At the site of injury, the interaction between macrophages and intestinal epithelial cells is mediated by mTORC1 activation, and this plays a significant role in promoting chronic intestinal inflammation. Both macrophages and intestinal epithelial cells, upon mTORC1 activation, release pro-inflammatory cytokines in the lamina propria [100-102]. This, in turn, stimulates unnecessary proliferation and inflammation of differentiated intestinal

epithelial cells in undamaged areas of the epithelium, perpetuating the inflammatory stress [99, 103].

Kaur et al. [103] reported that mTORC1 activity positively correlated with the expression of both anti-inflammatory and proinflammatory cytokines specifically TNF-α, IL-6, IL-8, and IL-10 in non-stimulated and LPS-stimulated THP-1 macrophages. Notably, LPS significantly augmented the cytokine gene expression in an mTORC1-dependent manner. The study concluded that the activation of mTORC1 due to knockdown of TSC2 in THP-1 macrophages (THP-1shTSC2) enhanced the expression of TNF-α, IL-6, IL-8, and IL-10 in response to LPS stimulation. The mechanisms underlying this phenomenon was attributed to the interaction of mTORC1 with various signal transduction pathways, including MAPK, NFκB, mTORC2, and CREB, which play crucial roles in macrophage polarization [103-105].

5. ROLE OF mTORC1 IN INTESTINAL EPITHELIAL BARRIER FUNCTION

Emerging evidence suggests that mTORC1 signaling is involved in the regulation of tight junction barrier function, by stimulating intestinal stem cells which are crucial for maintaining the integrity of the intestinal barrier [95-99]. Thus, mTORC1 activity might be playing a crucial role in maintaining the integrity of cellular barriers. Consistent with this notion, the activity of mTORC1 intestinal epithelial cells was found to influence the expression of TJ proteins [106].

The study showed that Raptor knockdown in Caco-2 cells (Caco-2 shRaptor cells) raised transepithelial electrical resistance (TEER, a measure of barrier integrity) and concomitantly increased permeability to 4 kDa and 500 kDa dextran molecules through both the paracellular and transcellular routes, respectively. Conversely, Caco-2 cells with

constitutively elevated mTORC1 activity (Caco-2 shTSC2 cells) were the least permeable to FITC-dextran 4 kDa despite having the lowest TEER [106]. The heightened permeability of Caco-2 shRaptor cell monolayers was due to the decreased expression of TJ proteins. In contrast, Caco-2 shTSC2 cells exhibited significantly higher expression levels of claudin-2, claudin-15, claudin-5, JAM-A, and β1 integrin compared to both Caco-2 shRaptor cells and control Caco-2 cells having baseline mTORC1 activity (Caco-2 shScramble cells). The findings suggested that the paracellular permeability of solutes larger than 8 Å in diameter, such as FITC-dextran 4 kDa (~14 Å), is contingent upon the presence of adequately sized spaces created by the diminished expression of TJs.

Conversely, the permeability of electrolytes, especially small ions, is further regulated by ion-selective claudin-2 and claudin-15 molecules within the TJs. Consequently, the low expression of claudin-2 and claudin-15 in Caco-2 shRaptor cells hindered the paracellular transport of ions resulting in higher TEER values and increased electrical resistance.

While this study provided insights into the involvement of mTORC1 in TJ barrier function, the identification of downstream effectors and targets of mTORC1 involved in TJ regulation remains an area of active investigation. Understanding the specific molecular mechanisms by which mTORC1 modulates the expression and localization of TJ proteins, as well as the actin cytoskeleton remodeling, could reveal novel therapeutic targets for conditions and diseases related to TJ dysfunction.

6. THERAPEUTIC INTERVENTIONS TARGETING mTORC1 IN MACROPHAGES TO PRESERVE INTESTINAL EPITHELIAL BARRIER FUNCTION

Considering the role of mTORC1 in mediating intestinal inflammation, some studies have suggested that inhibition of mTORC1 in IECs during inflammatory episodes might be beneficial [100, 102]. While inhibiting mTORC1 may be promising, it may also impede the repair process of damaged intestinal mucosa caused by insults such as dextran sulfate sodium (DSS) [101].

Kaur et al. [109] reported that curcumin (CUR), an anti-inflammatory polyphenol found in the rhizome of turmeric (*Curcuma longa*), effectively suppressed the LPS-induced immune response of THP-1-derived macrophages in an mTORC1-dependent manner. The study showed that CUR inhibited macrophage polarization towards the M1 or M2 subtype when exposed to LPS and/or under constitutively active mTORC1, thereby guiding monocytes into a quiescent M0 macrophage subtype [109]. These effects were brought about by CUR which acted on diverse cellular targets including mTORC2, MAPK, and CREB, which intricately interact with mTORC1 [109-111].

REFERENCES

1) Ayabe T, Satchell DP, Wilson CL, Parks WC, Selsted ME, Ouellette AJ. Secretion of microbicidal alpha-defensins by intestinal Paneth cells in response to bacteria. Nat Immunol, 2000. 1:113–118
2) Pelaseyed T, Bergstrom JH, Gustafsson JK, Ermund A, Birchenough GM, Schutte A et al. The mucus and mucins of the goblet cells and enterocytes provide the first defense line of the gastrointestinal tract and interact with the immune system. Immunol Rev, 2014. 260:8–20

3) Van Der Flier LG, Clevers H. Stem cells, self-renewal, and differentiation in the intestinal epithelium. Annu Rev Physiol, 2009. 71:241–260.
4) Noah TK, Donahue B, Shroyer NF. Intestinal development and differentiation. Exp Cell Res, 2011. 317:2702–2710.
5) Scoville, D. H., & Sato, T. Current view: intestinal stem cells and signaling. Gastroenterology, 2008. 134(3), 849-864.
6) Ivanov AI. Structure and regulation of intestinal epithelial tight junctions: current concepts and unanswered questions. Adv Exp Med Biol, 2012. 763:132–148.
7) Bischoff, S. C., Barbara, G., Buurman, W., Ockhuizen, T., Schulzke, J.-D., Serino, M., Wells, J. M. Intestinal permeability–a new target for disease prevention and therapy. BMC gastroenterology, 2014. 14(1), 189.
8) Cheng P, Yao J, Wang C, Zhang L, Kong W. Molecular and cellular mechanisms of tight junction dysfunction in the irritable bowel syndrome. Mol Med Rep, 2015. 12(3):3257.
9) Turner J. Intestinal mucosal barrier function in health and disease. Nat Rev Immunol, 2009. 9:799-809.
10) Anderson, J.M. Molecular structure of tight junctions and their role in epithelial transport. News Physiol Sci, 2001. 16: p. 126-30.
11) Anderson, J.M. and C.M. Van Itallie. Physiology and function of the tight junction. Cold Spring Harb Perspect Biol, 2009. 1(2): p. a002584.
12) France, M.M. and J.R. Turner. The mucosal barrier at a glance. J Cell Sci, 2017. 130(2): p. 307-314.
13) Krug, S.M., et al. Claudin-17 forms tight junction channels with distinct anion selectivity. Cell Mol Life Sci, 2012.69(16): p. 2765-78.
14) Tanaka, H., et al. Claudin-21 Has a Paracellular Channel Role at Tight Junctions. Mol Cell Biol, 2016. 36(6): p. 954-64.
15) Van Itallie, C.M., et al. The density of small tight junction pores varies among cell types and is increased by expression of claudin-2. J Cell Sci, 2008.121(Pt 3): p. 298-305.

16) Yu, A.S., et al. Molecular basis for cation selectivity in claudin-2-based paracellular pores: identification of an electrostatic interaction site. J Gen Physiol, 2009. 133(1): p. 111-27.

17) Buschmann, M.M., et al (2013). Occludin OCEL-domain interactions are required for maintenance and regulation of the tight junction barrier to macromolecular flux. Mol Biol Cell. 24(19): p. 3056-68.

18) Aihara, E et al (2016). Epithelial Regeneration After Gastric Ulceration Causes Prolonged Cell-Type Alterations. Cell Mol Gastroenterol Hepatol. 2(5): p. 625-647.

19) Suzuki, T. (2013). Regulation of intestinal epithelial permeability by tight junctions. Cellular and molecular life sciences, 70(4), 631-659.

20) L. Barron, R.C. Sun, B. Aladegbami, C.R. Erwin, B.W. Warner, J. Guo, Intestinal epithelial-specific mTORC1 activation enhances intestinal adaptation after small bowel resection, Cell. Mol. Gastroenterol. Hepatol. 3 (2017) 231–244.

21) Q. Qin, X. Xu, X. Wang, H. Wu, H. Zhu, Y. Hou, et al., Glutamate alleviates intestinal injury, maintains mTOR and suppresses TLR4 and NOD signaling pathways in weanling pigs challenged with lipopolysaccharide, Sci. Rep. 8 (2018) 15124.

22) J.B. Ewaschuk, G.K. Murdoch, I.R. Johnson, K.L. Madsen, C.J. Field, Glutamine supplementation improves intestinal barrier function in a weaned piglet model of Escherichia coli infection, Br. J. Nutr. 106 (2011) 870–877.

23) D. Yi, Y. Hou, L. Wang, W. Ouyang, M. Long, D. Zhao, et al., L-glutamine enhances enterocyte growth via activation of the mTOR signaling pathway independently of AMPK, Amino Acids 47 (2015) 65–78.

24) Y. Zhou, P. Rychahou, Q. Wang, H.L. Weiss, B.M. Evers, TSC2/mTORC1 signaling controls paneth and goblet cell differentiation in the intestinal epithelium, Cell Death Dis. 6 (2015), e1631.

25) Kerckhoffs AP, Akkermans LM, de Smet MB, Besselink MG, Hietbrink F, Bartelink IH, Busschers WB, Samsom M, Renooij W: Intestinal permeability in irritable bowel syndrome patients: effects of NSAIDs. Dig Dis Sci. 2010, 55:716–723.

26) Lam YY, Ha CW, Campbell CR, Mitchell AJ, Dinudom A, Oscarsson J, Cook DI, Hunt NH, Caterson ID, Holmes AJ, Storlien LH: Increased gut permeability and microbiota

change associate with mesenteric fat inflammation and metabolic dysfunction in diet-induced obese mice. PLoS One. 2012, 7:e34233.

27) Buckley A, Turner JR. Cell biology of tight junction barrier regulation and mucosal disease. Cold Spring Harb Perspect Biol (2018) 10: a029314. doi: 10.1101/cshperspect.a029314.

28) Guo C, Shen J. Cytoskeletal organization and cell polarity in the pathogenesis of crohn's disease. Clin Rev Allergy Immunol (2021) 60:164–74. doi: 10.1007/s12016-020-08795-5.

29) Sugita K, Kabashima K. Tight junctions in the development of asthma, chronic rhinosinusitis, atopic dermatitis, eosinophilic esophagitis, and inflammatory bowel diseases. J Leukoc Biol (2020) 107:749–62. doi: 10.1002/JLB.5MR0120-230R.

30) Helander HF, Fandriks L. Surface area of the digestive tract - revisited. Scand J Gastroenterol. 2014. 49: 681–689.

31) Van Der Flier LG, Clevers H. Stem cells, self-renewal, and differentiation in the intestinal epithelium. Annu Rev Physiol, 2009. 71:241–260.

32) Peterson LW, Artis D. Intestinal epithelial cells: Regulators of barrier function and immune homeostasis. Nat Rev Immunol. 2014. 14: 141–153.

33) Elphick D, Mahida Y. Paneth cells: Their role in innate immunity and inflammatory disease. Gut 2005; 54:1802-9.

34) Salzman N, Hung K, Haribhai D, et al. Enteric defensins are essential regulators of intestinal microbial ecology. Nat Immunol. 2010;11(1):76-83.

35) Shan M, Gentile M, Yeiser J, et al. Mucus enhances gut homeostasis and oral tolerance by delivering immunoregulatory signals. Science. 2013; 342:447-53.

36) Miguel A. Garcia, W. James Nelson, and Natalie Chavez. Cell–Cell Junctions Organize Structural and Signaling Networks. Cold Spring Harb Perspect Biol 2018;10:a029181.

37) Galipeau HJ, Verdu EF. The complex task of measuring intestinal permeability in basic and clinical science. Neurogastroenterology & Motility. 2016;28(7):957-65.

38) Garcia MA, Nelson WJ, Chavez N. Cell-cell junctions organize structural and signaling networks. Cold Spring Harb Perspect Biol (2018) 10:a029181.

39) Rubsam M, Broussard JA, Wickstrom SA, Nekrasova O, Green KJ, Niessen CM. Adherens junctions and desmosomes coordinate mechanics and signaling to orchestrate

tissue morphogenesis and function: An evolutionary perspective. Cold Spring Harb Perspect Biol (2018) 10:a029207.

40) Srinivasan B, Kolli AR, Esch MB, Abaci HE, Shuler ML, Hickman JJ. TEER measurement techniques for in vitro barrier model systems. Journal of laboratory automation. 2015 ;20(2):107-26.

41) Benson K, Cramer S, Galla HJ. Impedance-Based Cell Monitoring: Barrier Properties and Beyond. Fluids Barriers CNS. 2013; 10:5.

42) Zihni C, Mills C, Matter K, Balda MS. Tight junctions: From simple barriers to multifunctional molecular gates. Nat Rev Mol Cell Biol. 2016;17: 564–580.

43) Van Itallie CM, Anderson JM. Architecture of tight junctions and principles of molecular composition. Semin Cell Dev Biol. 2014; 36: 157–165.

44) Furuse M, Sasaki H, Fujimoto K, Tsukita S. A single gene product, claudin-1 or -2, reconstitutes tight junction strands and recruits occludin in fibroblasts. J Cell Biol. 1998; 143: 391–401.

45) Sapone A, Lammers KM, Casolaro V, Cammarota M, Giuliano MT, De Rosa M, Stefanile R, Mazzarella G, Tolone C, Russo MI, et al. 2011. Divergence of gut permeability and mucosal immune gene expression in two gluten-associated conditions: Celiac disease and gluten sensitivity. BMC Med 9: 23.

46) Dörfel M, Huber O. Modulation of tight junction structure and function by kinases and phosphatases targeting occludin. J Biomed Biotechnol 2012; 2012:807356.

47) Hartsock A, Nelson W. Adherens and tight junctions: Structure, function and connections to the actin cytoskeleton. Biochim Biophys Acta 2008;1778:660-9.

48) Escaffit F, Boudreau F, Beaulieu J. Differential expression of claudin-2 along the human intestine: Implication of GATA-4 in the maintenance of claudin-2 in differentiating cells. J Cell Physiol 2005;203:15-26.

49) Liu Y, Nusrat A, Schnell F, et al. Human junction adhesion molecule regulates tight junction resealing in epithelia. J Cell Sci 2000;113:2363-74.

50) Laukoetter M, Nava P, Lee W, et al. JAM-A regulates permeability and inflammation in the intestine in vivo. J Exp Med. 2007;204:3067-76.

51) Amasheh S, Meiri N, Gitter A.H, Schoneberg T, Mankertz J, Schulzke J.D, Fromm M. Claudin-2 expression induces cation-selective channels in tight junctions of epithelial cells. J Cell Sci. 2002; 115: 4969–4976.

52) Günzel D, Stuiver M, Kausalya PJ, Haisch L, Krug SM, Rosenthal R, Meij IC, Hunziker W, Fromm M, Muller D. Claudin-10 exists in six alternatively spliced isoforms that exhibit distinct localization and function. J Cell Sci. 2009;122: 1507–1517.

53) Inai T, Kamimura T, Hirose E, Iida H, Shibata Y. The protoplasmic or exoplasmic face association of tight junction particles cannot predict paracellular permeability or heterotypic claudin compatibility. Eur J Cell Biol. 2010; 89: 547–556.

54) Krug SM, Günzel D, Conrad MP, Rosenthal R, Fromm A, Amasheh S, Schulzke JD, Fromm M. Claudin-17 forms tight junction channels with distinct anion selectivity. Cellular and molecular life sciences. 2012; 69:2765-78.

55) Tamura A, Hayashi H, Imasato M, Yamazaki Y, Hagiwara A, Wada M, Noda T, Watanabe M, Suzuki Y, Tsukita S. Loss of claudin-15, but not claudin-2, causes Na+ deficiency and glucose malabsorption in mouse small intestine. Gastroenterology. 2011;140(3):913-23.

56) Alexandre MD, Jeansonne BG, Renegar RH, Tatum R, Chen YH. The first extracellular domain of claudin-7 affects paracellular Cl- permeability. Biochem Biophys Res Commun. 2007; 357: 87–91.

57) Hou J, Renigunta A, Konrad M, Gomes AS, Schneeberger EE, Paul DL, Waldegger S, Goodenough DA. Claudin-16 and claudin-19 interact and form a cation-selective tight junction complex. J Clin Invest. 2008; 118: 619–628.

58) Milatz S, Krug SM, Rosenthal R, Günzel D, Müller D, Schulzke JD, Amasheh S, Fromm M. Claudin-3 acts as a sealing component of the tight junction for ions of either charge and uncharged solutes. Biochimica et Biophysica Acta (BBA)-Biomembranes. 2010;1798(11):2048-57.

59) Wen H, Watry DD, Marcondes MC, Fox HS. Selective decrease in paracellular conductance of tight junctions: role of the first extracellular domain of claudin-5. Mol Cell Biol. 2004; 24: 8408–8417.

60) Sas D, Hu M, Moe OW, Baum M. Effect of claudins 6 and 9 on paracellular permeability in MDCK II cells. American Journal of Physiology-Regulatory, Integrative and Comparative Physiology. 2008 ;295(5):R1713-9.

61) Angelow S, Schneeberger EE, Yu AS. Claudin-8 expression in renal epithelial cells augments the paracellular barrier by replacing endogenous claudin-2. J Membr Biol 2007; 215: 147–159.

62) Hou J, Renigunta A, Konrad M, Gomes AS, Schneeberger EE, Paul DL, Waldegger S, Goodenough DA. Claudin-16, and claudin-19 interact and form a cation-selective tight junction complex. J Clin Invest. 2008; 118: 619–628.

63) Hou J, Renigunta A, Yang J, Waldegger S. Claudin-4 forms paracellular chloride channel
in the kidney and requires claudin-8 for tight junction localization. Proc Natl Acad Sci USA.
2010; 107: 18010–18015.

64) Christina M. Van Itallie and James M. Anderson. The Role of Claudins in Determining Paracellular Charge Selectivity. Proc Am Thorac Soc. 2004; 1 (38–41).

65) Van Itallie C, Rahner C, Anderson JM. Regulated expression of claudin-4 decreases paracellular conductance through a selective decrease in sodium permeability. J Clin Invest 2001; 107:1319–1327.

66) McCarthy KM, Francis SA, McCormack JM, Lai J, Rogers RA, Skare IB, Lynch RD, Schneeberger EE. Inducible expression of claudin-1–myc but not occludin–VSV-G results in aberrant tight junction strand formation in MDCK cells. J Cell Sci 2000; 113:3387–3398.

67) Furuse M, Furuse K, Sasaki H, Tsukita S. Conversion of Zonulae occludentes from tight to leaky strand type by introducing claudin-2 into Madin-Darby canine kidney I cells. J Cell Biol 2001;153:263–272.

68) Wolf MTF, Karle SM, Zalewski I, Loeys B, Fuchshuber A, Otto E, Hildebrandt F. Further confirmation of the MCKD1 locus at chromosome 1q21 and exclusion of the claudin 6–like gene as a candidate gene. J Am Soc Nephrol 2002; 13:305A–306A.

69) Ben Yosef T, Belyantseva IA, Saunders TL, Hughes ED, Kawamoto K, Van Itallie CM, Beyer LA, Halsey K, Gardner DJ, Wilcox ER, et al. Claudin 14 knockout mice,

a model for autosomal recessive deafness DFNB29, are deaf due to cochlear hair cell degeneration. Hum Mol Genet 2003; 12:2049–2061.

70) Ikenouchi J, Furuse M, Furuse K, Sasaki H, Tsukita S, Tsukita S. Tricellulin constitutes a novel barrier at tricellular contacts of epithelial cells. The Journal of cell biology. 2005 ;171(6):939-45.

71) Mariano C, Sasaki H, Brites D, Brito MA. A look at tricellulin and its role in tight junction formation and maintenance. European journal of cell biology. 2011;90(10):787-96.

72) Umeda K, Ikenouchi J, Katahira-Tayama S, Furuse K, Sasaki H, Nakayama M, Matsui T, Tsukita S, Furuse M, Tsukita S. ZO-1 and ZO-2 independently determine where claudins are polymerized in tight-junction strand formation. Cell. 2006;126(4):741-54.

73) Shen L, Weber CR, Raleigh DR, Yu D, Turner JR. Tight junction pore and leak pathways: a dynamic duo. Annual review of physiology. 2011; 73:283-309.

74) Wyatt J, Vogelsang H, Hübl W, Waldhoer T, Lochs H. Intestinal permeability, and the prediction of relapse in Crohn's disease. The Lancet. 1993;341(8858):1437-9.

75) Yu AS, Cheng MH, Angelow S, Günzel D, Kanzawa SA, Schneeberger EE, Fromm M, Coalson RD. Molecular basis for cation selectivity in claudin-2–based paracellular pores: identification of an electrostatic interaction site. Journal of General Physiology. 2009;133(1):111-27.

76) Van Itallie CM, Holmes J, Bridges A, Gookin JL, Coccaro MR, Proctor W, Colegio OR, Anderson JM. The density of small tight junction pores varies among cell types and is increased by expression of claudin-2. Journal of Cell Science. 2008 ;121(3):298-305.

77) Weber CR, Raleigh DR, Su L, Shen L, Sullivan EA, Wang Y, Turner JR. Epithelial myosin light chain kinase activation induces mucosal interleukin-13 expression to alter tight junction ion selectivity. J Biol Chem. 2010; 285:12037–12046.

78) Raleigh DR, Boe DM, Yu D, Weber CR, Marchiando AM, Bradford EM, Wang Y, Wu L, Schneeberger EE, Shen L, Turner JR. Occludin S408 phosphorylation regulates tight junction protein interactions and barrier function. J Cell Biol. 2011; 193:565–582.

79) Mankertz J, Amasheh M, Krug SM, Fromm A, Amasheh S, Hillenbrand B, Tavalali S, Fromm M, Schulzke JD. TNFα up-regulates claudin-2 expression in epithelial HT-29/B6 cells via phosphatidylinositol-3-kinase signaling. Cell and tissue research. 2009;336(1):67-77.

80) Prasad S, Mingrino R, Kaukinen K, Hayes KL, Powell RM, MacDonald TT, Collins JE. Inflammatory processes have differential effects on claudins 2, 3 and 4 in colonic epithelial cells. Laboratory investigation. 2005;85(9):1139-62.

81) Amasheh M, Fromm A, Krug SM, Amasheh S, Andres S, Zeitz M, Fromm M, Schulzke JD. TNFα-induced and berberine-antagonized tight junction barrier impairment via tyrosine kinase, Akt and NFκB signaling. Journal of cell science. 2010;123(23):4145-55.

82) Takigawa M, Iida M, Nagase S, Suzuki H, Watari A, Tada M, Okada Y, Doi T, Fukasawa M, Yagi K, Kunisawa J. Creation of a Claudin-2 Binder, and Its Tight Junction–Modulating Activity in a Human Intestinal Model. Journal of Pharmacology and Experimental Therapeutics. 2017;363(3):444-51.

83) Meijers B, Farré R, Dejongh S, Vicario M, Evenepoel P. Intestinal barrier function in chronic kidney disease. Toxins. 2018;10(7):298.

84) Menard S, Cerf-Bensussan N, Heyman M. Multiple facets of intestinal permeability and epithelial handling of dietary antigens. Mucosal immunology. 2010;3(3):247-59.

85) Liang GH, Weber CR. Molecular aspects of tight junction barrier function. Current opinion in pharmacology. 2014; 19:84-9.

86) Peterson TR, Laplante M, Thoreen CC, Sancak Y, Kang SA, Kuehl WM, et al. DEPTOR Is an mTOR Inhibitor Frequently Overexpressed in Multiple Myeloma Cells and Required for Their Survival. Cell. 2009;137(5):873–86.

87) Hara K, Maruki Y, Long X, Yoshino K ichi, Oshiro N, Hidayat S, et al. Raptor, a binding partner of target of rapamycin (TOR), mediates TOR action. Cell. 2002;110(2):177–89.

88) Nojima H, Tokunaga C, Eguchi S, Oshiro N, Hidayat S, Yoshino KI, et al. The mammalian target of rapamycin (mTOR) partner, raptor, binds the mTOR substrates p70 S6 kinase and 4E-BP1 through their TOR signaling (TOS) motif. J Biol Chem. 2003;278(18):15461–4.

89) Kim DH, Sarbassov DD, Ali SM, Latek RR, Guntur KV, Erdjument-Bromage H, Tempst P, Sabatini DM. GβL, a positive regulator of the rapamycin-sensitive pathway required for the nutrient-sensitive interaction between raptor and mTOR. Molecular cell. 2003;11(4):895-904.

90) Sancak Y, Thoreen CC, Peterson TR, Lindquist RA, Kang SA, Spooner E, et al. PRAS40 Is an Insulin-Regulated Inhibitor of the mTORC1 Protein Kinase. Mol Cell. 2007;25(6):903–15.

91) Laplante M, Sabatini DM. mTOR signaling at a glance. Journal of Cell Science. 2009;122(20):3589-94.

92) Tee AR, Manning BD, Roux PP, Cantley LC, Blenis J. Tuberous Sclerosis Complex gene products, Tuberin and Hamartin, control mTOR signaling by acting as a GTPase-activating protein complex toward Rheb. Curr Biol. 2003;13(15):1259–68.

93) Inoki K, Zhu T, Guan K-L. TSC2 mediates cellular energy response to control cell growth and survival. Cell. 2003;115(5):577–90.

94) Laplante M, Sabatini DM. mTOR signaling in growth control and disease. Cell. 2012;149(2):274-93.

95) Q. Qin, X. Xu, X. Wang, H. Wu, H. Zhu, Y. Hou, et al., Glutamate alleviates intestinal injury, maintains mTOR and suppresses TLR4 and NOD signaling pathways in weanling pigs challenged with lipopolysaccharide, Sci. Rep. 8 (2018) 15124.

96) J.B. Ewaschuk, G.K. Murdoch, I.R. Johnson, K.L. Madsen, C.J. Field, Glutamine supplementation improves intestinal barrier function in a weaned piglet model of Escherichia coli infection, Br. J. Nutr. 106 (2011) 870–877.

97) D. Yi, Y. Hou, L. Wang, W. Ouyang, M. Long, D. Zhao, et al., L-glutamine enhances enterocyte growth via activation of the mTOR signaling pathway independently of AMPK, Amino Acids 47 (2015) 65–78.

98) Y. Zhou, P. Rychahou, Q. Wang, H.L. Weiss, B.M. Evers. TSC2/mTORC1 signaling controls paneth and goblet cell differentiation in the intestinal epithelium, Cell Death Dis. 6 (2015), e1631

99) H. Kaur, R. Moreau, Role of mTORC1 in intestinal epithelial repair and tumorigenesis, Cell. Mol. Life Sci. 76 (2019) 2525–2546.

100) S. Al-Ghadban, S. Kaissi, F.R. Homaidan, H.Y. Naim, M.E. El-Sabban. Crosstalk between intestinal epithelial cells and immune cells in inflammatory bowel disease, Sci. Rep. 6 (2016) 29783.

101) Y.T. Xiao, W.H. Yan, Y. Cao, J.K. Yan, W. Cai, Neutralization of IL-6 and TNF-alpha ameliorates intestinal permeability in DSS-induced colitis, Cytokine 83 (2016) 189–192.

102) S. Wang, Q. Ye, X. Zeng, S. Qiao, Functions of macrophages in the maintenance of intestinal homeostasis, J. Immunol. Res. 2019 (2019), 1512969.

103) Harleen Kaur, Anjeza Erickson and Régis Moreau. Divergent regulation of inflammatory cytokines by mTORC1 in THP-1–derived macrophages and intestinal epithelial Caco-2 cells. Life Sciences 284 (2021) 119920.

104) Haloul M, Oliveira ERA, Kader M , Wells JZ , Tominello TR , El Andaloussi A , et al. mTORC1-mediated polarization of M1 macrophages and their accumulation in the liver correlate with immunopathology in fatal ehrlichiosis. Sci Rep 2019; 9:14050.

105) Banerjee D, Sinha A, Saikia S, Gogoi B, Rathore AK, Das AS, et al. Inflammation-induced mTORC2-Akt-mTORC1 signaling promotes macrophage foam cell formation. Biochimie. 2018; 151:139–49.

106) Harleen Kaur, Regis Moreau. Raptor knockdown concurrently increases the electrical resistance and paracellular permeability of Caco-2 cell monolayers. Life Sciences 308 (2022) 120989.

107) S. Wang, Q. Ye, X. Zeng, S. Qiao, Functions of macrophages in the maintenance of intestinal homeostasis, J. Immunol. Res. 2019 (2019), 1512969.

108) Y.T. Xiao, W.H. Yan, Y. Cao, J.K. Yan, W. Cai, Neutralization of IL-6 and TNF-alpha ameliorates intestinal permeability in DSS-induced colitis, Cytokine 83 (2016) 189–192.

109) Kaur H, Moreau R. Curcumin steers THP-1 cells under LPS and mTORC1 challenges toward phenotypically resting, low cytokine-producing macrophages. The Journal of Nutritional Biochemistry. 2021; 88:108553.

110) Kaur H, Moreau R. Curcumin represses mTORC1 signaling in Caco-2 cells by a two-sided mechanism involving the loss of IRS-1 and activation of AMPK. Cellular Signaling. 2021; 78:109842.

111) Aoki H, Takada Y, Kondo S, Sawaya R, Aggarwal BB, Kondo Y. Evidence that curcumin suppresses the growth of malignant gliomas in vitro and in vivo through induction of autophagy: role of Akt and extracellular signal-regulated kinase signaling pathways. Molecular pharmacology. 2007;72(1):29-39.

CHAPTER 2

EFFECTS OF MACROPHAGE PROINFLAMMATORY SECRETAGOGUES ON THE EPITHELIAL BARRIER FUNCTION OF CACO-2 CELL MONOLAYERS WITH MANIPULATED mTORC1 ACTIVITY

1. INTRODUCTION

The intestinal epithelium plays a crucial role in absorbing nutrients and protecting against harmful substances, therefore, to maintain its function, intestinal homeostasis is essential [1]. Achieving intestinal homeostasis involves coordinated interactions between different types of cells in the intestinal mucosa, including epithelial and immune cells [2]. The primary components of the intestinal epithelial barrier are the intestinal epithelial cells (IECs), goblet cells that produce mucus, M cells, and immune cells such as macrophages and dendritic cells found in the lamina propria [3-5]. The delicate interplay between these cells, along with the gut microbiota, determines intestinal homeostasis.

The integrity of the intestinal epithelial barrier relies on TJs that connect enterocytes, forming a semi-permeable and selective mucosal barrier. However, insult or injury can damage epithelial cells, leading to the release of damage-associated molecular patterns (DAMPs), ATP, or formylated peptides by IECs. These released molecules attract circulating monocytes to the injured epithelium [6, 7]. During this process, the lamina propria monocytes (M0 macrophages) can engulf luminal antigenic substances before they pass through the intestinal barrier and upon activation by these antigens, monocytes differentiate into proinflammatory M1 macrophages or anti-inflammatory M2 macrophages [7]. The M2 macrophages aid in epithelial repair, regeneration, and matrix remodeling by secreting IL-10 and TGF-β. In contrast, M1 macrophages secrete

proinflammatory cytokines such as TNF-α, IL-6, IL-1β, and IL-8, promoting chronic intestinal inflammation, TJ disruption, and tissue damage [6-9].

Macrophages release secretagogues that exert a direct influence on the IECs, eliciting a response characterized by the production of their own cytokines [6]. This reciprocal interaction between macrophages and IECs, mediated by the activation of mTORC1, plays a significant role in promoting chronic intestinal inflammation [10, 11]. Considering the role of mTORC1 in mediating intestinal inflammation, some studies have suggested that inhibition of mTORC1 in IECs during inflammatory episodes may potentially be beneficial [8]. While inhibiting mTORC1 may be promising, it can also impede the repair process of damaged intestinal mucosa caused by insults like dextran sulfate sodium (DSS) [7]. Thus, it is pertinent to investigate further the intricate role played by mTORC1 signaling in the context of inflammation-induced loss of intestinal epithelial barrier function.

Accumulating evidence supports a correlation between intestinal inflammation and a decrease in TEER, and this reduction is associated with increased claudin-2 expression along the inflamed crypt epithelium [12-15]. Furthermore, it was shown that suppressing the activity of Raptor (a protein involved in mTORC1 signaling), in Caco-2 cells led to increased resistance to the movement of small, charged ions across the epithelium [16]. This effect was attributed to the degradation of claudin-2 facilitated by autophagy [17, 18]. Consequently, many studies revolve around enhancing the function of TJ barrier by inhibiting inflammatory cytokines, with a view to reducing the expression of claudin-2 which is believed to be upregulated by inflammatory cytokines [19-27]. However, many of these studies focus on inhibiting one or two proinflammatory

cytokines to investigate the association between suppression of claudin-2, inflammatory cytokines, and improvement of inflammation-induced TJ barrier dysfunction. This approach, however, does not reflect the complexity resulting from combined effects of multiple cytokines that characterize intestinal inflammatory disorders, such as inflammatory bowel disease, where intestinal epithelial cells are exposed to a diverse array of proinflammatory cytokines. Investigating the complexity of these combined effects is crucial to expanding our current knowledge. Therefore, it is crucial to model the conditions of intestinal epithelial cells exhibiting low claudin-2 expression, exposed to macrophage secretions. Such studies will broaden our understanding of the interplay among these cytokines, and associated molecular pathways, and pave the way for targeted therapeutic strategies.

Numerous lines of evidence have demonstrated that upon stimulation with lipopolysaccharide (LPS), an endotoxin found in bacteria, macrophages produce various cytokines including TNFα, IL1β, IL6, IL8, IL10, IL12, IL15, and TGFβ, which impact the immune response [28-30]. Furthermore, Kaur et al. [10] revealed that upon LPS stimulation, THP-1 derived macrophages with constitutively active mTORC1 signaling (TPH-1shTSC2 macrophages) expressed significantly more IL-10, an anti-inflammatory cytokine, as well as proinflammatory cytokines TNF-α, IL-6, IL-1β, and IL-8, in comparison with THP-1 derived macrophages with depleted or baseline mTORC1 activity [10, 31]. Notably, these proinflammatory cytokines are associated with inflammatory bowel disease (IBD), as they influence the expression of TJ proteins and alter epithelial permeability [32, 33]. Moreover, their participation in the activation of diverse intracellular signaling pathways serves to underscore their impact. For instance,

TNF-α is known to trigger the activation of multiple signaling pathways, including mitogen-activated protein kinase (MAPK), c-Jun N-terminal kinase (JNK), and nuclear factor kappa B (NF-κB) [10, 11]. Similarly, IL-6 activates the JNK pathway, while IL-1β stimulates the NF-κB pathway [32, 33, 38]. Thus, understanding the intricate interplay between these proinflammatory cytokines and intracellular signaling pathways may reveal valuable therapeutic targets in the treatment of cytokine-induced TJ barrier disruption.

Building upon these findings, we asked whether the level of mTORC1 activity in Caco-2 cell monolayers would influence their epithelial barrier function when exposed to macrophage-produced proinflammatory stimuli. Therefore, a model was developed to simulate the conditions of intestinal epithelial cells when exposed to macrophage-produced inflammatory stimuli that have the potential to disrupt TJs.

In this study, we investigated the response of Caco-2 cells with manipulated mTORC1 activity, to inflammatory signals from THP-1 derived macrophages exhibiting heightened mTORC1 activity. Furthermore, recognizing the involvement of various intracellular signaling pathways in the disruption of epithelial barrier function, we investigated the impact of exposing Caco-2 cells at the apical side to specific inhibitors for selected cellular targets such as RSK, p70S6K, PI3K, MEK/ERK, p38 MAPK, JNK, and IKKβ, along with basolateral exposure to macrophage-produced inflammatory signals.

2. MATERIALS AND METHODS

2.1 Reagents

We obtained phorbol-12-myristate 13-acetate (PMA) from ThermoFisher Scientific (Cat #J63916.M), LPS from Sigma (#L4391-1MG, 99+% purity), FITC-dextran 4 kDa (#FD4) from Sigma-Aldrich, CUR from Santa Cruz Biotechnologies (#SC-200509, purity ≥95%), recombinant human TNF-α from PeproTech (#300-01A, used at a final concentration of 10 or 50ng/mL, stock was made in 1% BSA/PBS), human TNF-α neutralizing (D1B4) rabbit IgG monoclonal antibody (mAb) from Cell Signaling Technology (#7321S, used at a final concentration of 4.3ng/µL, stock was made in 1% BSA/PBS), control rabbit IgG monoclonal antibody (mAb) from PeproTech (#500-P00, used at a final concentration of 4.3µg/mL, stock was made in 1% BSA/PBS), BI-D1870, a selective RSK inhibitor from Cayman Chemical (#15264, used at a final concentration of 100 nM), LYS6K2, a selective inhibitor of p70S6K, from Cayman Chemical (#15320, used at a final concentration of 1µM), LY294002, a selective PI3K inhibitor from Cayman Chemical (#70920, , used at a final concentration of 50 nM), U0126, a selective MEK/ERK inhibitor from Cayman Chemical (#70970, used at a final concentration of 1 µM), SB 203580, a selective p38 MAPK inhibitor from Cayman Chemical (#13067, used at a final concentration of 2µM), SP600125, a selective JNK inhibitor from Cayman Chemical (#10010466, used at a final concentration of 1 µM) and IMD 0354, a selective IKKβ inhibitor from Cayman Chemical (#17290, used at a final concentration of 1µM). The inhibitors were dissolved in sterile dimethyl sulfoxide (DMSO, ATCC #4-X); DMSO final concentration was 0.1% DMSO.

2.2 Cell culture

Human monocytic leukemia THP-1 cells (ATCC #TIB202) were used as a model for blood-derived macrophages. These cells, when treated with PMA, undergo differentiation into macrophage-like cells that closely resemble primary human macrophages, overcoming limitations such as limited lifespan and individual variability [10, 31]. THP-1 cells were cultured in RPMI 1640 media (Gibco #A1049101) supplemented with 0.05 mM 2-mercaptoethanol (Gibco #21985-023), 0.25% Pen/Strep (ATCC #30-2300), and 10% FBS (low endotoxin, Gibco #16000044), subsequently referred to as complete RPMI. Human colorectal adenocarcinoma Caco-2 epithelial cells (ATCC #HTB-37) were used due to their potential to differentiate into polarized enterocytes with selectively permeable monolayers [16, 39]. Caco-2 cells were cultured in Eagle's minimum essential medium (EMEM, Corning #10009CV) supplemented with 10% fetal bovine serum (FBS, low endotoxin, Gibco #16000044) and 1% Pen/Strep (Gibco #151-140-122), subsequently referred to as complete EMEM. All cells were cultured at 37°C under 5% CO2 in a humidified incubator.

2.3 Lentivirus-mediated short-hairpin RNA gene editing

THP-1 monocytes with constitutively active mTORC1 were generated by using lentiviral shRNA to knock down TSC2 (cell line referred to as THP-1shTSC2 cells or TTHP-1). Caco-2 cells with constitutively downregulated mTORC1 activity were generated by using lentiviral shRNA to knock down *Raptor* (cell line referred to as Caco-2shRaptor or R Caco-2 cells); Caco-2 cells in which at mTORC1 activity was at baseline (control cells) were generated by using a scramble shRNA (cell line referred to as Caco-2shScramble or S Caco-2 cells). pLKO.1-TSC2 (Addgene plasmid #15478) was from

Do-Hyung Kim [35]. pLKO.1-Raptor (Addgene plasmid #1858) and pLKO.1 scramble shRNA (Addgene plasmid #1864) were from David Sabatini [36]. Details about lentivirus production were described previously [37].

2.4 Quantitative Real-Time PCR (qRT-PCR)

qRT-PCR was performed as previously described by Kaur et al. [10, 26]. Briefly, total RNA was isolated from cells using Aurum Total RNA Mini Kit (BioRad) that includes a DNase I incubation step. First strand cDNA was synthesized with oligo(dT) and random primers using BioRad iScript. All qRT-PCR were performed on a BioRad CFX96 Real-Time PCR Detection System using BioRad Sso Advanced SYBR Green supermix. Amplicon authenticity was confirmed by melt curve analysis. PCR efficiencies were assessed with serial dilutions of the template (0.0064–100 ng cDNA/reaction) and 0.3 μM of each primer and plotting quantification cycle (Cq) vs. log amount of template. PCR efficiencies between target genes and housekeeping genes were comparable, thus unknown amounts of target in the sample were determined relative to cyclophilin A (PPIA). Primer sequences are provided in Table 2.1.

Table 2.1 Oligonucleotide primer sequences used for qRT-PCR.

Gene	Sequence (5'→3') [a]	Product size (bp)	GenBank[b]	Ref.
TNFα	CCCAGGGACCTCTCTCTAATCA GCTTGAGGGTTTGCTACAACATG	104	NM_000594.4	[10, 26]
PPIA	TTCATCTGCACTGCCAAGAC TGTCCACAGTCAGCAATGGT	152	NM_001300981.1	[10, 37]

[a]Shown as sense primer followed by antisense primer. [b] GenBank accession number.

2.5 Validation of TNF-α mRNA in THP-1shTSC2 macrophages and preparation of conditioned media.

Stably transduced THP-1shTSC2 monocytes cultured in complete RPMI were differentiated into macrophages by treating them with phorbol-12-myristate 13-acetate (PMA) for 3 days [10, 31]. PMA was dissolved in sterile DMSO and used at a final concentration of 200nM. Subsequently, THP-1shTSC2 macrophages were incubated with 1µg of lipopolysaccharide (LPS) per mL of media or curcumin as described in Fig. 1A. CUR was dissolved in sterile DMSO and used onto cells at a final concentration of 20µM as previously described [31, 44]. Following incubation with LPS or LPS+CUR, the gene expression of TNF-α was assessed. Total RNA extracted from cells was subjected to qRT-PCR to determine TNF-α mRNA levels, which were then normalized using the housekeeping gene, PPIA. Once validated, conditioned media were generated from THP-1shTSC2 macrophages treated with LPS and LPS+CUR, as outlined in Fig. 1A. The conditioned media collected was centrifuged at 4000 rpm for 10 minutes at 4°C, and the supernatant was stored at -80°C until further use.

2.6 Western blotting

Cells were washed with ice-cold PBS and scraped in RIPA buffer supplemented with 1 mM DTT, Halt protease and phosphatase inhibitor cocktails (Thermo Scientific). Soluble protein concentration of clarified supernatants was determined using the Pierce BCA Assay. Cellular proteins were heat denatured (95 °C, 5 min) in Laemmli sample buffer, resolved by reducing SDS-PAGE, and transferred onto nitrocellulose membrane.

Antibodies against p70S6 kinase (p70S6K, #2708) and phospho-p70 S6 kinase (Thr389, #9234) were obtained from Cell Signaling Technology, while β-actin antibody (#A0072) was from GenScript. The blots were incubated with HRP-conjugated secondary antibodies and visualized using enhanced chemiluminescence (Plus-ECL, PerkinElmer) on a ProteinSimple imaging system. Band densitometry was determined using LI-COR Image Studio Lite software.

2.7 Measurement of transepithelial electrical resistance (TEER)

Stable S and R Caco-2 cells were seeded on Transwell® inserts (Corning #3401, 12-mm polycarbonate membrane, 0.4-μm pore, 12 inserts/plate) at a density of 50,000 cells. Over the course of 25 days, Caco-2 cells underwent spontaneous differentiation into nutrient-absorbing enterocytes specialized in absorbing nutrients, forming a functional barrier that was both tight and selectively permeable. Throughout this period, the cells were cultured in complete EMEM, with the media being refreshed every other day. The cells were maintained at 37°C under 5% CO2 in a humidified incubator. Transepithelial electrical resistance (TEER) was measured 24 hours after each media change, using a Millicell® ERS-2 system (Millipore). The TEER values were expressed as Ω (resistance) x cm2 (surface area of the insert) after subtracting the blank resistance value of inserts without cells.

2.8 Permeability assay

Paracellular transport was assessed on days 13, 15, 16, 18, and 20 after seeding. FITC-dextran 4 kDa (500 μL of 0.5 mg/mL HBSS) was added to the apical chamber of the Transwell® inserts, while the basolateral chamber was filled with 1.5 mL of HBSS

(Gibco™ #14175095). Basolateral samples (100 µL) were collected at 20, 40, 60, and 80 minutes after the addition of FITC-dextran 4 kDa to the apical chamber. An equal volume of fresh HBSS was added to the basolateral chamber after each sampling. The collected basolateral samples were diluted (1:3) with HBSS and loaded onto opaque flat bottom plates. Fluorescence measurements were obtained using a Synergy H1 hybrid multi-mode reader (BioTek) at excitation and emission wavelengths of 480 nm and 528 nm, respectively.

2.9 Statistical analysis

Results are expressed as means ± SEM based on three independent biological replicates. Statistical significance was determined by unpaired two-tailed Student's *t-test* or one-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons test using GraphPad Prism software. All statistical tests were performed at the 5% significance level.

3. RESULTS

3.1 Validation of Raptor knockdown in Caco-2 cells and elevated TNF-α mRNA expression in THP-1shTSC2-derived macrophages.

For Raptor knockdown validation, stable Caco-2 cells were cultured on 6 well plates at a density of 94,000 cells/well; until 80% confluence. Results showed that both Caco-2shScramble (referred to as S Caco-2 cells) and Caco-2shRaptor (referred to as R Caco-2 cells) maintained the expected mTORC1 activity. Specifically, the mTORC1 activity, assessed by measuring phosphorylated p70S6K at Thr389 by Western blotting, was 72% lower in R Caco-2 cells compared to S Caco-2 cells (Fig. 1; $P < 0.017$).

To validate the exacerbated expression of TNF-α in THP-1 shTSC2 macrophages and the remediation brought about by CUR, macrophages were stimulated with LPS, vehicle or LPS+CUR, and the abundance of TNF-α mRNA was quantified by qRT-PCR. Results showed that TNF-α mRNA levels were significantly higher in LPS-treated macrophages versus vehicle controls. However, CUR treatment reduced the expression of TNF-α by 97% (Fig. 1; $P < 0.0001$).

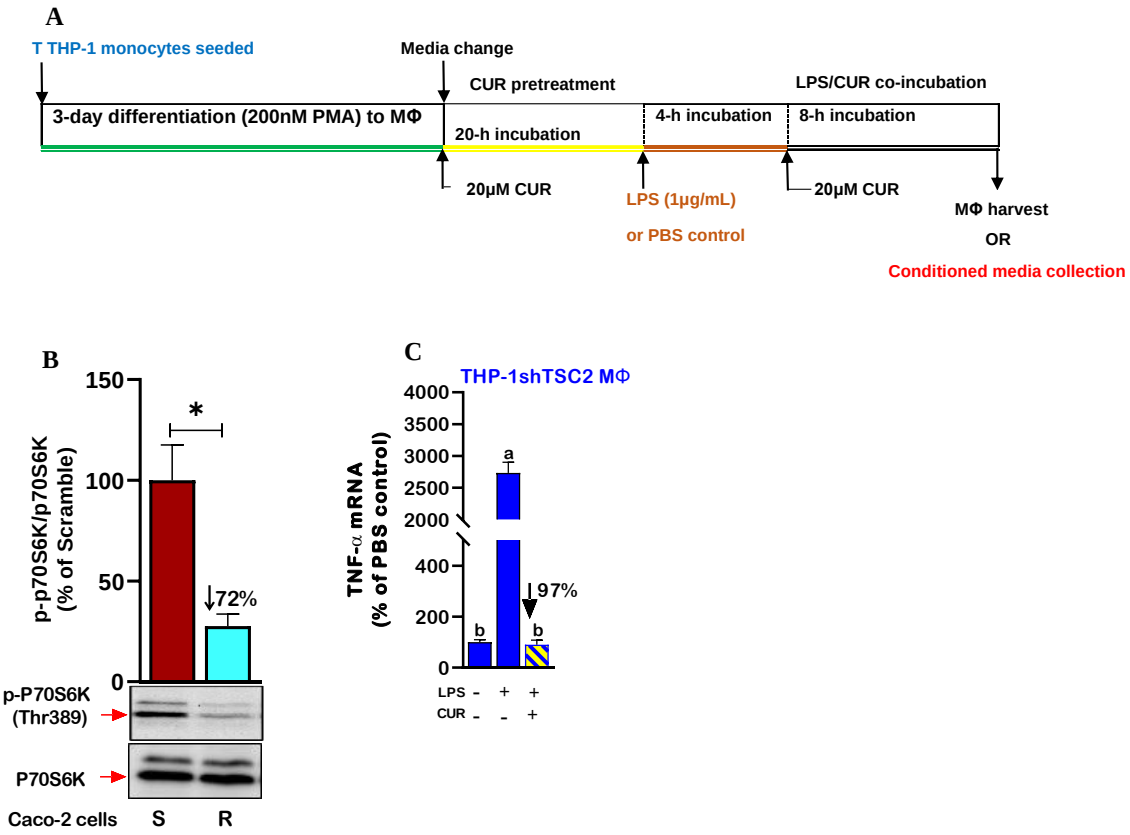


Fig. 1. Validation of Raptor knockdown in Caco-2 cells and effect of CUR on TNF-α mRNA expression in THP-1shTSC2 macrophages.

(A) Experimental timeline (B) Validation of Raptor knockdown in stably transduced Caco-2 epithelial cells (R Caco-2 cells). Scramble shRNA treated cells (S Caco-2 cells) were used as control cells. Cellular proteins were extracted in modified RIPA buffer and analyzed for p70S6K, phosphorylated p70S6K (Thr389), and β-actin by western blotting. * $P< 0.05$, Student's *t*-test, n=3. (C) T THP-1 macrophages were incubated with 1 μg LPS/mL media or (PBS control), co-incubated with CUR as outline in the experimental timeline. Following incubation with LPS or LPS+CUR, conditioned media were obtained, and total RNA was extracted for TNFα mRNA expression which was measured by qRT-PCR and normalized to housekeeping gene, PPIA. Data were analyzed by one-way ANOVA followed by Tukey's multiple comparisons test. Data not sharing a common letter are statistically different, $P < 0.05$.

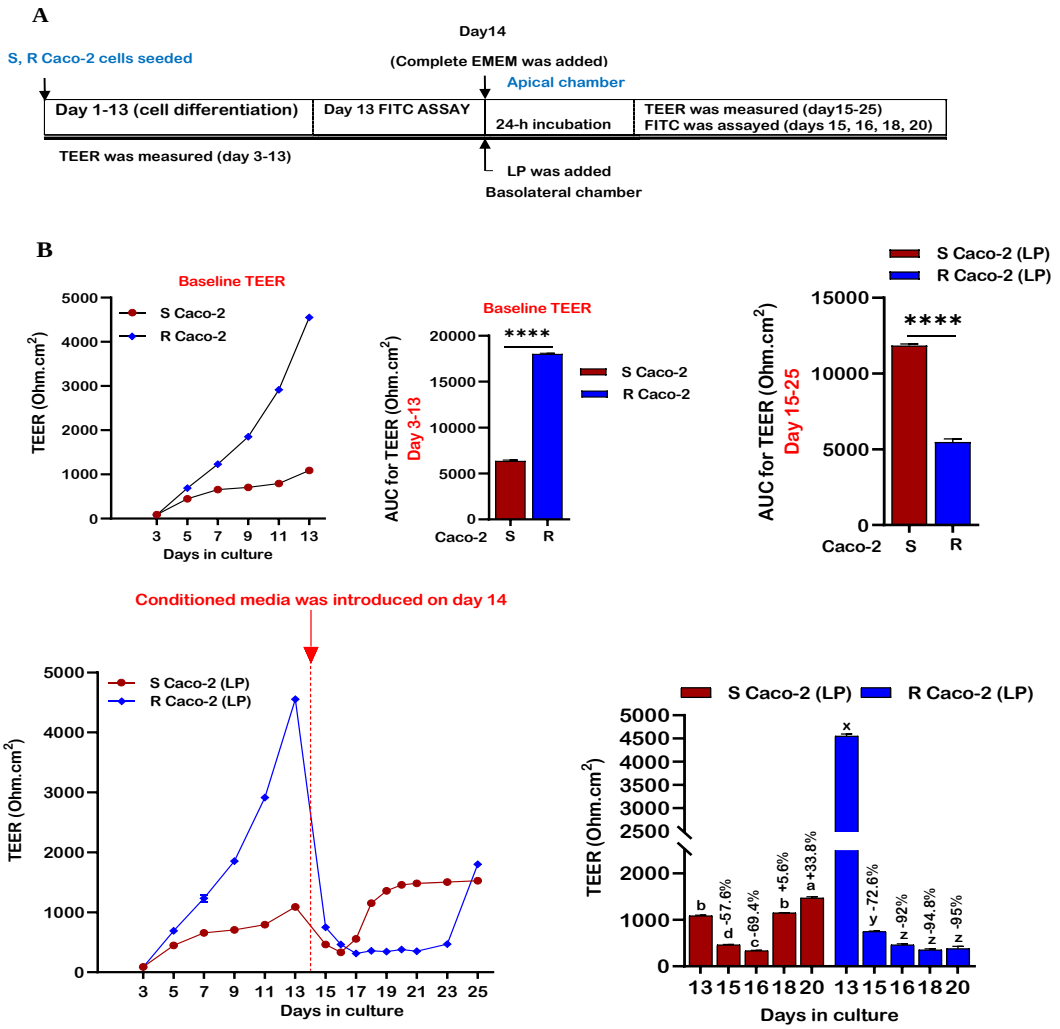
3.2 Disruption of Caco-2 monolayer barrier function by macrophage-derived conditioned media

To study the impact of conditioned media from LPS-treated THP-1shTSC2 macrophages (referred to as treatment LP) on the function of the intestinal epithelial barriers using Caco-2 cell monolayers with manipulated mTORC1 activity, we repeatedly measured TEER at 1-or 2-day intervals in S and R Caco-2 cells from day 3 to 25 post-seeding. On day 13, 15, 16, 18, and 20, TEER was measured prior to evaluating the transport of FITC-dextran 4 kDa (molecular radius ~ 14 Å). On day 13 post-seeding (prior to the addition of LP), R Caco-2 cells exhibited a 122% higher TEER than S Caco-2 cells (Fig. 2B; AUC, $P<0.0001$), along with a twofold rise in FITC-dextran 4 kDa permeability (Fig. 2C; AUC, $P<0.02$). Subsequently, on day 14, R and S Caco-2 cells were basolaterally exposed to LP, while complete EMEM was added to the apical chamber.

On day 15, both S and R Caco-2 cells showed substantial decrease of their TEER, with reduction of 57.6% and 72.6%, respectively. The decline in TEER observed in S Caco-2 cells on days 15 and 16 was significantly lower than their baseline TEER on day 13 (Fig. 2B; AUC, $P<0.0001$). S Caco-2 cells recovered their initial TEER as early as day 18 and subsequently surpassed their baseline TEER by day 20 (Fig. 2B; AUC, $P<0.0001$). Furthermore, on day 15, the apical-to-basal flux of FITC-dextran 4 kDa in S Caco-2 cells increased transiently (Fig. 2C; AUC, $P<0.005$), but the increase was significantly lower compared to that observed in R Caco-2 cells (Fig. 2C; AUC, $P<0.05$). From day 15, the TEER of R Caco-2 cells collapsed, and FITC-dextran permeability

increased up to three-fold compared to their baseline permeability recorded on day 13 (Fig. 2C; AUC, P<0.0001).

Collectively, the results showed that Raptor-depleted Caco-2 cell monolayers (constitutively downregulated mTORC1 activity) displayed elevated TEER compared to control cells (S Caco-2 cells), but TEER collapsed precipitously in the presence of macrophage-derived conditioned media (LP). It demonstrated that macrophage-derived secretagogues disrupted the epithelial transport characteristics of Caco-2 cells particularly in Caco-2 cells with depleted mTORC1 activity (R Caco-2 cells).



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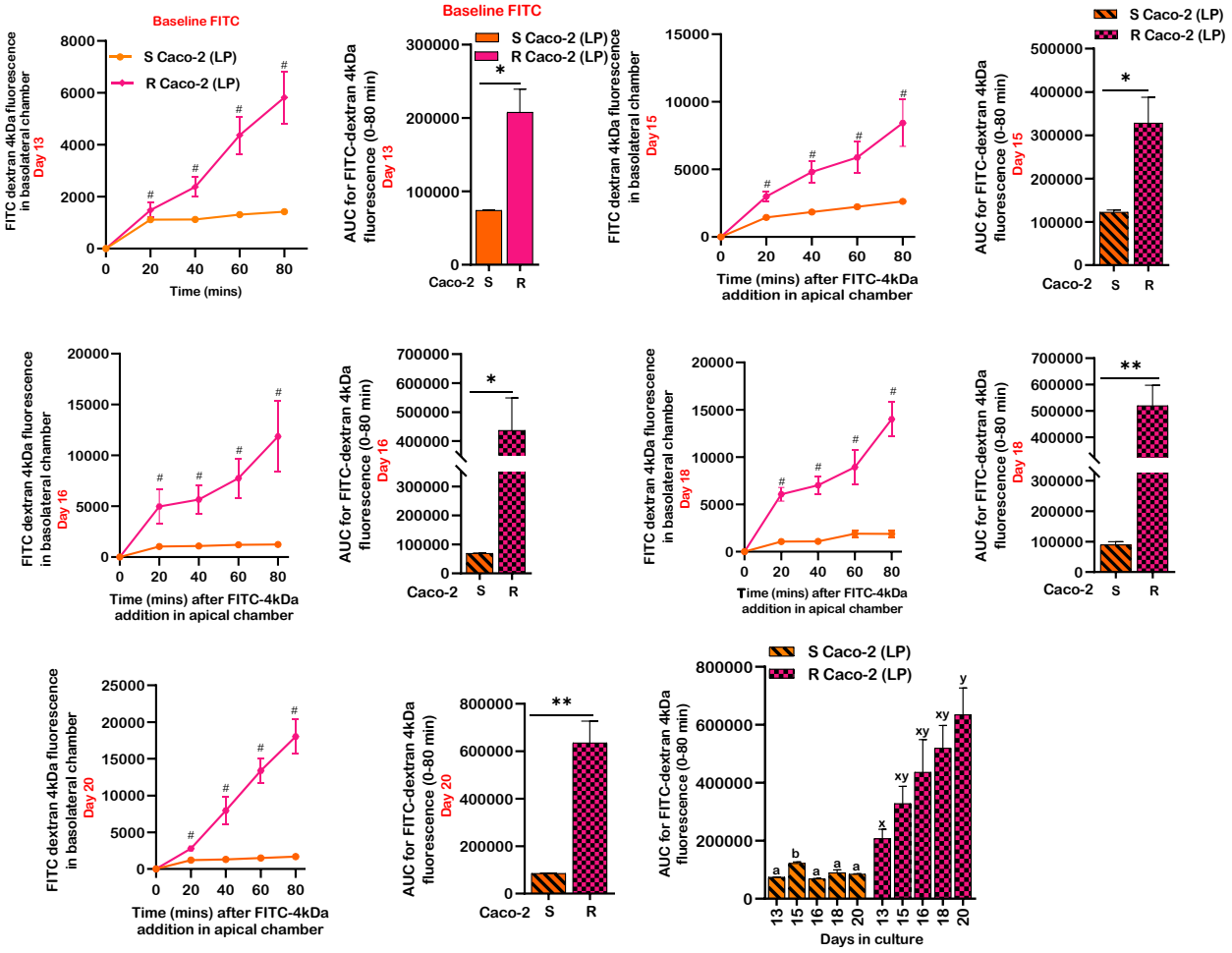


Fig. 2. Macrophage-produced-inflammatory factors negatively affected the epithelial barrier function of Raptor-depleted Caco-2 cell monolayers. To induce differentiation, stable Caco-2 cells (S, R) were cultured on Transwell® inserts for 14 days. (A) Experimental timeline (B) TEER of S, R Caco-2 cells was measured over time, and the area under the curve (AUC) was calculated for the period spanning from day 3 to day 13, and day 15-25 (*n* = 3 biological replicates). On day 14 post-seeding, conditioned media from LPS stimulated THP-1shTSC2 macrophages (LP) was added to the basolateral chamber of S Caco2 cells (LP) and R Caco2 cells (LP) to induce inflammatory stress whereas complete EMEM medium was added to the apical chamber (C) The paracellular transport of FITC-dextran 4 kDa was assessed on day 13, 15, 16, 18 and 20 (*n* = 3 biological replicates). Data in (B) and (C) were analyzed using Student's *t*-

test and one-way ANOVA for repeated measures, followed by Tukey's multiple comparisons test. Data not sharing a common letter are significantly different, $*P < 0.05$. [#]FITC-dextran 4 kDa permeability is significantly higher in R versus S Caco-2 cell monolayers at the indicated time points.

3.3 Dose-response relationship between conditioned media from LPS stimulated THP-1shTSC2 macrophages and the TEER of Raptor-depleted Caco-2 cell monolayers

First, to exclude the possible effect of RPMI – the growth medium of THP-1 derived macrophages – on the TEER of Caco-2 cells, we subjected R Caco-2 cell monolayers to pH-equilibrated complete RPMI media. To that end, complete RPMI media was placed in the CO_2 cell incubator for 32 hours, which is the time RPMI was in contact with THP-1 derived macrophages, as indicated in Fig. 1A. Caco-2 cells received pH-equilibrated complete RPMI in the basolateral chamber on day 14 post-seeding and complete EMEM medium was added to the apical chamber. From days 15 to 18, we compared the TEER values obtained from R Caco-2 cells that received complete EMEM media in the basolateral chamber with those that received complete RPMI media in the basolateral chamber. The results showed that there was no statistically significant difference in TEER values between the two groups (Fig. 3B) indicating that complete RPMI medium itself did not contribute to the drop in TEER of R Caco-2 cells.

Based on these results, we investigated the effects on TEER of varying proportions of conditioned media (CM) derived from LPS-stimulated THP-1shTSC2 macrophages (abbreviated LP in the figures) when added to the basolateral chamber of

Caco-2 cells. We tested the following proportions of LP-to-EMEM (%): 50/50, 25/75, 5/95, 1/99 and 0.1/99.9, and evaluated their effects on the TEER of R Caco-2 cell monolayers. Complete EMEM was added to the apical chamber. To that end, on day 14, R Caco-2 cells were exposed to the varying concentrations of LP including undiluted LP (abbreviated as ULP) and after 24 hours (day 15), we observed that TEER was dose dependently disrupted by LP. Specifically, ULP, 50%LP, 25%LP, and 5%LP brought down TEER of R Caco-2 cells by 90.7%, 90.8%, 84.9%, and 85.9%, respectively. and their effects were not significantly different from each other (Fig. 3C). Note that table 2.2 shows residual TEER as % of the respective TEER recorded on day 13. However, 1%LP caused a significant drop in TEER with reductions of 65% and 70.4% drop in TEER on days 15 and 16, respectively, whereas 0.1%LP decreased TEER by less than 10% on day 15 (Table 2.2; Fig. 3C). Collectively, these findings showed that CM from LPS-stimulated THP-1shTSC2 macrophages (i) disrupted TEER over a wide concentration range, (ii) the disrupting factors contained in LP were potent and/or highly concentrated, and (iii) dilution down to 1% was required to observe partial reversal of TEER collapse. In subsequent experiments, we used a proportion of LP to EMEM of 1/99 (abbreviated 1%LP), as it confers conditions to test interventions to mitigate the drop in Caco-2 TEER such as CUR, TNF-α blocking antibody, and pharmacological inhibitors.

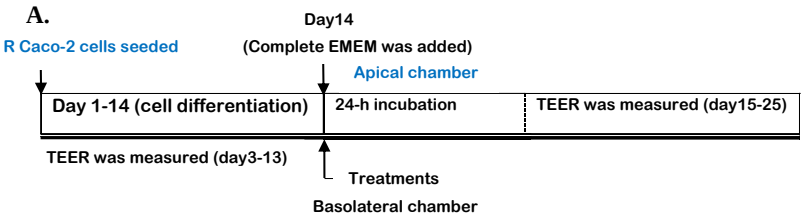
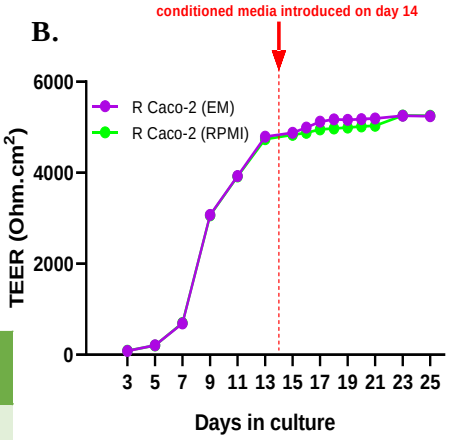
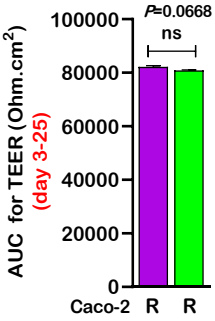
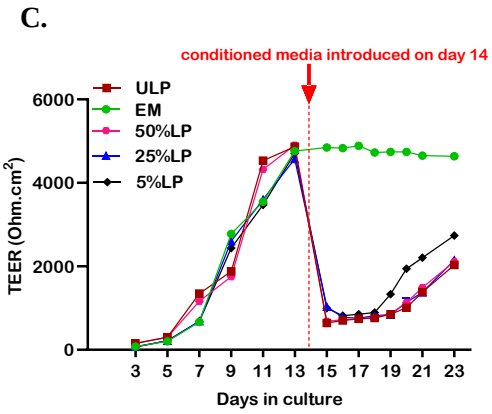


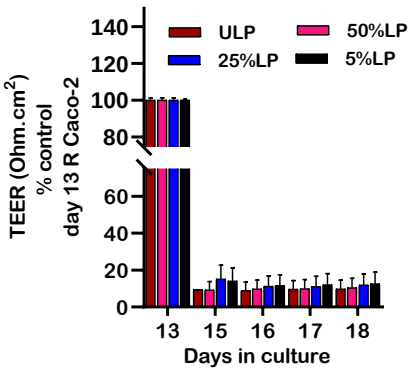
Table 2.2. Relative TEER level (%) on day 15-18 after exposing R Caco-2 cell monolayers to varying proportions of LP in complete EMEM

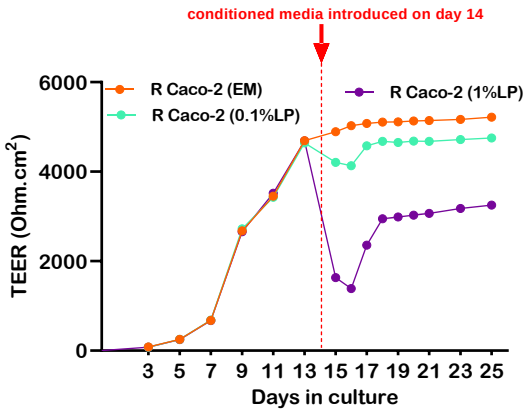
Days	R Caco-2 (ULP)	R Caco-2 (50%LP)	R Caco-2 (25%LP)	R Caco-2 (5%LP)	R Caco-2 (1%LP)	R Caco-2 (0.1%LP)
%	% MEAN	% MEAN	% MEAN	% MEAN	% MEAN	% MEAN
13	100	100	100	100		
14	Conditioned media was added to the basolateral chambers of Caco-2shRaptor					
15	9.3	9.2	15.1	14.1	35	90.6
16	9.0	9.7	11.3	11.6	29.6	88.4
17	9.6	9.9	11.1	12.1	50.2	98.5
18	9.7	10.4	11.9	12.6	62.8	99.3











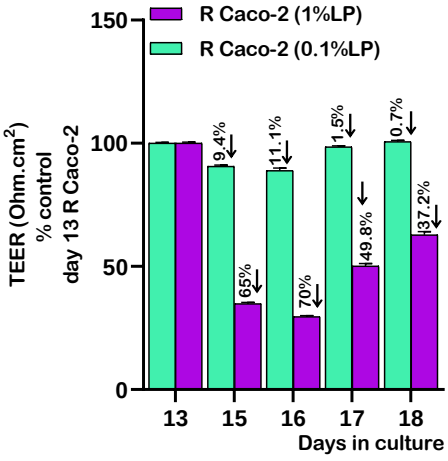


Fig. 3. Dose-dependent disruption of TEER in Raptor-depleted Caco-2 cell monolayers by the conditioned media of LPS treated THP-1shTSC2 macrophages (LP).

(A) Experimental timeline (B) TEER of R Caco-2 cells was measured over time, and the area under the curve (AUC) was calculated for the period from day 3 to day 25 (n = 3 biological replicates). On day14 post-seeding, complete EMEM media in the basolateral chamber was replaced with pH-equilibrated complete RPMI media. Independent wells of R Caco2 (EM) cells received complete EMEM media whereas R Caco-2 (RPMI) cells received pH-equilibrated complete RPMI media. (C) Comparison of the effect of varying concentrations of LP- the conditioned media derived from LPS treated THP-1shTSC2 macrophages. On day 14 post-seeding, R Caco-2 (EM) received only complete EMEM media to the basolateral chambers. R Caco-2 (0.1%LP) cells received 0.1% diluted conditioned media from LPS treated THP-1shTSC2 macrophages. R Caco-2 (1%LP) received 1% diluted conditioned media from LPS treated THP-1shTSC2 macrophages. R Caco-2 (5%LP) received 5% diluted conditioned media from LPS treated THP-1shTSC2 macrophages. R Caco-2 (25%LP) received 25% diluted conditioned media from LPS treated THP-1shTSC2 macrophages. R Caco-2 (50%LP) received 50% diluted conditioned media from LPS treated THP-1shTSC2 macrophages. R Caco-2 (ULP) received undiluted conditioned media from LPS treated THP-1shTSC2 macrophages. Data were analyzed by one-way ANOVA, followed by Tukey's multiple comparisons test. Values not sharing a common letter are significantly different ($P < 0.05$), ns= not significant (n = 3).

3.4 Treating THP-1shTSC2 macrophages with curcumin (CUR) mitigated the disruption of Caco-2 monolayer barrier function.

Previous studies showed that CUR inhibited the polarization of macrophages into M1 or M2 subtypes when exposed to LPS and/or constitutively active mTORC1 [31]. This inhibition led monocytes into a quiescent macrophage subtype. Based on these findings and our earlier observation that 1%LP induced a substantial drop in TEER of R Caco-2 cells (Fig. 3C), we aimed to investigate the impact of conditioned media derived from THP-1shTSC2 macrophages treated with a combination of LPS and CUR (1%LC) on the TEER of R Caco-2 cell monolayers and compare the effect with 1%LP.

After 24 hours of introducing the conditioned media from LPS+CUR-treated THP-1shTSC2 macrophages (1%LC), i.e., day 15, we observed that 1%LC significantly

counteracted the effect of macrophage-produced inflammatory secretagogues by ~40% and by ~50% on day 16 (Fig.4B; AUC, P<0.0001). These findings suggested that CUR mitigated the disruption R Caco-2 monolayer barrier function, resulting in a reduced drop in TEER compared to 1%LP. Overall, these findings highlight the potential protective effects of CUR in mitigating the disruption of barrier function in R Caco-2 cells.

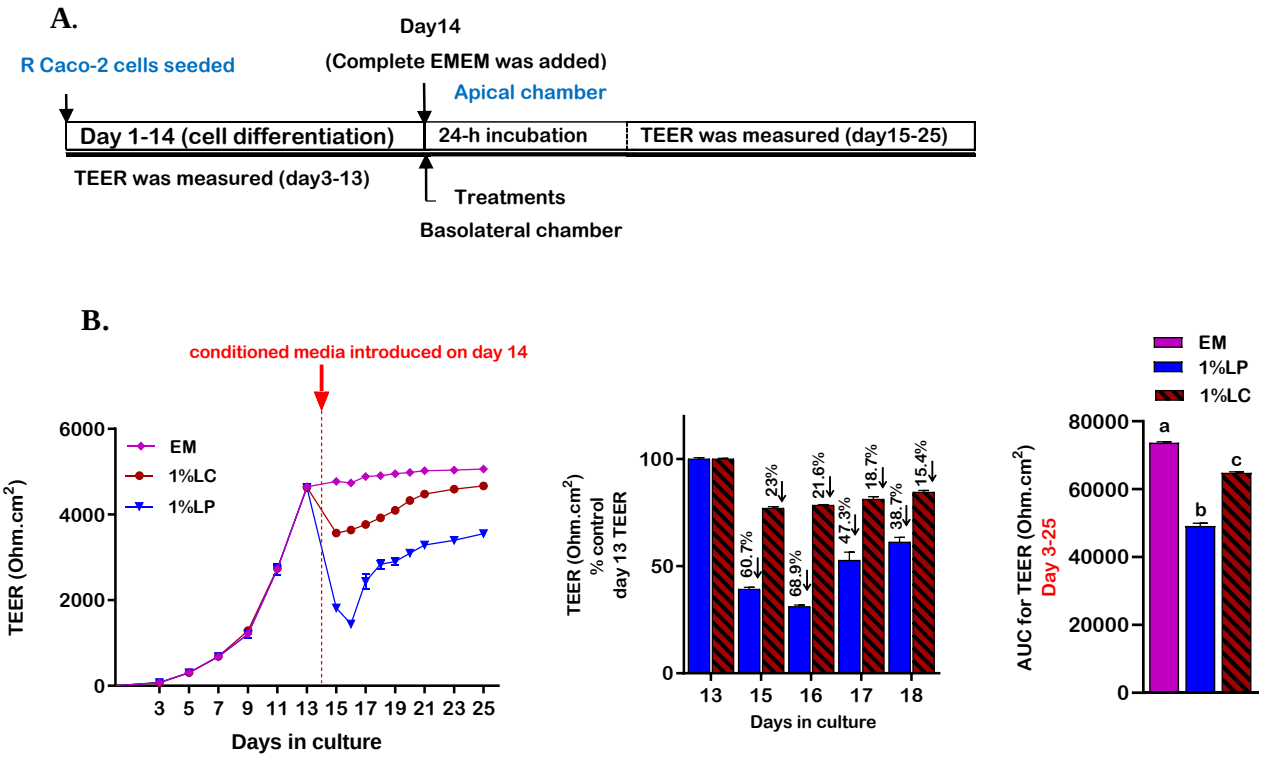


Fig. 4. LPS+CUR-treated THP-1shTSC2 macrophages (LC) mitigated drop in TEER of Raptor-depleted Caco-2 cell monolayers

(A) Experimental timeline (B) On day 14, R Caco-2 cells received complete EMEM in the apical chamber and the following treatments in the basolateral chamber; R Caco-2 (EM) received complete EMEM, R Caco-2 (1%LP) received 1% diluted CM from LPS-treated THP-1shTSC2 macrophages, R Caco-2 (1%LC) received 1% diluted CM from LPS+CUR-treated THP-1shTSC2 macrophages. Data were analyzed by one-way ANOVA, followed by Tukey's multiple comparisons test. Values not sharing a common letter are significantly different (P < 0.05), (n = 3).

3.5 TNF-α blocking antibody prevented the drop in TEER of Raptor-depleted Caco-2 cell monolayers

We previously reported that THP-1shTSC2-derived macrophages expressed elevated levels of TNF-α when treated with LPS [10]. Therefore, we aimed to investigate whether recombinant human TNF-α could induce a drop in TEER in R Caco-2 cells in a manner analogous to that observed with 1%LP. To that end, on day 14 post-seeding, we tested two concentrations of recombinant TNF-α, 10 and 50 ng/mL, added into the basolateral chamber of R Caco-2 monolayers. After treatment, we measured TEER from day 15-25 and observed a concentration-dependent decline in TEER of 18% and 27% at 10 ng/mL TNF-α on day 15 and 16, and of 31% and 47% at 50 ng/mL TNF-α on day 15 and 16 (Fig. 5B; $P<0.05$).

Furthermore, we sought to evaluate the effects of blocking recombinant TNF-α with a specific antibody on the epithelial barrier integrity of R Caco-2 cell monolayers. On day 14, post-seeding, media was changed, and R Caco-2 cell monolayers were treated basolaterally with either recombinant human TNF-α (50 ng/mL medium) pre-incubated with TNF-α blocking antibody (4.3 μg/mL medium) or recombinant human TNF-α (50 ng/mL medium) pre-incubated with control unrelated rabbit IgG mAb (4.3 μg/mL medium). Subsequently, TEER was measured from day 15 to day 25. Note that the treatments were removed on day 16 with a media change after the measurement of TEER. Results showed that the TNF-α blocking antibody completely prevented the drop in TEER caused by recombinant TNF-α in R Caco-2 cells compared to control rabbit IgG mAb (Fig. 5C; AUC, $P<0.0001$).

Motivated by this observation, we asked whether the TNF-α blocking antibody would mitigate the drop in TEER induced by 1%LP. To that end, we treated R Caco-2

monolayers basolaterally with either 1%LP, 1%LPAT (1%LP preincubated with 4.3 μg/mL TNF-α blocking antibody), 1%LPIG (1%LP pre-incubated with 4.3 μg/mL control rabbit IgG mAb), or EMEM and measured TEER until day 25. On days 15 and 16, the TNF-α blocking antibody (treatment 1%LPAT) mitigated the drop in TEER by 12% and 22%, respectively versus control rabbit IgG (treatment 1%LPIG). The TNF-α blocking antibody significantly improved the TEER AUC encompassing day 3 through day 25 compared with the control rabbit IgG or 1%LP treatment (Fig. 5D; AUC, $P<0.0001$). These results underscored the therapeutic potential of TNF-α blocking antibody for preserving the epithelial barrier integrity and mitigating TEER disruptions in Caco-2 cells exposed to TNF-α and macrophage-produced pro-inflammatory secretagogues.

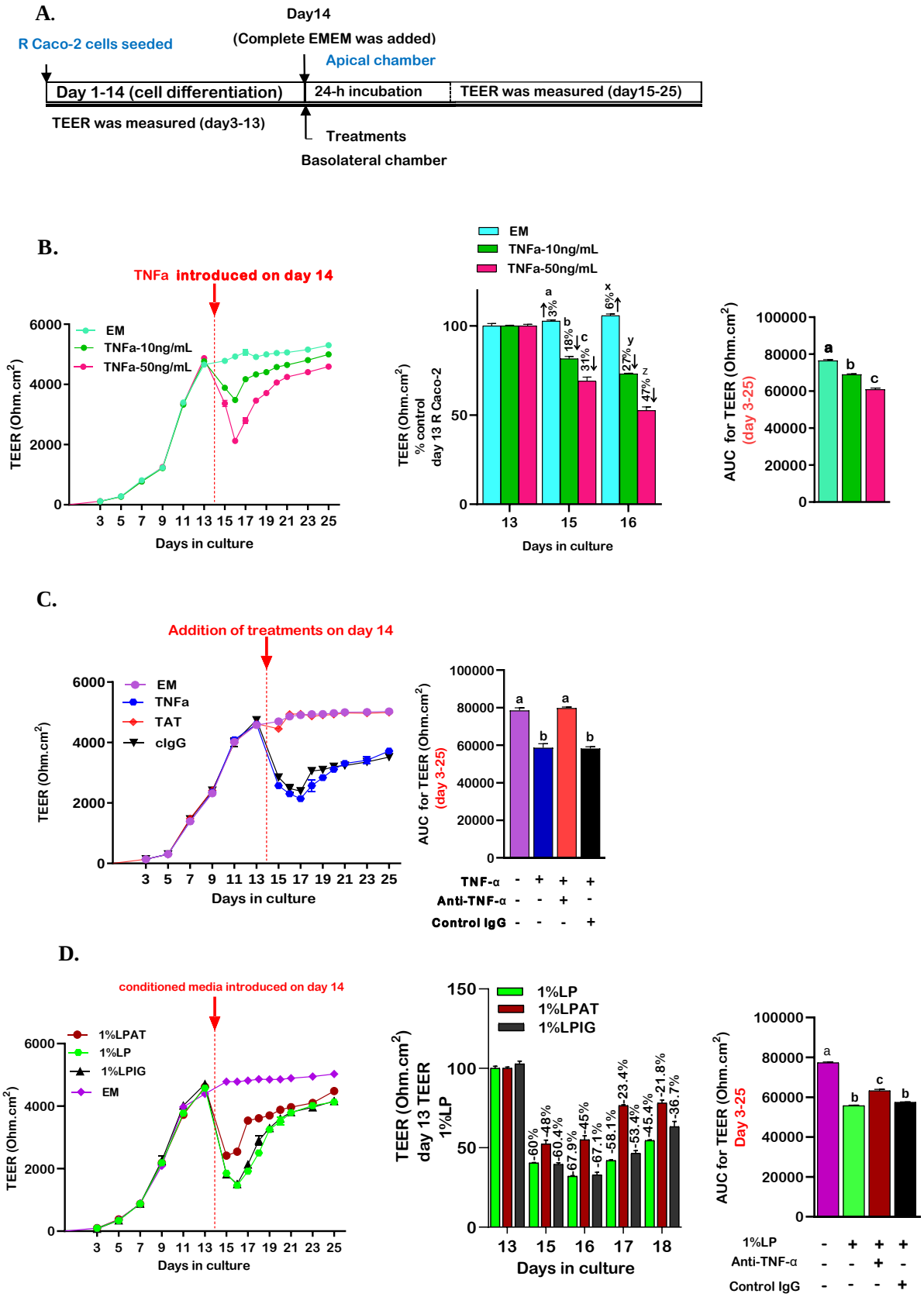

A.
Day14
(Complete EMEM was added)
R Caco-2 cells seeded
Apical chamber
Day 1-14 (cell differentiation)
24-h incubation
TEER was measured (day15-25)
TEER was measured (day3-13)
Treatments
Basolateral chamber
B.
TNFa introduced on day 14
EM
TNFa-10ng/mL
TNFa-50ng/mL
TEER (Ohm.cm2)
Days in culture
TEER (Ohm.cm2) % control day 13 R Caco-2
AUC for TEER (Ohm.cm2) (day 3-25)
C.
Addition of treatments on day 14
EM
TNFa
TAT
cIgG
TEER (Ohm.cm2)
Days in culture
AUC for TEER (Ohm.cm2) (day 3-25)
TNF-α
Anti-TNF-α
Control IgG
D.
conditioned media introduced on day 14
1%LPAT
1%LP
1%LPIG
EM
TEER (Ohm.cm2)
Days in culture
TEER (Ohm.cm2) day 13 TEER 1%LP
AUC for TEER (Ohm.cm2) Day 3-25
1%LP
Anti-TNF-α
Control IgG

Fig. 5. TNF-α blocking antibody mitigated the drop in TEER of Raptor-depleted Caco-2 cell monolayers. (A) Experimental timeline (B) On day 14 post-seeding, there was addition of TNF-α at 10ng/mL and 50ng/mL to the basolateral chamber of R Caco-2 cell monolayers cultured on Transwell® inserts. R Caco2 (EM): complete EMEM media was added to the basolateral chamber. R Caco-2 (TNFa-10ng/mL): TNFα at 10ng/mL media was added to the basolateral side. R Caco-2 (TNFa-50ng/mL): TNFα at 50ng/mL medium was added to the basolateral side. (C) On day 14, R Caco-2 cells received complete EMEM to the apical chamber, followed by basolateral treatment as follows; R Caco-2 (EM) received only complete EMEM media, R Caco-2 (TAT) received TNF-α at 50ng/mL medium and TNF-α blocking antibody at 4.3µg/mL medium, R Caco-2 (cIgG) received TNF-α at 50ng/mL medium and control rabbit IgG monoclonal antibody (mAb) at 4.3µg/mL medium. (D) On day 14, R Caco-2 cells received complete EMEM to the apical chamber, followed by basolateral treatment as follows; R Caco-2 (EM) received only complete EMEM media, R Caco-2 (1%LP) received 1% of the conditioned media from LPS-treated THP-1shTSC2 macrophages, R Caco-2 (1%LPAT) received 1% diluted conditioned media from LPS-treated THP-1shTSC2 macrophages along with TNF-α blocking antibody, R Caco-2 (1%LPIG) received 1% diluted conditioned media from LPS treated THP-1shTSC2 macrophages and control rabbit IgG monoclonal antibody (mAb), Data were analyzed by one-way ANOVA, followed by Tukey's multiple comparisons test. Values not sharing a common letter are significantly different ($P < 0.05$), ($n = 3$).

3.6 Effects of selected pharmacological inhibitors of protein kinases on macrophage conditioned media-induced drop in TEER in Raptor-depleted Caco-2 cell monolayers

Previous studies have reported the relationship between epithelial barrier function and various intracellular signaling pathways [47-51]. Specifically, the MEK (MAPK/ERK kinase) and ERK (extracellular signal-regulated kinase), phosphoinositide 3-kinase (PI3K) and ribosomal S6 kinase (RSK) have been recognized as crucial players in the inflammatory responses of epithelial cells, with their activation being triggered by cytokines or growth factors (Fig. 6). Moreso, p70S6K, also known as ribosomal protein S6 kinase beta-1 (S6K1), a downstream effector of mTORC1, has been reported to

influence the inflammatory response by regulating the translation and expression of key pro-inflammatory proteins [40]. In light of this knowledge, we examined the effects of a cocktail of pharmacological inhibitors (inhibitor cocktail-1) targeting RSK, p70S6K, PI3K, and MEK/ERK on the drop in TEER induced by 1% diluted CM from LPS-treated THP-1shTSC2 macrophages (1%LP). On day 14 post-seeding, inhibitor cocktail-1 was added to the apical chamber of R Caco-2 cells, and, after an hour, 1%LP was added in the basolateral chamber. Subsequently, TEER was measured from day 15 to day 25. Inhibitor cocktail-1 modestly counteracted 1%LP-induced drop in TEER by 19% and 11% on day 15 and 16, respectively (Fig. 7B), and did not significantly improve the TEER AUC (Fig. 7B, AUC, $P > 0.05$). The data suggested that other intracellular signaling pathways were active. It is reasonable to infer that these additional pathways play a pivotal role in mediating the deleterious effects on TEER, thereby contributing significantly to the overall disruption of the epithelial barrier. Although displaying a certain improvement on day 15 and 16 in attenuating the drop of TEER induced by 1%LP, the inhibition of RSK, PI3K, p70S6K, and ERK alone was not sufficient to markedly counteract the adverse effects.

Motivated by these findings, we investigated the effects of another inhibitor cocktail (inhibitor cocktail-2) targeting p38 MAPK (mitogen-activated protein kinase), JNK (c-Jun N-terminal kinase), and IKKβ (inhibitor of κB kinase beta) on 1%LP-induced drop in TEER of R Caco-2 cells. As above, we monitored TEER levels and found that inhibitor cocktail-2 significantly counteracted 1%LP-induced drop in TEER by 46% and 34% on day 15 and 16, respectively (Fig. 7C). The TEER AUC was also significantly improved by inhibitor cocktail-2 (Fig. 7C, AUC, $P<0.001$). These findings indicate that

the counteractive effect of inhibitor cocktail-2 over 1%LP-induced drop in TEER was superior to that of inhibitor cocktail-1. It implied that specific signaling pathways were identified and may play crucial roles in regulating the TEER of Caco-2 cell monolayers exposed of macrophage-produced secretagogues.

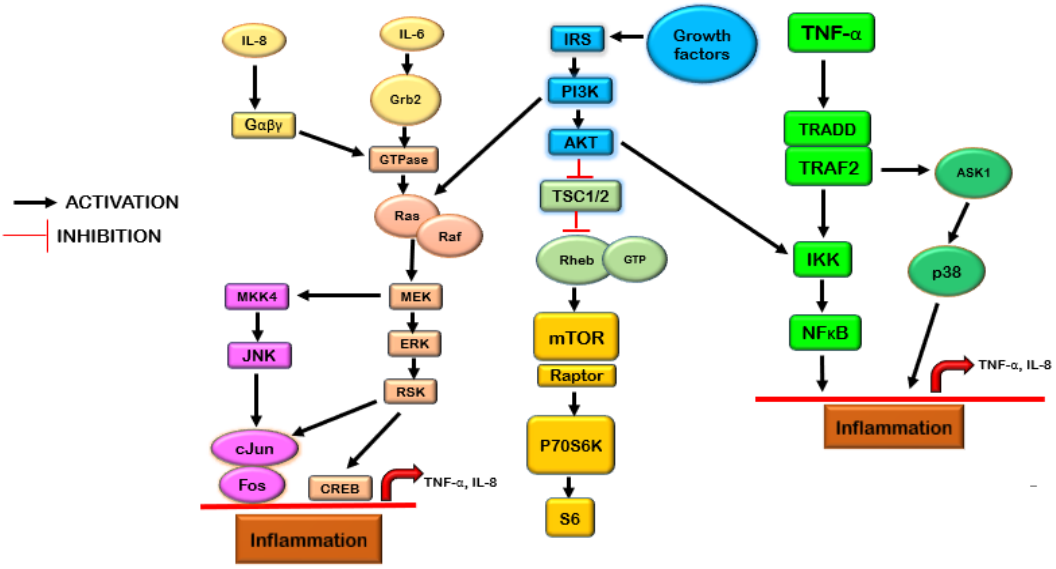


Fig. 6. Protein kinase activation amplifies intestinal barrier disruption. Macrophage-produced conditioned media, a complex mix of growth factors and cytokines, activates protein kinases. These kinases, through crosstalk enhance the generation of proinflammatory signals, disrupting intestinal barriers. For example, upon activation, the IRS/PI3K/AKT pathway can phosphorylate IKKβ, which subsequently phosphorylates and degrades IκB proteins. Through this process NFκB is released and migrates into the nucleus. Once inside the nucleus, NFκB binds to specific DNA sequences, promoting the transcription and subsequent production of proinflammatory cytokines.

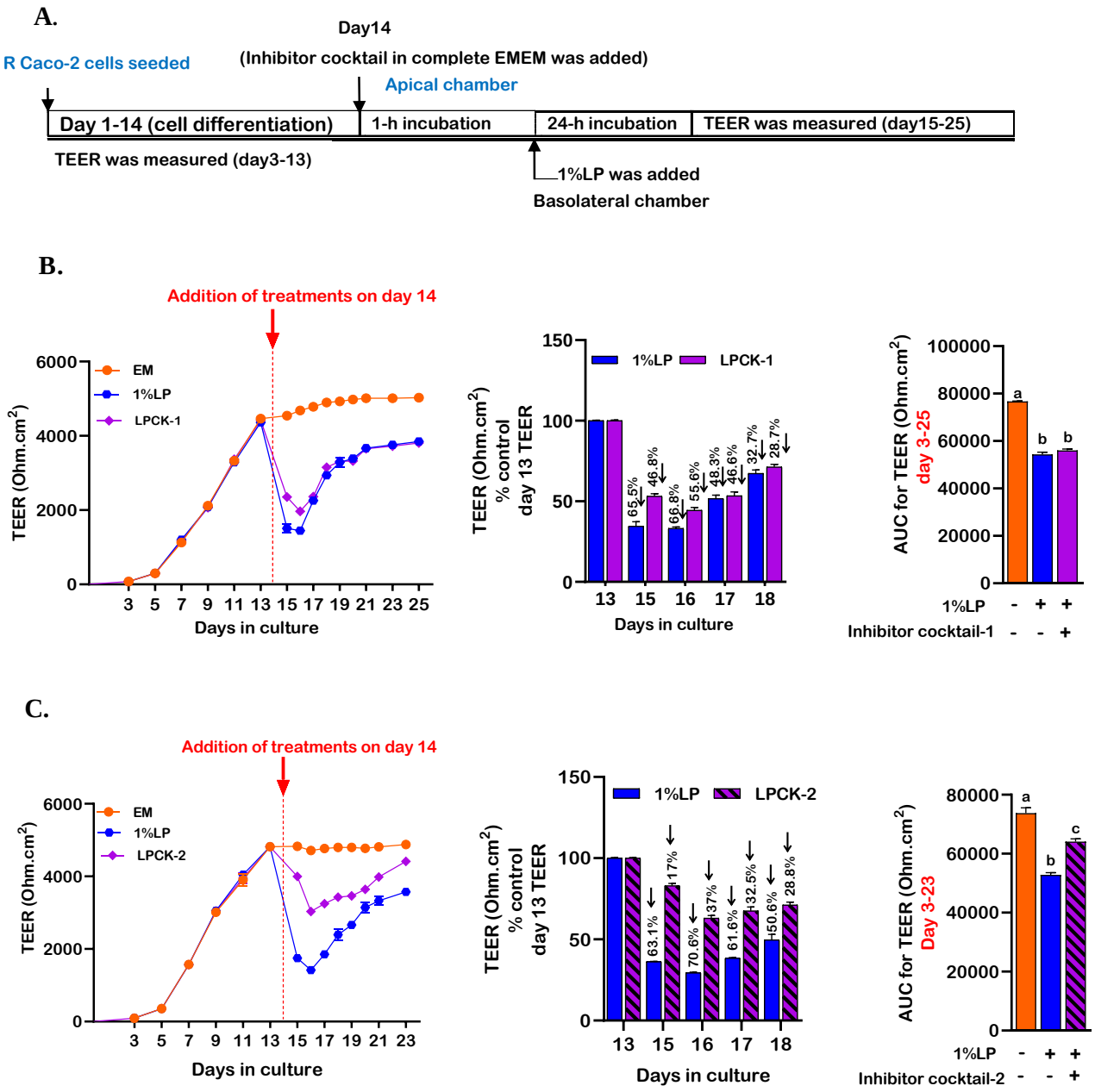


Fig. 7. Cocktail of inhibitors for intracellular signaling pathway counteracted 1%LP-induced drop in TEER of Raptor-depleted Caco-2 cell monolayers.

(A) Experimental timeline (B) On day 14 post-seeding, there was apical treatment of R Caco-2 cells with inhibitor cocktail-1 (LPCK-1) containing RSK inhibitor (100 nM), p70S6K inhibitor (1µM), PI3K inhibitor (50 nM), ERK inhibitor (1 µM). Also, there was apical treatment of R Caco-2 cells with inhibitor cocktail-2 (LPCK-2) containing p38 MAPK inhibitor (2µM), JNK inhibitor (1µM) and IKKβ (1µM). Then, an hour later, there was basolateral treatment of R Caco-2 cells as follows: R Caco-2 (EM) received

complete EMEM media, R Caco-2 (1%LP) received 1% diluted conditioned media from LPS-treated THP-1shTSC2 macrophages, R Caco-2 (LPCK-1) and R Caco-2 (LPCK-2) received 1% diluted conditioned media from LPS-treated THP-1shTSC2 macrophages. Data were analyzed by one-way ANOVA, followed by Tukey's multiple comparisons test. Values not sharing a common letter are significantly different ($P < 0.05$), ($n = 3$).

4. DISCUSSION

Multiple studies have provided evidence of the involvement of mTORC1 activation in perpetuating intestinal inflammation [2, 5, 6, 8, 9]. Activation of mTORC1 influences the production of proinflammatory cytokines in macrophages and intestinal epithelial cells. Research evidence suggests that resident and peripheral macrophages play a crucial role in regulating gut immune response, but upon injury, the inflammatory cues they release prompt intestinal epithelial cells to produce their own cytokines in a coordinated interaction [7, 11]. This phenomenon was shown to dependent upon mTORC1 activity whereby upon LPS challenge, macrophages with constitutively active mTORC1 (THP-1shTSC2 macrophages) exhibited higher expression of proinflammatory cytokines TNF-α, IL-6, and IL-1β than THP-1 macrophages with either constitutively downregulated or baseline mTORC1 activity [10].

Furthermore, it has been shown that mTORC1 regulates intestinal barrier function as evidenced by elevated transepithelial electrical resistance (TEER) and para/transcellular permeability in Raptor-depleted Caco-2 cells (constitutively downregulated mTORC1 activity) compared Caco-2 cells with constitutively active mTORC1 or baseline mTORC1 activity [21].

Based on these findings, we asked whether the level of mTORC1 activity in Caco-2 cell monolayers would influence their epithelial barrier function when exposed to proinflammatory stimuli present in the conditioned media from LPS-treated THP-1shTSC2 macrophages. Raptor-depleted (R Caco-2 cells) and cells with baseline mTORC1 activity (S Caco-2 cells) were basolaterally exposed to the conditioned media. Additionally, considering the involvement of multiple intracellular signaling pathways in epithelial barrier disruption, we explored the effects of pharmacological inhibitors of specific cellular targets (RSK, PI3K, p70S6K, ERK, p38 MAPK, JNK, and IKKβ).

The present studies revealed that R Caco-2 cells exhibited a 122% higher TEER compared to S Caco-2 cells. However, R Caco-2 cells also had increased permeability to FITC-dextran 4 kDa compared to S Caco-2 cells. This observation is consistent with a previous study by Kaur et al. [21] which reported that the depletion of claudin-2 and claudin-15 molecules in R Caco-2 cells likely hindered ion paracellular transport, leading to increased paracellular electrical resistance and TEER. Furthermore, in the present work, we found that the initially elevated TEER in R Caco-2 cells collapsed when exposed to macrophage-produced secretagogues (treatment LP).

It is worth noting that a decrease in TEER can be attributed to various factors, as highlighted in previous study [31]. Firstly, an increase in paracellular permeability to ions can contribute to this phenomenon. Secondly, alterations in transcellular ion flux, mediated by changes in plasma membrane channels or pumps, can also impact TEER. Lastly, uncontrolled cell death within the monolayer is another contributing factor [31]. It is plausible that any of these events individually or in combination may be responsible for the decline of TEER observed in R Caco-2 cells following basolateral exposure to LP.

Additionally, S Caco-2 cells concurrently displayed a substantial drop in TEER and increased flux of FITC-dextran 4 kDa following basolateral exposure to LP, thus suggesting alterations to the flux of molecules via transcellular and paracellular routes. Interestingly, unlike R Caco-2 cells, S Caco-2 cells exhibited a significant recovery after 24 hours, as evidenced by improved leakiness to FITC-dextran 4 kDa and an increase in TEER. Moreover, the TEER of S Caco-2 cells after recovery was significantly higher than both their baseline and that of R Caco-2 cells. Previous studies have shown that autophagy plays a crucial role in protecting intestinal cells against cellular stress as evidenced by its role in enhancing the levels of cellular occludin thereby mitigating LPS and TNF-α induced losses of TJ proteins [22, 23]. It is hypothesized that a stress-coping mechanism may be at play in S Caco-2 cells enabling them to recover quickly from the adverse effects of pro-inflammatory stressors. Autophagy was elevated in R Caco-2 cells [21] but it did not prove advantageous in relation to TJ proteins because Raptor depletion in these cells correlated with reduced expression of both pore forming and pore sealing TJ proteins [21]. The reduced expression of pore sealing TJ proteins provides an explanation for the observed increase in permeability to FITC-dextran 4 kDa in R Caco-2 cell monolayers. Overall, the present studies indicated that inflammatory mediators exert a significant and disruptive influence on Caco-2 epithelial barrier function, particularly in conjunction with constitutively downregulated mTORC1 activity. Moreso, the present studies underscored the role of macrophage-produced inflammatory secretagogues in modulating epithelial barrier function, which could have far-reaching implications under various pathological conditions that challenge intestinal epithelium homeostasis.

Furthermore, the present study demonstrated that the secretagogues produced by macrophages in the conditioned media (LP) could trigger a significant loss of TEER even at 1% dilution. CM contain an array of mediators including proteins released from the cell surface as well as intracellular proteins secreted through non-classical pathways or exosomes. This collection of secretagogues includes components such as enzymes, growth factors, cytokines, hormones, and other soluble mediators. [45]. It is thought that the TEER disrupting factors present in LP reached high concentrations hence, several-fold dilution was needed.

Additionally, the current studies provided evidence that CM from LPS+CUR treated THP-1shTSC2 macrophages (LC) preserved some aspects of Caco-2 monolayer barrier function by significantly lessening the drop in TEER. This attenuating effect of CUR expands upon earlier work [31], which reported that CUR represses the inflammatory response of THP-1 derived macrophages exposed to LPS. CUR-mediated inhibition was attributed to its ability to mitigate crosstalks between mTORC1, mTORC2, MAPK, and CREB, all of which participate in macrophage polarization towards either the M1 or M2 lineage. We speculate that this was at play in the present study. In sum, our results support the notion that CUR preserves intestinal barrier integrity under conditions that induce TJ dysfunction.

Numerous indications suggest that intestinal epithelial cells actively respond to inflammatory mediators produced by hyperactive macrophages by releasing their own cytokines. Kaur et al. reported on the involvement of mTORC1 in regulating this phenomenon by showing that Caco-2 cells with constitutively downregulated mTORC1 activity (R Caco-2 cells) had higher expression of TNFα and IL-10 in response to

secretagogues present in the conditioned media derived from LPS-treated THP-1 derived macrophages [10]. Mechanistically, the suppression of mTORC1 activity may alleviate the cross-pathway inhibition of mTORC1 onto the MAPK/Akt signaling pathway, which is induced by the knockdown of Raptor. This, in turn, leads to the NF-κB dependent and independent expression of inflammatory cytokines, including TNF-α [10]. It is plausible that this mechanism is at play in the present study, as evidenced by significant drop of TEER in R Caco-2 cells. In the presence of TNF-α blocking antibody, the collapse of TEER induced by TNF-α was abrogated. TNF-α blocking antibody was also protective against 1%LP-induced drop in TEER and supported its subsequent recovery. The mechanism of action may involve interferences with the binding of macrophage-derived TNF-α to its specific receptors (TNFR) expressed in the basolateral membrane of intestinal epithelial cells, and in turn hinders the recruitment of crucial adaptor proteins TRADD and TRAF2 (Fig. 6 and Fig. 8). TRADD and TRAF2 are crucial mediators of intracellular signaling pathways, including NF-κB signaling whose activation leads to NF-κB translocation to the nucleus and the transcription of numerous proinflammatory genes including those involved in the disruption of the intestinal epithelial barrier. Overall, our findings indicate that TNF-α blocking antibody was highly effective in preserving the integrity of R Caco-2 cell monolayers when exposed to TNF-α or macrophage-derived secretagogues.

In an effort to identify potential Caco-2 cellular targets responsible for the collapse of TEER, the study focused on intracellular signaling pathways previously reported to be activated by pro-TJ disruptive mediators [10, 47-51]. While an inhibitor cocktail targeting RSK, PI3K, p70S6K, and ERK displayed a modest counteractive effect

on the drop in TEER induced by 1%LP, an inhibitor cocktail targeting p38 MAPK, JNK, and IKKβ showed a remarkable counteractive effect on the drop in TEER. These results suggested that p38 MAPK, JNK, and IKKβ are involved in orchestrating the disruption of TEER in R Caco-2 cells exposed to LP. Further research will be needed to tease out the relative contribution of these protein kinases.

5. CONCLUSIONS

In conclusion, this study investigated the effects of conditioned media from THP-1shTSC2 macrophages treated with LPS or a combination of LPS + CUR on the integrity of intestinal epithelial barrier in differentiating and polarizing Caco-2 cell monolayers. The experiments were conducted using Caco-2 cells with either baseline mTORC1 activity (S Caco-2 cells) or constitutively downregulated mTORC1 activity (R Caco-2 cells). The results revealed that R Caco-2 cells, howbeit exhibited increased TEER, they also displayed increased permeability to FITC-dextran compared to S Caco-2 cells. The initially increased TEER in R Caco-2 cells was not sustained when exposed to conditioned media from LPS-treated THP-1shTSC2 macrophages (treatment LP) suggesting alterations to trans/paracellular flux of ions. In contrast, S Caco-2 cells were minimally affected by LP and recovered quickly following exposure. These findings suggested the possible recruitment of stress coping mechanisms in S Caco-2 cells that restored or enhanced the levels of pore sealing TJ proteins such as cellular occludin levels, thus minimizing the effect of LP. By comparison, factors present in the conditioned media may have adversely affected the epithelial barrier of R Caco-2 cell

monolayers upon exposure to LP due to their constitutively low levels of pore sealing TJ proteins.

Moreover, it is noteworthy that the conditioned media obtained from THP-1shTSC2 macrophages treated with LPS+CUR (treatment LC) exhibited a significant capacity to prevent the detrimental disruption of epithelial barriers in R Caco-2 cells. This finding underscores the potential protective effects of CUR and positions it as a promising candidate for preserving the integrity of the intestinal barrier, particularly in circumstances characterized by the dysfunction of TJs. Thus, CUR holds considerable promise as a therapeutic agent for mitigating the adverse effects associated with compromised intestinal barrier function.

Additionally, it is worth noting that the utilization of TNF-α blocking antibody demonstrated remarkable efficacy in safeguarding the integrity of epithelial barriers within Raptor-depleted Caco-2 cell monolayers. Overall, the present study demonstrated substantial potential of TNF-α blocking antibody as a therapeutic intervention for preserving and protecting the structural integrity of TJs under conditions characterized by the influence of TNF-α or macrophage-produced secretagogues.

The study also identified potential cellular targets, including p38 MAPK, JNK, and IKKβ, as regulators of epithelial barriers in Raptor-depleted Caco-2 cell monolayers, under the influence of macrophage-produced secretagogues (Fig. 8). These findings emphasize the complexity of the signaling pathways involved in epithelial barrier disruption and provide insights into potential therapeutic targets for enhancing intestinal barrier integrity.

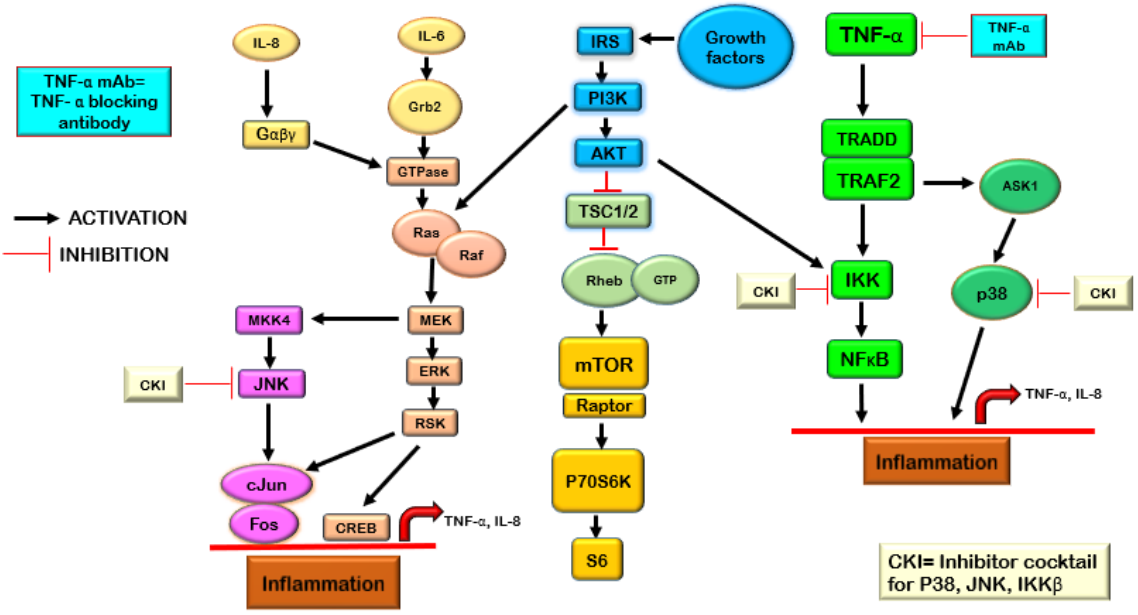


Fig. 8. Proposed molecular mechanisms for mitigating the loss of transepithelial electrical resistance (TEER). Preserving epithelial barrier integrity in a Raptor-depleted intestinal epithelial cell model exposed to macrophage-produced secretagogues necessitates the inhibition of TNF-α, p38, JNK, and IKKβ. Blocking TNF-α would hinder the recruitment of TRADD, TRAF2, and the triggering of subsequent signaling molecules like IKK, NFKB, ASK1, and p38. Additionally, combined inhibition of p38, JNK, and IKK would impede the generation of proinflammatory cytokines, thus effectively counteracting the detrimental impacts of macrophage-produced secretagogues on the integrity of the intestinal epithelial barriers.

FUTURE DIRECTIONS

1) Characterization of the secretagogues present in the CM. Such an exploration would be vital for gaining a comprehensive understanding of the underlying mechanisms involved in the disruption of epithelial barriers and could provide valuable insights for potential therapeutic interventions.
2) The contribution of apoptosis to the loss of transepithelial electrical resistance (TEER) in Raptor-depleted Caco-2 cell monolayers requires exploration. By delving into the role of apoptosis in this phenomenon, we can unravel valuable insights into

the interplay between Raptor depletion, apoptosis, and TEER disruption, which could have significant implications for our understanding of disruption of epithelial barrier function mediated by macrophage-produced secretagogues.

3) Employing proteomics could prove instrumental in pinpointing proteins expressed in Raptor-depleted Caco-2 cells upon basolateral exposure to macrophage-produced secretagogues. This approach will aid in distinguishing protein profiles and identifying TJ proteins involved in TEER loss, particularly in the presence of macrophage-produced secretagogues, ultimately enhancing our comprehension of the pathophysiology of disrupted epithelial barrier function.

REFERENCES

1) Tordesillas L, Berin MC. Mechanisms of oral tolerance. *Clin Rev Allergy Immunol*. 2018;55(2):107–17.
2) H. Kaur, R. Moreau, Role of mTORC1 in intestinal epithelial repair and tumorigenesis, Cell. Mol. Life Sci. 76 (2019) 2525–2546.
3) T. Ayabe, D.P. Satchell, C.L. Wilson, W.C. Parks, M.E. Selsted, A.J. Ouellette, Secretion of microbicidal α-defensins by intestinal paneth cells in response to bacteria, Nat. Immunol. 1 (2000) 113.
4) T. Pelaseyed, J.H. Bergstrom, J.K. Gustafsson, A. Ermund, G.M. Birchenough, A. Schutte, et al., The mucus and mucins of the goblet cells and enterocytes provide the first defense line of the gastrointestinal tract and interact with the immune system, Immunol. Rev. 260 (2014) 8–20.
5) Corr SC, Gahan CC, Hill C. M-cells: origin, morphology and role in mucosal immunity and microbial pathogenesis. FEMS Immunol Med Microbiol. 2008;52(1):2–12.
6) S. Al-Ghadban, S. Kaissi, F.R. Homaidan, H.Y. Naim, M.E. El-Sabban. Crosstalk between intestinal epithelial cells and immune cells in inflammatory bowel disease, Sci. Rep. 6 (2016) 29783.

7) S. Wang, Q. Ye, X. Zeng, S. Qiao, Functions of macrophages in the maintenance of intestinal homeostasis, J. Immunol. Res. 2019 (2019), 1512969.
8) Y.T. Xiao, W.H. Yan, Y. Cao, J.K. Yan, W. Cai, Neutralization of IL-6 and TNF-alpha ameliorates intestinal permeability in DSS-induced colitis, Cytokine 83 (2016) 189–192.
9) L. Mao, A. Kitani, W. Strober, I.J. Fuss, The role of NLRP3 and IL-1beta in the pathogenesis of inflammatory bowel disease, Front. Immunol. 9 (2018) 2566.
10) Harleen Kaur, Anjeza Erickson and Régis Moreau. Divergent regulation of inflammatory cytokines by mTORC1 in THP-1–derived macrophages and intestinal epithelial Caco-2 cells. Life Sciences 284 (2021) 119920.
11) Haloul M , Oliveira ERA , Kader M , Wells JZ , Tominello TR , El Andaloussi A , et al. mTORC1-mediated polarization of M1 macrophages and their accumulation in the liver correlate with immunopathology in fatal ehrlichiosis. Sci Rep 2019;9:14050 .
12) Covarrubias AJ, Aksoylar HI, Horng T. Control of macrophage metabolism and activation by mTOR and Akt signaling. Seminars in immunology. Elsevier; 2015. p. 286–96.
13) Banerjee D, Sinha A, Saikia S, Gogoi B, Rathore AK, Das AS, et al. Inflammation-induced mTORC2-Akt-mTORC1 signaling promotes macrophage foam cell formation. Biochimie. 2018;151:139–49.
14) Zhang B, Wei YZ, Wang GQ, Li DD, Shi JS, Zhang F. Targeting MAPK path- ways by naringenin modulates microglia M1/M2 polarization in lipopolysaccharide-stimulated cultures. Front Cell Neurosci 2018;12:531.
15) Ruffell D, Mourkioti F, Gambardella A, Kirstetter P, Lopez RG , Rosenthal N , et al. A CREB-C/EBPbeta cascade induces M2 macrophage-specific gene expression and promotes muscle injury repair. Proc Natl Acad Sci U S A 2009; 106:17475–80.
16) Hallowell RW, Collins SL, Craig JM , Zhang Y , Oh M , Illei PB , et al. mTORC2 sig-nalling regulates M2 macrophage differentiation in response to helminth infection and adaptive thermogenesis. Nat Commun 2017; 8:14208.
17) Guan Y, Zhang L, Li X, Zhang X , Liu S , Gao N , et al. Repression of mammalian target of rapamycin complex 1 inhibits intestinal regeneration in acute inflammatory bowel disease models. J Immunol 2015; 195:339–46.

18) Shyam Prasad, Roberto Mingrino, Katri Kaukinen, Katherine L Hayes, Robert M Powell1, Thomas T MacDonald and Jane E Collins. Inflammatory processes have differential effects on claudins 2, 3 and 4 in colonic epithelial cells. Laboratory Investigation (2005) 85, 1139–1162.
19) Suzuki T, Yoshinaga N, and Tanabe S. Interleukin-6 (IL-6) regulates claudin-2 expression and tight junction permeability in intestinal epithelium. J Biol Chem (2011) .286:31263–31271.
20) Mutsumi Takigawa, Manami Iida, Shotaro Nagase, Hidehiko Suzuki, Akihiro Watari, Minoru Tada, Yoshiaki Okada, Takefumi Doi, Masayoshi Fukasawa, Kiyohito Yagi, Jun Kunisawa, and Masuo Kondoh. Creation of a claudin-2 binder and its tight junction–modulating activity in a human intestinal model. J Pharmacol Exp Ther (2017). 363:444–451.
21) Harleen Kaur, Regis Moreau. Raptor knockdown concurrently increases the electrical resistance and paracellular permeability of Caco-2 cell monolayers. Life Sciences 308 (2022) 120989.
22) Prashant K. Nighot, Chien-An Andy Hu, and Thomas Y. Ma. Autophagy Enhances Intestinal Epithelial Tight Junction Barrier Function by Targeting Claudin-2 Protein Degradation. J. biol. Chem (2015). 290 (11):234–7246.
23) Saha K, Subramenium Ganapathy A, Wang A, Michael Morris N, Suchanec E, Ding W, Yochum G, Koltun W, Nighot M, Ma T, Nighot P. Autophagy reduces the degradation and promotes membrane localization of occludin to enhance the intestinal epithelial tight junction barrier against paracellular macromolecule flux. Journal of Crohn's and Colitis. 2023;17(3):433-49.
24) Zheng, L. et al. Microbial-derived butyrate promotes epithelial barrier function through IL-10 receptor-dependent repression of claudin-2. J. Immunol (2017). 199, 2976–2984.
25) Raju, P. et al. Inactivation of paracellular cation-selective claudin-2 channels attenuates immune-mediated experimental colitis in mice. J. Clin. Invest (2020). 130, 5197–5208.
26) Koch, S. et al. Protein kinase CK2 is a critical regulator of epithelial homeostasis in chronic intestinal inflammation. Mucosal Immunol. 6, 136–145 (2013).
27) Ma, J. et al. Identification of selective claudin cation channel blockers [abstract]. Gastroenterology 160 (Suppl. 3), S33–S34 (2021).

28) Sweet MJ, Hume DA. Endotoxin signal transduction in macrophages. J Leukoc Biol. 1996; 60:8–26.

29) Rossol M, Heine H, Meusch U, et al. LPS-induced cytokine production in human monocytes and macrophages. Crit Rev Immunol. 2011; 31:379–446.

30) Tucureanu MM, Rebleanu D, Constantinescu CA, Deleanu M, Voicu G, Butoi E, Calin M, Manduteanu I. Lipopolysaccharide-induced inflammation in monocytes/macrophages is blocked by liposomal delivery of Gi-protein inhibitor. International journal of nanomedicine. 2018; 13:63.

31) Kaur H, Moreau R. Curcumin steers THP-1 cells under LPS and mTORC1 challenges toward phenotypically resting, low cytokine-producing macrophages. The Journal of Nutritional Biochemistry. 2021; 88:108553.

32) Rana Al-Sadi, Shuhong Guo, Dongmei Ye, Karol Dokladny, Tarik Alhmoud, Lisa Ereifej, Hamid M. Said, and Thomas Y. Ma. Mechanism of IL-1b Modulation of Intestinal Epithelial Barrier Involves p38 Kinase and Activating Transcription Factor-2 Activation. J Immunol (2013) 190 (12): 6596–6606.

33) Cotton JA, Platnich JM, Muruve DA, Jijon H, Buret AG, Beck PL. Interleukin-8 in gastrointestinal inflammation and malignancy: induction and clinical consequences. Int.J Interferon, Cytokine and Mediator Research (2016) :8 13–34.

34) S. Tedesco, F. De Majo, J. Kim, A. Trenti, L. Trevisi, G.P. Fadini, et al., Convenience versus biological significance: are PMA-differentiated THP-1 cells a reliable substitute for blood-derived macrophages when studying in vitro polarization? Front. Pharmacol. 9 (2018) 71.

35) E. Vander Haar, S.I. Lee, S. Bandhakavi, T.J. Griffin, D.H. Kim. Insulin signalling to mTOR mediated by the Akt/PKB substrate PRAS40, Nat. Cell Biol. 9 (2007) 316–323.

36) D.D. Sarbassov, D.A. Guertin, S.M. Ali, D.M. Sabatini. Phosphorylation, and regulation of Akt/PKB by the rictor-mTOR complex. Science. 307 (2005) 1098–1101.

37) J.L. Roberts, B. He, A. Erickson, R. Moreau. Improvement of mTORC1-driven overproduction of apoB-containing triacylglyceride-rich lipoproteins by short-chain fatty acids, 4-phenylbutyric acid and (R)-alpha-lipoic acid, in human hepatocellular carcinoma cells, Biochim. Biophys. Acta 2016 (1861) 166–176.

38) Carrozzino, F.; Pugnale, P.; Feraille, E.; Montesano, R. Inhibition of basal p38 or JNK activity enhances epithelial barrier function through differential modulation of claudin expression. Am. J. Physiol. Cell Physiol. 2009, 297, C775–C787.
39) Gao Y, Li S, Wang J, Luo C, Zhao S, Zheng N. Modulation of intestinal epithelial permeability in differentiated Caco-2 cells exposed to aflatoxin M1 and ochratoxin A individually or collectively. Toxins. 2017. 27;10(1):13.
40) Wu D, Cheng J, Sun G, Wu S, Li M, Gao Z, Zhai S, Li P, Su D, Wang X. p70S6K promotes IL-6-induced epithelial-mesenchymal transition and metastasis of head and neck squamous cell carcinoma. Oncotarget. 2016;7(24):36539.
41) Madara JL. Regulation of the movement of solutes across tight junctions. Annual review of physiology. 1998 Mar;60(1):143-59.
42) Konsoula, R.; Barile, F.A. Correlation of in vitro cytotoxicity with paracellular permeability in Caco-2 cells. Toxicol. Vitr. 2005, 19, 675–684.
43) Corazza, F.G.; Ernesto, J.V.; Nambu, F.A.N.; de Carvalho, L.R.; Leite-Silva, V.R.; Varca, G.H.C.; Calixto, L.A.; Vieira, D.P, Andreo-Filho, N.; Lopes, P.S. Papain-cyclodextrin complexes as an intestinal permeation enhancer: Permeability and in vitro safety evaluation. J. Drug Deliv. Sci. Technol. 2020, 55, 101413.
44) Kaur H, Moreau R. Curcumin represses mTORC1 signaling in Caco-2 cells by a two-sided mechanism involving the loss of IRS-1 and activation of AMPK. Cellular Signaling. 2021; 78:109842.
45) Dowling P, Clynes M. Conditioned media from cell lines: A complementary model to clinical specimens for the discovery of disease-specific biomarkers. Proteomics. 2011; 11(4):794-804.
46) Aoki H, Takada Y, Kondo S, Sawaya R, Aggarwal BB, Kondo Y. Evidence that curcumin suppresses the growth of malignant gliomas in vitro and in vivo through induction of autophagy: role of Akt and extracellular signal-regulated kinase signaling pathways. Molecular pharmacology. 2007;72(1):29-39.
47) Carracedo A, Ma L, Teruya-Feldstein J, Rojo F, Salmena L, Alimonti A, Egia A, Sasaki AT, Thomas G, Kozma SC, Papa A. Inhibition of mTORC1 leads to MAPK pathway activation through a PI3K-dependent feedback loop in human cancer. The Journal of clinical investigation. 2008;118(9):3065-74.

48) K. Moelling, K. Schad, M. Bosse, S. Zimmermann, M. Schweneker, Regulation of raf-akt cross-talk, J. Biol. Chem. 277 (2002) 31099–31106.

49) N. Buzzi, A. Colicheo, R. Boland, A.R. de Boland, MAP kinases in proliferating human colon cancer Caco-2 cells, Mol. Cell. Biochem. 328 (2009) 201–208.

50) M.C. Mendoza, E.E. Er, J. Blenis, The ras-ERK and PI3K-mTOR pathways: cross-talk and compensation, Trends Biochem. Sci. 36 (2011) 320–328.

51) Carrozzino, F.; Pugnale, P.; Feraille, E.; Montesano, R. Inhibition of basal p38 or JNK activity enhances epithelial barrier function through differential modulation of claudin expression. Am. J. Physiol.-Cell Physiol.2009, 297, C775–C787.