Original Research *Highlight article*

TGF-β receptor I inhibitor may restrict the induction of EMT in inflamed intestinal epithelial cells

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Impact Statement

Considering previous efforts, the underlying mechanisms of inflammatory bowel disease (IBD) development are still not fully understood. According to the literature, the multifunctional transforming growth factor–β1 (TGF-β1) signaling is a complicated pathway associated with the development of inflammation diseases, including IBD, as well as virtually targeting all cell types residing in the gut, including leukocytes, stromal cells, and epithelial cells. Consequently, we assessed the TGF-β1 signaling activity in mucosal cells of active lesions in patients suffering from IBD, followed by the inhibition of TGF-β1 signaling in an *in vitro* co-culture model. We showed that the TGF-β receptor I (TβRI) inhibitor could suppress the canonical and PI3K/AKT (non-canonical) TGF-β1 pathways, leading to the prevention of the epithelial–mesenchymal transition (EMT) initiation as a hallmark of IBD. In addition, the TβRI inhibitor exerts a protective effect on the tight junction impairment. We suggested that TGF-β1 signaling suppression could be a novel therapeutic target to manage IBD.

Abstract

Despite the extensive body of research, understanding the exact molecular mechanisms governing inflammatory bowel diseases (IBDs) still demands further investigation. Transforming growth factor–β1 (TGF-β1) signaling possesses a multifacial effect on a broad range of context-dependent cellular responses. However, long-term TGF-β1 activity may trigger epithelial–mesenchymal transition (EMT), followed by fibrosis. This study aimed to determine the role of epithelial TGF-β1 signaling in inflammatory bowel disease (IBD) pathogenesis. The expression of TGF-β1 signaling components and EMT-related and epithelial tight junction markers was examined in IBD patients ($n = 60$) as well as LPS-induced Caco-2/RAW264.7 co-culture model using quantitative real-time polymerase chain reaction (qRT-PCR), Western blotting, and immunofluorescence staining. Furthermore, the effect of A83-01, as a TGF-β receptor I (TβRI) inhibitor, on the inflamed epithelial cells was evaluated *in vitro*. To evaluate the cytotoxic effects of the TβRI inhibitor, a cell viability assay was performed by the MTS method. Considering the activation of canonical and non-canonical TGF-β1 signaling pathways in IBD patients, expression results indicated that administering A83-01 in inflamed Caco-2 cells substantially blocked the expression level of *TGF-β1*, *SMAD4*, and *PI3K* and the phosphorylation of p-SMAD2/3, p-AKT, and p-RPS6 as well as prevented downregulation of *LncGAS5* and *LncCDKN2B*. Further analysis revealed that the inhibition of TGF-β1 signaling in inflamed epithelial cells by the small molecule could suppress the EMT-related markers as well as improve the expression of epithelial adherens and tight junctions. Collectively, these findings indicated that the inhibition of the TGF-β1 signaling could suppress the induction of EMT in inflamed epithelial cells as well as exert a protective effect on preserving tight junction integrity. There is a pressing need to determine the exact cellular mechanisms by which TGF-β1 exerts its effect on IBD pathogenesis.

Keywords: Inflammatory bowel disease, TGF-β signaling, Epithelial–mesenchymal transition, PI3K signaling, A83-01, Long noncoding RNAs

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Introduction

Inflammatory bowel disease (IBD), classified into two main entities, namely, ulcerative colitis (UC) and Crohn's disease (CD), remains a lifelong disease predominantly observed in industrialized countries and is thought to be triggered by multiple environmental factors in genetically susceptible individuals.[1] An impaired mucosal barrier, along with

disturbed commensal gut flora, results in the intestinal immune system dysregulation. Xenobiotic receptors are chemical-sensing transcription factors that play crucial roles in the transcriptional regulation of intestinal inflammation, and dysregulation of xenobiotic sensing is correlated to an increased risk of IBD.[2] Although notable progress has been made in understanding IBD development in recent decades, and a great number of investigations have been conducted to interfere with IBD, the exact molecular mechanism underlying IBD remains intractable.[3]

Transforming growth factor–β (TGF-β), a multifunctional signaling protein whose receptors are widely expressed, has wide-ranging effects on a variety of cell types, especially, although not exclusively, immune cells and epithelial cells.[4] There are three TGF-β sub-family members, namely, TGF-β1, β2, and β3, among which TGF-β1 is the most abundant isoform in most tissues, particularly immune system. According to previous studies, TGF-β1 is generally increased in response to injury and stress and by inflammation induction, while TGF-β2 and TGF-β3 are mostly involved in the regulation of developmental processes.[5] Furthermore, TGF-β1 is strongly found in both developing embryos and adults, and involved in widespread multifocal inflammatory diseases, whereas both TGF-β2 null mice and TGF-β3 null mice could not survive after birth due to the severe developmental defects in the heart, lung, and ear.[6–8]

There are contradictory studies about TGF-β1 function which is thought to depend on TGF-β1-secreting tissue.[4,9] Accumulating evidence demonstrates that TGF exerts a dual effect on different types of cancers and inflammatory disorders. There are several controversial issues regarding the effect of TGF-β1 on IBD pathogenesis. Early work carried out by Babyatsky *et al.*[10] reported that gut mucosa from patients with UC and CD displays a high level of *TGF-β1*. Furthermore, *TGF-β* expression was shown to be higher in blood samples of active IBD patients.[11] Contrarily, Monteleone *et al.*[12] implicated that phospho-SMAD3 in mucosa lamina propria mononuclear cells are significantly downregulated than those found in normal controls. Indeed, TGF-β1 can activate both SMAD-dependent (canonical) and SMAD-independent (non-canonical) signaling pathways to exert its biological effects.[13,14] More importantly, TGF-β1 is a crucial regulator to drive epithelial–mesenchymal transition (EMT) which could result in the induction of tight junction degradation in epithelial cells, activation of myofibroblasts, and excessive production of extracellular matrix (ECM).[15] EMT is defined by disintegration of cell–cell adhesion with the loss of epithelial markers and the rise of mesenchymal markers (also known as EMT markers).[16] TGF-induced EMT is mostly mediated by *SMAD*-level activities, leading to increased expression of *E-cadherin* transcriptional repressors such as *SNAIL*, *ZEB*, and *TWIST* that interact with other transcription regulators in the nucleus. Therefore, blockade of activated TGF pathways seems to be an appealing strategy to prevent the EMT onset in colitis, which may efficiently improve the damaged epithelial tight junction architecture in colitis.

Long noncoding RNAs (LncRNAs) are defined as RNA transcripts longer than 200 nucleotides and considered as novel regulators of gene expression.[17] Although there are few data on the mechanism of action of many LncRNAs, recent findings have indicated that they are involved in a variety of biological processes, including chromatin remodeling, regulation of gene expression, and protein activity modulation.[18] It has recently been proposed that LncRNAs may contribute to IBD pathophysiology. Dysregulation of 47 LncRNAs has been reported in IBD, highlighting their potential role in the control of inflammatory pathways.[19] Nowadays, a wide range of studies investigated the regulatory role of SMAD-dependent TGF-β signaling LncRNAs in fibrosis and inflammation.[20] Meanwhile, despite numerous studies on the role of LncRNAs in diseases, their contribution to TGF signaling activity in IBD remains largely unknown.

Therefore, the aim of this study is to determine the activity status of the TGF-β1 signaling in the inflamed tissues of IBD patients and examine whether the inhibition of TGF-β1 signaling through small molecules improves impaired epithelial adherens and junction in IBD with a focus on the control of EMT pathways. To this end, the expression of canonical and non-canonical TGF-β1 signaling genes as well as some TGF signaling-related LncRNAs was first evaluated in mucosal biopsies of IBD patients. Thereafter, the cytotoxicity assay was performed to achieve the optimum concentration of A83-01. Then, the effect of TGF-β receptor I inhibitor (TβRI inhibitor) on the expression of EMT-related, epithelial adherens and tight junction markers was assessed in the LPS-induced Caco-2/RAW264.7 co-culture model.

Materials and methods

Patients

A total of 60 cases and controls, including 16 CD patients, 20 UC patients, and 24 healthy controls, were enrolled in this study. Colonic biopsies were collected from patients with moderate/severe active disease based on the pathologic and endoscopic reports in 2019–2021 at the gastrointestinal and liver diseases clinic of Taleghani hospital associated with Shahid Beheshti University of Medical Sciences (SBMU). Healthy controls showed endoscopy with normal findings and no gastrointestinal disease. The study excluded patients suffering from other autoimmune diseases (including psoriasis, primary sclerosing cholangitis [PSC], autoimmune hepatitis [AIH], primary biliary cholangitis [PBC], and cirrhosis) or having a history of using antibiotics during the last three months. Written informed consent was obtained from all subjects. The ethics committee of SBMU approved the protocols used in this study. Samples were stored at –80°C. Clinical information and patient characteristics were retrieved from participants.

Cell line culture and reagents

Caco-2 and RAW264.7 cell lines were purchased from the Iranian Biological Resource Center (IBRC). They were cultured at 37°C and 5% CO_2 in DMEM medium (Gibco) that was supplemented with 10% fetal bovine serum (FBS) (Gibco), 2 mM L-glutamine (Gibco), 10 mM HEPES, and 1% nonessential amino acids (Gibco).

The co-culture model of Caco-2/RAW264.7 cells

The co-culture model was established according to the previous study.[21] Cells were harvested to confirm the establishment of an *in vitro* IBD model. To evaluate the role of the TGF-β1 signaling pathway in IBD, 2 μM of A83-01, as a TGF-β signaling inhibitor, was used for 24 h on the apical side of the Transwell insert, while inflammation was induced in macrophage RAW264.7 cells. Wells treated with 0.1% dimethyl sulfoxide (DMSO) were used as controls.

Cell viability assay

MTS assay was used to assess the effect of TGF-β1 receptor I inhibitor, A83-01 on the viability of Caco2 cell viability, and determine the optimized dose, according to the manufacturer's protocol (Promega). In this regard, 10^4 Caco-2 cells were plated into a 96-well plate and treated overnight with the A-83-01 small molecule at concentrations of 0, 0.5, 1, 1.5, 2, 3, and 4 μM. DMSO reagent was used in the control group. The absorbance at 490 nm was determined using a multi-well plate reader (ELx800; BioTek).

Real-time quantitative polymerase chain reaction

Total RNA from biopsy specimens and cultured cells was extracted using TRIzol (Invitrogen) according to the manufacturer's instructions. For this purpose, genomic DNA was first decontaminated using RNase-free DNase (Qiagen), and RNA was eluted with 15–25 μL RNase-free water and was reverse-transcribed to cDNA. The expression levels of candidate genes were determined by real-time quantitative polymerase chain reaction (RT-qPCR) in a Rotor Gene Q System (Qiagen) as described before.[22] The relative expression of candidate genes was achieved using the comparative Ct (ΔΔCt) method. The expression of individual values was normalized to that of the GAPDH as a housekeeping gene. Supplementary Table 1 indicates the primer sequences used in this study.

Immunofluorescence analysis

After the fixation of Caco-2 cells in 4% paraformaldehyde solution for 20 min at room temperature (RT), the cells were blocked and incubated with primary antibodies, VIM diluted 1:100 and E-cadherin diluted 1:100, overnight, followed by treatment with fluorescence-conjugated secondary antibodies for 30 min. The nucleus of the cells was counterstained by 40,6-diamidino-2-phenylindole (DAPI) (D9542; Sigma-Aldrich). Fluorescent cells were visualized and digital images were captured using a microscopy camera (Olympus BioScapes). Supplementary Table 2 indicates the list of antibodies used in this study.

Immunoblotting assay

Protein extraction was performed using radioimmunoprecipitation assay (RIPA) buffer (BIO Basic, USA). Protein concentration was then measured by BCA assay kit (Thermo Scientific). Proteins (30 μg) were separated on 12% SDS-polyacrylamide gel and transferred to polyvinylidene difluoride (PVDF) membrane. They were then blocked for 2 h at RT and incubated overnight with primary antibodies. After washing, horseradish peroxidase (HRP)-conjugated secondary antibodies were used for 1 h. Chemiluminescent peroxidase substrate was used to visualize protein-specific bands by an imaging system (VILBER). Relative densities were determined as the ratio of sample signal intensity to α-actin intensity. Antibodies are listed in Supplementary Table 2.

Statistical analysis

Comparisons among multiple groups were performed using one-way analysis of variance (ANOVA) and Tukey's post hoc tests. The Student's *t*-test was used for two group comparisons. Statistical analyses were performed using GraphPad 6 Prism (GraphPad, La Jolla, CA, USA). The association between any two genes was obtained by calculating Pearson correlation coefficients (PCCs) using cor function in R, and cor.mtest from the corrplot package was utilized to estimate the *P* values of the expression levels of gene pairs in each condition. The correlation plots were also generated by R package corrplot based on the PCCs of gene pairs with a *P* value ≤ 0.05. The results are presented as mean ± SEM ($n = 3$). *P* values ≤ 0.05 were considered statistically significant.

Results

SMAD-dependent TGF-β signaling is significantly activated in the inflamed colonic mucosa of IBD patients

A total of 60 patients were classified into three clinical phenotypes, where groups included 20 UC and 16 CD patients as well as 24 healthy controls. Supplementary Table 3 shows relevant characteristics of phenotypes and comparative outcome groups. There was no remarkable difference in age and gender across the three groups. Interestingly, no significant differences were observed in disease activity index (clinical or endoscopic scores) between CD and UC groups, representing similar levels of inflammation.

To further clarify the role of TGF-β1 signaling in the IBD disease, we first assessed the expression of TGF-β1 signaling—related genes in inflamed colon tissues of IBD patients using qRT-PCR. Results showed that the expression of *TGF-β1* and *SMAD4* transcripts was upregulated in the inflamed UC and CD tissues compared with healthy controls (Figure 1(A)).

According to the RNA immunoprecipitation (RIP) assays for SMAD protein–associated LncRNAs screening, *LncGAS5* (*GAS5*) showed a high affinity in binding to *SMAD3* and *SMAD4* and physically associated with the endogenous SMAD3 in 10T1/2 cells.[23] Hence, *GAS5* expression was also evaluated by qRT-PCR and showed a substantial decrease in active lesions of IBD patients compared with healthy controls (Figure 1(A)).

Considering the overexpression of canonical TGF-β1 signaling components in the active lesions derived from patients with UC and CD, this study examined the effect of the TβRI inhibitor, A83-01, on the inflamed Caco-2 cells. Cells treated with DMSO were used as a control group. We performed MTS assay to evaluate the viability of Caco-2 cells in the presence of various concentrations (0–4 μM) of A83-01 for 24 h.

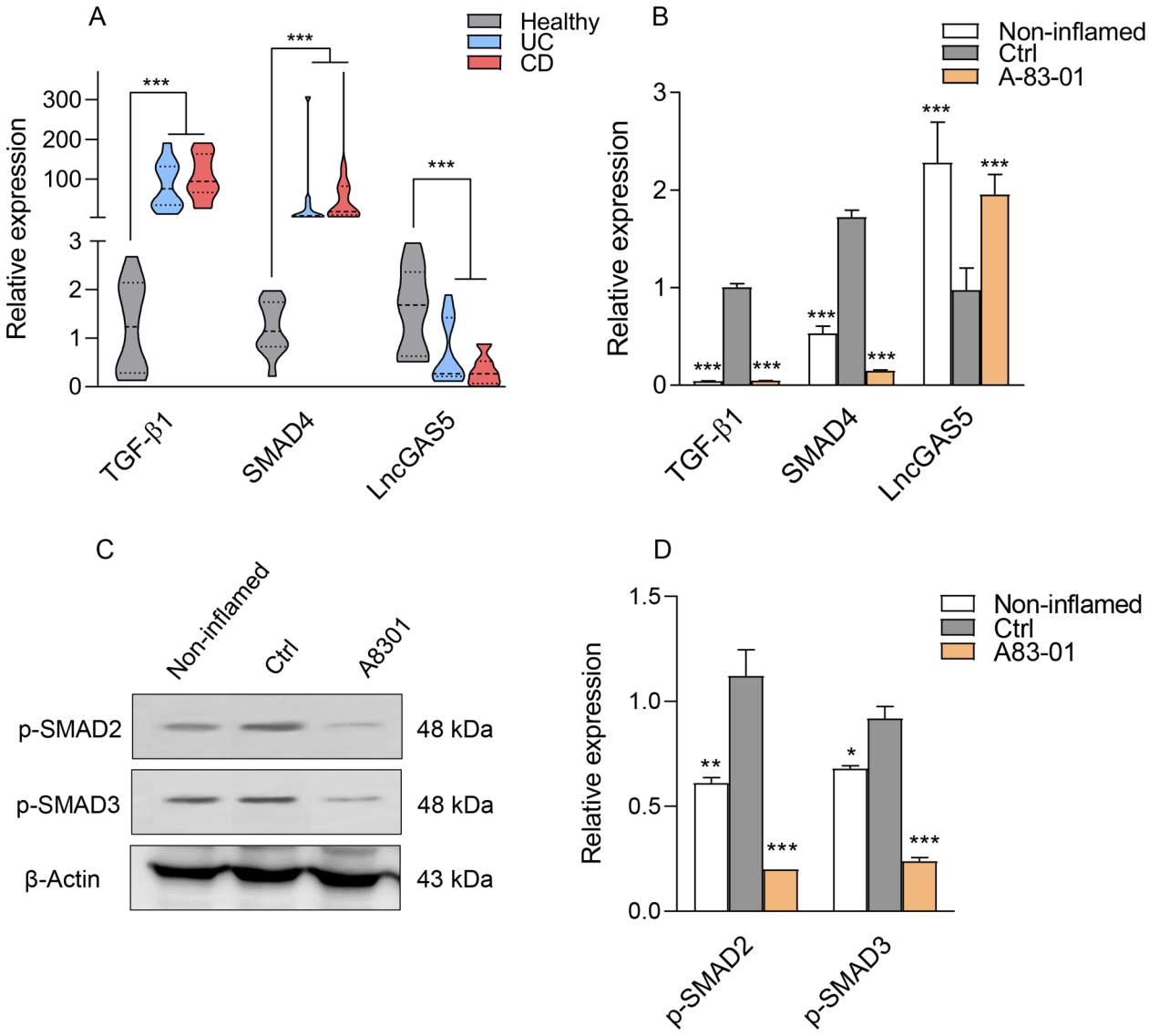

Figure 1. SMAD-dependent TGF-β1 signaling in IBD patients and Caco-2/RAW264.7 co-culture model. (A) Expression levels of *TGF-β1*, *SMAD4*, and *LncGAS5* transcripts in CD and UC patients and healthy controls (B) and in the Caco-2/RAW264.7 co-culture model after treatment with 2 μM of A83-01. LPS-treated and LPS-untreated cells in the co-cultured system were considered as the control (Ctrl) and non-inflamed groups. (C) Protein levels for SMAD-dependent TGF-β1 signaling molecules, p-SMAD2 and p-SMAD3 in LPS-induced Caco-2/RAW264.7 co-culture model treated with A83-01. β-actin was used as a loading control. (D) The histogram represented the intensity of proteins for each group which was normalized to the β-actin band. Results are presented as the mean ± SEM. *In vitro* analyses were performed in three biological replicates. *, **, and *** refer to the adjusted *P* value < 0.05, < 0.01, and < 0.001, respectively.

As the concentration of A83-01 increased, cell viability decreased in a dose-dependent pattern. A83-01 at 2 μM concentration had no significant cytotoxic effect on cells (Supplementary Figure 1); Therefore, 2 μM was selected as the optimum concentration for further analysis. According to previous reports, the effect of A83-01 on cell viability is dependent on the cell type and pathophysiological condition and different doses have been used in studies, such as 25 μM for A549 cells and MCF-7 cells[24] and 1 μM for human endometrial mesenchymal stem cells[25] and postnatal Nkx2.5 cardiomyoblast.[26] Thus, we applied the MTS test as a time- and cost-effective assay to select the optimum dose.

To decipher the role of TGF-β1 signaling in IBD pathogenesis, we used a previously established co-culture system including intestinal epithelial Caco-2 cells and RAW264.7 macrophage cells in a Transwell® plate.[21] Briefly, the intestinal physiological environment was simulated through differentiation of Caco-2 cells for 21 days into a monolayer of enterocyte-like cells, at which point RAW264.7 macrophages were pre-seeded on the basolateral side in Transwell plates, allowing interaction between the two cell monolayers. Subsequently, LPS (at a concentration of 1.2 μg/mL) was added to stimulate RAW264.7 macrophages on the basolateral side for 24 h, resulting in the overexpression of tumor necrosis factor–α (TNF-α) and interleukin-6 (IL-6) within 24 h (data not shown). LPS-treated and LPS-untreated RAW264.7 cells in the co-cultured system were considered as the control and non-inflamed groups, respectively. Then, qRT-PCR was performed to validate the increased expression of *TGF-β1*, *SMAD4*, and *GAS5* in inflamed enterocyte-like cells (Figure 1(B)) . Over 24 h, A83-01 (at a 2 μM concentration) was added to the apical side of the LPS-induced Caco-2/RAW264.7

co-culture model. Figure 1(B) demonstrates that the small molecule A83-01 could prevent the overexpression of *TGF-β1* in the inflamed epithelial cells *in vitro*. Furthermore, A83-01 significantly decreased *SMAD4* expression induced in the inflamed epithelial cells at the transcript level (Figure 1(B)). Subsequently, the suppression effect of A83-01 was evaluated on the phosphorylation of SMAD2/3 family proteins, as downstream markers of the *TGF-β1* pathway. Increased p-SMAD2/3 in the inflamed epithelial cells was completely blocked by A83-01, as illustrated in Figure 1(C) and (D). Because *GAS5* can bind to SMAD3 and dephosphorylate, we sought to determine whether the suppression of the TGF-β1 pathway affects the expression level of *GAS5* in the inflamed enterocyte-like cells. Interestingly, our results (Figure 1(B)) showed that suppression of TGF-β1 pathway could prominently lead to increased transcriptional levels of *GAS5*. Our findings demonstrated that TβRI inhibitors as a therapeutic option could decrease SMAD-dependent TGF-β1 signaling activated in IBD.

TβRI inhibitor prevents the activation of SMAD-independent TGF-β signaling in inflamed intestinal epithelial cells

To investigate the role of the SMAD-independent TGF-β signaling in the development of colitis, the activity of phosphatidylinositol-3-kinase (PI3K)/AKT pathway as one of the SMAD-independent pathways was assessed. PI3K/AKT signaling is involved in the regulation of cell growth, cell proliferation, and inflammation through downstream signal transduction molecules such as p-70S6K and p-RPS6.[27] *PI3K* mRNA was found to be highly expressed in the inflamed epithelial tissue of UC and CD patients compared with healthy controls (Figure 2(A)), while *LncCDKN2B* (*CDKN2B*) as a key regulatory LncRNA of the PI3K/AKT pathway was decreased (Figure 2(A)). Likewise, there was a similar trend toward increased *PI3K* and decreased *CDKN2B* transcripts in the LPS-induced Caco-2/RAW264.7 co-culture model. Treatment of inflamed epithelial cells by A83-01 led to a decrease in *PI3K* and an increase in *CDKN2B*, showing to be statistically significant (Figure 2(B)). However, no changes in *AKT* were found in both patients and *in vitro* IBD model. Therefore, the phosphorylation level of AKT and its downstream target, p-RPS6, was analyzed by Western blotting in the *in vitro* co-culture model. As shown in Figure 2(C) and (D)), treatment with A83-01 suppressed the phosphorylation of AKT and subsequently RPS6 in the inflamed group compared with the control.

TβRI inhibitor suppresses EMT induced in inflamed intestinal epithelial cells

To understand the contribution of TGF-β1 signaling to promote EMT in the inflamed mucosal biopsies of IBD patients, the expression of EMT markers was investigated. As illustrated in Figure 3(A), a broad panel of mesenchymal markers, including *VIM*, *SNAI1*, *SNAI2*, *CTNNB1*, *ACTA2*, and *CDH2*, were upregulated in active lesions of UC and CD patients compared with the healthy control, while the expression of *CDH1* was downregulated. These data suggested that increased activity of *TGF-β1* signaling in inflamed colonic tissues may trigger the expression of mesenchymal marker inducers, thereby causing reduction of epithelial markers. The R package corrplot was used to visualize correlations across genes. We intended to remove the nonsignificant correlations according to the confidence interval of 0.95. None of the correlations were removed due to the significant *P* value of less than 0.05. Figure 3(B) and (C)) demonstrates the correlation of any gene pairs in UC and CD conditions. In these plots, color and ellipticity represent the correlations. The correlation pattern between genes was similar due to the interconnected nature of these conditions. The strongest negative correlations were between ZO-1-SNAI1 and OCCLUDIN-LncH19 in UC and CD, respectively. The most positively correlated genes in UC were *PI3K* and *CTNNB1*, while they were *CDH2* and *SNAI1* in CD.

Afterward, we sought whether the TβRI inhibitor affects the induction of EMT pathway in the LPS-induced Caco-2/RAW264.7 co-culture model. Our results revealed that A83-01 treatment could markedly decrease mRNA expression levels of *VIM*, *SNAI1*, *SNAI2*, *CTNNB1*, *ACTA2*, and *CDH2* (Figure 4(A)). Similarly, decrease in the level of Vim, β-CATENIN, and α-SMA proteins was detected in the inflamed epithelial cells in response to the A83-01 treatment (Figure 4(B) and (C))). The immunostaining analysis also confirmed lower expression of VIM (green), a main marker of EMT, in the A83-01-treated cells (Figure 5(A)). Contrary to mesenchymal markers, the expression level of *CDH1*, a well-established marker of cell adhesion, remarkably increased at both transcript and protein levels in the A83-01-treated epithelial cells compared with the control group (Figure 4(A) and (B)), consistent with results obtained from the E-CADHERIN immunostaining (red) (Figure 5(B)). Based on increasing levels of *LncRNAH19* (*H19*) in many inflammatory diseases and organ fibrosis, as well as its novel regulatory role in the EMT progression,[28,29] we first examined the mRNA expression of *H19* in patient groups. The expression level of *H19* exhibited the greatest increment in active lesions of patients with UC and CD compared with the healthy control (Figure 3(A)). Furthermore, the expression level of *H19* was assessed in the LPS-induced Caco-2/RAW264.7 co-culture model upon treatment with A83-01. Intriguingly, *H19* was downregulated following A83-01 treatment (Figure 4(A)), indicating its involvement in the *TGF-β1*-induced EMT in the inflamed epithelial cells. Overall, these results suggested that the TβRI inhibitor maintains the epithelial characteristics of intestinal cells in inflammatory conditions by limiting the EMT induction.

The protective effect of TβRI inhibitor on the impaired epithelial barrier

We assessed the expression of epithelial-related markers, including *CLAUDIN7*, *ZO-1*, and *OCCLUDIN*, in the inflamed colon tissues. In agreement with previous studies,[30] we indicated that expression levels of *CLAUDIN7*, *ZO-1*, and *OCCLUDIN* decreased in active tissue lesions from patients with UC and CD compared with the healthy control (Figure 5(C)). We, therefore, aimed to assess the possible association of epithelial adherens and tight junction

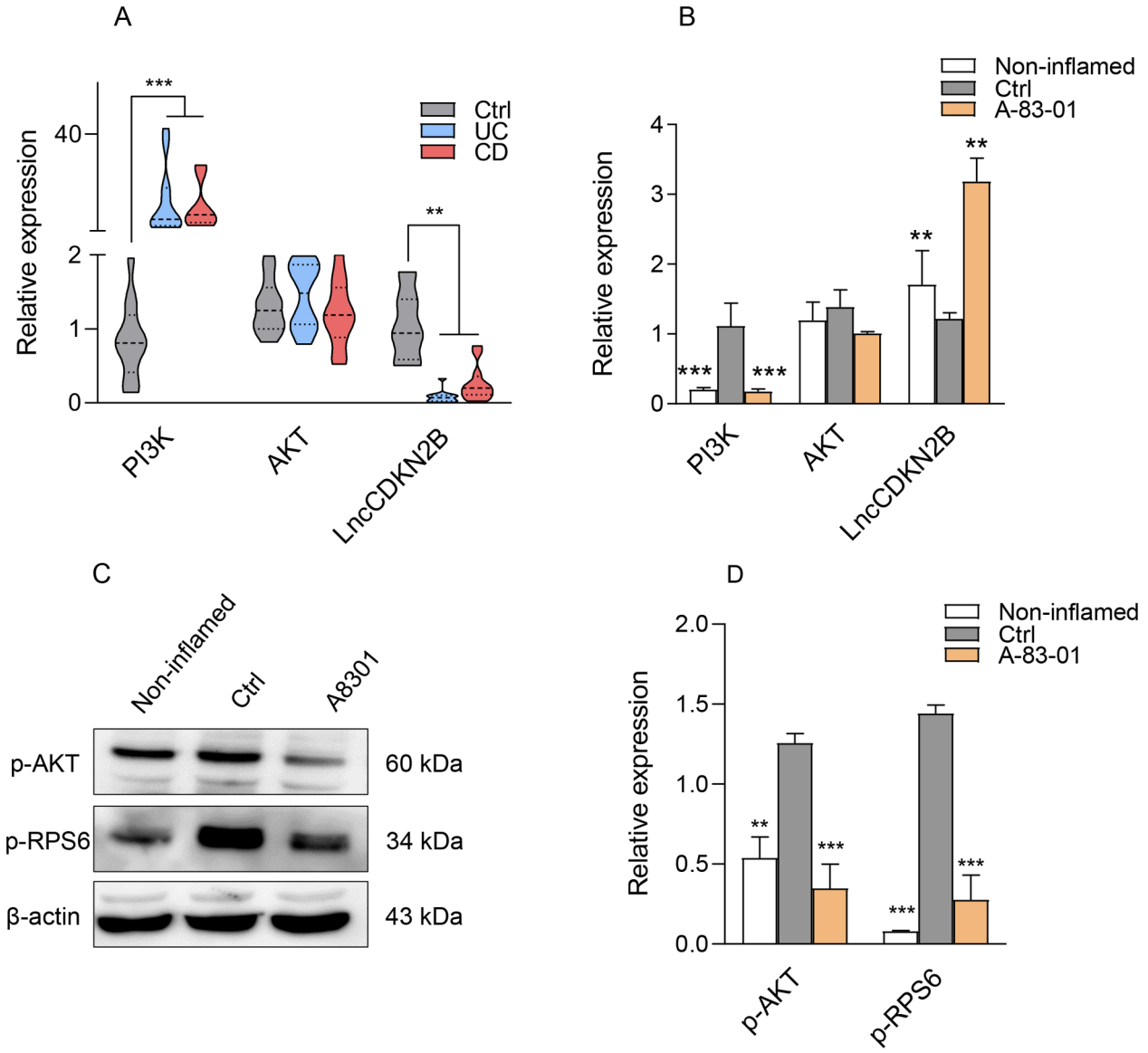

Figure 2. PI3K/AKT signaling in IBD patients and Caco-2/RAW264.7 co-culture model. (A) Expression levels of *PI3K*, *AKT*, and *LncCDKN2B* transcripts in CD and UC patients and healthy controls (B) and in the Caco-2/RAW264.7 co-culture model after treatment with 2 μM of A83-01. LPS-treated and LPS-untreated cells in the co-cultured system were considered as the control (Ctrl) and non-inflamed groups. (C) Protein changes of p-AKT and p-RPS6 after treatment with A83-01. β-actin was used as a loading control. (D) The histogram represented the intensity of proteins for each group which was normalized to the β-actin band. Results are presented as the mean ± SEM. *In vitro* analyses were performed in three biological replicates. *, **, and *** refer to the adjusted *P* value <0.05, <0.01, and <0.001, respectively.

markers with the suppression of TGF-β1 signaling in the co-culture model. Intriguingly, mRNA levels of *OCCLUDIN*, *CLAUDIN7* and *ZO-1* in the LPS-induced Caco-2/RAW264.7 co-culture model upon treatment with A83-01 sharply enhanced compared with those in the control (Figure 5(D)). These findings revealed that the TGF-β1 signaling suppression may play an effective role in preventing the disruption of tight junctions and maintaining the epithelial barrier in inflammatory conditions.

Discussion

The results obtained from this study offers an insight into the role of SMAD-dependent and SMAD-independent TGF-β1 pathways in intestinal epithelial cells undergoing inflammation conditions which may reconcile some of the apparently contradicting results in patients suffering from IBD. We revealed how TGF-β1 signaling suppression leads to targeting the EMT process as well as preventing the impairment of epithelial tight junctions in the *in vitro* IBD model (Figure 6).

According to previous studies, the TGF-β1 pathway plays a vital role in the pathogenesis of inflammatory disorders.[4] The upregulation of *TGF-β1*, *SMAD4*, and *PI3K* transcripts in inflamed biopsies of IBD patients confirmed previously published results,[31,32] while the expression level of *AKT* transcript did not alter because AKT serves as a serine/threonine kinase, which is induced by the phosphorylation. In addition, expression trends of *TGF-β1*, *SMAD4*, *AKT*, and *PI3K* in our LPS-induced Caco-2/RAW264.7 co-culture model were similar to those observed in patients. In agreement with our study, a study conducted by Huang *et al.*[32] reported that the development of UC is closely related to the activation

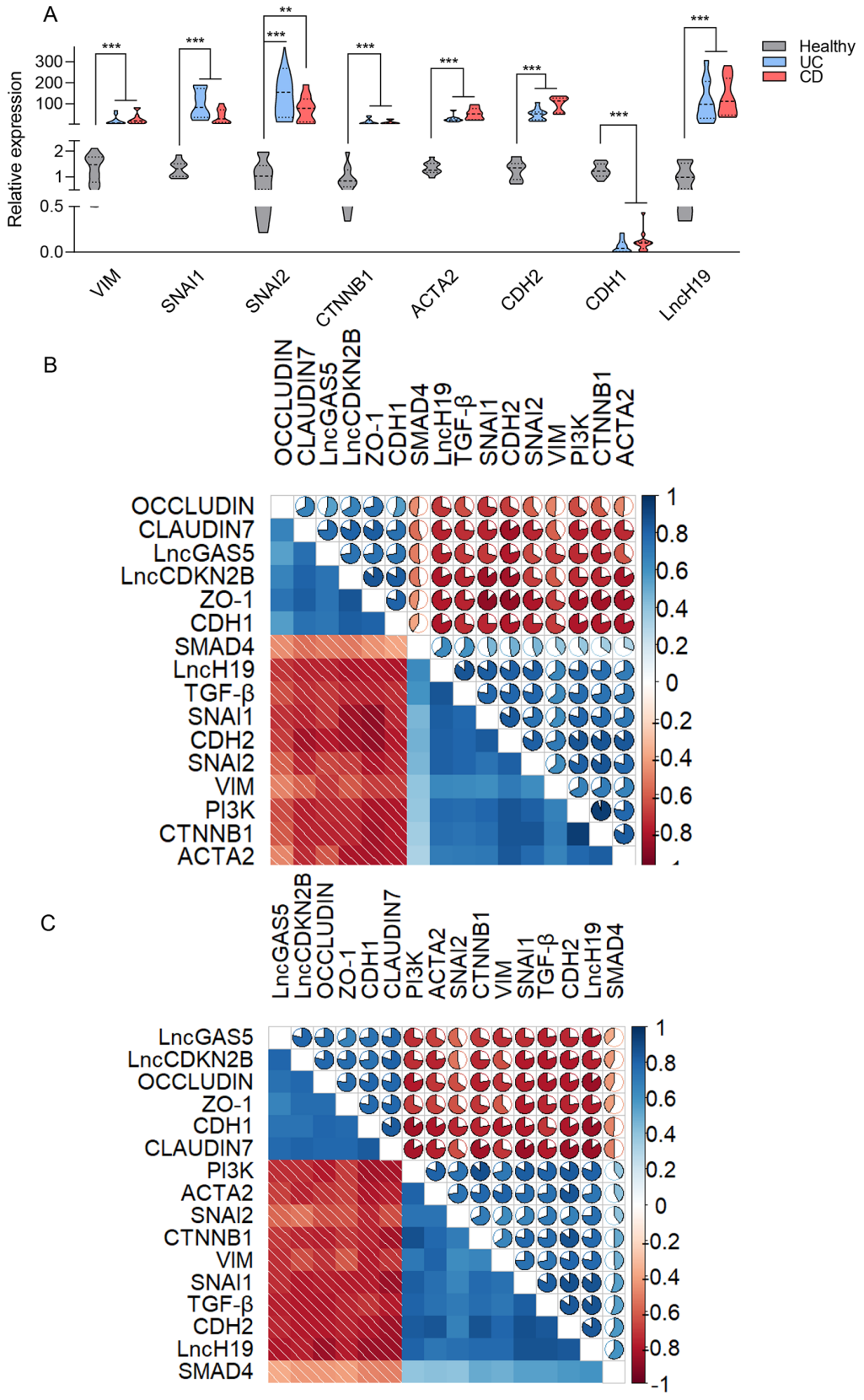

Figure 3. TGF-β1 signaling correlates with the EMT pathway in IBD. (A) Changes in the levels of *VIM*, *SNAI1*, *SNAI2*, *CTNNB1*, α-*SMA*, *CDH2*, *CDH1*, and *LncH19* mRNA in CD and UC patients were detected by qRT-PCR. Correlation analysis of gene pairs of (B) UC and (C) CD conditions. Correlations are indicated by the ellipticity and color of the ellipses as well as the numbers. The bar on the right represents the correlation values of PCCs. All the correlations have a significant *P* value of less than 0.05. Results are presented as the mean ± SEM. *In vitro* analyses were performed in three biological replicates. *, **, and *** refer to the adjusted *P* value < 0.05, < 0.01, and < 0.001, respectively.

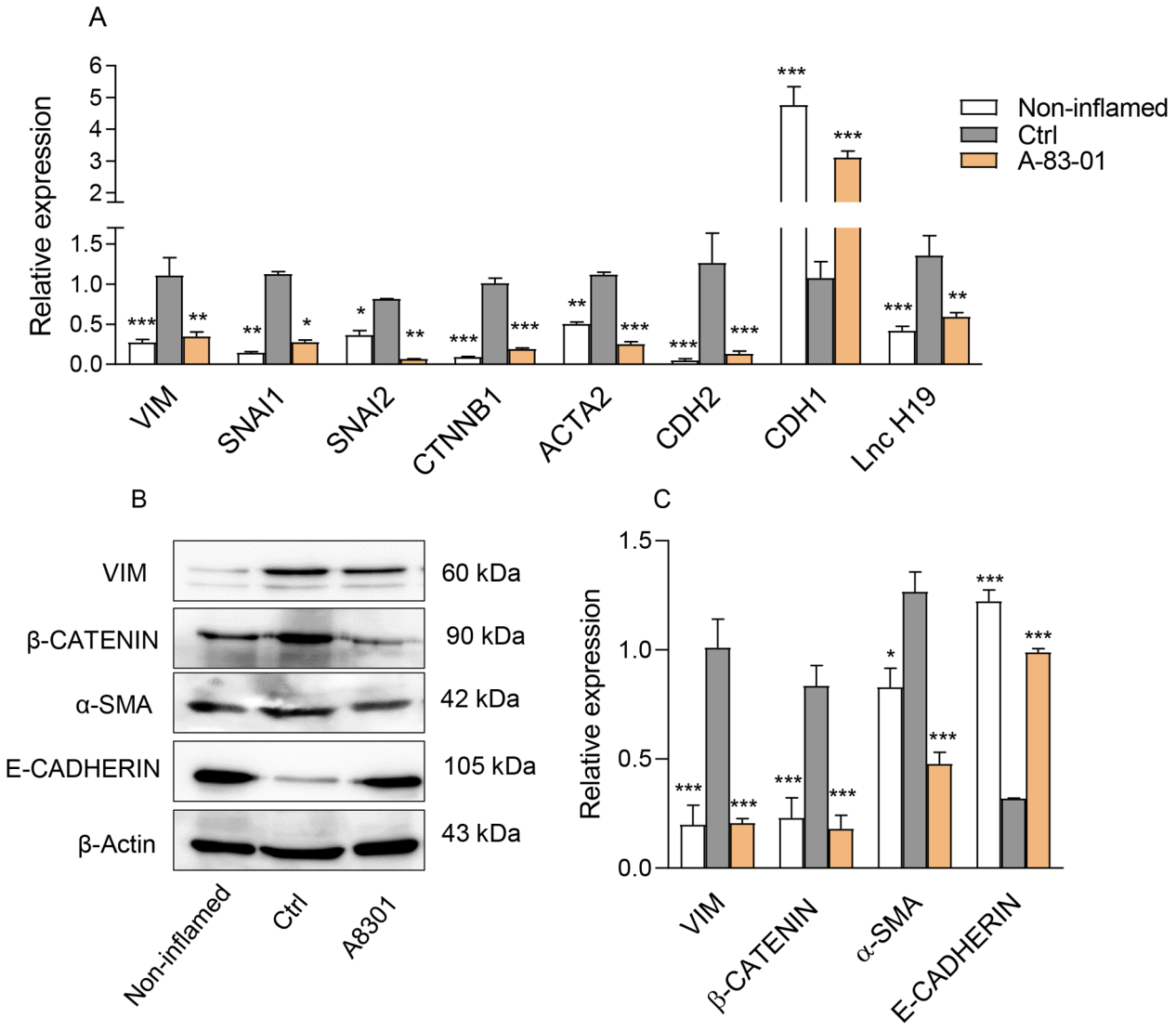

Figure 4. TGF-β1 signaling affected the EMT pathway in the Caco-2/RAW264.7 co-culture model. (A) Expression changes of EMT-related markers at the transcript level in the Caco-2/RAW264.7 co-culture model after treatment with 2 μM of A83-01. LPS-treated and LPS-untreated cells in the co-cultured system were considered as the control (Ctrl) and non-inflamed groups. (B) Protein expression levels of VIM, β-CATENIN, α-SMA, and E-CADHERIN in the Caco-2/RAW264.7 co-culture model. β-actin was used as a loading control. (C) The histogram represented the intensity of proteins for each group which was normalized to the β-actin band. Results are presented as the mean ± SEM. *In vitro* analyses were performed in three biological replicates. *, **, and *** refer to the adjusted *P* value <0.05, <0.01, and <0.001, respectively.

of the *PI3K/AKT* signal transduction pathway, an SMAD-independent TGF-β1 signaling pathway, in the colon mucosa of UC patients and colitis mice. Therefore, overexpression of these transcripts as well as p-SMAD2, p-SMAD3, p-AKT, and p-RPS6 at the protein level highlighted the activation of both SMAD-dependent and SMAD-independent TGF-β1 pathways in response to the immune activation. Furthermore, our results demonstrated a noticeable decrease in *GAS5* and *CDKN2B* expression, known as regulators of TGF-β1 pathway important mediators, in active lesions from patients with CD and UC. In this line, findings conducted by Lucafò *et al*.[33] were also based on the dramatically lower expression of *GAS5* in inflamed CD and UC tissues compared with the adjacent normal part. Interestingly, as reported in the literature, GAS5 is involved in the regulation of TGF/SMAD signaling pathway. It has been shown that *GAS5* suppresses TGF/SMAD3 signaling and inhibits TGF-β-induced smooth muscle cell (SMC) differentiation. Indeed, Smad3 phosphorylation/dephosphorylation turnover is affected through directly binding GAS5 into the Smad3 promoter in 3T3 cells.[23]

Our results revealed that upregulation of the TGF-β1 pathway in IBD patients was negatively correlated with the *GAS5* expression. In addition, according to the study performed by Mirza *et al*.,[34] *CDKN2B* expression was reduced in inflamed CD and UC patients compared with the healthy control, which was also in agreement with our findings. A substantial decrease in *CDKN2B* expression has been also found when Caco-2 and HT29 colonic epithelial cells were stimulated with TNF-α or IL-1β.[35,36] Furthermore, Nanda *et al*.[37] illustrated that *CDKN2B* deficiency is accompanied by an increase in the TGF-β1 production in the endothelial and smooth muscle cells. enzyme-linked immunosorbent assay (ELISA)-based assays showed that knockdown of CDKN2B

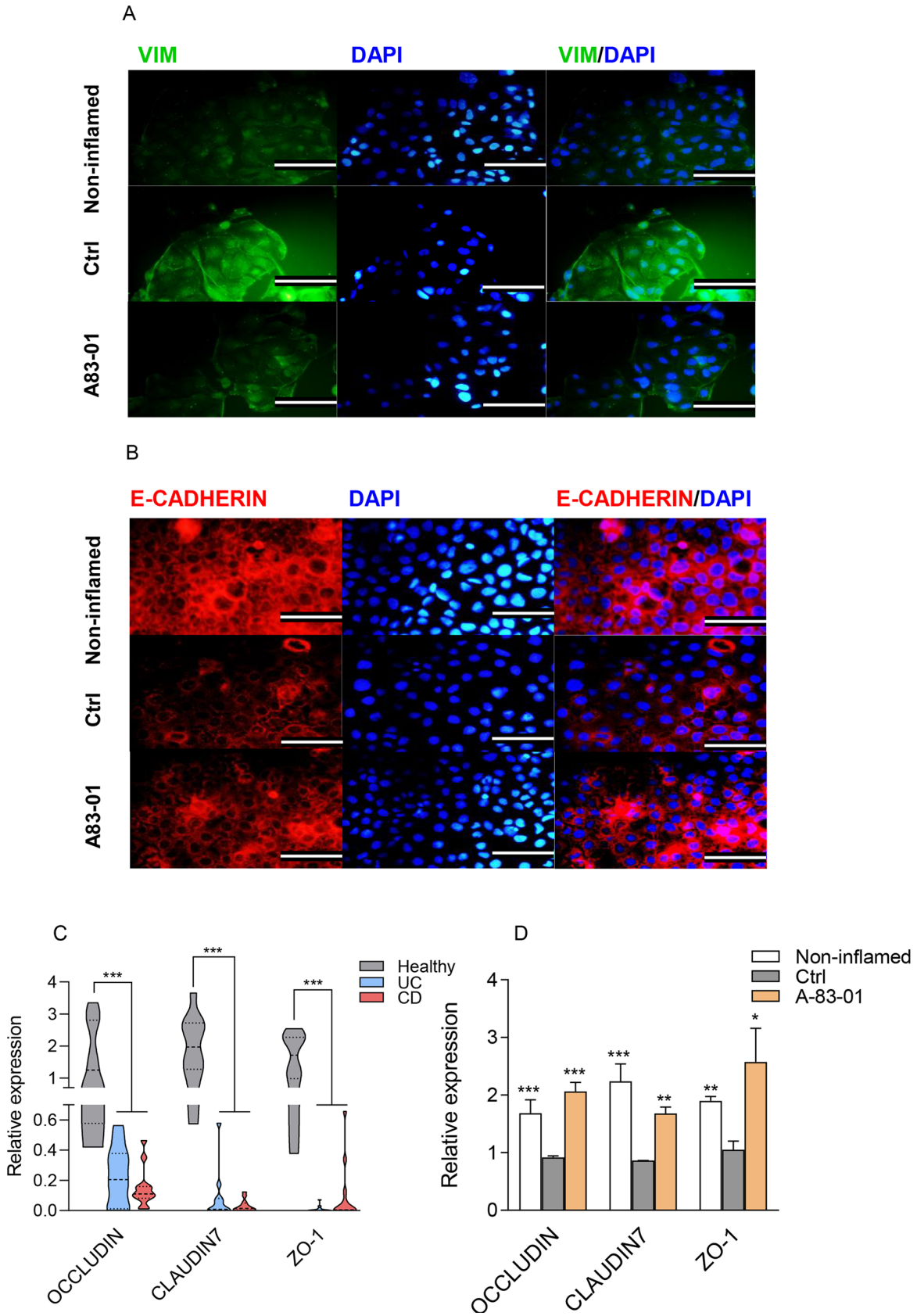

Figure 5. TGF-β1 signaling affected the EMT pathway, epithelial adherens, and tight junctions in IBD. (A, B) Immunofluorescence analyses of EMT-related proteins, VIM (green) and E-CADHERIN (red) in the Caco-2/RAW264.7 co-culture model after treatment with 2 μM of A83-01. (Scale bar: 50 μm). (C) The *OCCLUDIN*, *CLAUDIN7*, and *ZO-1* transcript levels in CD and UC patients and healthy controls (D) and in the Caco-2/RAW264.7 co-culture model after treatment with 2 μM of A83-01. LPS-treated and LPS-untreated cells in the co-cultured system were considered as the control (Ctrl) and non-inflamed groups.

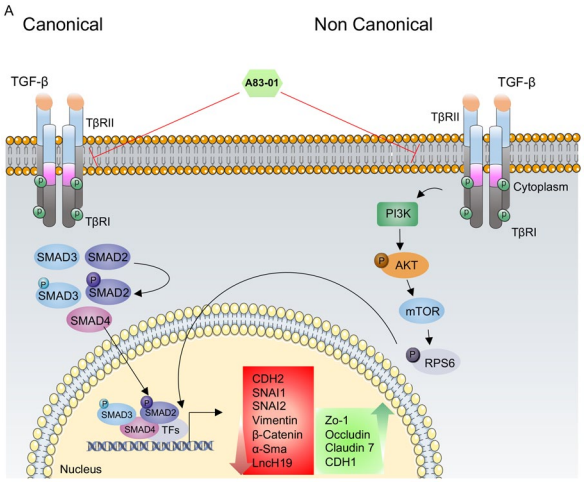

Figure 6. Schematic of SMAD-independent and SMAD-independent TGF-β1 signaling pathways in IBD. (A) In the canonical pathway, the TGF-β1 ligand binds to the TβRII receptor. Activated TβRI can phosphorylate a group of transcription factors, including SMAD2 and SMAD3 on C-terminal serine. Consequently, a trimeric complex containing SMAD2, SMAD3, and SMAD4 was formed. This complex translocates into the nucleus, where it induces or inhibits target gene expression. In addition to the canonical TGF-β1 signaling pathway, TGF-β type I and II receptors can also activate downstream non-canonical pathways, including PI3K/AKT signaling pathway, in an SMAD-independent manner.

in hypoxic cells resulted in an increase in the TGF-β1 expression compared with the control group. Furthermore, the enhancement of SMAD3 as a downstream factor in the TGF-β1 pathway has been shown in CDKN2B-deficient cells. Presumably, TGF-β1 pathway activation may act as a molecular brake for the *GAS5* and *CDKN2B* in the inflamed epithelial tissue as well as the LPS-induced Caco-2/RAW264.7 co-culture model.

We further identified that targeting TβRI by A83-01 not only inhibited LPS-induced overexpression of TGF-β1 signaling components and the phosphorylation of SMAD2, SMAD3, AKT, and RPS6 proteins but also enhanced the expression of *GAS5* and *CDKN2B* in inflamed intestinal epithelial cells. The inhibition of the PI3K/AKT pathway was found to be an ideal therapeutic option in a variety of inflammatory disorders.[38] Anti-colitis effect of Arctigenin, a pharmaceutical compound, has been reported through the phosphorylation blockade of p-RPS6 and p-70S6K in dextran sulfate sodium (DSS)-induced mice[39] which is in agreement with our findings.

Considering the downregulation of *CDKN2B* and *GAS5* in the inflamed epithelial cells and the recovery effect of A83-01 on their expression, it seems preserving their expression by small molecules, oligonucleotide, or drug candidates could be a proper therapeutic strategy,[40,41] although more systematic studies are needed. In this line, Shi *et al.*[42] showed lower expression of *GAS5* in patients with type 2 diabetes mellitus (T2DM) and introduced a peptidomimetic ligand, as a binding probe to *GAS5*, which highlighted the importance of the proper GAS5 level in glucose uptake and metabolism.

Due to increased TGF-β1 signaling in both IBD patients and the *in vitro* model, we decided to assess the EMT process and markers responsible for preserving epithelial characteristics of epithelial-like cells undergoing inflammation conditions. EMT-related markers were upregulated in active CD and UC patients as well as the LPS-induced Caco-2/RAW264.7 co-culture model and correlated with the activated TGF-β signaling. In a seminal study aiming to elucidate the molecular process underlying UC development, Zhao *et al.*[43] found an increase in the EMT-related markers in active lesions of UC patients. Our findings support the hypothesis that TGF-β1 activation may contribute to the induction of EMT in inflamed intestinal tissues. Interestingly, we revealed that treatment with A83-01 could suppress the EMT pathway as well as improve the epithelial tight junction markers. In this context, a decrease in the TGF-β1 signaling pathway members has been shown to prevent EMT induction and decrease fibrosis in the kidney nephropathies.[44] Furthermore, the higher expression of *H19* in inflamed epithelial cells was completely suppressed after the A83-01 treatment. In a study, Chen *et al.*[28] also demonstrated that inflamed UC patients exhibit overexpression of *H19* and decreased expression of tight junction markers, including *ZO-1* and *Occludin*. On the contrary, we demonstrated that upregulation of *H19* could be associated with the induced-EMT pathway in inflamed epithelial cells, which is consistent with the previous observations that the higher expression of *H19* is connected to the EMT process in carcinogenesis and embryogenesis.[45] Yang and colleagues illustrated that TGF-β1 enhances H19 expression through PI3K/AKT pathway. They established an stable mammary alveolar cell-T (MAC-T) cell clones to assess the relationship between H19- and TGF-induced EMT in MAC-T cells and found that overexpression of H19 sharply promotes TGF-β1-induced EMT in MAC-T cells.[29]

TGF-β, activin, nodal, and bone morphogenetic proteins (BMPs) are members of the TGF-β superfamily and as multifunctional cytokines play important regulatory roles in a wide range of cellular processes. These ligands bind to type II serine/threonine kinase receptors at the cell surface, leading to the phosphorylation of type I receptors (also, termed activin receptor-like kinases (ALKs)).[46] A83-01 small molecule serves directly as a potent selective inhibitor of TβRI (ALK5), activin/nodal type I receptor (ALK4), and nodal type I receptor (ALK7).[47,48] In this study, we focused on the inhibitory effect of A83-01 on the TGF-β1 pathway, although it can influence signaling pathways induced by other ligands that share receptors TβRI and Alk4 and Alk7, which needs further investigation.

These *in vitro* results strongly highlighted the function of A83-01 in preventing the EMT initiation in inflamed epithelial cells by repression of the SMAD-dependent and SMAD-independent TGF-β1 signaling pathways, as well as noted the contribution of three LncRNAs, namely, *LncGAS5*, *CDKN2B* and *H19*, in IBD pathogenesis.

Conclusions

The function of TGF-β1 signaling is a complicated phenomenon depending on the cell type. However, insights into its pathological effect during IBD are currently unclear. In this study, we indicated that both canonical and non-canonical TGF-β1 signaling pathways are activated in inflamed intestinal epithelial cells resulting in EMT activation, along with the epithelial tight junction disruption, as a hallmark of

IBD. Based on the results, TGF-β1 signaling inhibition could attenuate the induction of EMT in inflamed epithelial cells and exert a protective effect on the tight junction integrity. In this scenario, attempts to suppress epithelial TGF-β1 signaling function for remodeling intestinal epithelial cells would be an ideal candidate to control this disease. Furthermore, we highlighted the possible important role of three EMT-related LncRNAs, namely, *H19*, *GAS5*, and *CDKN2B*, in the IBD pathogenesis, which may have implications for better IBD management, although further *in vitro* and *in vivo* mechanistic studies are necessary.

AUTHORS' CONTRIBUTIONS

MG contributed to investigation, methodology and writing—original draft; MA contributed to review & editing; SS contributed to resources and consultation; SF contributed to review and bioinformatics analysis; HA contributed to resources and consultation; and AM contributed to conceptualization, investigation, supervision, and writing—review & editing. All authors reviewed and approved the manuscript.

DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

ETHICAL APPROVAL

Tissue biopsies were obtained with patients' consent and the study was confirmed by the Research Ethics Committee of the Research Institute for Gastroenterology and Liver Diseases, SBMU.

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SUPPLEMENTAL MATERIAL

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