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The protective effect of trefoil factor 3 on the intestinal tight junction barrier is mediated by toll-like receptor 2 via a PI3K/Akt dependent mechanism

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ABSTRACT

Trefoil factor peptides are highly conserved secreted molecules characterized by heat and enzymatic digestion resistance. Intestinal trefoil factor 3 (TFF3) protects and repairs the gastrointestinal mucosa and restores normal intestinal permeability, which is dependent on the integrity of the tight junction (TJ) barrier and the TJ associated proteins claudin-1, zona occludens-1 (ZO-1) and occludin. Despite the important role of intestinal barrier dysfunction in the pathogenesis of inflammatory bowel diseases, the underlying mechanisms and associated molecules remain unclear. In the present study, we show that TFF3 and toll-like receptor 2 (TLR2) are functionally linked and modulate intestinal epithelial permeability via a mechanism that involves the PI3K/Akt pathway. We used the Caco-2 cell model to show that TLR2 and TFF3 inhibit the IL-1β induced increase in permeability and release of proinflammatory cytokines, and that this effect is mediated by activation of PI3K/Akt signaling. TLR2 silencing downregulated the expression of TFF3 and overexpression of TLR2 and TFF3 increased the levels of phospho-Akt. TFF3 overexpression significantly upregulated the expression of ZO-1, occludin and claudin-1 and this effect was abrogated by inhibition of the PI3K/Akt pathway. Taken together, our results indicate that TLR2 signaling selectively enhances intestinal TJ barrier integrity through a mechanism involving TFF3 and the activation of the PI3K/Akt pathway.

1. Introduction

The intestinal mucosa is lined with epithelial cells forming an active barrier that protects the subepithelial tissue against pathogenic bacteria through the expression of proinflammatory genes, secretion of inflammatory cytokines and the recruitment of inflammatory cells [1]. Dysfunction of the intestinal barrier or increased intestinal permeability is an important event in the pathogenesis of different diseases, and the main determinant of permeability is the integrity of the intestinal epithelial tight junction (TJ) [2]. TJs are adhesion complexes that form a morphological and functional border and are composed of transmembrane proteins including claudins, occludin and junctional adhesion molecules or JAMs [3] that interact with junctional adaptors such as the ZO proteins. These proteins form a network linking TJs to the actin cytoskeleton, which is critical for the function of TJs in the regulation of epithelial permeability. ZO-1, the first identified TJ protein, regulates cell proliferation and interacts with ZONAB, which modulates cell cycle progression by interacting with CDK4 [4]. Claudins and occludin regulate the diffusion of ions and solutes across the epithelium [5].

The trefoil factor (TFF) family of peptides is characterized by the presence of one or several trefoil factor domains, which are three-leafed-clover shaped structures joined by disulfide bonds [6]. TFF peptides are highly conserved secretory products of the normal mucous epithelium such as that of the gastrointestinal and respiratory tracts, and they play a role in wound healing and mucosal protection, as suggested by their expression in response to mucosal damage [7] and in patients with inflammatory bowel diseases [8]. In mammals, three TFFs have been identified (TFF 1–3), and TFF3, the most widely expressed member, plays a role in the stabilization of tight junctions by upregulating claudin-1 and promoting the redistribution of ZO-1 to the intercellular membrane and its binding to occludin [6,9].

Toll-like receptors (TLRs) are transmembrane pattern recognition receptors that are essential for microbial recognition and the initiation of inflammatory and immune defense responses [10,11]. TLR2 maintains the functional integrity of the tight junction barrier and protects against apoptosis in the intestinal epithelium, decreasing mucosal damage in the intestine [12]. TLR2 induces the expression of TFF3 by intestinal goblet cells (GC), which confers antiapoptotic protection of the intestinal

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mucosa, as demonstrated by the defect in GC-derived TFF3 production and the exacerbation of inflammatory stress-induced damage of the intestine in response to TLR2 deficiency [13].

In the present study, the mechanism underlying the IL-1β induced increase in intestinal TJ permeability was examined by investigating the roles of the TFF3 peptide and TLR2 in the maintenance of intestinal mucosal homeostasis. The association between TFF3 and TLR2 and their effect on the modulation of the TJ-associated proteins ZO-1, occludin and claudin-1 was examined, as well as the role of the phosphatidylinositol 3-kinase (PI3K) signaling pathway.

2. Materials and methods

2.1. Cell culture

The Caco-2 cell line was obtained from the American Type Culture Collection and grown in Dulbecco's minimal essential medium supplemented with 15% fetal bovine serum, 1% penicillin–streptomycin-amphothericin solution and 1% NEAA solution at 37 °C in a CO_2 incubator water jacketed with HEPA Class 100 (Forma Series II, Thermo Electron Corporation, Ohio, USA). Cells were harvested at 80–85% confluency and seeded on 0.4 μm pore polycarbonate filters (Millipore Corporation, Bedford, MA) at a density of 75,000 cells per well for 21–22 days to achieve a consistent monolayer.

2.2. Transepithelial electrical resistance measurement

Caco-2 monolayers were treated with IL-1β (Endogen, Cambridge, MA) at a concentration of 10 ng/mL for various times (0, 12, 24, 48, and 72 h) and transepithelial electrical resistance (TEER) was measured on day 5 and every two days up to 21 days using an epithelial voltohm meter (World Precision Instruments, Sarasota, FL). The TEER value was calculated using the following equation as described previously [14]:

$$\mathrm{TEER} = (R_{\mathrm{monolayer}} - R_{\mathrm{blank}}) \times A$$

where $R_{\mathrm{monolayer}}$ is the resistance of the cell monolayer and the filter membrane, R_{blank} is the resistance of the filter membrane and A is the surface area of the membrane (0.7 cm^2 in 24-well plates).

2.3. Vector construction and transfection

The small interfering RNA (siRNA) target sequence against TLR2 (siTLR2, GTATTGAACTGGACTTCTCC) was designed and synthesized by Ambion (Austin, Texas, USA). For transfection, 15 μL of 20 μM stock siTLR2 solution was mixed with 300 μL Opti-MEM medium (Invitrogen, Carlsbad, CA) and added to a solution containing 15 μL Lipofectamine 2000 (Invitrogen), incubated at room temperature for 20 min and gently overlaid onto Caco-2 cell monolayers in 3 mL of medium. Human TLR2 and TFF3 DNA sequences were obtained by PCR amplification from the Caco-2 DNA genome. Primers for TLR2 were as follows: forward, 5′-GCCCATTGCTCTTTCACTGC-3′, reverse, 5′-GCCACTCCAGGTAGGTCTTG-3′; primers for TFF3 were as follows: forward, 5′-CTGTGCAAACAACGGTGCAT-3′, reverse, 5′-AGGCACGAAGAACTGTCCTC-3′. Plasmids were prepared using an endotoxin-free plasmid Maxi-prep kit (Qiagen, Valencia, CA). Transfection was performed using Lipofectamine 2000. Cells were harvested after 48 h and subjected to different assays.

2.4. Western blot analysis

Caco-2 monolayers were treated with IL-1β at a concentration of 10 ng/mL for various times (0, 12, 24, 48, and 72 h), lysed with lysis buffer, centrifuged and the protein concentration of lysates was determined using the Bio-Rad Protein Assay kit (Bio-Rad Laboratories). Aliquots containing 10–20 μg of protein were treated with Laemmli gel loading buffer, boiled for 7 min and proteins were separated by SDS–PAGE and transferred to nitrocellulose membranes overnight. Membranes were incubated in blocking solution (5% dry milk in TBS-Tween 20 [TBS-T]) for 2 h and then in the indicated primary antibodies in blocking solution. After washing in TBS-T, membranes were incubated with the appropriate secondary antibodies and bands were visualized using the Santa Cruz Western Blotting Luminol Reagent (Santa Cruz Biotechnology) and Kodak BioMax MS film (Fisher Scientific). The following antibodies, all purchased from Abcam, were used: primary antibodies, anti-TLR2 (1/1000), anti-TFF3 (1/1000), anti–phospho S473-Akt (1/5000), anti-Akt (1/1000), anti-ZO-1 (1/500), anti-occludin (1/250), anti-claudin-1 (1/500), anti-β-actin (1/1000); secondary antibodies, goat polyclonal anti-rabbit IgG–H&L-pre-adsorbed (HRP) at 1/2000 dilution. β-Actin served as loading control.

For Western blot analysis, Caco-2 monolayer cells were transfected with siTLR2, TFF3 DNA, or TLR2 DNA for 96 h, and treated or not with 10 ng/ml IL-1β for 48 h, or pretreated or not with LY294002 (10 μM) 1 h prior to IL-1β treatment.

2.5. Quantitative real-time PCR

Caco-2 cells were treated as described and total RNA was isolated using the RNeasy kit (Qiagen) following the manufacturer's protocol. Total RNA concentration was determined by measuring absorbance at 260/280 nm and cDNA was generated by reverse transcription using 2 μg of total RNA and reverse transcription kit (Applied Biosystems, Branchburg, NJ). Real-time PCR was conducted using an ABI prism 7900 sequence detection system and Taqman universal PCR master mix kit (Applied Biosystems). The primers used were as follows: TLR2, forward: 5′- CCGTG GAATGTTTGGAACTGC-3′, reverse: 5′-ATGCAGCCTCCGGATTGTTA-3′; TFF3, forward: 5′-TCTGCTGAGGAGTACGTGGG-3′, reverse: 5′-AGGGGCTTGAAACACCAAGG-3′; GAPDH was used as an internal control, forward: 5′- GACCACAGTCCATGCCATCA-3′, reverse: 5′-GTCAGGTCCACCACTGACAC-3′.

2.6. ELISA

Cells were treated as described for western blot analysis and the culture medium was collected. The amounts of IL-8, TNF-α and IL-6 in the culture medium were measured by ELISA (R&D Systems, USA).

2.7. Statistical analysis

Data are expressed as the mean ± standard error from at least 3 separate experiments performed in triplicate. Comparisons among groups were undertaken using one-way analysis of variance (ANOVA) with Scheffe's *post hoc* test or Student's *t*-tests, as appropriate. A p value of <0.05 was used to indicate statistical significance.

3. Results

3.1. Effect of IL-1β on tight junction permeability and TLR2 and TFF3 expression

Tight junctions establish a polarity of the epithelial cell layer by forming a seal that separates the luminal compartment from the basolateral surface [15]. TEER was measured in polarized Caco-2 cells to evaluate the integrity of the monolayer formed. TEER values >450 Ω/cm^2 reflect the development of functional polarity [16]. TEER was assessed in a Caco-2 cell monolayer on day 5 and

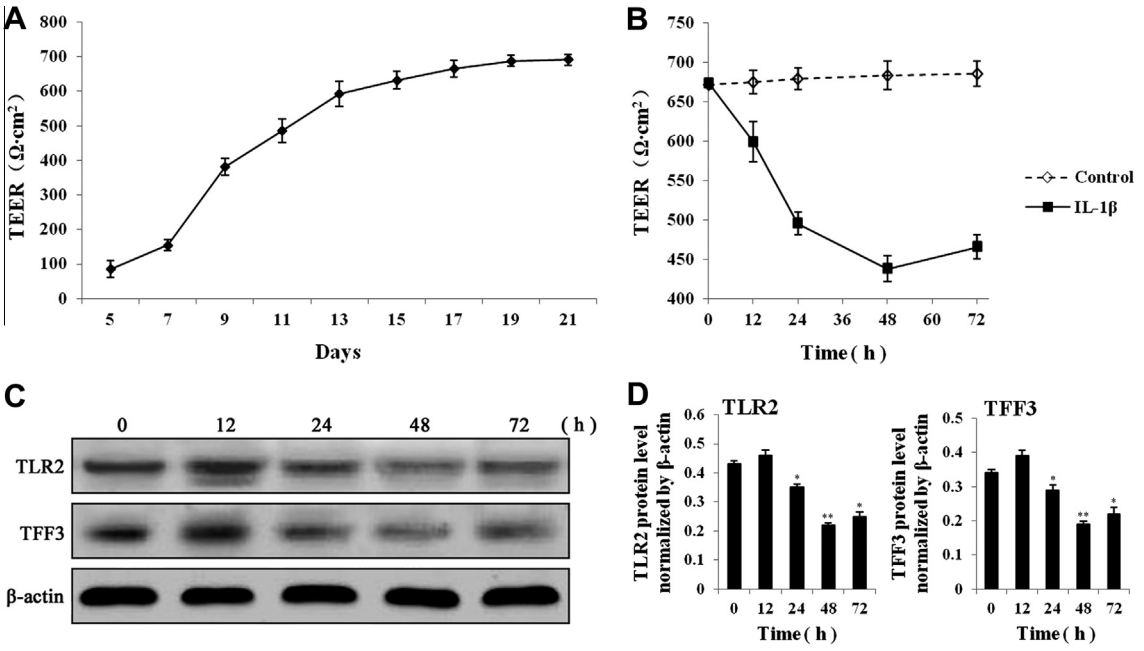

Fig. 1. Effect of IL-1β on tight junction permeability and TLR2 and TFF3 expression. (A) Transepithelial electrical resistance (TEER) measured in the Caco-2 cell model. (B) Caco-2 monolayers were treated with IL-1β at 10 ng/mL for the indicated times and TEER was measured. (C) Caco-2 monolayers were treated with IL-1β at 10 ng/mL for the indicated times and the protein levels of TLR2 and TFF3 were assessed by western blotting with β-actin as the loading control. (D) Quantitative analysis of TLR2 and TFF3 protein levels normalized to β-actin. All values are the means ± SD of three replicates. $^{*}p < 0.05$, $^{**}p < 0.01$ vs. at 0 h.

then every 2 days up to 21 days, which showed a gradual increase in resistance from day 5 to day 17, when a plateau was reached and maintained until day 21 (Fig. 1A). To assess the effect of IL-1β on TJ permeability, which has been shown to be associated with inflammatory conditions of the gut, Caco-2 cells were treated with 10 ng/mL IL-1β for different times. The results showed a progressive time-dependent decrease in TEER, which reached the maximal drop at 48 h and then increased slightly at 72 h (Fig. 1B). To determine the relation between changes in TJ permeability and the expression of TLR2 and TFF3, Caco-2 monolayers were treated with

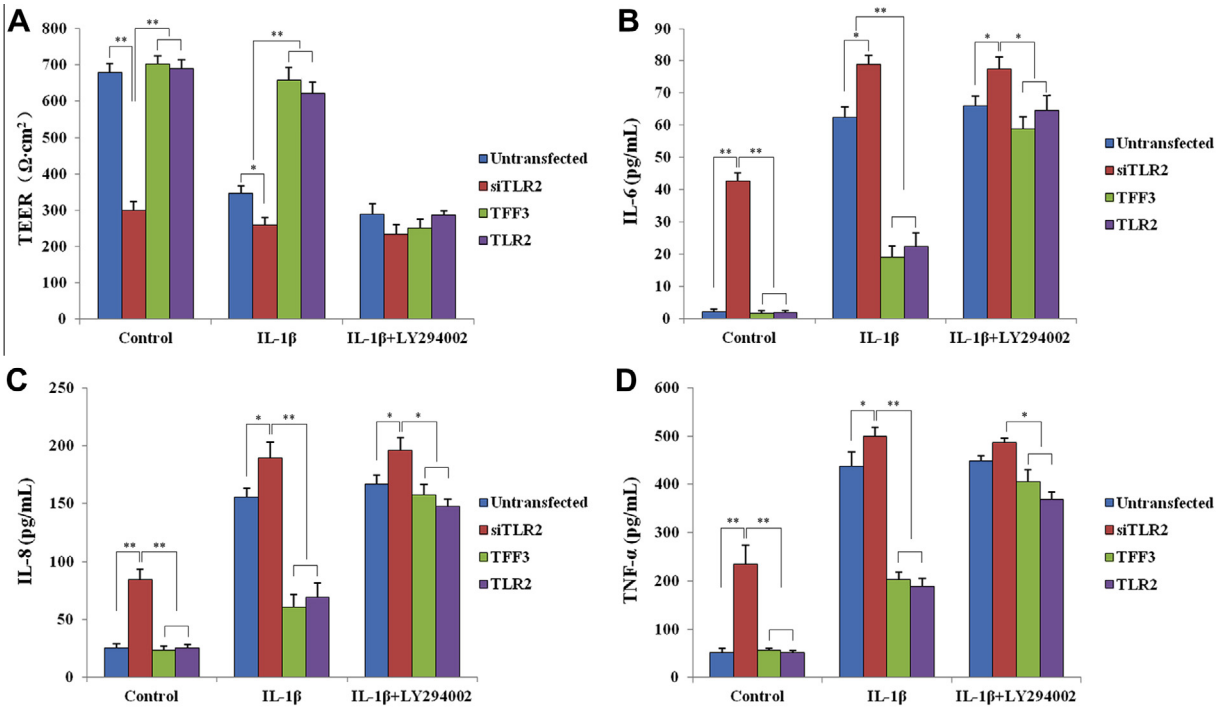

Fig. 2. Effect of TLR2 and TFF3 on transepithelial electrical resistance (TEER) and the release of proinflammatory cytokines. (A) TEER was measured in cells transfected with siRNA against TLR2 or DNA plasmids overexpressing TLR2 or TFF3, and treated or not with IL-1β (10 ng/mL) for 48 h, and pretreated or not with LY294002 (10 μM) for 1 h prior to IL-1β treatment. (B–D) Cells were treated as described above and the levels of IL-6 (B), IL-8 (C) and TNF-α (D) in the culture medium were measured by ELISA. All values are the means ± SD of three replicates. $^{*}p < 0.05$, $^{**}p < 0.01$.

10 ng/mL IL-1β for different times and the expression levels of the TLR2 and TFF3 proteins were determined by western blotting with β-actin as a loading control. As shown in Fig. 1C, TLR2 and TFF3 protein levels increased slightly at 12 h and then decreased progressively until 48 h, showing a slight increase at 72 h, which was similar to the pattern of TEER changes in response to IL-1β treatment (Fig. 1C). Quantitation of protein levels by densitometry showed that TLR2 and TFF3 levels decreased by approximately 50% and 40%, respectively ($p < 0.01$), after 48 h of IL-1β stimulation (Fig. 1D).

3.2. TLR2 and TFF3 inhibit the IL-1β induced increase in permeability and release of proinflammatory cytokines via the PI3K/Akt pathway

To examine the effect of TLR2 and TFF3 on IL-1β induced changes in Caco-2 monolayer permeability, cells were subjected to siRNA mediated silencing of TLR2 or transfected with a TLR2 or TFF3 expressing plasmid and TEER was measured in the presence of IL-1β with or without pretreatment with the PI3 kinase inhibitor LY294002. Silencing of TLR2 significantly decreased TEER whereas overexpression of TLR2 and TFF3 increased TEER to levels similar to those of untreated control cells in the presence or absence of IL-1β (Fig. 2A). However, pretreatment with the PI3 kinase inhibitor LY294002 abolished the effect of TFF3 and TLR2 overexpression in the presence of IL-1β, suggesting the involvement of the PI3K/Akt pathway. IL-1β increased the release of the proinflammatory cytokines IL-6, IL-8 and TNF-α, and this effect was significantly enhanced by silencing of TLR2 ($p < 0.05$) (Fig 2B, C and D). Conversely, overexpression of TLR2 and TFF3 significantly inhibited the IL-1β induced upregulation of IL-6, IL-8 and TNF-α ($p < 0.01$) and this effect was abolished by pretreatment with LY294002. Taken together, these results indicate that TLR2 and TFF3 may play a protective role against IL-1β induced increase in TJ permeability, and this effect is dependent on the activity of the PI3K/Akt pathway.

3.3. Correlation between TLR2 and TFF3 levels and PI3K/Akt pathway activity

We next examined the effect of IL-1β on the mRNA and protein levels of TLR2 and TFF3 in Caco-2 cells treated as described above. IL-1β and LY294002 downregulated the mRNA expression of TLR2; however, overexpression of TFF3 significantly upregulated the mRNA levels of TLR2 in the presence of the PI3K inhibitor LY294002 (Fig. 3A). Knockdown of TLR2 downregulated the expression of TFF3 (Fig. 3B), and inhibition of the PI3K/Akt

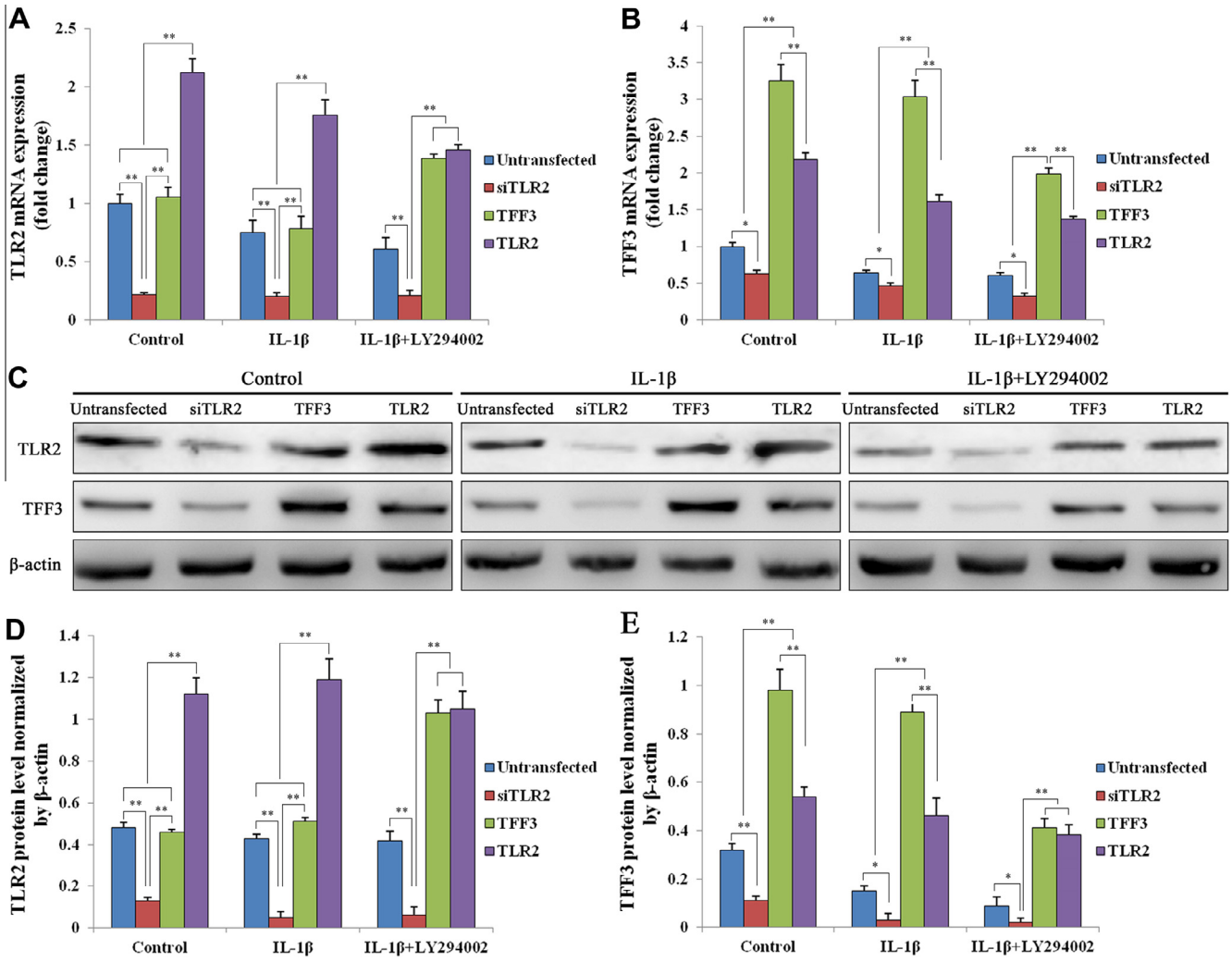

Fig. 3. TLR2 and TFF3 mRNA and protein levels. (A and B) mRNA expression of TLR2 (A) and TFF3 (B) was detected by quantitative real time PCR in cells overexpressing TLR2 or TFF3 or silenced TLR2 in the presence or absence of IL-1β and LY294002 as described above. GAPDH was used as a control. (C) Western blot detection of TLR2 and TFF3 levels in cells overexpressing TLR2 or TFF3 or silenced TLR2 in the presence or absence of IL-1β and LY294002. β-actin was used as the loading control. (D–E) Quantitative analysis of TLR2 (D) and TFF3 (E) protein levels normalized to β-actin levels. All values are the means ± SD of three replicates. $^{*}p < 0.05$, $^{**}p < 0.01$.

pathway downregulated the mRNA expression of TLR2 and TFF3 even in cells transfected with TLR2 and TFF3 overexpressing plasmids (Fig. 3A and B). Assessment of the protein expression of TLR2 and TFF3 by western blotting showed a similar expression pattern as that determined by qRT-PCR with the exception of the effect of IL-1β on TLR2 expression in the presence of LY294002, which showed a significant increase in untransfected cells (Fig. 3C). Fig. 3D and E shows the quantitation of the western blotting results. Taken together, these results suggest a correlation between the expressions of TLR2 and TFF3 in response to inflammation and their dependence on the activity of the PI3K/Akt pathway.

3.4. The PI3K/Akt pathway mediates the effect of TLR2 and TFF3 on tight junction integrity

To further examine the association between TLR2, TFF3 and the activation of Akt, the levels of phospho-Akt were examined in control and transfected cells. The results showed that silencing of TLR2 downregulated pAkt levels whereas overexpression of TLR2 or TFF3 increased the levels of pAkt in the presence or absence of IL-1β and without affecting the levels of total Akt ($p < 0.01$) (Fig. 4A and B). This effect was abrogated by pretreatment with LY294002. The levels of ZO-1, claudin-1 and occludin, which mediate the effect of TFF3 on the stabilization of TJs, were examined in Caco-2 cells treated as described above. Silencing of TLR2 downregulated the expression of ZO-1, claudin-1 and occludin, whereas overexpression of TLR2 or TFF3 had the opposite effect (Fig. 4D). Treatment with LY294002 downregulated the expression of ZO-1, claudin-1 and occludin, and partly abolished the effect of TLR2 and TFF3, particularly with respect to the protein levels of claudin-1. Fig. 4E–G show the quantitation of the western blotting results.

4. Discussion

Defects in the integrity of the intestinal epithelial TJ barrier are associated with the development of various inflammatory diseases

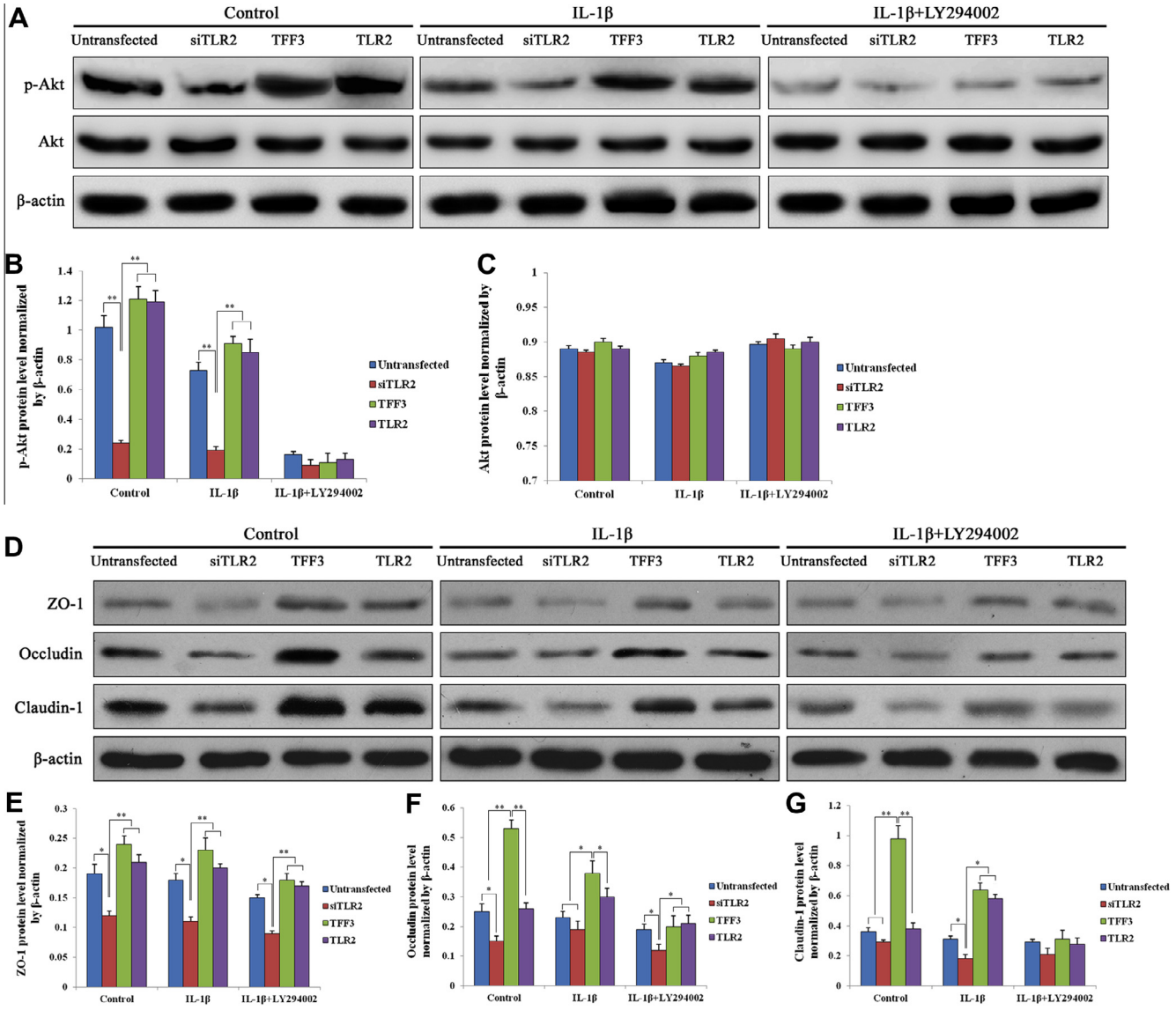

Fig. 4. Akt activation and expression of tight junction related proteins in correlation with TLR2 and TFF3 expression. (A) Western blot detection of Akt and phospho-Akt levels in cells overexpressing TLR2 or TFF3 or silenced TLR2 in the presence or absence of IL-1β and LY294002. β-Actin was used as the loading control. (B–C) Quantitative analysis of p-Akt (B) and Akt (C) protein levels normalized to β-Actin levels. (D) Western blot detection of the levels of ZO-1, occludin and claudin-1 in cells overexpressing TLR2 or TFF3 or silenced TLR2 in the presence or absence of IL-1β and LY294002. β-actin was used as the loading control. (E–G) Quantitative analysis of ZO-1 (E), occludin (F) and claudin-1 (G) protein levels normalized to β-actin levels. All values are the means ± SD of three replicates. $^{*}p < 0.05$, $^{**}p < 0.01$.

of the intestine, and the cytokine-induced increase in TJ permeability has significant proinflammatory effects [17,18]. The effect of IL-1β on the induction of epithelial TJ permeability has been described previously, and several pathways have been proposed to explain the role of proinflammatory cytokines on intestinal barrier dysfunction [19–21]. However, the precise mechanisms underlying the modulation of intestinal TJ barrier function and the interactions between the molecules involved remain unclear. In the present study, we show that TLR2 regulates intestinal TJ barrier integrity through the modulation of TFF3 and its protective effect against epithelial cell damage, and we examined the underlying mechanisms, in particular the involvement of the PI3K/Akt pathway.

We used the Caco-2 cell model to examine the involvement of TLR2 and TFF3 in the regulation of TJ permeability. Our results showed that IL-1β downregulated TLR2 and TFF3 and increased Caco-2 TJ permeability, confirming the involvement of TLR2 in the regulation of the TJ barrier and its association with TFF3. The involvement of TLR signaling in the regulation of TJ barrier integrity has been shown in several studies [10,22–24]. TLR2 increases transepithelial resistance by inducing the redistribution of ZO-1, which results in the stabilization of gap junctional intercellular communication [24], and it is necessary to maintain TJ integrity in response to inflammatory stress [12]. TLR2 protects the intestinal epithelial layer against stress-induced mucosal damage in ulcerative colitis [25]. Furthermore, TLR2 regulates the expression of TFF3, which functions in wound healing and repair of the intestinal mucosa [13], in the colon, consistent with our findings showing that TLR2 and TFF3 function together in the modulation of TJ barrier integrity.

In the present study, silencing of TLR2 downregulated TFF3 expression, confirming their association, and decreased the levels of phospho-Akt. Treatment with a PI3 kinase inhibitor downregulated the expression of TFF3 and TLR2, and decreased the levels of the TJ associated proteins ZO-1, claudin-1 and occludin, which was similar to the effect of TLR2 silencing. Overexpression of TFF3 significantly upregulated the expression of ZO-1, occludin and claudin-1, and this effect was decreased by inhibition of the PI3K/Akt pathway. Different cytokines have been shown to have differential effects on TJ protein expression, although the exact role of TJ proteins in intestinal TJ barrier function is controversial. TNF-α downregulates ZO-1, claudin-1 and occludin in Caco-2 cells [26], whereas IFN-γ decreases ZO-1 and occludin but increases claudin-1 levels in T-84 cells [27]. In Caco-2 cells, a cytomix consisting of TNF-α, IFNγ, and IL-1β downregulates ZO-1 and occludin expression but increases claudin-1 levels [28]. The involvement of PI3K/Akt signaling in the effect of TLR2 on TJ stability has been discussed in several studies [10,24,29]. Our data confirmed the involvement of the PI3K/Akt pathway in the TFF3 and TLR2 modulation of the levels of TJ-associated proteins. However, further studies are necessary to elucidate the precise mechanisms underlying the PI3K/Akt pathway-dependent effects of TFF3 and TLR2 on TJ barrier integrity and the involvement of TJ-associated proteins.

A growing body of evidence supports the role of TLR signaling in the maintenance of intestinal homeostasis, which is of particular importance in chronic inflammatory conditions of the gut [30,31]. Adequate intestinal immunity is dependent on a balance between proinflammatory responses and protective inflammatory responses. Furthermore, inflammatory bowel diseases are associated with an increased risk for colorectal cancer, and proinflammatory cytokines produced during chronic intestinal inflammation in response to bacteria promote cell proliferation and survival and therefore tumorigenesis [32,33]. In the present study, overexpression of TFF3 and TLR2 abolished the IL-1β induced upregulation of the proinflammatory cytokines IL-6, IL-8 and TNF-α, and silencing of TLR2 had the opposite effect. In addition, the decrease in the secretion of these proinflammatory cytokines induced by TLR2 and TFF3 was abolished by inhibition of PI3K. These results indicate that TFF3 and TLR2 may play a protective role against inflammatory responses in the gut mediated by the modulation of the expression of proinflammatory cytokines via a mechanism involving PI3K/Akt signaling, which warrants further investigation in particular in light of the association between inflammatory responses, alterations in intestinal homeostasis and tumorigenesis.

In conclusion, the present study showed that the IL-1β induced increase in intestinal TJ permeability involves the modulation of the expression and activity of TLR2 and TFF3 via a mechanism dependent on the PI3K/Akt pathway. The protective effect of TFF3 on intestinal barrier integrity and the preservation of mucosal homeostasis by TLR2 involve the modulation of the TJ associated proteins ZO-1, occludin and claudin-1 and the regulation of proinflammatory cytokines, although the precise mechanism underlying their involvement remains to be elucidated.

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