Glucocorticoids regulate barrier function and claudin expression in intestinal epithelial cells via MKP-1

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Glucocorticoids constitute potent anti-inflammatory agents and are first-line therapeutics for the induction of remission in moderate to severe IBD (4). They effectively control symptoms in collagenous colitis (27), but detrimental side effects prohibit their long-term use. Glucocorticoid therapy restores increased intestinal permeability in CD (55), an observation commonly interpreted to reflect the overall attenuation of inflammation. However, studies in various endothelial and nonintestinal epithelial cell lines provided evidence for the ability of glucocorticoids to stimulate tight junction sealing in the absence of inflammatory stimuli (14, 16, 36, 60). A recent report furthermore described upregulation of multiple tight junction proteins by corticosteroids in immature enterocytes as part of their well-known ability to promote intestinal matura-

THE INTESTINAL EPITHELIUM forms the largest mucosal surface of the human body, separating the highly antigenic environment of the intestinal lumen from the milieus intérieur. Integrity of this barrier is of pivotal importance for homeostasis and compromised in a variety of pathological conditions, most prominently inflammatory bowel diseases (IBD) (7, 39, 44). Importantly, IBD-associated barrier dysfunction does not merely reflect the inflammatory epithelial destruction during phases with high disease activity, as increased intestinal permeability precedes clinical relapse in patients with inactive disease (56). Moreover, several studies reported structural and functional barrier abnormalities in IBD during remission and in macroscopically normal mucosa (30, 54). As, in addition, several animal models with targeted barrier defects show either overt inflammation or increased susceptibility to experimental colitis (19, 22, 43), sound evidence suggests a prominent role for barrier dysfunction as a cofactor in IBD pathogenesis.

At the molecular level, IBD-associated barrier dysfunction is linked to increased expression of myosin light chain kinase (MLCK) and elevated levels of phosphorylated MLC (5). In addition, changes in numerous tight junction components have been reported in IBD, with upregulation of the pore-forming transmembrane protein claudin-2 being the most consistent finding both in Crohn’s disease (CD) and ulcerative colitis. Conversely, expression of other tight junction proteins with putative sealing properties, such as occludin, claudin-4, -5, and -8, was reduced in some but not all studies (18, 32, 52, 59). Increased paracellular permeability ensuing from these changes has been proposed to result in unrestrained delivery of luminal material to the lamina propria, where an inflammatory response is initiated. Subsequent secretion of proinflammatory cytokines, such as TNF-α, IFN-γ, and IL-13, causes additional damage to the intestinal barrier, forming a vicious circle of barrier dysfunction and inflammation (44). Increased paracellular permeability furthermore contributes to the pathogenesis of diarrhea via a leak-flux mechanism resulting from the passive back leak of ions and water into the intestinal lumen (39), and, of note, the existence of such mechanism has also been proposed in collagenous colitis, a condition with macroscopically intact mucosa but reduced expression of occludin and claudin-4 (9).


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is accompanied by changes in ion permeability and claudin expression patterns. Moreover, we provide evidence for a novel signaling pathway involving MAPK phosphatase-1 (MKP-1) to control tight junction permeability in intestinal epithelial cells (IECs), pointing to a previously unrecognized mode of action potentially contributing to the therapeutic efficacy of glucocorticoids in the treatment of IBD and microscopic colitis.

MATERIALS AND METHODS

Cell culture. Caco-2 cells were grown in DMEM containing 10% FBS, 1% nonessential amino acids, 10 mM HEPES, and 1% penicillin-streptomycin and subcultured every 3–4 days before reaching confluency. Cell culture media and supplements were obtained from Life Technologies (Darmstadt, Germany), and cells from passages 10–35 were used for all experiments. IFN-γ, TNF-α, and IL-1β were obtained from Cell Signaling Technology (Danvers, MA) and applied to the basal compartment of the Transwell system after a 35-day differentiation period.

Electrophysiological and permeability measurements. For measurement of transepithelial electrical resistance (TEER), 1 × 10⁵ cells were seeded on polycarbonate Transwell inserts with a diameter of 12 mm and a pore size of 0.4 μm (Corning Costar, Cambridge, MA). TEERs were recorded using the Millicell system (Millipore, Billerica, MA), and resistances obtained over a blank filter were subtracted from the obtained values. For assessment of Lucifer Yellow or dextran permeability (both obtained from Sigma, Taufkirchen, Germany), Transwell inserts were washed with HBSS followed by apical application of the tracer compounds. After 2 h of gentle agitation at 37°C, basal tracer concentration was measured using a fluorescence plate reader (Perkin Elmer, Rodgau, Germany). Dilution potentials were measured as previously described (8) and used to determine relative sodium-to-chloride permeability according to the Goldmann-Hodgkin-Katz equation.

Cell proliferation assay. Cell proliferation was determined using the XTT assay (Roche Diagnostics, Mannheim, Germany) according to the manufacturer’s instructions.

RNA isolation and quantitative RT-PCR. RNA was prepared with Trizol (Life Technologies), and, following removal of residual genomic DNA (TURBO DNA-free, Life Technologies), cDNA was constructed using the iScript kit (Bio-Rad Laboratories, Munich, Germany). Quantitative RT-PCRs were carried out on an Mx3005P light cycler (Stratagene, La Jolla, CA) using SybrGreen and EvaGreen master mixes from Fermentas (St. Leon-Rot, Germany) and Bio-Rad as well as primers listed in Table 1. All reactions were carried out in technical triplicates, and a standard curve derived from pooled samples as well as primers listed in Table 1. All reactions were carried out in technical triplicates, and a standard curve derived from pooled samples was used to determine primer efficiency and to ensure assay linearity. Reactions lacking cDNA and samples in which reverse transcriptase was used to determine primer efficiency and to ensure assay linearity. Western blotting. Cells were lysed in urea buffer supplemented with protease and phosphatase inhibitors as previously described (15). Proteins were separated using tricine SDS-PAGE, and Western Blotting was performed according to standard procedures. Primary antibodies were obtained from Life Technologies (Santa Cruz Biotechnology [β-actin, MKP-1, and glucocorticoid receptor (GR); Santa Cruz, CA]; horseradish peroxidase–coupled secondary antibodies were from DAKO (Hamburg, Germany). Stripping of Western blot membranes was performed for 30 min at 50°C in a buffer containing 2% SDS and 0.7% β-mercaptoethanol. Densitometric quantification was performed using the AlphaEase software (Alpha Innotech San Leandro, CA) or ImageJ (U.S. National Institutes of Health, Bethesda, MD).

Immunofluorescence. Immunofluorescence was performed on Transwell inserts fixed in methanol at −20°C. After being blocked in PBS containing 3% BSA and 0.2% Triton X-100, filters were incubated with primary antibodies used at a dilution of 1:100 (claudin-2) or 1:200 (ZO-1, occludin, claudin-4) overnight. After repeated washing, fluorophore-coupled secondary antibodies (Life Technologies) were applied for 1 h at room temperature, and cells were washed again in PBS/Triton X-100. Mounting was performed using the ProLong antifade kit (Life Technologies), and images were collected using the AxioVision software on an Axio M1 imager equipped for fluorescence (Carl Zeiss, Jena, Germany).

Statistics. Electrophysiological and densitometry data were analyzed in SPSS (IBM, Armonk, NY) employing Student’s two-tailed t-test for comparisons of two groups and one-way ANOVA with Dunnet and Bonferroni correction for multiple testing when comparing more than two groups. Quantitative RT-PCR data were analyzed using the REST software tool (31). Graphs were prepared using GraphPad Prism (GraphPad Software, San Diego, CA); error bars depict the SE unless otherwise noted.

RESULTS

Glucocorticoids increase transepithelial electrical resistance in a GR-dependent manner. To test whether glucocorticoid hormones modulate barrier characteristics in IECs, Caco-2 monolayers were treated with dexamethasone, and TEER was recorded to assess paracellular ion permeability. TEER remained relatively constant within the first 3 wk after being seeded both in control and dexamethasone-treated cells, and no differences between groups were observed. However, once Caco-2 cells had undergone complete differentiation, a process known to take up to 21 days in culture (38), cells treated with dexamethasone (0.1–10.0 μM) displayed a dose-dependent increase in TEER that continued to rise until reaching a plateau.

Table 1. Primer sequences

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer Sequence</th>
<th>Reverse Primer Sequence</th>
<th>PCR Product</th>
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<tr>
<td>Claudin-2</td>
<td>CTCGGGAATTGATCTGCGGTG</td>
<td>AGTTGAGGGCTATCACTCGG</td>
<td>190 bp</td>
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<tr>
<td>Claudin-4</td>
<td>CTCGGGAATTGATCTGCGGTG</td>
<td>AAGGAGGAGGAAAACCCCA</td>
<td>278 bp</td>
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<tr>
<td>GR</td>
<td>TCTTCGAGGATCCTGAAGGCGGTCG</td>
<td>ATGATTTCCAAGCTAATCTCGGG</td>
<td>254 bp</td>
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<tr>
<td>MKP-1</td>
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<td>GGTAGCTATGACAGCTAGAGAG</td>
<td>288 bp</td>
</tr>
<tr>
<td>RPLP0</td>
<td>GCAATGTTGACCTGTTCTTTG</td>
<td>GCCCTGACCTTCTCAAGCA</td>
<td>142 bp</td>
</tr>
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| GR, glucocorticoid receptor; MKP, MAPK phosphatase.
~35 days after being seeded (Fig. 1A). In fully differentiated control cells, resistances also slowly increased with time but were consistently significantly lower than in steroid-stimulated cells. Significant increases in TEER were also observed upon treatment with hydrocortisone (relative TEER after 35 days compared with cells treated with vehicle 1.53 ± 0.10, n = 4, P < 0.01) and budesonide (relative TEER 1.46 ± 0.11, n = 4, P < 0.05, Fig. 1B). This effect depended on activation of the GR, as cotreatment with the GR antagonist RU-486 (10 μM) abrogated the dexamethasone-induced increase in TEER (Fig. 1C). Of note, Western blotting and quantitative RT-PCR revealed upregulation of the GR during Caco-2 differentiation both on the protein and RNA level (Fig. 1, D and E), providing an explanation for the steroid unresponsiveness of these cells before terminal differentiation.  

Because it has been established that paracellular permeability toward ions and larger solutes can be regulated independently (40), passage of the fluorescent tracer Lucifer Yellow (LY, molecular weight ~450 kDa, median diameter 9.9 Å) across a monolayer of fully differentiated Caco-2 cells was quantified. Although dexamethasone significantly increased TEER, permeability of LY was not changed (Fig. 1F). Similar results were obtained with fluorescence-labeled dextran of 4 kDa (data not shown).

Immunofluorescence against the tight junction transmembrane protein occludin and the adapter molecule ZO-1 revealed membranous colocalization of these proteins, and no differences in cell morphology or the subcellular distribution patterns of these proteins were observed between dexamethasone-treated cells and controls (Fig. 2). Similarly, barrier-modulat-

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**Fig. 1. Glucocorticoids and tight junction permeability.**  
A: mean transepithelial electrical resistances (TEERs) ± SD of Caco-2 cells treated with dexamethasone (Dex, 0.1–10.0 μM). Significance levels have been omitted for reasons of clarity; however, all treatment groups exhibited significantly increased TEERs compared with cells treated with vehicle alone (0.1% ethanol; control) with P < 0.05 after 22 and P < 0.001 after 30 days in culture. n = 4 in all groups. B: mean TEER of Caco-2 cells treated either with vehicle (Con) or 1 μM hydrocortisone (Hc), budesonide (Bs), or dexamethasone (Dex) for 35 days (n = 4). C: mean TEERs ± SD of Caco-2 cells treated with vehicle, dexamethasone (10 μM), RU-486 (10 μM), or a combination of both compounds (n = 4). D: glucocorticoid receptor (GR) expression as assessed by Western blotting at different time points after seeding. E: GR expression as assessed by quantitative RT-PCR. Data were standardized to the mean expression level at day 1 (n = 4). F: relative TEER and Lucifer Yellow (LY) permeability of Caco-2 cells treated with dexamethasone for 35 days compared with controls (n = 4). *P < 0.05, **P < 0.01, ***P < 0.001.
ing effects of dexamethasone were not associated with increased proliferation, as the number of viable cells was in fact slightly reduced upon glucocorticoid stimulation as evidenced by the XTT assay (relative number of viable cells compared with cells treated with vehicle alone 0.91 ± 0.01, n = 30, P < 0.001). Taken together, these data suggest that glucocorticoids selectively modulate ion permeability without affecting the passage of larger solutes or changing principal tight junction architecture.

Dexamethasone selectively modulates expression of claudin-2 and -4 without altering their subcellular distribution. Paracellular ion permeability is determined by the expression pattern of tight junction transmembrane proteins, in particular those belonging to the claudin family (40, 45). We therefore investigated whether dexamethasone altered expression of these molecules. Our analysis focused on claudin-1, -2, -4, -5, -7, -8, and occludin, as changes in the expression of these proteins have been previously reported in IBD (18, 32, 52, 59) or collagenous colitis (9). As demonstrated in Fig. 3, dexamethasone induced a significant reduction in claudin-2 as assessed by Western blotting (relative abundance compared with cells treated with vehicle 0.37 ± 0.07, n = 4, P < 0.005), whereas claudin-4 was significantly increased in these cells (relative abundance 1.91 ± 0.13, n = 4, P < 0.01). In contrast, occludin, claudin-1, -7, and -8 displayed no significant changes upon dexamethasone stimulation, whereas claudin-5 was not detectable in fully differentiated Caco-2 cells.

Consistent with this, quantitative RT-PCR revealed down-regulation of claudin-2 (relative expression in cells treated with dexamethasone compared with cells treated with vehicle 0.78 ± 0.05, n = 6, P < 0.01), as well as increased expression of claudin-4 (relative expression 1.25 ± 0.05, n = 6, P < 0.01, Fig. 3C), indicating that dexamethasone at least partly acted on the transcriptional level. This was confirmed in Caco-2 cells stably transfected with a luciferase reporter for the human claudin-4 promoter. Dexamethasone treatment of these cells significantly increased luciferase activity in two independent clones after 30 days (relative reporter activity compared with cells treated with vehicle 1.48 ± 0.09, n = 5, P < 0.01), whereas reporter activation was not changed in cells stably transfected with the empty vector (data not shown). Again, terminal differentiation was required, as no increase in promoter activity was observed before this point (Fig. 3D).

As claudin-2 has been shown to form cation-selective paracellular pores (2, 46, 58), whereas claudin-4 restricts sodium permeability in Madin-Darby canine kidney cells (48), we next investigated whether altered expression of these proteins was associated with changes in ion selectivity. Indeed, paracellular cation flux was reduced in Caco-2 cells treated with dexamethasone as reflected by a highly significant decrease in sodium-to-chloride permeability (PNa⁺/PCl⁻ 3.21 ± 0.21 vs. 5.32 ± 0.16, n = 3, P < 0.001). This was antagonized by RU-486 (PNa⁺/PCl⁻ 5.50 ± 0.35 and 5.66 ± 0.07 with RU-486 or a combination of dexamethasone and RU-486, respectively), confirming a role for the GR in the dexamethasone-induced alteration of paracellular ion selectivity (Fig. 3E).

We next assessed the impact of dexamethasone on claudin-2 and -4 trafficking. Indirect immunofluorescence revealed membranous colocalization, with ZO-1 as the predominant staining pattern for claudin-2 although focal cell contacts lacking claudin-2 were frequently found in fully differentiated control cells (Fig. 4A, arrows). Dexamethasone induced an almost complete loss of claudin-2 from the vast majority of cell contacts; however, rarely focal junctions with preserved claudin-2 staining remained detectable (Fig. 4A, arrowhead). Claudin-4 predominantly colocalized with occludin and displayed a more homogenous membrane staining that appeared more intense in dexamethasone-treated cells, indicating that increased abundance of this protein as observed by Western blotting indeed reflected a net gain of junctional claudin-4 (Fig. 4B).

Of note, dexamethasone-induced alterations in claudin expression appeared to intensify changes observed during spontaneous Caco-2 differentiation, as time course analyses revealed a profound reduction of claudin-2 abundance, as well as increased claudin-4 expression in these cells following terminal differentiation (Fig. 4C). Immunofluorescence furthermore demonstrated that this was accompanied by a progressive focal loss of claudin-2 staining from single ZO-1-positive junctions over time (Fig. 4D), whereas the majority of claudin-4 remained homogenously distributed along tight junction strands with Caco-2 differentiation (Fig. 4E).

Barrier effects of dexamethasone depend on MKP-1. The MAPK phosphatase MKP-1 is an important mediator of the anti-inflammatory effects exerted by glucocorticoids (12). Quantitative RT-PCR revealed a highly significant upregulation of MKP-1 in fully differentiated Caco-2 cells treated with dexamethasone (relative expression compared with cells treated with vehicle 2.66 ± 0.09, n = 6, P < 0.0001) that coincided with the observed increase in TEER (Fig. 5A). Western Blotting confirmed this observation on the protein level (Fig. 5B). As MKP-1 targets the MAPKs p38 and p44/42 (50), we furthermore assessed activation of these pathways. Figure 5C demonstrates that dexamethasone reduced the phosphorylation of both MAPKs without altering the total amount of these proteins, suggesting that increased expression of MKP-1 indeed results in increased enzymatic activity.

We next sought to determine the functional relevance of these findings by employing the MKP-1 inhibitor triptolide (11). As shown in Fig. 5D, triptolide dose dependently antagonized the effect of dexamethasone on paracellular ion permeability with 10 nM of the inhibitor being sufficient to completely prevent the steroid-induced increase in TEER (relative...
TEERs compared with controls 2.2 ± 0.13, 1.4 ± 0.87, and 1.04 ± 0.066, with dexamethasone and 0.5, and 10 nM triptolide, respectively). Similarly, coadministration of sanguinarine (10 μM), a structurally unrelated MKP-1 inhibitor with an IC\textsubscript{50} of 10 μM (49), resulted in a highly significant reduction in TEER compared with cells treated with dexamethasone alone (relative TEER 1.38 ± 0.10). In line with this, Western blotting revealed that both inhibitors antagonized the steroid-induced changes in claudin-2 and -4 expression (Fig. 5E). Pharmacological inhibition of p38 and p44/42 either alone or in combination using the specific inhibitor compounds BIRB796 (500 nM), U0126 (10 μM), or SB203580 (10 μM; data not shown), however, failed to increase TEER in Caco-2 cells (Fig. 5F), suggesting that blocking of these downstream MAPKs is not sufficient to recapitulate the effects of glucocorticoids on paracellular permeability.

**Impact of dexamethasone on the decline in TEER and changes in claudin expression induced by IL-1β or a combination of IFN-γ and TNF-α.** Proinflammatory cytokines play a pivotal role in mediating barrier dysfunction in IBD (44). To assess the impact of dexamethasone on the decline in TEER under these conditions, Caco-2 cells were allowed to differentiate for 35 days in the presence or absence of dexamethasone (10 μM) followed by stimulation with a combination of IFN-γ (10 nM) and TNF-α (5 nM) or IL-1β (10 nM). As shown in Fig. 6A, both of these treatments resulted in a highly significant decline in TEER within 48 h (mean TEER 324 ± 11 Ω/cm\textsuperscript{2} without cytokines vs. 223 ± 9 Ω/cm\textsuperscript{2} and 264 ± 10 Ω/cm\textsuperscript{2} with IFN/TNF and IL-1β, respectively, n = 11, P < 0.001). TEER values were consistently significantly higher in cells treated with dexamethasone (mean TEER 496 ± 10 Ω/cm\textsuperscript{2} without cytokines vs. 368 ± 12 Ω/cm\textsuperscript{2} and 401 ± 14 Ω/cm\textsuperscript{2}...
with IFN/TNF and IL-1β, respectively, \( n = 11, P < 0.001 \) vs. cells receiving these cytokines in the absence of dexamethasone); however, the relative decline in TEER upon cytokine stimulation was similar between groups both with IFN/TNF (relative TEER compared with baseline 0.69 ± 0.025 and 0.74 ± 0.015 for vehicle and dexamethasone, respectively, \( n = 11, P = 0.14 \)) and IL-1β (0.82 ± 0.02 vs. 0.81 ± 0.02, \( n = 11, P = 0.59 \)).

Treatment with IL-1β resulted in a highly significant up-regulation of claudin-2 after 48 h (Fig. 6B), and, although claudin-2 expression was significantly lower in dexametha-
sone-treated cells compared with controls, its relative increase in abundance was similar between both groups (2.01 \pm 0.11 and 1.80 \pm 0.22 in control and dexamethasone-treated cells, respectively, n = 3, P = 0.44, Fig. 6D). Combined treatment with IFN and TNF also increased claudin-2 expression, although to a lesser extent, in control cells (relative protein abundance compared with cells without cytokines 1.60 \pm 0.12, n = 3, P < 0.01), which was significantly attenuated by dexamethasone (relative protein abundance 1.13 \pm 0.12, n = 3, P = 0.37 vs. dexamethasone-treated cells without cytokines and P < 0.05 vs. control cells with IFN/TNF, Fig. 6D).

In contrast to our observations with claudin-2, IL-1β did not affect claudin-4 expression either in controls or dexamethasone-treated cells (Fig. 6C), whereas combined treatment with IFN/TNF slightly increased claudin-4 abundance in both groups (relative protein abundance 1.45 \pm 0.12 and 1.40 \pm 0.12 for controls and dexamethasone-treated cells, respectively, n = 3, P < 0.05 vs. cells without cytokines and P = 0.79 for controls vs. dexamethasone-treated cells, Fig. 6D).

**DISCUSSION**

Glucocorticoids are the mainstay for inducing remission in IBD and the treatment of collagenous colitis (4, 27). Barrier
dysfunction constitutes an important factor in the pathogenesis of these conditions (39, 44), and it has been noticed that steroids restore increased intestinal permeability in patients suffering from CD (55). By demonstrating that glucocorticoids time and dose dependently reduce paracellular ion permeability in Caco-2 cells, we provide novel evidence that this observation might not be solely the result of an overall attenuation of inflammation but could reflect an epithelial-specific steroid effect.

Within recent years, evidence for a differential regulation of paracellular permeability toward ions and larger solutes has emerged. According to this concept, ions traverse the intercellular space via pores formed by claudin proteins, whereas flux of solutes larger than 4 Å occurs via the so-called leak pathway formed by temporary breaks in the tight junction strands (3, 40). Our observation that dexamethasone increased TEER without affecting LY or dextran permeability suggests that the main effect of glucocorticoids in IECs is to reduce flux via the pore pathway. In line with this, Caco-2 cells displayed an altered claudin expression pattern upon dexamethasone stimulation, whereas expression of MLCK and MLC phosphorylation, two major determinants of leak dysfunction in IBD (44), remained unchanged (unpublished observations). This is in contrast to numerous studies demonstrating that glucocorticoids reduce both pore and leak permeability in endothelial and mammary epithelial cells (14, 16, 36). Thus it appears that, depending on the biological context, corticosteroids may act via multiple mechanisms to modulate barrier characteristics. This is further emphasized by the observation that glucocorticoid stimulation of IECs did not alter expression or localization of occludin or ZO-1, as reported in other cell types (14, 36, 41).

Our finding that reduced claudin-2 expression paralleled the increase in TEER is in good agreement with numerous studies demonstrating that this protein increases paracellular conductance by forming pores within the tight junction strands (2, 17, 47). Conversely, claudin-4 forms a sealing tight junction component whose overexpression has been associated with increased TEER in various cell types (46, 48). Therefore, both downregulation of claudin-2 and increased claudin-4 expression constitute plausible mechanisms to account for the increase in TEER observed in our model. Similarly, as previous studies demonstrated claudin-2 to facilitate cation-flux, whereas claudin-4 restricts sodium permeability (2, 46, 48, 58), the reduction in sodium-to-chloride permeability found upon dexamethasone

Fig. 6. Glucocorticoids and barrier disruption by proinflammatory cytokines. A: Caco-2 cells were differentiated for 35 days with 10 μM dexamethasone (dashed line) or vehicle (0.1% ethanol; control; solid line) and stimulated with IL-1β (10 ng/ml), a combination of IFN-γ (10 ng/ml) and TNF-α (5 ng/ml; IFN/TNF) or no cytokines (Con) all applied to the basal compartment of the Transwell system for 48 h. Mean TEERs ± SD are shown; significance levels are given compared with cells receiving the same treatment with respect to steroid stimulation (dexamethasone or vehicle) but no cytokines (Con group), n = 11 in each group. B: Western blots showing the abundance of claudin-2 and claudin-4 (C) after 48 h of cytokine stimulation as described above. Three biological replicates are shown for each group; matching bands for β-actin are shown as a loading control. Note that exposure times are different for dexamethasone-treated cells and controls to correct for differences in the basal abundance of these proteins. D: densitometric quantification of claudin-2 and -4 in cells treated as above (n = 3). *P < 0.05, **P < 0.01, ***P < 0.001.
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Time-course experiments revealed both downregulation of claudin-2 and upregulation of claudin-4 in spontaneously differentiating Caco-2 cells in culture, supporting the idea that the promotion of intestinal maturation by glucocorticoids might not be restricted to immature enterocytes (23). Of note, numerous studies reported overexpression of claudin-2 (32, 52, 59) and downregulation or redistribution of claudin-4 in IBD (18, 29) although evidence for the latter is less unequivocal with other studies failing to replicate these findings (32, 59). Claudin-4 downregulation has also been reported in collagenous colitis (9), raising the possibility that reversal of altered claudin expression might contribute to barrier protection by glucocorticoids in IBD or microscopic colitis.

Although the approach employed in our study enabled us to specifically investigate glucocorticoid effects on IECs, data from animal models demonstrated that, in vivo, corticosteroids also participate in the stress-induced breakdown of the intestinal barrier (26). As this was abrogated by eradicating the gut microflora (42), it appears that glucocorticoids might also have detrimental effects on barrier function by changing characteristics of the intestinal microbiome. Dissecting the molecular pathways mediating barrier protection in IECs is therefore crucial for developing strategies to reverse barrier dysfunction in IBD and microscopic colitis.

In this respect, our study revealed two signaling intermediates involved in the barrier-modulating steroid effects. First, activation of the GR was required, as both increased TEER and altered ion selectivity were antagonized by RU-486. Following the conception that Caco-2 differentiation resembles changes of enterocytes along the crypt-villus axis (37), our observation that the GR was induced in Caco-2 cells with time suggests that the primary glucocorticoid targets in IECs are located apically along this axis. This is supported by in vivo evidence demonstrating increasing GR expression from the crypt to the villus in the small intestine and from the basal to the apical parts of colonic crypts (28, 33). Importantly, claudin-2 preferentially localizes to small and large intestinal crypts in normal mucosa (34), whereas expression in surface colonocytes has been frequently reported in IBD (32, 52). This implies the possibility that glucocorticoids might selectively act on pathologically altered claudin-2 expression patterns in IBD while having less impact on normal mucosa although further colocalization studies will be needed to confirm this hypothesis in vivo.

Apart from the GR, our study identified the MAPK phosphatase MKP-1 as a novel regulator of barrier function in IECs. Although MKP-1 has been attributed an important role in mediating the anti-inflammatory effects of glucocorticoid hormones (12), its impact on tight junction permeability has not been investigated up to now. A recent report, however, demonstrated that knockout of MKP-1 accelerated colitis development in the IL-10 knockout model (25), suggesting a potential role for this molecule in IBD pathogenesis. It has been established that MKP-1 acts by dephosphorylating ERK, p38, and JNK (50). In line with this, we found that dexamethasone reduced phosphorylation of ERK and p38, whereas we were not able to detect significant amounts of activated JNK in Caco-2 cells not exposed to proinflammatory stimuli (unpublished observations). However, whereas changes in TEER and claudin expression were reversed by MKP-1 inhibition, blockade of p38 and ERK either alone or in combination failed to recapitulate the barrier-modulating effects of dexamethasone. Thus, in line with the evolving perception of nonclassical MKP substrates (20, 21), our data might point to MAPK-independent signaling pathways regulating tight junction permeability downstream of MKP-1 in IECs.

Numerous studies have implicated proinflammatory cytokines such as IFN, TNF, or IL-1β in the barrier defect observed in IBD (10, 44). In vitro, these cytokines primarily induce a leak-type barrier defect that is mediated via activation of MLCK and interference with the junctional actomyosin ring (1, 61). This rapid and reversible leak-type barrier loss occurs independently of the claudin expression pattern, and, in line with our notion that glucocorticoids reduce permeability of the pore pathway without affecting leak flux, treatment with dexamethasone was not sufficient to prevent the decline in TEER induced by IFN/TNF or IL-1β. These findings contrast with a previous report demonstrating that glucocorticoids abrogate the increase in IEC tight junction permeability upon stimulation with TNF by inhibiting the upregulation of MLCK (6). The reason for this discrepancy is presently unclear but might relate to different culture conditions requiring different cytokine-stimulation protocols as in the aforementioned study. TNF alone was sufficient to reduce TEER in Caco-2 cells, whereas we and others observed the need for concurrent or sequential stimulation with IFN in this cell line (15, 51). Moreover, it could be speculated that prolonged exposure of IECs to glucocorticoids in our experimental setting might lead to adaptive changes not observed with a short-term glucocorticoid protocol. Changes of the claudin expression pattern on the other hand are commonly regarded as a means to control the long-term characteristics of paracellular pore permeability (44), and hence it appears conceivable that reduced expression of claudin-2 and increased claudin-4 abundance resulted in significantly higher TEERs upon IFN/TNF and IL-1β stimulation in dexamethasone-treated cells. Generally, the role of proinflammatory cytokines in regulating claudin expression is not well understood and appears to be highly dependent on the cell type and the experimental protocol used (15, 24, 32, 53). In this regard, overexpression of claudin-2 has been most consistently observed upon stimulation of IECs with IL-13 (18, 32, 53). However, as opposed to HT29 or T84 cells, IL-13 failed to elicit any effect on barrier function in the Caco-2 line in our hands, which is in line with a previous report (35) and precluded us from testing the impact of dexamethasone on this phenomenon. However, we here report the novel finding that IL-1β also induces claudin-2 expression in IECs as previously described for rat hepatocytes (57). Whereas the functional significance of this observation remains to be established, it is noteworthy that the relative increase in claudin-2 expression upon IL-1β stimulation was similar in glucocorticoid-treated cells and controls and that its induction by IFN/TNF was significantly attenuated by dexamethasone, suggesting that the signaling pathways regulating basal, IFN/TNF-, and IL-1β-mediated claudin-2 expression differ in their sensitivity to glucocorticoid modulation.

Taken together, by describing an epithelial-specific barrier modulating effect of glucocorticoids, our data suggest a novel previously unrecognized mode of action potentially contributing to the efficacy of these compounds in the treatment of IBD and microscopic colitis. Further study of the molecular mechanisms involved may contribute to the identification and de-
Development of pharmacological strategies to restore impaired intestinal barrier function that lack the detrimental side effects of conventional steroids.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

Author contributions: A.F. conception and design of research; A.F., M.G., and U.-F.P. interpreted results of experiments; A.F. prepared figures; and F.W. performed experiments; A.F., M.G., and U.-F.P. analyzed data; A.F., M.G., and U.-F.P. edited and revised manuscript.

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