Adalimumab prevents barrier dysfunction and antagonizes distinct effects of TNF-α on tight junction proteins and signaling pathways in intestinal epithelial cells

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INFLAMMATORY BOWEL DISEASES (IBDs), such as Crohn’s disease; ulcerative colitis; claudin; tumor necrosis factor-α; intestinal barrier; IBD

MATERIALS AND METHODS

Cell culture. Caco-2 BBE cells were cultured in DMEM containing 10% FBS, 1% nonessential amino acids, and 10 mM HEPES as well as penicillin-streptomycin and subcultured every 3–4 days before reaching confluence. T-84 cells cultured in DMEM-F-12 containing 10% FBS, 10 mM HEPES, and penicillin-streptomycin were subcultured once a week. All cell culture media and supplements were 10% FBS, 10 mM HEPES, and penicillin-streptomycin were subcultured on coverslips was used as previously described (43). Briefly, cells were fixed in methanol at −20°C for 20 min and blocked in PBS containing 3% BSA and 0.2% (vol/vol) Triton X-100 for 1 h followed by incubation with primary antibodies against zonula occludens (ZO)-1 and occludin (Life Technologies) used at a dilution of 1:200 overnight. After primary antibodies, 1% nonfat dry milk, and 0.2% (vol/vol) Triton X-100 for 1 h followed by treatment with TNF-α (Cell Signaling Technology, Danvers, MA), and adalimumab (Abbott Laboratories, Wiesbaden, Germany) applied to the basal compartment of the transwell system as indicated. TEERs were recorded using the Millicell electrical resistance system (Millipore, Billerica, MA) used at a dilution of 1:200 overnight. After repeated washes with PBS containing 0.1% Triton X-100, fluorescence microscope. 

Protein analyses. T-84 and Caco-2 cells grown on transwell inserts or on cells grown on coverslips (Sarstedt, Nürnberg, Germany). Cells were fixed in methanol at −20°C for 20 min and blocked in PBS containing 3% BSA and 0.2% (vol/vol) Triton X-100 for 1 h followed by incubation with primary antibodies against zonula occludens (ZO)-1 and occludin (Life Technologies) used at a dilution of 1:200 overnight. After repeated washes with PBS containing 0.1% Triton X-100, fluorescence microscope. 

Viability assays. Neutral red uptake assays were performed to assess the cytotoxicity of IFN-γ, TNF-α, and adalimumab as previously described (35).

Immunofluorescence. Standard immunofluorescence was performed either directly on transwell inserts or on coverslips on an Axio M1 imager equipped for fluorescence (Carl Zeiss, Jena, Germany). To visualize the redistribution of claudin-1 upon cytokine stimulation, a modified protocol designed to preserve intracellular vesicles was used as previously described (43). Briefly, cells were fixed in methanol, air dried, and rehydrated with 100 μM bis(sulfosuccinimidyl) suberate in PBS with 0.1% N-octylgluceraldehyde (PBS+). After being quenched in ethylenediamine, monolayers were blocked in 1% nonfat dry milk and 1% fish gelatin in PBS+ and incubated with primary and secondary antibodies as described above. Z-stack images were taken on a Keyence BZ-9000 microscope.

RESULTS

Adalimumab prevents the TEER decline induced by TNF-α, but not IFN-γ, in intestinal epithelial cells. To investigate the impact of adalimumab on the barrier-disrupting effects of IFN-γ and TNF-α, two well-established intestinal epithelial barrier models were used. In line with previous observations (48), treatment of fully differentiated Caco-2 cells with either IFN-γ (5–10 ng/ml) or TNF-α (5–20 ng/ml) alone did not result in changes of TEER within 24 h (data not shown). However, when Caco-2 monolayers were preconditioned for 24 h with IFN-γ (10 ng/ml) to induce the expression of TNF-α receptors (49), the subsequent addition of TNF-α (5 ng/ml) induced a significant drop in TEER within 6 h (mean TEER: 148 ± 10 Ω·cm² compared with 230 ± 6 Ω·cm² in nontreated cells; n = 9, P < 0.001; Fig. 1A). This was reversed by coadministration with 10 μg/ml adalimumab, a concentration commonly found in sera from patients lacking anti-adalimumab antibodies (6) (mean TEER: 229 ± 8 Ω·cm², n = 9), whereas the antibody alone had no effect on TEER (mean TEER: 235 ± 8 Ω·cm², n = 9). Increasing doses of cytokines (20 ng/ml IFN-γ and 20 ng/ml TNF-α) and prolonged stimulation for 24 h substantially worsened barrier dysfunction (mean TEER: 37 ± 7 Ω·cm², n = 8, P < 0.0001); however, even under these conditions the decline in TEER was prevented by adalimumab [mean TEER: 241 ± 5 Ω·cm², not significant (NS); Fig. 1B].

We next attempted to determine the minimum concentration of adalimumab required to antagonize the barrier-disrupting effect of TNF-α. Using low cytokine concentrations (10 ng/ml IFN-γ and 5 ng/ml TNF-α) and short incubation times (6 h), the reduction in TEER upon TNF-α treatment was fully prevented by adalimumab in concentrations down to 100 ng/ml in Caco-2 cells, whereas 10 ng/ml only partially inhibited the TNF-α-induced barrier breakdown and lower concentrations were not effective (Fig. 1C).
In contrast to these observations in Caco-2 cells, treatment of T-84 cells with low concentrations of IFN-γ/H9253 (2.5 ng/ml) was sufficient to induce a substantial decline in TEER (relative TEER: 3,162 ± 155 Ω·cm² compared with 4,809 ± 271 Ω·cm², n = 8, P < 0.001), and adalimumab was not effective in reversing this effect (mean TEER: 3,242 ± 185 Ω·cm², n = 8, NS vs. cells treated with IFN-γ only; Fig. 1D). However, stimulation of IFN-γ-preconditioned cells with TNF-α (5 ng/ml) resulted in a further decrease of TEER within 6 h (mean TEER: 2,570 ± 150 Ω·cm², n = 15, P < 0.05 vs. cells treated with IFN-γ only; Fig. 1E). Concomitant administration of adalimumab prevented this effect (mean TEER: 3,060 ± 255 Ω·cm², n = 8, NS vs. cells treated with IFN-γ only), whereas similar to our observations in Caco-2 cells, adalimumab alone did not affect TEER in T-84 cells in the absence of TNF-α (mean TEER: 3,242 ± 185 Ω·cm², n = 8, NS vs. cells treated with IFN-γ only). Titration
experiments revealed that, similar to the Caco-2 cell line, an adalimumab concentration of 100 ng/ml was sufficient to prevent barrier disruption by TNF-α; however, concentrations down to 1 ng/ml still partially reversed the TNF-α-induced TEER loss in T-84 cells, whereas 100 pg/ml of the antibody did not elicit any relevant effects. In line with previous observations (48), neither TNF-α nor IFN-γ displayed relevant cytotoxicity within 6 h when used in low concentrations, and no reduction in the number of viable cells was observed within this timeframe. Similarly, adalimumab did not elicit any cytotoxic response in either cell line (Fig. 2).

**Adalimumab prevents structural changes of the tight junctions induced by TNF-α.** Next, we assessed the impact of TNF-α and adalimumab on tight junction structure using immunofluorescence against the integral tight junction transmembrane protein occludin (16) and the adapter molecule ZO-1. Paralleling the observed decline in TEER, treatment of IFN-γ-primed Caco-2 monolayers with TNF-α induced profound changes in tight junction architecture within 6 h, as evidenced by irregular undulations in the staining pattern for both molecules (Fig. 3A). In addition, we observed the appearance of intracellular vesicles staining positive for occludin but not for ZO-1 in both cell lines, as previously described (10, 25, 49). Adalimumab prevented these changes when coadministered with TNF-α but did not affect ZO-1 or occludin distribution in the absence of TNF-α. In line with the aggravated loss of TEER, stimulation of Caco-2 cells with higher cytokine concentrations (20 ng/ml IFN-γ and 20 ng/ml TNF-α) for 24 h substantially intensified these structural alterations, and a highly fragmented membrane staining pattern for occludin was observed (Fig. 3B). In addition, visual assessment suggested a reduction in total cell number, presumably reflecting apoptotic and/or necrotic cell death (10) that was accompanied by an increase in size of the remaining cells to maintain an intact monolayer (24). Concurrent administration of adalimumab prevented these alterations.

Since this study aimed to investigate the effects of adalimumab, TNF-α, and IFN-γ on barrier function, lower cytokine concentrations and shorter treatment periods were used for subsequent experiments to avoid potential cytotoxic effects of IFN-γ and TNF-α. Of note, even though treatment with IFN-γ alone induced a significant decline in TEER in the T-84 cell line, occludin and ZO-1 staining revealed no abnormalities in these cells. Stimulation of IFN-γ-primed T-84 cells with TNF-α resulted in changes similar to those seen in Caco-2 cells, and adalimumab was equally effective in preventing these alterations (Fig. 3C).

**Adalimumab prevents the TNF-α-induced phosphorylation of MLC in Caco-2 and T-84 cells and antagonizes distinct alterations in the expression pattern of tight junction proteins in both cell lines.** The cytoskeleton and perijunctional actomyosin ring are of pivotal importance for the short-term regulation of tight junction permeability (21). In particular, increased phosphorylation of MLC has a crucial role for the barrier-disrupting effect of proinflammatory cytokines (46). We therefore assessed the effect of TNF-α and adalimumab on MLC phosphorylation using Western blot analysis. As shown in Fig. 4A, TNF-α induced an increase in MLC phosphorylation in Caco-2 and T-84 cells primed with IFN-γ that paralleled the observed decline in TEER. This was prevented by the concurrent administration of adalimumab, demonstrating its ability to antagonize the activation of the MLC pathway by TNF-α.

Changes in the expression of tight junction transmembrane proteins represent an additional way to modulate paracellular permeability. In particular, occludin and members of the claudin family of proteins, such as claudin-1, claudin-2, and claudin-4, are of central importance for barrier regulation, and alterations in the mucosal expression of these proteins have been frequently observed in IBDs (33, 51, 53). We therefore assessed the impact of TNF-α and adalimumab on the expression of these proteins. Caco-2 and T-84 cells grown on filter inserts and primed with IFN-γ (10 and 2.5 ng/ml, respectively) for 24 h were treated with TNF-α (5 ng/ml), and protein extracts were obtained after 6 h when TEER measurements indicated a significant decline in barrier function. Western blot analysis revealed that under these conditions, the expression of occludin, claudin-2, and claudin-4 was not altered upon stimulation with either TNF-α, adalimumab, or both compounds in Caco-2 cells (Fig. 4A). In contrast, the abundance of claudin-1 increased slightly in Caco-2 cells stimulated with TNF-α (relative protein abundance: 1.42 ± 0.12, n = 4, P < 0.05), and, remarkably, this was also observed in cells treated with a combination of TNF-α and adalimumab (relative protein abundance: 1.57 ± 0.14, n = 4, P < 0.05). In contrast to these findings in the Caco-2 cell line, we observed a substantial reduction in the amount of occludin, claudin-1, claudin-2, and claudin-4 in T-84 cells treated with TNF-α (relative protein abundance: 0.25 ± 0.13, 0.24 ± 0.16, 0.40 ± 0.09, and 0.30 ± 0.23, respectively, n = 3–4, P < 0.05), whereas the abundance of the adapter molecule ZO-1 was not changed. Cotreatment with adalimumab restored expression of these proteins, whereas adalimumab alone did not affect their abundance. Quantitative RT-PCR revealed significant parallel alterations in the mRNA expression of these genes (Fig. 4B), suggesting that changes on the level of transcription are at least partially responsible for these effects.
Similarly, quantitative RT-PCR confirmed the upregulation of claudin-1 in Caco-2 cells treated with TNF-α (relative expression compared with cells primed with IFN-γ only: 1.48, \( n=6 \), \( P<0.05 \)) or a combination of TNF-α and adalimumab (relative expression: 1.47, \( n=6 \), \( P<0.05 \); Fig. 4B). We therefore used immunofluorescence to determine whether these changes were accompanied by alterations in the subcellular distribution pattern of claudin-1 in Caco-2 cells. Using a protocol designed to preserve intracellular vesicles (43) and Z-stacks, we observed that treatment with TNF-α changed the subcellular localization of this molecule from a more membrane-associated pattern in IFN-γ-primed cells to a diffuse cytoplasmic staining upon TNF-α stimulation. Although adalimumab did not prevent claudin-1 upregulation in our system, its redistribution was reversed by coadministration of the antibody (Fig. 4C).

Adalimumab prevents the activation of signaling pathways implicated in the mediation of TNF-α-induced barrier dysfunction. As previous studies suggested a potential role for p38 MAPK both in the regulation of MLC phosphorylation in vivo (14) and

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**Fig. 3. Effect of TNF-α and Ada on tight junction structure.**

**A:** Caco-2 cells primed with 10 ng/ml IFN-γ for 24 h were treated with TNF-α (5 ng/ml), Ada, or both compounds for 6 h as indicated followed by staining of the filter inserts for zonula occludens (ZO)-1 and occludin. Note the internalization of occludin (arrow, top IFN + TNF image) and undulations within the membrane staining (arrow, bottom IFN + TNF image) in cells treated with TNF-α but not the combination of TNF-α and Ada. Scale bars = 20 μm.

**B:** ZO-1 and occludin staining in Caco-2 cells primed for 24 h with 20 ng/ml IFN-γ followed by stimulation with TNF-α (20 ng/ml) or the combination of TNF-α and Ada for 24 h. Note the fragmented occludin staining at the membrane and the increased cell size upon TNF-α treatment that was antagonized by the addition of Ada. Scale bars = 20 μm.

**C:** ZO-1 and occludin staining in T-84 cells primed for 24 h with IFN-γ (2.5 ng/ml) followed by stimulation with TNF-α (5 ng/ml), Ada, or both compounds for 6 h. Scale bars = 10 μm. Representative results from 3 independent experiments are shown for each group.
increased intestinal permeability after cytokine stimulation in vitro (50), we analyzed the activation of this pathway in our system. As shown in Fig. 5A, TNF-α induced phosphorylation of p38 in both Caco-2 and T-84 cells primed with IFN-γ without altering the total amount of this protein. Adalimumab prevented these changes but did not affect p38 phosphorylation or expression in the absence of TNF-α.

Another key signaling pathway downstream of TNF-α is the activation of transcription by members of the NF-κB family of proteins, and evidence has been provided demonstrating that this activation might have an important role in the mediation of barrier dysfunction both in Caco-2 cells (22) and T-84 cells (9). As TNF-α-induced activation of NF-κB has been previously linked to phosphorylation of the p65/RelA subunit on Ser536 (37, 47), we used an antibody that specifically recognizes this residue to assess the impact of TNF-α and adalimumab on NF-κB activation. The results shown in Fig. 5B demonstrate that TNF-α increased phosphorylation of p65/RelA within 6 h,
of TEER upon TNF-α/H9251 cells primed with IFN-γ/H9253 in the presence of the NF-κB inhibitor BIRB796 (500 nM) did not alter TEER in Caco-2/H9262 cells. This suggests that the inhibition of NF-κB partially reversed the TNF-α-induced TEER loss (mean TEER: 3,470 ± 32 Ω·cm² with BAY 11–7082 compared with 3,060 ± 126 Ω·cm² with vehicle alone, n = 4, P < 0.05). Our data furthermore suggested a potential impact of PI3K signaling in the T84 cell line, where we used LY-294002 (10 μM), a potent and selective PI3K inhibitor. Treatment with this compound resulted in a highly significant increase in TEER in IFN-γ-primed cells (mean TEER: 5,185 ± 152 Ω·cm² with LY-294002 compared with 3,675 ± 130 Ω·cm² with vehicle alone, n = 4, P < 0.001); however, subsequent stimulation with TNF-α induced a loss of TEER comparable to that seen in the absence of the inhibitor (relative TEER compared with IFN-γ-primed cells: 0.8 ± 0.01 with LY-294002 compared with 0.83 ± 0.03 with vehicle alone, n = 4, NS).

DISCUSSION

Barrier dysfunction represents a hallmark finding in IBDs that has been proposed to contribute both to the pathogenesis and symptoms of these disorders (11, 41, 46). Adalimumab, a fully human therapeutic antibody directed against TNF-α, has a significant improvement of barrier function upon TNF-α stimulation (mean TEER: 128 ± 3 Ω·cm², n = 4, P < 0.05). In contrast, BIRB796 did not elicit any significant effect in T-84 cells either in the presence or absence of TNF-α, whereas similar to our findings in the Caco-2 line, inhibition of NF-κB partially reversed the TNF-α-induced TEER loss (mean TEER: 3,470 ± 32 Ω·cm² with BAY 11–7082 compared with 3,060 ± 126 Ω·cm² with vehicle alone, n = 4, P < 0.05). As our data further supported a potential impact of PI3K signaling in the T84 cell line, we also used LY-294002 (10 μM), a potent and selective PI3K inhibitor. Treatment with this compound resulted in a highly significant increase in TEER in IFN-γ-primed cells (mean TEER: 5,185 ± 152 Ω·cm² with LY-294002 compared with 3,675 ± 130 Ω·cm² with vehicle alone, n = 4, P < 0.001); however, subsequent stimulation with TNF-α induced a loss of TEER comparable to that seen in the absence of the inhibitor (relative TEER compared with IFN-γ-primed cells: 0.8 ± 0.01 with LY-294002 compared with 0.83 ± 0.03 with vehicle alone, n = 4, NS).

Finally, various studies (9, 23, 28) have implicated activation of PI3K signaling in the barrier-disrupting effects of proinflammatory cytokines and the regulation of claudin expression. We therefore investigated the activation of this pathway by determining phosphorylation of the PI3K downstream target Akt. As shown in Fig. 5C, TNF-α induced a profound increase in Akt phosphorylated both on Thr308 and Ser473 in T-84 cells, whereas there was no effect on the total abundance of this protein. Again, this increase was prevented by the concurrent administration of adalimumab, whereas the antibody had no effect on Akt phosphorylation in the absence of TNF-α. In contrast, PI3K signaling appeared not to be affected by TNF-α or adalimumab in Caco-2 cells as treatment with either of these compounds resulted in no changes of Akt phosphorylation in this cell line.

Inhibition of p38, NF-κB, and PI3K signaling suggests distinct roles for these signaling pathways in the mediation of TNF-α-induced barrier dysfunction. To test the functional significance of our findings, we next used specific inhibitor compounds for the signaling pathways identified above. As shown in Fig. 6A, treatment of Caco-2 cells with the p38 inhibitor BIRB796 (500 nM) did not alter TEER in Caco-2 cells primed with IFN-γ but resulted in an almost complete loss of TEER upon TNF-α stimulation (mean TEER with BIRB796: 41 ± 2 Ω·cm² compared with 113 ± 4 Ω·cm² with vehicle alone, n = 4, P < 0.001). Treatment with the NF-κB inhibitor BAY 11–7082 (10 μM) resulted in a small but
demonstrated clinical efficacy in the treatment of IBDs (19, 34), yet its impact on barrier function has not been systematically investigated until now. We therefore used the Caco-2 and T-84 cell lines, representing well-established in vitro models of the intestinal epithelial barrier, to evaluate the interaction of adalimumab and the proinflammatory cytokines TNF-α and IFN-γ in the control of barrier properties.

In line with the notion that IFN-γ upregulates the receptors for TNF-α and in agreement with previous studies (10, 17, 49), we found that barrier disruption by TNF-α required the presence of IFN-γ. After IFN-γ priming, both Caco-2 and T-84 cells exhibited unequivocal evidence of barrier disruption within 6 h after TNF-α stimulation, as indicated by 1) a significant decline in TEER, 2) the appearance of irregular membrane undulations in the plane of the tight junctions, 3) the internalization of occludin, and 4) increased phosphorylation of MLC, all of which have been previously associated with the barrier defect induced by these cytokines in vitro (48). Concurrent administration of adalimumab effectively prevented all of these changes, demonstrating its ability to antagonize barrier disruption by TNF-α on structural and functional levels, whereas no cytokine-independent effects of the antibody were observed in these experiments. In addition, adalimumab prevented the reduction in cell number observed with higher cytokine concentrations and longer treatment periods, suggesting that it effectively antagonizes the induction of necrotic and/or apoptotic cell death under these conditions (10).

Of note, while adalimumab has been demonstrated to induce apoptosis in monocytes and lymphocytes (4, 42), no reduction in the number of viable cells was observed in either of our epithelial models upon stimulation with adalimumab in concentrations up to 100 μg/ml (higher concentrations not tested; data not shown). However, our experimental system only allowed for the assessment of direct cytotoxic effects of the antibody on intestinal epithelial cells. Complement- or immune cell-mediated cytoxicity was not investigated in our study. Similarly, our model cannot account for potential changes of the cytokine milieu elicited by interference of adalimumab with the intricate network of lamina propria immune cells, such as the induction of T cell apoptosis resulting from impaired interactions with intestinal macrophages (4).

While stimulation with TNF-α resulted in comparable changes in both model systems, low concentrations of IFN-γ were sufficient to induce a substantial decline in TEER in the absence of TNF-α in T-84 cells but not in Caco-2 cells. The mechanism underlying this effect appears to be different from that of TNF-α as, concurrent with previous research, the TEER reduction in IFN-γ-stimulated T-84 cells was not accompanied by alterations in occludin trafficking or increased MLC phosphorylation (9, 10). The reason for the differential response in the two cell lines has not yet been clarified but might reflect general differences in signaling patterns as suggested by the observation that activation of the cellular energy sensor AMP-activated protein kinase is required for the barrier-disrupting effects of IFN-γ in T-84 cells (40) whereas in Caco-2 cells, this molecule seems to have a barrier augmenting function (30). In contrast to our observations with TNF-α, the IFN-γ-induced decline in TEER was not reversed by adalimumab, indicating that its barrier protective effect appears to be TNF-α specific.

The molecular events underlying the regulation of intestinal permeability have been the subject of intense investigation over the past years. In contrast to the well-established importance of the actin cytoskeleton and the MLC pathway for the short-term regulation of paracellular permeability (21, 46), the contribution of transmembrane tight junction components such as occludin or members of the claudin family remains controversial. Although alterations in the expression of these proteins have been frequently reported in both CD and UC (33, 51, 53), it is presently unknown whether these changes merely represent a consequence of inflammation or whether they actively contribute to the barrier defects observed in IBDs. In vitro studies have reported changes in the expression of tight junction transmembrane proteins by IFN-γ and/or TNF-α based on longer (24–72 h) incubation periods and reported conflicting results depending on the cell line and experimental protocols used (23, 33, 52). We therefore analyzed the expression of occludin, claudin-1, claudin-2, and claudin-4 under conditions where a clear effect of TNF-α on TEER and tight junction structure was observed. Our finding that a profound downregulation of these proteins was observed in T-84 cells both on the protein and RNA level, whereas no (occludin, claudin-2, and claudin-4) or opposite effects (claudin-1) occurred in Caco-2 cells, suggest that reduced expression of these proteins is not universally required for the early effects of TNF-α on TEER and tight junction structure and furthermore emphasizes the differences between both cell lines. However, it should be stressed that these results could only reflect the direct impact of TNF-α on epithelial cells in vitro, whereas the in vivo situation might be considerably more complex, as demonstrated by the recent finding that activation of MLC kinase results in increased mucosal production of IL-13 with a subsequent upregulation of claudin-2 in a transgenic mouse model (52).

Despite this limitation, two aspects of our in vitro observations warrant special mention: first, TNF-α induced a downregulation of both “tightening” (such as claudin-1) and pore-forming (such as claudin-2) claudin isoforms (3) in T-84 cells. While the net effect of such changes is difficult to predict, this may indicate that instead of selectively modulating barrier characteristics, TNF-α could interfere with cell differentiation on a broader scale. In line with this, we found that TNF-α induced a significant downregulation of the homebox transcription factor cdx-2 (unpublished observations), one of the major regulators of intestinal epithelial cell differentiation (13), in this cell line.

Second, claudin-1 was upregulated both at the RNA and protein level upon TNF-α stimulation in Caco-2 cells. While it appears unlikely that increased claudin-1 expression is responsible for the observed barrier defect (2), we and others (1, 32) have found that TNF-α induced a redistribution of this protein from a predominately membranous to a more diffuse cytoplasmic localization. Upregulation of claudin-1 has been previously reported in IBDs in vivo (32, 51), and, of note, multiple studies (15, 26, 51) have linked increased expression and altered subcellular localization of this protein to IBD-associated dysplasia and colorectal carcinogenesis. Thus, our observations in Caco-2 cells mimic these in vivo findings and corroborate a recent report (32) in which TNF-α induced an upregulation of claudin-1 in the nontransformed rat intestinal IEC-18 cell line. Our observation that coadministration of adalimumab did not antagonize the effect of TNF-α on claudin-1 expression but prevented its translocation to the cytosol is surprising and might indicate that distinct signaling path-
ways with different sensitivities to adalimumab control expression and localization of this molecule upon TNF-α exposure. The pivotal importance of MLC in the mediation of the TNF-α-induced barrier breakdown is well established, but the contribution of other signaling pathways is less clear. We therefore tested the activation of three candidate pathways in response to TNF-α in our system. We found that phosphorylation of MAPK p38 paralleled the early reduction in TEER upon TNF-α stimulation in both T-84 and Caco-2 cells, thereby supporting earlier reports that implicated this pathway in the regulation of MLC phosphorylation in vivo (14) and cytokine-induced barrier disruption in the Caco-2 model (50). However, whereas in the latter study inhibition of p38 with SB-203580 prevented the increase in macromolecular permeability induced by a mixture of IFN-γ, TNF-α, and IL-1β, treatment of Caco-2 cells with the more specific p38 inhibitor BIRB796 (5) not only failed to antagonize the TNF-induced decline in TEER but resulted in an almost complete loss of TEER in our study, thereby suggesting a rather protective role of p38 signaling in this cell line. Whether this discrepancy is due to a differential regulation of leak and pore tight junction permeability (44), a different spectrum of kinases inhibited by the respective inhibitor compounds, or specific characteristics of the cytokines used remains to be elucidated. In addition, the absent effect of BIRB796 in T-84 cells suggests that the impact of p38 on barrier dysfunction depends on the cell line. Thus, despite the fact that our data do not support a crucial role for p38 in the TNF-α-induced barrier disruption, the precise function of this signaling pathway remains to be investigated in future studies.

Activation of the NF-κB axis also correlated with barrier disruption in both cell lines and was sensitive to inhibition by adalimumab. Pharmacological inhibition of NF-κB partially prevented the TNF-α-induced barrier loss in both cell lines, thereby confirming an earlier report (1) obtained in the HT-29 cell line. Finally, PI3K signaling has been previously linked to barrier disruption and claudin dysregulation downstream of proinflammatory cytokines (9, 23, 28). Our results suggest that sustained activation of PI3K, as indicated by increased phosphorylation of Akt, is dispensable for the barrier defect in Caco-2 cells, whereas it correlated with the decline in TEER upon TNF-α stimulation in T-84 cells. Blockade of PI3K raised basal TEERs in this cell line but failed to prevent the TNF-α-induced barrier breakdown, supporting an earlier report (1) demonstrating that isolated inhibition of this pathway is insufficient to prevent barrier loss upon TNF-α stimulation. Thus, although adalimumab prevented Akt phosphorylation in response to TNF, additional functional experiments will be needed to address the precise impact of PI3K signaling on intestinal barrier function.

Taken together, our study identified, for the first time, distinct molecular mechanisms for the ability of adalimumab to reestablish the intestinal barrier disrupted by proinflammatory cytokines at the structural and functional level. This protective effect may explain its ability to induce mucosal healing in select patients with IBDs—a worthwhile new goal beyond symptom relief in the management of CD and UC (29).

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DISCLOSURES

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AUTHOR CONTRIBUTIONS


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