Investigation of the role of a macromolecular complex of CFTR-NHERF2-LPA2 in the fluid hemostasis and inflammatory responses in intestinal epithelial cells

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Abstract

CFTR is a cAMP-regulated chloride channel located in the apical surface of intestinal epithelial cells; where it forms a macromolecular complex with NHERF2 and LPA2. CFTR has been shown to play a role in the pathogenies of several types of secretory diarrheas. Inflammatory bowel disease (IBD) is a chronic condition of intestine characterized by severe inflammation and mucosal destruction, genetic analysis has shown that LPA contribute to IBD and patients of cystic fibrosis also display the phenotype of diarrhea. The purpose of this study is to investigate if this complex plays a role in the pathogenesis of IBD, especially in the intestinal fluid homeostasis and inflammatory responses.

To investigate the role(s) of CFTR-NHERF2-LPA2 complex in the pathogenesis of IBD, we first identified the existence of this complex in intestinal epithelial cells; our results showed that (1) CFTR, NHERF2, LPA2 are expressed in these cells evidenced by western blotting and Q-PCR; (2) NHERF2 and LPA2 can be co-immunoprecipated with CFTR; (3) NHERF2 and LPA2 co-localize with CFTR at the plasma membrane of these cells; (4) NHERF2 and LPA2 interact with CFTR evidenced by proximity ligation assay. We then explored the role of this complex in maintaining the integrity of tight junction and inflammatory responses in these cells. Our preliminary data showed that inhibition of CFTR disrupted the tight junction and elicited the secretion of IL-8, while intriguing LPA2 increased the expression of IL-8. Our data also show that RNA knockdown LPA2 can decrease the expression of IL-8. These data suggest that CFTR-NHERF2-LPA2 might play a role in the fluid hemostasis and inflammatory responses of intestinal epithelium, thus could play a role in the pathogenesis of IBD.

Introduction

Intestinal epithelial cells (IEC) can form a continuous physical barrier that separate mammalian hosts from the external environment[1][2], and has diverse functions including physical segregation of commensal bacteria and the integration of microbial signals. IEC are crucial mediators to regulate the normal immunological function of intestinal[3]. Dysregulated epithelial barrier function may lead to inflammatory bowel disease which is associated with the increased bacterial translocation. And increasing evidences also indicate that loss of epithelial barrier function contributes to systemic immune activation, which promote the onset of immunological diseases[4]. Therefore, comprehensive understanding the immune regulatory properties of intestinal epithelial cells could aid to develop new strategies to prevent and treat human infection and inflammatory and metabolic diseases.

CFTR is a chloride channel that localized in the apical membrane of epithelial cells, and has been shown to play important role in the cystic fibrosis diseases[5]. CFTR is critical for remaining the normal function of epithelial cells in fluid homeostasis, airway fluid clearance, and airway submucosal glands secretion[6]. CFTR has been shown to form macromolecular complexes with other proteins at the plasma membrane of gut epithelia, which functionally couple LPA2 signaling to CFTR-mediated chloride transport[7]. People have shown that LPA can inhibit CFTR-mediated chloride transport through the LPA2-mediated Gi pathway, and LPA inhibits CFTR-dependent cholera toxin-induced mouse intestinal fluid secretion in vivo[8].

In this paper we show that the CFTR-LPA2 pathway plays important role in regulating the immunity function of intestinal epithelial cells. The interaction of CFTR-NHERF2-LPA2 can be detected in the

intestinal epithelial cells. And in vitro assays show that inhibiting CFTR or intriguing LPA2 in the intestinal epithelial cell could disrupt the epithelial cell junction and elevate the expression of IL-8 both in mRNA and protein level. In vivo experiment also show that inhibit CFTR or intrigue LPA can affect the fluid homeostasis of intestinal. Our results found that the CFTR-LPA2 pathway play important role in regulating the normal function of intestinal epithelial cells.

Interaction of CFTR-NHERF2-LPA2 in mICC12 cells

As m-ICC12 cells are intestinal epithelial cells providing physical and biochemical barrier between commensal and pathogenic microorganisms[9], they can sense and respond to microbial stimuli to reinforce their barrier function and to participate in the coordination of appropriate immune responses, ranging from tolerance to anti-pathogen immunity. Thus, IECs maintain a fundamental immune regulatory function that influences the development and homeostasis of mucosal immune cells.

To investigate the function of CFTR-NHERF2-LPA2 complex in m-ICC12 cells, we first conformed the interaction of the three molecules in mouse intestinal cells. The CFTR- IP results show that, the CFTR, NHERF2 and ~~CFTR,~~ LPA2 can form a complex, this indicated that the three molecules interact with each other in intestinal epithelial cells. The immunostaining results also demonstrated that NHERF2 and LPA2 are co-localized with CFTR. The above data show that the CFTR, NHERF2 and LPA2 form micro complex in mouse intestinal cells. We also detected the mRNA level of LPA receptors in m-ICC12 cells by Q-PCR, the results show that the expression levels of LPA_2 and LPA_3 were relatively high compared with other LPA receptors. This indicates LPA2 may play more crucial role in regulating the normal function of intestinal epithelial cells.

CFTR-NHERF2-LPA2 complex regulates the integrity of tight junction in intestinal epithelial cells

The intestinal epithelium has complex mechanisms to control and regulate bacterial interactions with the mucosal surface. Apical tight junction proteins are critical in the maintenance of epithelial barrier function and control of par acellular permeability[10]. The characterization of alterations in tight junction proteins are key players in epithelial barrier function in inflammatory bowel diseases is rapidly enhancing our understanding of critical mechanisms in disease pathogenesis as well as novel therapeutic opportunities. The disruption of integrity of the epithelial barrier may intrigue the onset of inflammatory bowel diseases[11]. The leaky intestinal epithelial barrier is mainly attributed to defects of the TJs and IEC loss[10]; the deficient TJs are the primary cause for the compromised intestinal epithelial barrier[3].

IBD is a chronic condition of intestine characterized by severe inflammation and mucosal destruction. In the normal intestine, epithelial tight junctions provide barrier function to prevent the diffusion of bacterial, toxins, allergens from the gut lumen into intestinal tissue and systemic circulation. Genetic analysis has shown that LPA contribute to IBD and patients of cystic fibrosis also display the phenotype of diarrhea[12]. Drugs perturb CFTR-containing macromolecular complexes in the intestinal can disrupt the TJ structure and also affected the immune function of epithelial cells.

To detect whether CFTR-LPA2 pathway play important function in the cellular junction in the intestinal epithelial cells. We add chemicals that can disrupt or intrigue CFTR-LPA2 pathway to the epithelial cells and then using ZO-1 antibody to detect the cellular junction integrity. Results show that, ImCc12 cells treated with CFTR-inh172, C143 and LPS can disrupt the cellular junction. We also repeated this experiment in human cell line HT-29cells, the result show that, the cells that treated with CFTR, C143 and LPS for 20 hours display disrupted cellular junction. This indicate that, the CFTR-LPA2 pathway play important roles keeping the cellular junction integrity in both human and mouse epithelial cells.

CFTR-NHERF2-LPA2 complex regulates IL-8 expression in intestinal epithelial cells

Previously, people have found that intestinal epithelial cells (IEC) can secret IL-8 in response to challenges from such as lipopolysaccharide with butyrate and IL-1[13]. IL-8 is a chemokine that stimulates migration of neutrophils from intravascular to interstitial sites and can directly activate neutrophils and regulate the expression of neutrophil adhesion molecules. To determine the function of CFTR-NHERF2-LPA2 in the process of IL-8 secretion, we treated mouse intestinal cell line and human colon cell line with molecules that block or intrigue CFTR-LPA2 pathway, and then detect the IL-8 in both mRNA and protein level. Q-PCR results show that, block CFTR through CFTRinh172 4 hours upregulate the mRNA level of IL-8 both in HT-29 cells and mICc12 cells. Intrigue LPA2 through LPS can also

upregulate the mRNA level of IL-8 both in HT-29 cells and mICc12 cells. This indicate the CFTR-LPA pathway play important role in regulating the IL-8 transcription. We also detected the IL-8 level through ELISA to confirm the function of CFTR-LPA pathway in regulating IL-8 level. The ELISA result displaying the similar results: blocking CFTR through CFTR-INH172 or intriguing LPA2 through LPS can elevate the IL-8 protein level in cell medium supernatant. These data indicates CFTR-LPA2 play important role in regulating the immunological function of intestinal epithelial cells.

To confirm the function of LPA2 in regulating the expression of IL-8 in epithelial cells, we reduced the LPA2 expression in HT-29 cells and mICC12 cells through RNA knock down. The western blot results show that the LPA2 expression can be successfully reduced through the transfection of LPA2 lentivirus, and the IL-8 expression is reduced when LPA2 is knocked down (figure 6). This experiment indicate that the IL-8 expression can be regulated through LPA2 receptors.

Discussion

Dysfunction of intestinal epithelial cells are related with some kind of immunological diseases, such as diarrhea, and intestinal bowel diseases. Restoring the epithelial cell function would be a novel therapy strategy for IBD therapy. Previously people has found that, CFTR-NHERF2-LPA2 form macrocomplex in intestinal epithelial cells, inhibition of CFTR through CFTR inh-172 attenuates diarrhea in DSS-induced colitis[14]. Chemicals that inhibit CFTR-LPA2 interaction can lead to diarrhea in mouse, this indicate the function of CFTR-LPA2 in intestinal. In this paper, we demonstrated this macrocomplex also formed in the intestinal epithelial cells in vitro. CFTR IP-western can drag down the NHERF2 and LPA2 in mouse intestinal cells, LPA assay also demonstrate the interaction of CFTR-LPA2 in the in vitro cell lines.

In healthy epithelial cells, the apical TJs construct a dynamic intestinal barrier that regulates the paracellular uptake of water, nutrients and electrolytes. TJ dysfunction can lead to the disruption of the intestinal barrier integrity, and can lead to inflammatory bowel diseases. Previous data has found that CFTR interacts with ZO-1 at tight junctions through its PDZ-binding domain and CFTR regulate tight junction assembly and control tubular genesis in cultured epididymal epithelial cells[15]. They also found that CFTRinh172 can significantly reduce the transepithelial electrical resistance. The most frequent CFTR gene mutation, F508del, has been shown to be associated with a disorganized actin cytoskeleton and altered tight junction permeability[16]. In our study, we demonstrate that CFTR is highly expressed in the intestinal epithelial cells, and the inhibition of CFTR or intriguing of LPA2 can lead to the disruption of tight junction of epithelial cells in both mouse and human. However, when we detected the TER of intestinal epithelial cells with the treatment of CFTR-inh172 and LPS, we did not find any differences.

IL-8 is a major chemokine in the onset of acute IBD and can recruit PMNs to the inflamed crypt and the intestinal lumen to amplify or sustain the inflammation[17]. In our present study, we demonstrated that inhibit CFTR or intrigue LPA2 elevated IL-8 expression at both the mRNA and protein levels. Together, in this paper we show for the first time that CFTR-LPA2 also involved in the intestinal inflammation.

Material and method

Cell culture and cell stimulation assays

The mouse intestinal cell line m-ICc12 cell were grown in medium which is composed of HAMF12(45%, Gibco), DMEM(45%, Gibco), glucose (1g/l, Sigma-Aldrich), insulin (5ug/ml, Sigma),Transferrin(5ug/ml, Sigma), HEPES(20mM, GIBCO), EGF(10ng/mL, Sigma), Trriodothyronine (1nM, Sigma), selenium(60nM, Sigma), dexamethasone (50nM, Sigma), 10% fetal calf serum and glutamine (2mM, Gibco). The human colon cell line HT-29 was grown in Dulbecco's modified Eagle's medium (DMEM; Gibco) containing 10% FBS (Gibco) and 1% P-S. All media compositions are mixed in a 500mL Filter unit under hood. Cocultures were incubated in a humidified incubator at 37°C and 5%CO2.

Immunofluorescent staining and microscopic imaging

Cells grown on CultureSlides (falcon) were fixed with 4% paraformaldehyde for 5 min at room temperature, permeablized with 0.1% tritonX-100 for 15min, blocked with 10% goat serum for 30 min, and then incubated with

antibodies at 4°C overnight. For the cell junction detection, cells were treated with 50uM C143 , 50uM Inh-172 or 50ug/mL LPS for 20 hours and then fixed with 4% PFA for 5 min at room temperature, permeablized with 0.1% tritonX-100 for 15min, blocked with 10% goat serum for 30 min, and then incubated with antibodies at 4°C overnight.

The antibodies used for staining are the following: anti-CFTR (ab2784, Abcam), anti-NHERF2 (sc-33615, Santa Cruz), anti-LPA2 (PA001514-R12-2723, Sydlabs), anti-ZO-1(OG215365, Thermo Fisher).The secondary antibodies were anti-mouse-594 (T20935, Invitrogen) and anti-rabbit-488, prepared according to manufacturer's instruction with predetermined dilutions. Slides were mounted with DAPI Fluoromount-G (0100-20, Southern biotech). Fluorescence images were acquired using a Zeiss 710 fluorescence microscope and then processed using Adobe Photoshop.

Immunoprecipitation and western blot

For immunoprecipitation, cell lysates were obtained by incubation of cells with Lysis buffer (1XPBS, pH 7.5, 0.2% TritonX-100 and protease inhibitors) for 30 minutes on ice and centrifuged for 15 minutes at 13,200 r.p.m. at 4°C to remove cellular debris. The supernatant was precleared with protein A/G agarose (PIERCE) at 4°C for 1 hr. The precleared supernatant was incubated with anti-CFTR (ACL-006, Alomone labs) at 4°C overnight followed by addition of 10μl protein G-Sepharose (GE Healthcare) and incubation for 1 hour at 4°C. Beads were washed three times with Lysis buffer, boiled in sample buffer and analyzed by western blotting. Normal mouse IgGs (Santa Cruz Biotechnology) as negative control. Western blotting was performed according to a standard protocol, using anti-NHERF2 (Santa Cruz), anti-LPA2 (PA001514-R12-2723, Sydlabs).

RNA isolation and Q-RT-PCR

RNA was extracted from fresh cells using miRNA Isolation kit (Thermo Fisher Scientific) following the manufacturer's protocol. Reverse transcription was performed using a Superscript III kit with oligo (dT) 20 primers (Invitrogen). Q-RT-PCR was performed using SYBR Green PCR Master Mix reagents (Qiagen). The following primers were used for DNA amplification: LPA1: forward 5′-CTATGTTCGCCAGAGGACTATG-3′, reverse 5′- GCAATAACAAGACCAATCCCG -3′. LPA2: forward 5′-CACACTCAGCCTAGTCAAGAC-3′, reverse 5′-GTACTTCTCCACAGCCAGAAC-3′. LPA3: forward 5′-GCCCGGTGTGCAATAAAA-3′, reverse 5′- CTTAAAAGCCCCAGAAGTGATG-3′. LPA4: forward 5′-ACAGGCATGAGCACATTCTC-3′, reverse 5′- TGGAGGCAGACGATCAGAG-3′. LPA5: forward 5′-CTCTGACCTTGTTGTTCCCTAC-3′, reverse 5′- GCACAGAGCTGTTGGTAGAA-3′. ATX: forward 5′-CCATGTGTACCAATCTTCCTGAG-3′, reverse 5′- CCTCCACCTTGCCTTCTC-3′. GAPDH: forward 5′-TGGAGGTGAAAGTCATGCTC-3′, reverse 5′- TGTTGGTAGAAGTCGTGTTGG -3′. hIL-8-1F: 5'-TTCTGCAGCTCTGTGTGAAGG-3' hIL-8-1R: 5'-ATGAATTCTCAGCCCTCTTC-3' hGAPDH: forward 5′-GGTGTGAACCATGAGAAGTATGA-3′, reverse 5′- GAGTCCTTCCACGATACCAAAG-3′. The relative mRNA levels of targeted genes were normalized to GAPDH (internal control gene).

Proximity ligation assay (PLA)

Cells grown on culture slides were incubated with Duolink blocking solution for 30 min at 37°C. CFTR and LPA2 primary antibodies were mixed (diluted 1:□200 in Duolink Antibody Diluent) and incubated overnight at 4°C. Following this, slides were washed in Duolink Wash Buffer (0.01 M Tris, 0.15 M NaCl, 0.05% Tween 20) twice at room temperature. After washing, PLA probes were applied in 1:10 dilution in blocking buffer for 1 h at 37°C, washed several times with wash buffer A and incubated for 30 min with the ligation reaction containing the circularization oligos and T4 ligase prepared according to the manufacturer's recommendation(Duolink Detection reagents Red, Sigma) at 37°C. Slides were washed in Wash Buffer A and incubated with Duolink Polymerase in Duolink Amplification Stock for 100 min at 37°C. Amplification-polymerase solution was removed, sections washed in Wash Buffer B and dried at room temperature in the dark. Finally, slides were mounted with Mounting Medium with DAPI. Images were acquired using a Zeiss 710 fluorescence microscope and then processed using Image J.

Transfection of lentiviral constructs Recombinant EDG-4 siRNA (h) Lentiviral Particle Gene Silencers (sc-39926-V) and negative control shRNA lentivirus were purchased from Santa Cruz. HT-29 cells and mICC12 cells (1×105 cells/well) were seeded in a 6-well plate. The EDG-4 and control siRNA (h) Lentiviral Particle lentiviruses were infected into HT-29 cells and mICC12 cells using Polybrene (Santa Cruz), according to the manufacturer's protocol. Cells were infected

with lentiviruses in the presence of 5 μg/ml Polybrene. The cells expressing the shRNA were selected via Puromycin dihydrochloride (sc-108071) selection.

Measurement of TER by Electrical Cell-Substrate Impedance Sensing System (ECIS)

HT-29 cells were grown to confluence on gold microelectrodes. TER was measured in an ECIS (Applied BioPhysics, Foster City, CA). The total electrical resistance measured dynamically across the epithelial monolayer was determined as the combined resistance between the basal surface of the cell and the electrode, reflecting alterations in cell-cell adhesion[18].

Measurement of IL-8 protein

Cells were treated with 50uM C143, 50uM Inh-172 or 50ug/mL LPS or 50uM Beck for 20 hours. Cell medium supernatants were collected and centrifuged to remove all cell debris (10000 g for 10 min at 4°C). IL-8 concentration was measured by an ELISA as described by manuscript (BioSource). The absorbance was measured at OD 450. Samples were run in quadruplicate, and IL-8 values were normalized to measured protein concentration to allow comparison among different samples. Experimental results were reported as average number ± SD.

Statistical analysis

Statistical significance between groups was assessed by Student's t test. Data are expressed as means standard deviations (SD). Triplicate determinations were performed in each experiment, and all experiments were repeated at least three times. A probability value of <0.05 was taken as the criterion for a significant difference.

Figure legend:

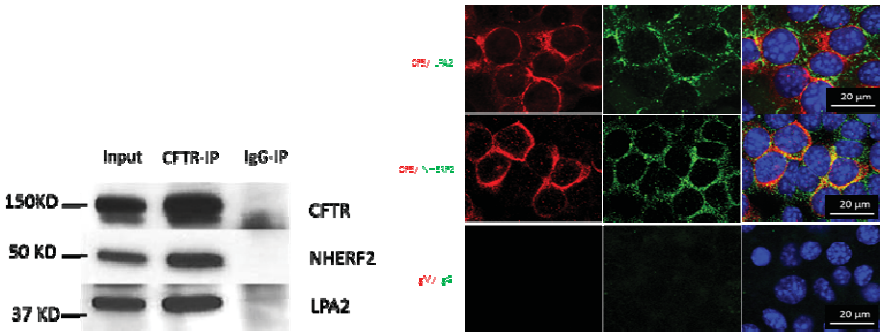

Fig.1 CFTR forms a complex with NHERF2 and LPA2 in mICc12 cells. (A) A representative blot showing that NHERF2 and LPA2 could be co-immunoprecipited with CFTR in mICc12 cells. Mouse IgG was used as a negative control of CFTR antibody for co-IP experiments. IP: immunoprecipitation. IB: immunoblotting. (B) NHERF2 and LPA2 co-localized with CFTR in mICc12 cells. The cells were permeabilized and stained with mouse α-CFTR and rabbit α-NHERF2 (or rabbit α-LPA2) antibodies, followed by incubation of fluorescence-labelled secondary antibodies and subjected to confocal microscopy. Mouse IgG and rabbit IgG were used as negative antibody controls. The nuclei were stained with DAPI.

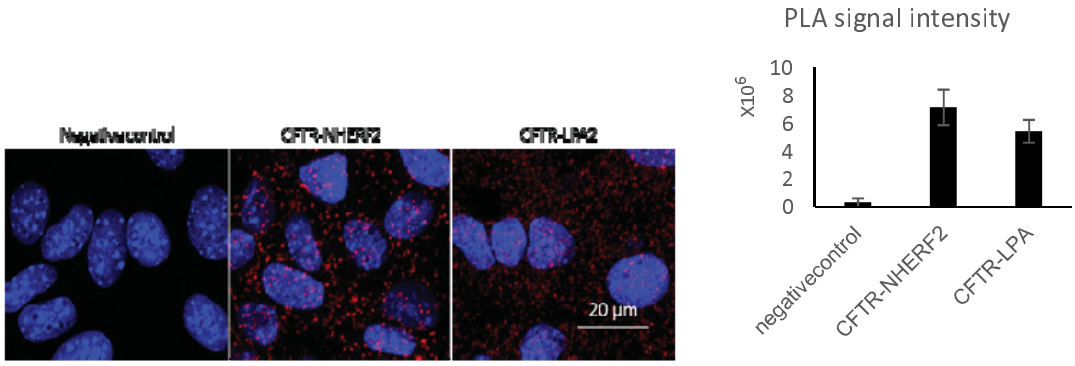

Fig2. Duolink proximity ligation assay to detect protein interactions of CFTR and NHERF2, CFTR and LPA2 in mouse intestinal cell line m-ICCl12. (A) Duolink® proximity ligation assay (PLA) showed the interactions between CFTR and NHERF2, and CFTR and LPA2 in mICc12 cells. The cells were treated with mouse α-CFTR and rabbit α-NHERF2 (or rabbit α-LPA2), followed by incubation with anti-mouse-minus probe and anti-rabbit-plus probe and followed the manufacturer's instructions. Each red spot represents an interaction between the binding partners. The nuclei were stained with DAPI. The corresponding IgGs were used as negative controls. (B) PLA data quantification using ImageJ software.

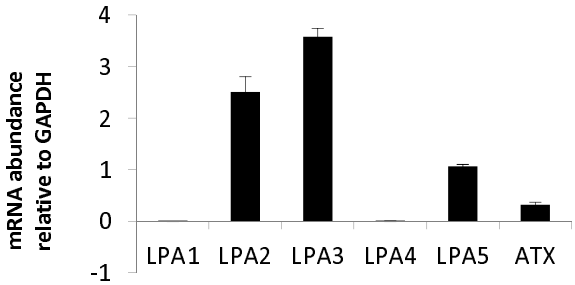

Fig.3 Normalized mRNA expression of LPA receptors in m-ICC12 cells.
Q-PCR were used to detect the gene expression level of LPA receptors (LPA1-LPA5). The results show that LPA2 and LPA3 were highly expressed in m-ICC12 cell line. The relative mRNA levels were normalized with GAPDH. Results were reported as means ± SD (error bars).

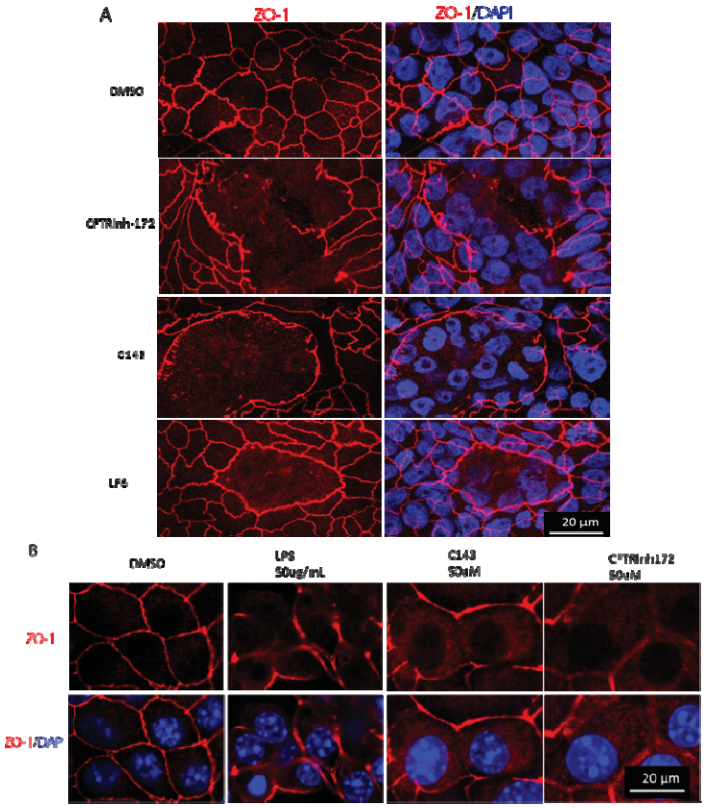

Fig.4 CFTR-NHERF2-LPA2 complex regulates the tight junction integrity in (A) HT-29 cells and (B) mICc12 cells.
CFTRinh-172 is a specific CFTR channel inhibitor. C143 is a specific LPA2 agonist. DMSO was used as a control. The cells were treated with the compounds for 20 hours, fixed, and then labeled with ZO-1 (red). The nuclei were stained with DAPI. Arrow heads indicate the sites where the junction were disrupted.

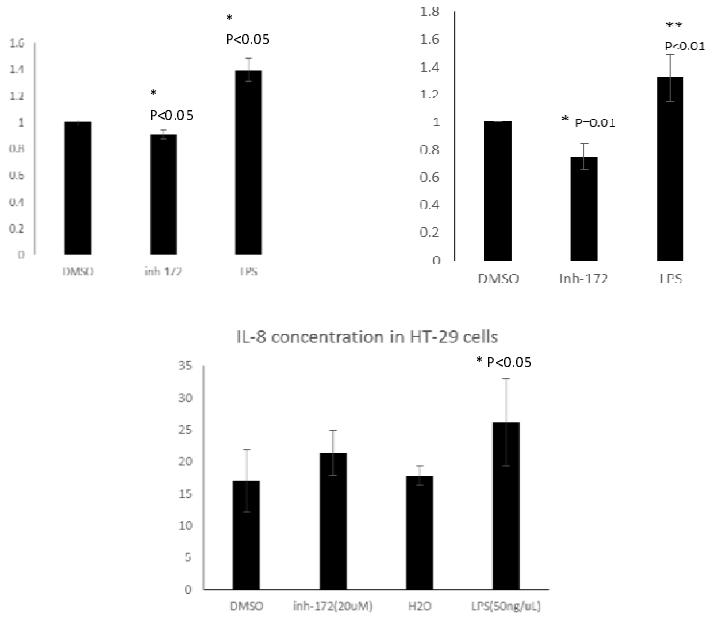

Fig. 5 CFTR-NHERF2-LPA2 complex regulates IL-8 expression in mICc12 cells and HT-29 cells. The relative IL-8 mRNA levels in mICc12 cells under different treatment conditions are shown in (A) and in HT-29 cells shown in (B). The relative secreted IL-8 levels in mICc12 cells under different conditions are shown in (C) and in HT-29 cells shown in (D). The secreted IL-8 protein was detected by using ELISA. The cells were lysed with Qiagene mRNA kit to extract mRNA and Q-PCR was used to quantify IL-8 mRNA levels. Effects of CFTR-LPA on IL-8 mRNA expression in HT-29 cells**.** Each assay was carried out in triplicate, with results reported as means ± SD (error bars).

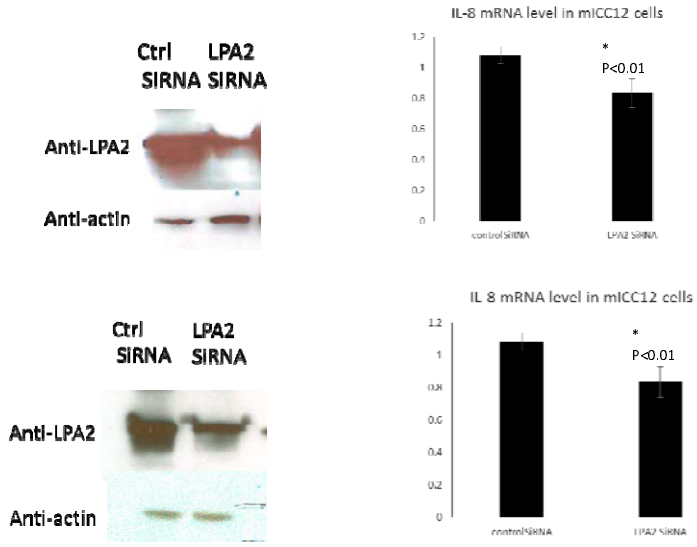

Fig. 6 LPA2 knock down can reduce the mRNA level of Il-8. (A)Western blot detect the LPA2 protein level in HT-29 cells. The LPA2 expression level was reduced by RNAi. (B) The relative IL-8 mRNA levels in HT29 cells transfected with LPA2 SiRNA lentivirus and control SiRNA lentivirus. The cells were lysed with Qiagene mRNA kit to extract mRNA and Q-PCR was used to quantify IL-8 mRNA levels. P=0.0012.

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