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High-Throughput Screen Identifies Host and Microbiota Regulators of Intestinal Barrier Function

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BACKGROUND & AIMS: The intestinal barrier protects intestinal cells from microbes and antigens in the lumen—breaches can alter the composition of the intestinal microbiota, the enteric immune system, and metabolism. We performed a screen to identify molecules that disrupt and support the intestinal epithelial barrier and tested their effects in mice. **METHODS:** We performed an imaging-based, quantitative, high-throughput screen (using CaCo-2 and T84 cells incubated with lipopolysaccharide; tumor necrosis factor; histamine; receptor antagonists; and libraries of secreted proteins, microbial metabolites, and drugs) to identify molecules that altered epithelial tight junction (TJ) and focal adhesion morphology. We then tested the effects of TJ stabilizers on these changes. Molecules we found to disrupt or stabilize TJs were administered mice with dextran sodium sulfate-induced colitis or *Citrobacter rodentium*-induced intestinal inflammation. Colon tissues were collected and analyzed by histology, fluorescence microscopy, and RNA sequencing. **RESULTS:** The screen identified numerous compounds that disrupted or stabilized (after disruption) TJs and monolayers of epithelial cells. We associated distinct morphologic alterations with changes in barrier function, and identified a variety of cytokines, metabolites, and drugs (including inhibitors of actomyosin contractility) that prevent disruption of TJs and restore TJ integrity. One of these disruptors (putrescine) disrupted TJ integrity in ex vivo mouse colon tissues; administration to mice exacerbated colon inflammation, increased gut permeability, reduced colon transepithelial electrical resistance, increased pattern recognition receptor ligands in mesenteric lymph nodes, and decreased colon length and survival times. Putrescine also increased intestine levels and fecal shedding of viable *C rodentium*, increased bacterial attachment to the colonic epithelium, and increased levels of inflammatory cytokines in colon tissues. Colonic epithelial cells from mice given putrescine increased expression of genes that regulate metal binding, oxidative stress, and cytoskeletal organization and contractility. Co-administration of taurine with putrescine blocked disruption of TJs and the exacerbated inflammation. **CONCLUSIONS:** We identified molecules that disrupt and stabilize intestinal epithelial TJs and barrier function and affect development of colon inflammation in mice. These agents might be developed for treatment of barrier intestinal impairment-associated and inflammatory disorders in patients, or avoided to prevent inflammation.

Keywords: Cytokine; IBD; Model; Microbiota.

Tightly regulated gut permeability is indispensable for the maintenance of intestinal homeostasis, healthy metabolism, and immune tolerance. A single gut epithelial layer along the small and large intestinal mucosa allows for vital absorptive functions to coexist with defense against an immense antigenic and microbial burden introduced by food and the indigenous gut microbiota.[1] Intestinal barrier function consists of multiple components, mostly contributed by intestinal epithelial cell subsets, a robust junctional complex consisting of tight junctions (TJs) and adherens junctions (AJ), which form a physical barrier, reinforced by the cytoskeleton,[2] as well as secretion by goblet cells of mucus that separates the epithelial layer from the luminal microbiota.[3] Additionally, the gut barrier is fortified by a complex and diverse mucosal immune system in conferring tolerance against food and microbiota-derived antigens, while preserving an ability to elicit an intense immune response when the barrier is breached.[4,5]

***Authors share co-first authorship; §Authors share co-senior authorship.**

Abbreviations used in this paper: **3D, 3-dimensional; AJ, adherens junction; ATRA, all-*trans*-retinoic acid; CCL, C-C motif chemokine ligand; DSS, dextran sulfate sodium; FA, focal adhesion; IBD, inflammatory bowel disease; IL, interleukin; LPS, lipopolysaccharide; TEER, transepithelial electrical resistance; TJ, tight junction; TNF, tumor necrosis factor; ZO, zonula occludens.**

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WHAT YOU NEED TO KNOW

BACKGROUND AND CONTEXT

The intestinal barrier protects intestinal cells from pathogens—breaches can alter the composition of intestinal microbes and the intestine immune system and metabolism.

NEW FINDINGS

This study identified molecules that disrupt and stabilize intestinal epithelial tight junctions and barrier function and affect development of colon inflammation in mice.

LIMITATIONS

This study was performed in cells, tissues, and mice; further studies of these agents are needed in humans.

IMPACT

The agents identified in this study might be developed for treatment of barrier intestinal impairment-associated and inflammatory disorders in patients, or avoided to prevent inflammation.

At the subcellular and molecular levels, the intestinal barrier intercellular integrity is maintained by robust adhesions to their neighbors and to the underlying extracellular matrix. Sealing of the epithelial monolayer is provided by the apical junctional complex, primarily by the TJs. TJs are composed of multiple strands of adhesive transmembrane molecules (eg, claudins, occludin, tricellulin, and junction adhesion molecules), connected via intracellular adaptor proteins (eg, zonula occludens [ZO]-1, ZO-2, and ZO-3, and cingulin) to the actin cytoskeleton.[6,7] At their basal aspects, TJs physically interact with and are mechanically reinforced by the cadherin-catenin–based AJ.[8] Focal adhesions (FAs) are cytoskeleton-associated multiprotein assemblies linking the cells via integrin-mediated adhesions to the extracellular matrix.[9]

Despite their crucial role in the epithelial biology, the specific molecular mechanisms regulating and modulating TJ and FA integrity remain poorly characterized.[10] Current evidence shows that TJ proteins can be dynamically regulated by intracellular signaling transduction molecules, such as small guanosine-5′-triphosphate–binding proteins, tyrosine kinases,[11] and extracellular stimuli, such as bacteria, dietary component,[12] hyperglycemia,[13] and some cytokines[11]; however, the underlying regulatory mechanism is still far less elucidated to allow development of novel and potential therapeutic strategies targeting or protecting the intestinal barrier function.

A disruption of gut barrier integrity generates a "leaky gut," allowing an aberrant interaction of the luminal contents with the intestinal mucosal immune system. This process, when perpetuated in genetically susceptible individuals, leads to local dysregulation of immune responses, which culminates in a chronic autoinflammatory state leading to diseases such as inflammatory bowel disease (IBD).[14] In addition to IBD, pathogenesis of celiac disease[15] or acute gut infectious diseases, such as intestinal *Citrobacter rodentium* infection in mice,[16] also involves significant disruption of intestinal permeability. Influx of bacterial ligands into the portal and systemic circulation through leaky gut triggers systemic inflammation in a broad range of target organs.

Gut leakiness is associated with a wide variety of extraintestinal diseases, including systemic lupus erythematosus,[17] cardiometabolic disease,[18] central nervous system disorders,[19] and aging-related disorders.[20] Although the direct cause-and-effect relationship between intestinal barrier dysregulation and these pathologic states is not yet confirmed, reversing gut leakiness may become an attractive and potent target for disease prevention and treatment.

In this study, we sought to uncover new molecular regulators of intestinal intercellular and cell-matrix adhesions as a means of comprehensively elucidating the regulation of this important but elusive first line of defense. We opted to develop an automated microscopy-based high-throughput screening pipeline of molecules that directly target the epithelial barrier in vitro by using cultured cell lines, such as CaCo-2. After validation and quantification of the effects on barrier integrity of known disruptors, such as tumor necrosis factor (TNF), interleukin 1β (IL-1β), and bacterial lipopolysaccharide (LPS),[21–23] we studied the barrier-regulating function of molecules of dietary and microbiota origin, such as taurine and histamine, previously shown to signal to gut epithelial cells.[24]

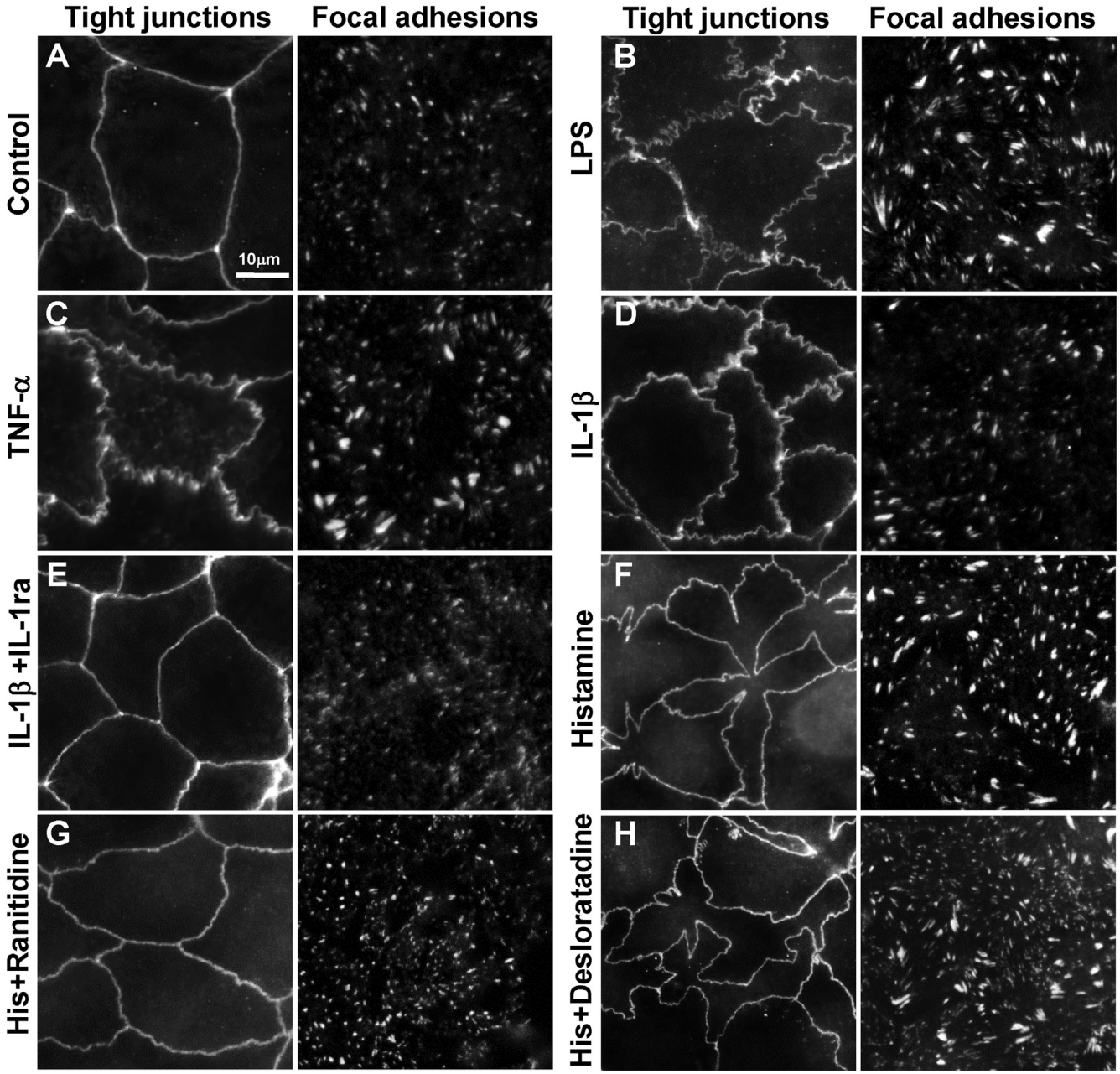
We then widened our search to screen for thousands of barrier-modulating compounds among a library of human-secreted molecules containing cytokines, growth factors, hormones, and other biologically active substances,[25] a metabolite library, and a drug library. These screens led to the discovery of multiple TJ disruptors, as well as stabilizers that blocked disruption and even restored TJ integrity in affected CaCo-2 monolayers.

By using in vivo mouse models, we then validated that putrescine administration induced a leaky gut phenomenon during both intestinal autoinflammation and infection. Strikingly, coadministration of a stabilizer, taurine, significantly reversed the disruptive effect of putrescine on intestinal barrier function during enteric infection, suggesting that therapeutic application of novel epithelial barrier stabilizers may be explored as future means of preventing or treating infectious, metabolic, and inflammatory human disease associated with gut barrier failure.

Methods

Cell Culture

CaCo-2 (ATCC HTB-37) and T84 (ATCC CCL-248) cells were purchased from American Type Culture Collection (Manassas, VA). Cells were routinely grown in Dulbecco's modified Eagle medium tissue culture medium supplemented with 10% fetal calf serum, penicillin (100 U/mL)/streptomycin (0.1 mg/mL), and 2 mmol/L GlutaMAX (Thermo Fisher, Waltham, MA). For the experiments described, single-cell clones of the parental CaCo-2 population, displaying uniform TJ patterns, were isolated and used. For the screening, cells were seeded onto glass-bottom 96-well plate (Thermo Fisher) coated with fibronectin or type I collagen (70,000 cells/well) and allowed to grow for 24 hours to form a confluent monolayer before the indicated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Focal adhesions
Tight junctions
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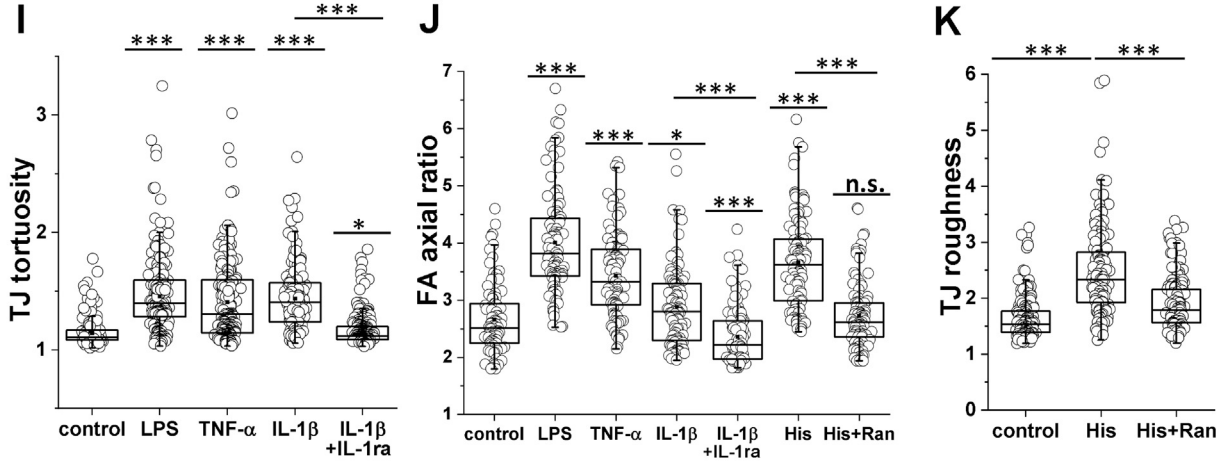
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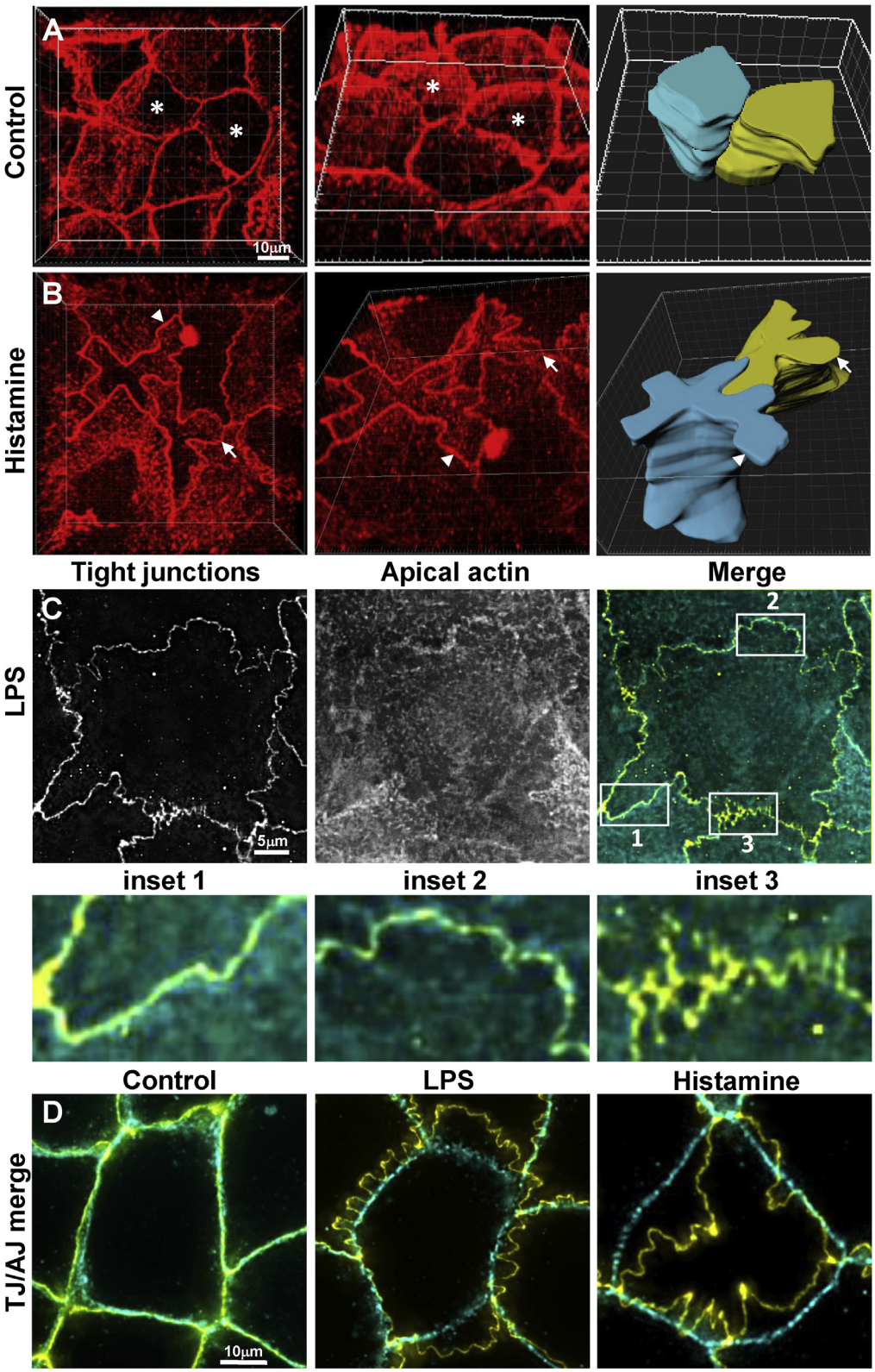
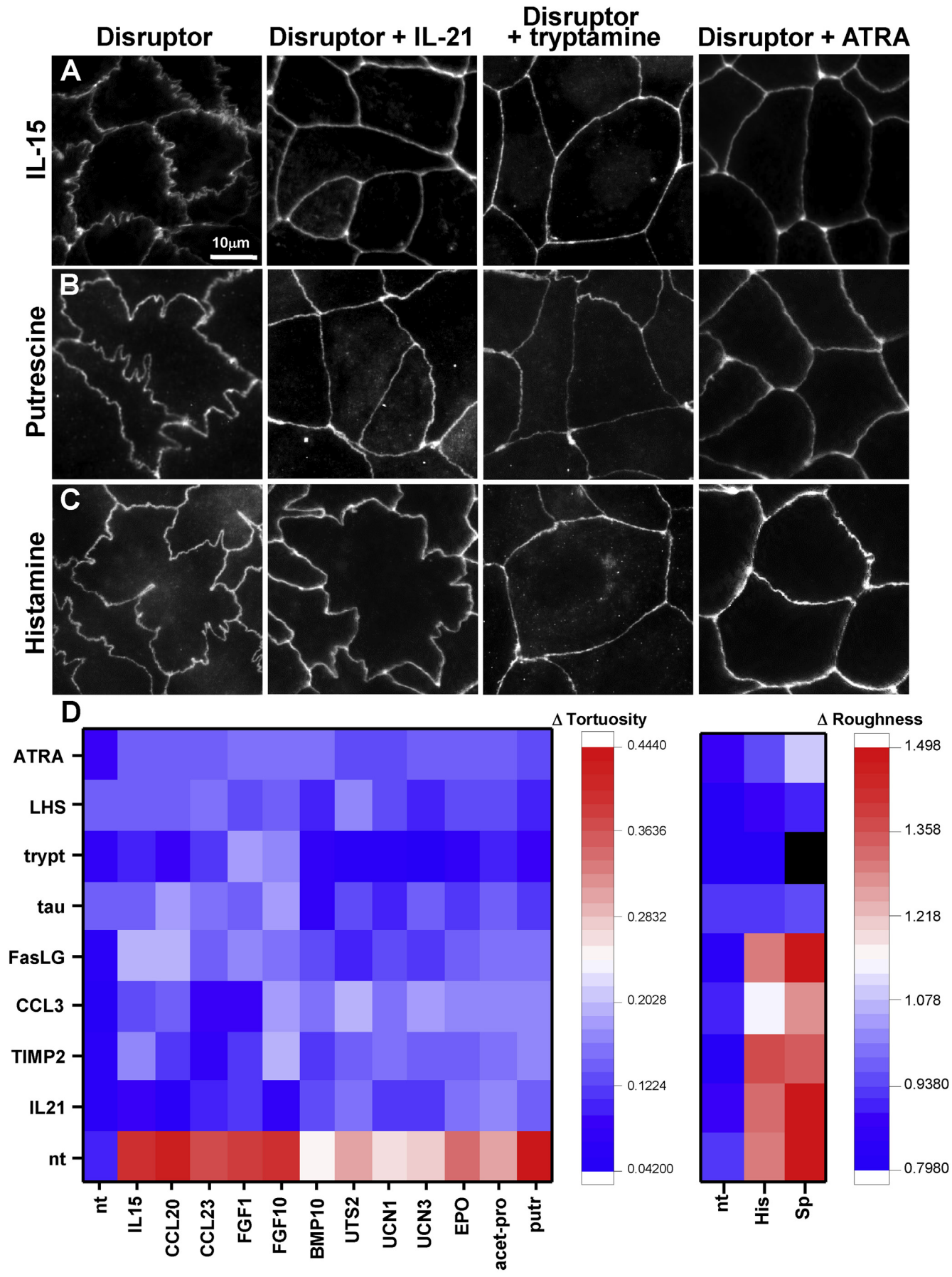


Figure 2. Type I and type II disruptors uncouple TJs from AJs at the apical cell domain. CaCo-2 cells (*A*) untreated or (*B*) treated for 24 hours with 10 mmol/L histamine were fixed, permeabilized, and double-labeled for cingulin (TJ) and β-catenin (AJ), both shown in *red*, with the TJ channel pseudocolored. A confocal stack covering the entire thickness of the monolayer is shown here as a z-projection (*left panel*) or a tilted side view (*middle panel*). 3D rendering was performed for the reconstruction of the TJ-AJ relationships (*right panel*). The *arrow* and *arrowhead* point to the same protrusions in histamine-treated cells, and the *asterisks* indicate the control cells used for the 3D rendering. (*C*) The apical domain of LPS-treated cells (300 ng/mL), labeled for ZO-1 to visualize TJs (*upper left* and pseudocolored in *yellow*) and with phalloidin to visualize AJ-associated actin (*middle panel* and pseudocolored in *light blue*). (*D*) The apical and subapical aspects of untreated CaCo-2 cells (*left panel*) and cells treated with LPS (*center*) or histamine (*right*) are shown.

Figure 1. Inflammatory IBD mediators affect TJ and FA integrity in cultured CaCo-2 cells. CaCo-2 cell were (*A*) incubated in regular medium, (*B*) treated with 300 ng/mL LPS, (*C*) 50 ng/mL TNF-α, (*D*) 30 ng/mL IL-1β, or (*F*) 10 mmol/L histamine (His). IL-1β and histamine were combined with their respective inhibitors (*E*) 100 ng/mL IL-1 receptor antagonist (IL-1ra), (*G*) 50 μmol/L ranitidine, or (*H*) 50 μmol/L desloratadine. (*I*) TJ disruption by essentially all type I disruptors was quantified by calculating its tortuosity. (*J*) FA elongation (quantified by axial ratio calculation) was used for quantifying FA deformation. (*K*) TJ roughness was measured for calculating the effect of histamine (type II disruptor). (*I*, *J*, and *K*) The *horizontal line* in the middle of each box indicates the median; the *top and bottom borders* of the box mark the 75th and 25th percentiles, respectively, and the *whiskers* mark minimum and maximum of all the data. *$P < .05$; ***$P <$ .001; n.s., not significant.


Disruptor
Disruptor + IL-21
Disruptor + tryptamine
Disruptor + ATRA
A
IL-15
10μm
B
Putrescine
C
Histamine
D
ATRA
LHS
trypt
tau
FasLG
CCL3
TIMP2
IL21
nt
nt
IL15
CCL20
CCL23
FGF1
FGF10
BMP10
UTS2
UCN1
UCN3
EPO
acet-pro
putr
Δ Tortuosity
0.4440
0.3636
0.2832
0.2028
0.1224
0.04200
Δ Roughness
1.498
1.358
1.218
1.078
0.9380
0.7980
nt
His
Sp

treatments were performed. The same experimental design was also applied to T84 cells.

Imaging

Immunofluorescence images of manually selected representative fields were acquired using a DeltaVision Elite wide field optical microscope (GE Healthcare, Waukesha, WI) equipped with 40×/1.3 UPlanFLN or 60×/1.42 PlanApo N oil objective (Olympus, Center Valley, PA). For multiwell plate scanning in screening experiments, the following automated imaging stations were used: a DeltaVision microscope with plate scanning regimen and a Hermes imaging station (IDEA Bio-Medical, Rehovot, Israel) or an ImagExpress microXL imaging station (Molecular Devices, San Jose, CA) equipped with 40× air objectives. In all cases, to cover the curvature of apical surface of the epithelial monolayer, 5-μm-thick z-stack was acquired around the TJs focus plane, and the resulting image was generated as a maximum projection of all optical sections (Supplementary Figure 1*A* and *B*).

DSS-Induced Colitis

Animal studies were conducted according to the Institutional Animal Care and Use Committee Standard Operating Procedure (IACUC SOPs). All experimental procedures were approved by the local Institutional Animal Care and Use Committee 20120815-2. Mice were administered with 2% (weight/volume) dextran sulfate sodium (DSS, molecular weight, 36,000–50,000 Da; MP Biomedicals, Solon, OH) in their drinking water for 5 or 7 days, followed by regular water. For details please view the Supplementary Methods.

Citrobacter rodentium *Infection*

We used a kanamycin-resistant and luciferase-expressing derivative of *C rodentium* DBS100 (ICC180) for infection, as previously described.[13] For details please view the Supplementary Methods.

Results

Effects of Known Inflammatory Bowl Disease–Associated Modulators on Tight Junction and Focal Adhesions Integrity in CaCo-2 Cells

A detailed description of the in vitro screening system is provided in the Supplementary Methods. We started our evaluation of barrier function–modulating molecules by examining the effects of the known gut barrier modulators LPS and TNF on TJ and FA integrity by treating cultured ZO-1 and zyxin-colabeled CaCo-2 cells for 24 hours with either molecule. Whereas the TJs of nontreated cells remained intact (Figure 1*A*), LPS and TNF both induced an apparent deformation of TJs, manifested by tortuous, often discontinuous morphology, which we refer to as "type I disruption" (Figure 1*B* and *C*). To quantify these effects, we measured the junctions' tortuosity and found that although most of the junctions were straight and intact, in nontreated cells (tortuosity value, 1.14 ± 0.11), LPS and TNF treatment resulted in a significant increase of junction distortion, manifesting as tortuosity values of 1.46 ± 0.31 and 1.41 ± 0.31, respectively (Figure 1*I*).

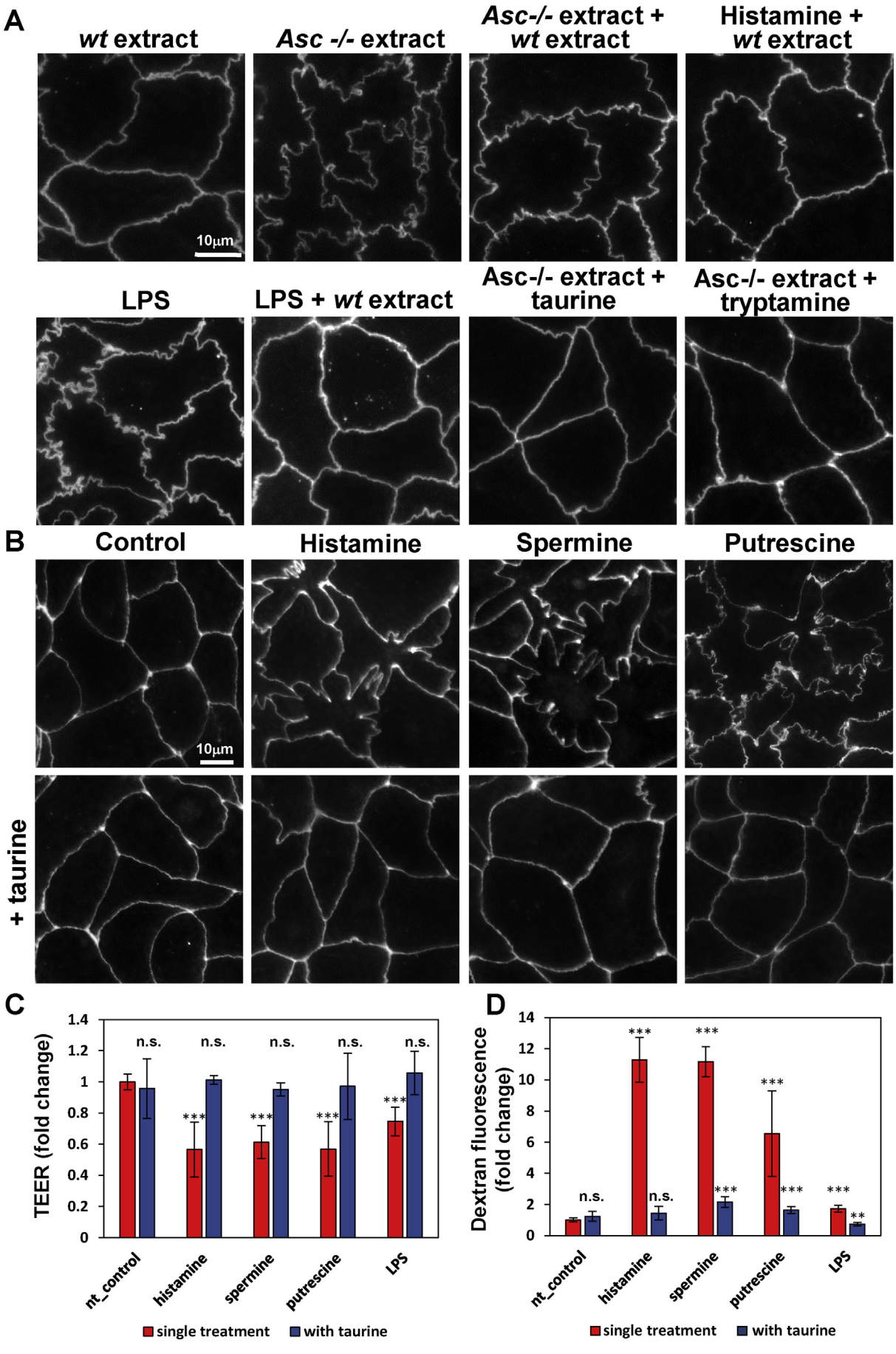
Interestingly, labeling of FAs revealed that treatments with both molecules resulted in a significant increase of FA length (axial ratio values of 4.00 ± 0.88 and 3.42 ± 0.73 instead of 2.66 ± 0.61 in control; Figure 1*A*, *B*, and *C*, *right panels*, quantified in Figure 1*J*). The inflammatory cytokine IL-1β induced a TJ distortion similar to that induced by LPS and TNF, whereas its effect on FAs was limited (Figure 1*D*; quantified in Figure 1*I* and *J*, respectively). The specificity of the IL-1β effect on TJ morphology was validated using an IL-1 receptor antagonist, which completely suppressed the IL-1β–induced TJ distortion (Figure 1*E*, quantified in Figure 1*I*).

We then tested the effect on TJ integrity of histamine, the microbiota-associated metabolite previously shown to be highly abundant in the feces of dysbiotic mice and to increase colitis severity in mice.[24] Unlike the type I distortive effects of LPS, TNF, and IL-1β, histamine treatment induced a dramatic change of TJ, as manifested by a major deformation of TJ geometry, leading to the development of multiple indentations and protrusions (Figure 1*F*). We named this phenotype "type II disruption." Morphometric quantification by "TJ roughness" (see Supplementary Methods) showed a remarkably increased TJ roughness, induced by histamine (Figure 1*K*). An addition of ranitidine, a specific antagonist of type 2 histamine (H_2) histamine receptor known to be expressed in the gastrointestinal tract,[26] significantly reduced the disruptive effect of histamine on TJ morphology (Figure 1*G*, quantified in Figure 1*K*), whereas antagonists of other histamine receptors (eg, H_1 antagonist desloratadine) were not able to block this effect (Figure 1*H*).

Similar to type I disruptors, type II disruptors also had a major effect on FA dimensions, manifested by an apparent increase in FA area (Figure 1*F*) as well as by a measurable increase in the FA axial ratio (Figure 1*J*). The H_2 antagonist efficiently abolished the histamine-induced increase of FAs, whereas H_1 antagonist did not (Figure 1*G* and *H*, *right panels*).

To obtain a comprehensive 3-dimensional (3D) characterization of TJ deformation in the whole cell context, we conducted a 3D deconvolution reconstruction microscopy of

Figure 3. TJs stabilizers can effectively block the effects of multiple disruptors. CaCo-2 cells were treated for 24 hours with different disruptor-stabilizer combinations, fixed, permeabilized, and labeled for ZO1 to visualize TJs. Selected disruptive molecules, (*A*) IL-15, (*B*) putrescine, and (*C*) histamine, were combined with stabilizers derived from human secreted molecules, bacterial metabolites, and pharmacologic agents (IL-21, tryptamine, and ATRA). (*D*) TJ disruption was quantified for 14 disruptors causing type I or type II phenotypes in combinations with 8 major TJ stabilizers, identified in the human secretome library, the bacterial metabolites library, and a pharmacologic stabilizer (ATRA). Junctional tortuosity (*left panel*) and roughness (*right panel*) were calculated for all combinations, displaying type I or type II phenotype, respectively. The heat maps present Δtortuosity and Δroughness values. BMP, bone morphogenetic protein; FasLG, Fas ligand G; EPO, erythropoietin; FGF, fibroblast growth factor; LHS, L-homoserine; TIMP, tissue inhibitor of metalloproteinase; UCN, urocortin; UTS, urotensin.


A
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Asc -/- extract
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Putrescine
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CaCo-2 cells treated with LPS or histamine and labeled for ZO-1 and F-actin or β-catenin. Whereas untreated cells featured a normal columnar morphology (Figure 2*A*), the type II phenotype induced by histamine was shown by 3D reconstruction to affect primarily the apical cell domain (Figure 2*B*), but not affecting the rest of the cell body. LPS treatment resulted in effects noted on TJ integrity along the "apical junctional belt" in the same cell, including relatively intact junctional regions, distorted type I regions, and visibly fragmented areas (Figure 2*C*, enlarged view in *insets 1*, *2*, and *3*, respectively).

The level of TJ distortion correlated with organization of apical F-actin, with intact areas uniformly decorated by F-actin, the type I regions characterized by interrupted, punctuate actin labeling, whereas the fragmented areas completely lost actin association (Figure 2*C*). A substantial uncoupling between TJ and AJ was demonstrated by double-labeling for TJs (anti–ZO-1) and AJ (anti–β-catenin), which are the 2 canonical components of the epithelial junctional complex, as an overlay of apical ZO-1 labeling and subapical β-catenin staining showed (Figure 2*D*).

In all, by testing known autoinflammation-associated molecules, we confirmed that the in vitro microscopy-based system is reliable for monitoring TJ and FA integrity in CaCo-2 cells and characterized 2 distinct morphologic variants of TJ disruption, thus enabling high-throughput screening and assessment of molecules involved in the regulation of intestinal barrier function.

High-Throughput Screening for Novel Tight Junction and Focal Adhesions Modulators in CaCo-2 Cells

Based on the effect of aforementioned inflammatory mediators on TJ and FA organization, we expanded to search for novel modulators of intestinal barrier function, by screening 3 different molecular libraries.

A human secreted molecule ("*secretome*") *library*. This library is composed of a variety of human-secreted molecules, containing cytokines, chemokines, growth factors, hormones, enzymes, and other biologically active molecules.[25] Of 298 molecules included in the library, we identified 10 that induced typical type I appearance of TJs (ie, increasing junctional tortuosity) and included cytokines (IL-15), chemokines (C-C motif chemokine ligand [CCL] 20 and CCL-23), growth factors (fibroblast growth factors 1 and 0, bone morphogenetic protein 10), neuropeptides (urotensin 2 and urocortin 1 and 3), and the hormone erythropoietin (Supplementary Figure 2*A* and *C* and Supplementary Table 1). Essentially all TJ disruptors discovered in this screen increased FA length and area (Supplementary Figure 2*A* and *D*). We also identified 4 molecules that significantly improved TJ integrity, referred to as TJ stabilizers, including IL-21, CCL-3, tissue inhibitor of metalloproteinase 2, and Fas ligand G (Supplementary Figure 2*B* and *C*). In addition to improvement of steady-state TJ morphology, these epithelial stabilizers possessed broad capacity to prevent TJ disruption caused by disrupting agents of different origins, as discussed below.

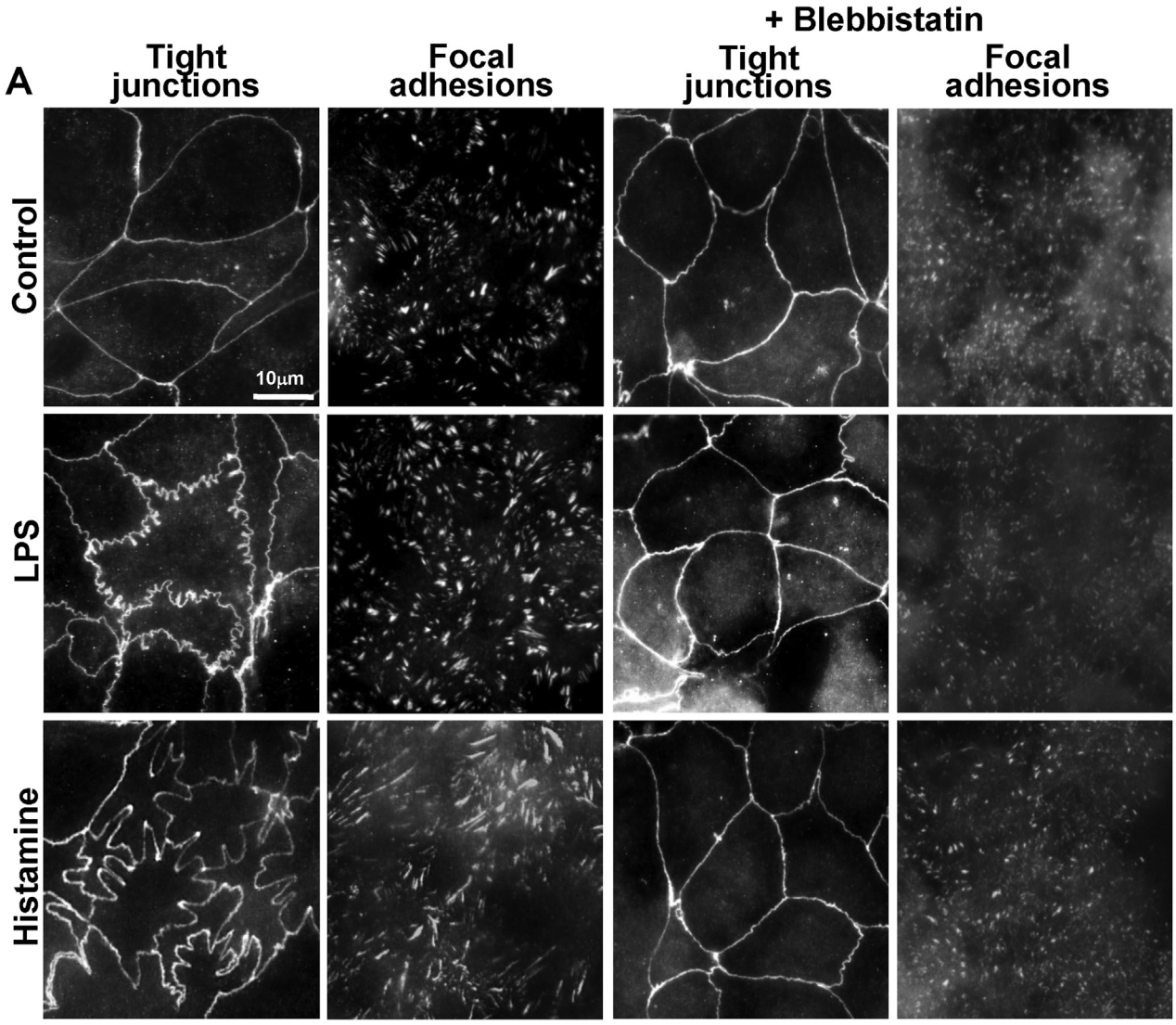
A microbiota-associated metabolite library. Despite the high variety (in the thousands) and concentrations (in the millimolar range) of many microbiota-associated metabolites at the host-microbiota interface, only a few, including taurine,[24] butyrate,[27] and microbial-specific indoles,[28] have been reported to modulate gut permeability. To identify novel modulating metabolites, we screened 25 molecules previously identified to be differentially abundant in healthy and dysbiotic mice (deficient in the inflammasome adaptor apoptosis-associated speck-like protein containing a C-terminal caspase recruitment domain [ASC][24]), based on their abundance, stability, and water solubility (Supplementary Table 2). We identified 3 potential disruptive metabolites (acetyl-proline, spermine, and putrescine) and 3 stabilizing metabolites (taurine, tryptamine, and L-homoserine) (Supplementary Results, Supplementary Figure 3).

Bioactive compounds library. This library contains 2956 pharmacologically active compounds. Screening identified more than 80 TJ stabilizers and disruptors, among them cell cycle and transcriptional modulators, kinase inhibitors, retinoic acid derivatives, specific flavonoids, and more (Supplementary Results, Supplementary Figure 4, and Supplementary Table 3).

Epithelial Stabilizers Are Dominant Over Multiple Disruptors in Restoring Tight Junction Integrity

After the identification of multiple TJ stabilizers that suppress the effects of specific disruptors, we assessed whether these stabilizers are specific only for a single disrupting agent or feature a broad stabilizing capacity against multiple TJ-disrupting molecules of different natures and origins. To this aim, we treated CaCo-2 cells with multiple disruptors (from the secretome, metabolite, and pharmaceutical library origins) in combination with multiple stabilizers of diverse origins and checked the cellular response in all combinations. Collectively, the tested stabilizing molecules commonly demonstrated a broad capacity to prevent TJ disruption, irrespective of the nature or signaling properties of the disrupting agent. As shown in Figure 3*A*–*C*, disruption caused by the inflammatory cytokine IL-15 or

Figure 4. (*A*) Healthy and dysbiotic fecal extracts act as TJ stabilizers and disruptors, respectively. (*B*) Epithelial disruptors/stabilizers differentially affect barrier function in vitro. (*A*) Aqueous extracts of feces of healthy wild-type (wt) or dysbiotic ($Asc^{-/-}$) mice were applied to cultured CaCo-2 cells individually, together, or in combination with specific TJ disruptors and stabilizers. (*B*) CaCo-2 cells were treated with indicated disruptors alone or in combination with taurine and fixed and stained for ZO1 to visualize TJs. The effects of selected epithelial disruptors, alone and in combination with taurine, (*C*) on TEER and (*D*) dextran permeability in CaCo-2 monolayer. Data are shown as the mean ± SD (*error bars*). $^{**}P < .01$, $^{***}P < .001$; n.s., not significant.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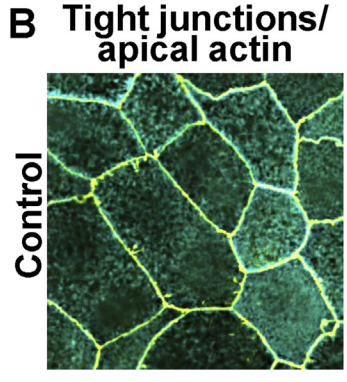
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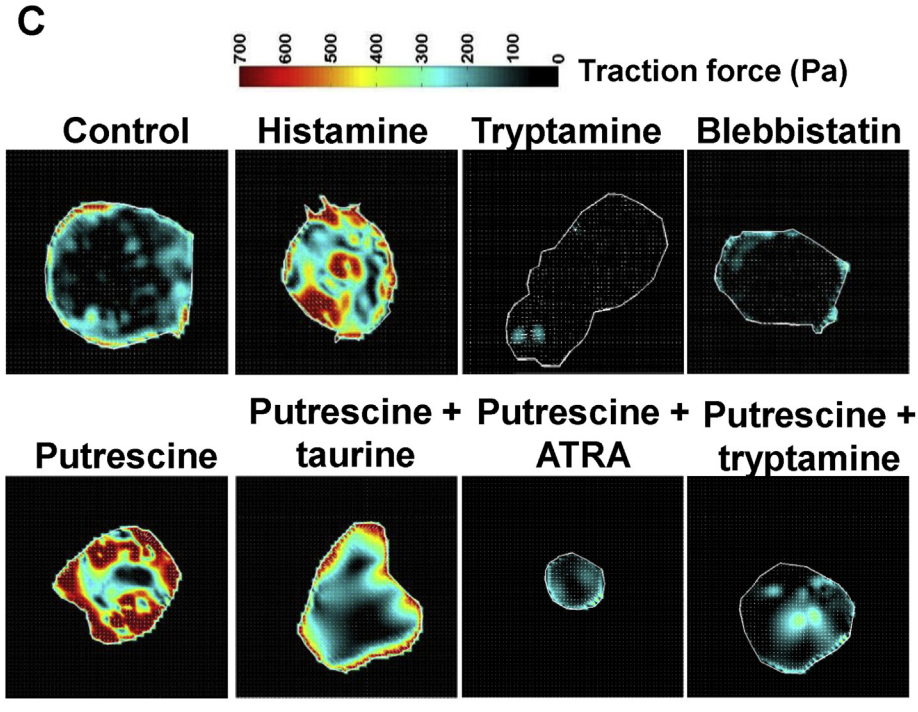
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Putrescine + ATRA
Putrescine + tryptamine

intestinal metabolites histamine and putrescine could be blocked by same subset of stabilizing agents. Quantifications performed for all tested combinations revealed that diverse modes on TJ disruption could be prevented by the tested stabilizers, although the degree of stabilization varied among compounds (Figure 3*D*).

Importantly, not all stabilizers were capable of preventing type II disruption induced by histamine and spermine (Figure 3*C* and *D*). The capacity of certain stabilizers to prevent disruption induced by multiple agents of different origin is an intriguing feature due to the apparent molecular diversity of the disruptors and their distinct biological activities (via cytokine receptors, growth factor receptors, histamine receptors, primary effects on cell cycle, cytoskeleton, and signaling machinery, etc). It suggests the possibility of a universal downstream executive mechanism of TJ disruption triggered via different pathways and the ability of stabilizing agents to effectively neutralize this mechanism. One example of such mechanism is further discussed below.

We have further demonstrated that epithelial stabilizers are capable not only of blocking disruption when administered simultaneously with different disruptors but also of restoring TJ integrity in cells displaying a preexisting disrupted phenotype (Supplementary Results and Supplementary Figure 5). To verify that these intestinal barrier function–modifying activities were not cell-line specific, we tested the effects of some newly identified disruptors and stabilizers by using the T84 cell line of gastrointestinal origin, which serves as a common model for assessing the epithelial barrier function.[29] Results showed that treatment of T84 cells with a type I disruptor (LPS) or with type II disruptors (histamine and putrescine) resulted in TJ disruption comparable to that observed in CaCo-2 cells and that in both cases, the deterioration of TJs was completely blocked by both taurine and all-*trans*-retinoic acid (ATRA) (Supplementary Results and Supplementary Figure 6).

We next assessed whether the dominant stabilizing capacity displayed by multiple molecules was also apparent using more complex physiologically relevant metabolite mixtures. To this aim, we tested the modulatory functions of aqueous fecal extracts from healthy mice and dysbiotic ($Asc^{-/-}$) mice at our vivarium.[30] The addition of fecal extract from healthy animals to regular culture medium did not induce detectable changes in TJ integrity (Figure 4*A*). In contrast, fecal extracts from $Asc^{-/-}$ mice caused a severe type I disruption, similar to that observed upon treatment with multiple disrupting agents. Strikingly, such TJ deformation was significantly reduced by the coaddition of fecal extracts from healthy mice, indicating that feces from healthy mice contain stabilizing components that can protect TJs from disruptors present in the feces of dysbiotic mice. We further tested the protective function of healthy fecal extracts against specific disruptive agents and noted that TJ alterations induced by LPS or histamine can be prevented by cotreatment with the fecal extracts from healthy mice. In addition, disruptive effects on TJs caused by the fecal extracts from dysbiotic mice could be prevented by cotreatment with the stabilizing metabolites taurine or tryptamine (Figure 4*A*).

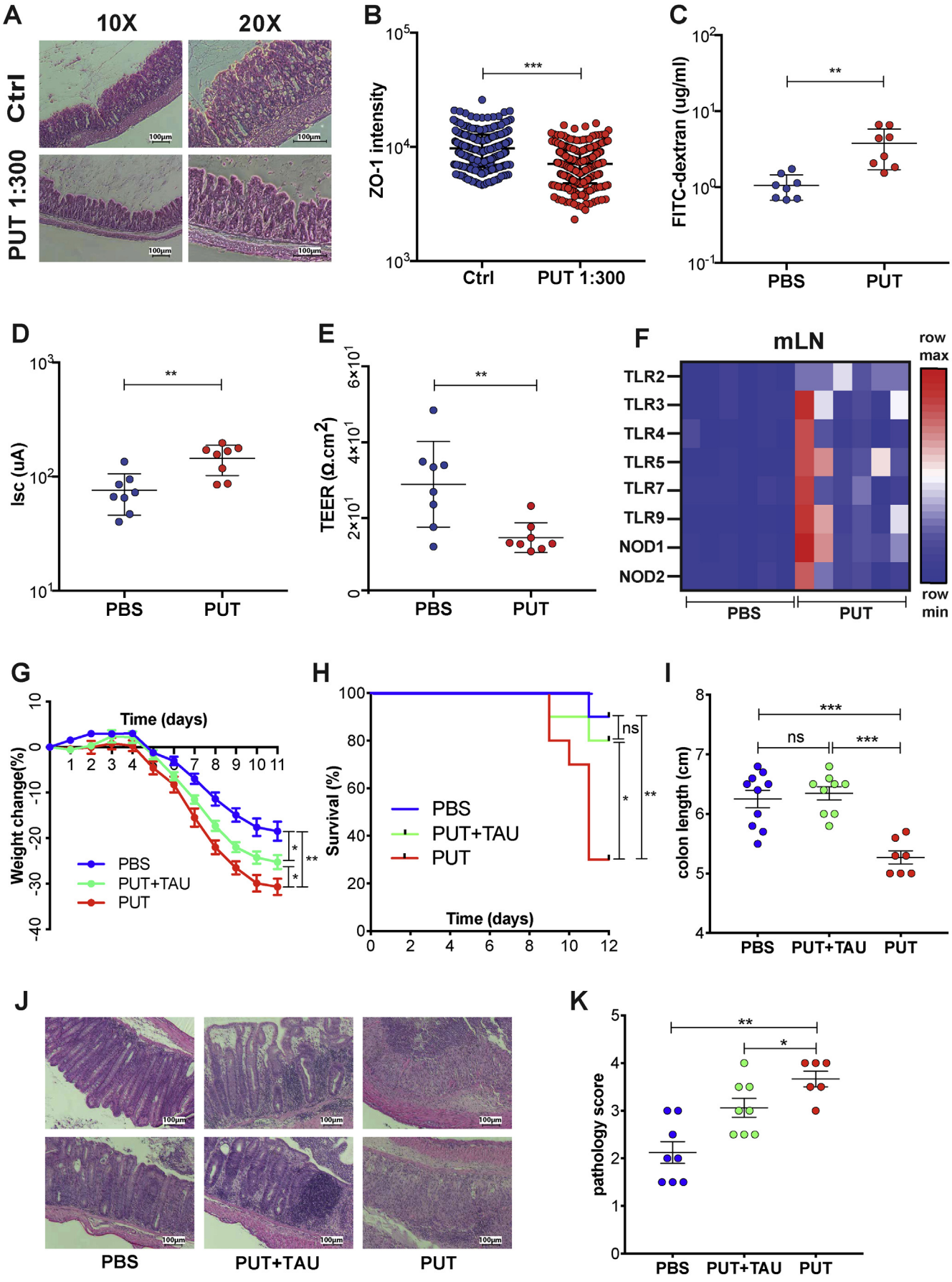
To validate that these dominant stabilizing effects on TJ morphology also impact transmembrane permeability, we measured the transepithelial electrical resistance (TEER) and permeability of fluorescent dextran across the CaCo-2 monolayer. Treatment with the various disruptors caused a major drop in the TEER and elevation of the permeability of dextran, reflecting an intestinal barrier functional alteration paralleling the morphologic disruption. Cotreatment with taurine abolished these functional barrier defects (Figure 4*B*–*D*). Interestingly, bacterial LPS caused TEER decrease but had limited effect on dextran leakage, suggesting that structural distortion of TJs may differentially affect permeability to ions and macromolecules.

Taken together, these data suggest that multiple simultaneously or sequentially administered purified stabilizers, or those present in fecal extracts from healthy mice, may reverse the morphologically and functionally disrupted intestinal barrier induced by purified disruptors or those present in fecal extracts of dysbiotic mice.

A Role for Actomyosin Contractility in the Regulation of Tight Junction Integrity

We next used our in vitro system in seeking a molecular mechanism of exemplary compounds impacting intestinal barrier function. A previous study showed that the activation of actomyosin contractility via phosphorylation of myosin regulatory light chain by myosin light-chain kinase can induce TJ disruption and increase epithelial permeability.[31] To examine whether this mechanism may explain the function of some of the epithelial disruptors identified in our screens, we tested the ability of blebbistatin, an actomyosin contractility inhibitor, to prevent TJ disruption using

Figure 5. Modulation of actomyosin contractility is involved in TJ disruption and stabilization processes. (*A*) CaCo-2 cells were treated for 24 hours with LPS or histamine alone or in combination with the actomyosin contractility blocker blebbistatin and were fixed and labeled for the TJ component cingulin and the FA molecule paxillin. (*B*) CaCo-2 cells untreated or treated with histamine for 24 hours were fixed and labeled for ZO-1 and phalloidin. Actin was imaged in the same focal planes as ZO-1 to determine the apical organization of the actin cytoskeleton (ZO-1 pseudocolored in *yellow* and actin in *blue*). (*C*) CaCo-2 cells were plated as individual islands on type I collagen–coated elastomeric hydrogel and treated for 24 hours with an individual disruptor (histamine, putrescine) or stabilizer (taurine, ATRA, tryptamine), or with the contractility inhibitor blebbistatin or with disruptor/stabilizer mixtures. Fluorescent beads embedded in the hydrogel were imaged before and after cell removal from the substrate. Beads displacement was used for calculating the traction forces applied by the cells to the underlying matrix (presented as a heat map, with the scale above the panel).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TEER (Ω.cm2)
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the hallmark type I disruptor LPS or the hallmark type II disruptor histamine. Indeed, cotreatment of CaCo-2 cells with blebbistatin abolished the TJ disruption induced by LPS or histamine alone (Figure 5*A*). Interestingly, blebbistatin also blocked the enlargement of FAs induced by the disruptors (Figure 5*A*). In addition to the type II disruption of TJ, histamine treatment induced the formation of robust radial F-actin bundles at the apical cell domain, which were not formed under untreated conditions (Figure 5*B*). Thus, the morphology of apical actin fibers upon treatment with disruptors suggests increased contractility applied to the apical junctional complex, which apparently leads to histamine-induced TJ deformation.

To further test the effect of selected epithelial disruptors and stabilizers on cellular contractility, we used traction force microscopy, a method allowing the microscopic measurement of contractile forces applied by cells to the underlying substrate.[32] Indeed, this analysis revealed an increased contractility upon treatment of CaCo-2 cells with the disruptive agent histamine and almost complete cellular relaxation upon treatment with the stabilizers tryptamine and blebbistatin (Figure 5*C*, *upper panel*). Another strong disruptor, putrescine, caused strong contraction of epithelial islands.

To directly assess whether disruptor-induced contraction can be reversed by the presence of stabilizers, we coincubated cells with putrescine and 3 stabilizers (taurine, tryptamine, and ATRA). Indeed, taurine significantly reduced putrescine-induced contraction to the level similar to control, whereas tryptamine and ATRA caused even more significant relaxation, reducing the contractility of cotreated cells below the control level (Figure 5*C*, *bottom panel*). We further confirmed the effect of actomyosin contractility on TJ integrity by assessing the effect of calyculin A, a pharmacologic activator of actomyosin contractility, on TJ morphology (Supplementary Results and Supplementary Figure 7).

In Vivo Validation of a Novel Metabolite Disrupting Intestinal Barrier Function

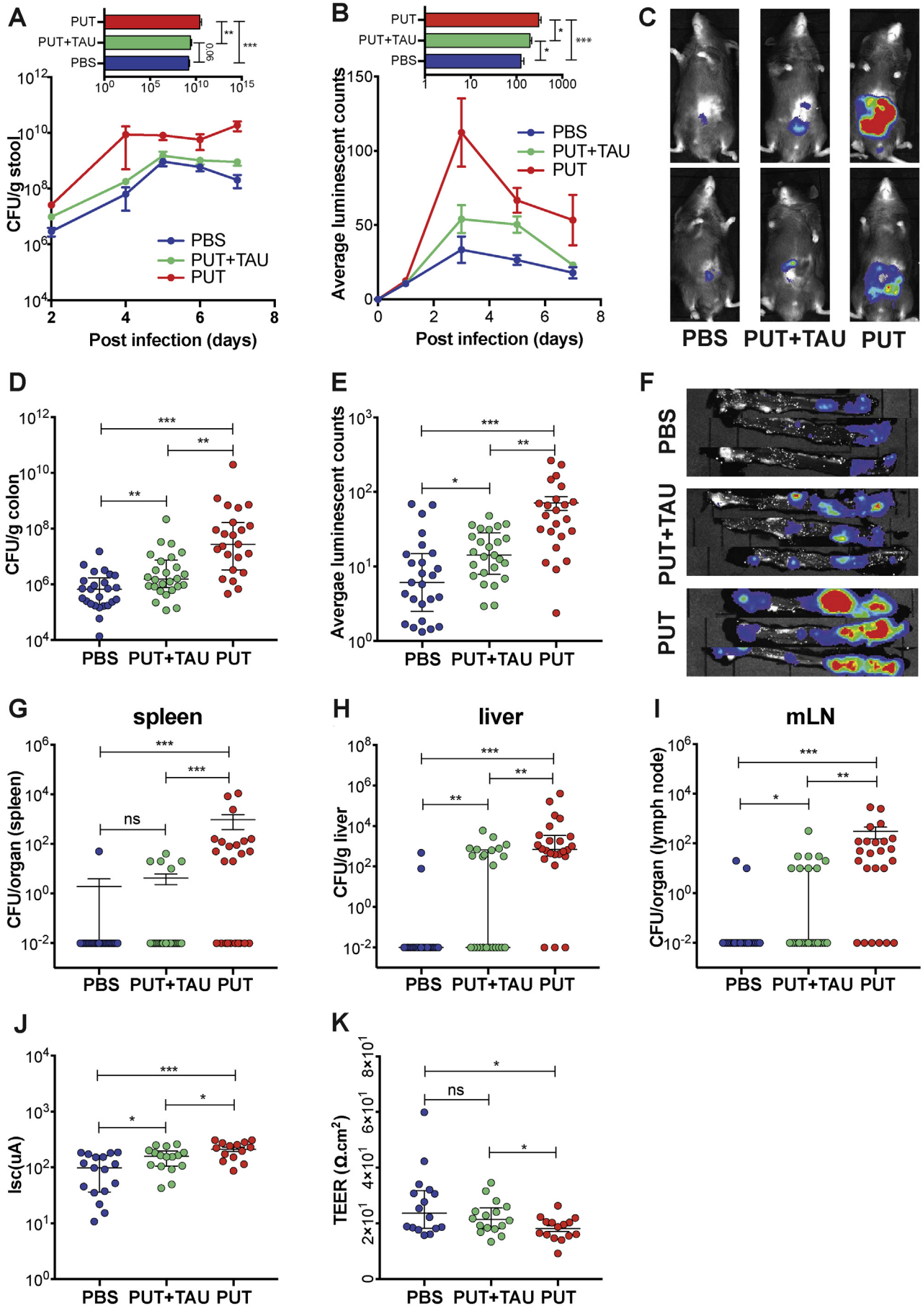
Based on the observed effect of the aforementioned TJ modulators, we sought to provide ex vivo and in vivo validation of an exemplary novel metabolite, putrescine, one of the most powerful TJ disruptors identified in our in vitro screen. To assess the effect of putrescine on steady-state intestinal barrier function, we implemented an ex vivo 3D colon culture system that can preserve the tissue architecture and mimic in vivo conditions.[33] Media containing different concentrations of putrescine were perfused continuously through the gut lumen for 2 hours (Supplementary Figure 8*A*). We detected dose-dependent histologic damage of colon tissues, as manifested by gradual epithelial detachment and crypt loss as the concentration of putrescine increased (Supplementary Figure 8*B*). Notably, at putrescine concentrations as low as 33.2 mmol/L, in which only mild tissue damage was observed and only mild histologic changes were documented, we could nonetheless detect a significantly decreased ZO-1 intensity compared with controls (Figure 6*A* and *B*; Supplementary Figure 8*C*), indicating that putrescine treatment disrupted TJ integrity in steady-state colon tissue, even in the absence of massive tissue damage.

To further characterize the in vivo effect of putrescine, we administered putrescine to naïve wild-type mice by oral gavage for 10 days, followed by induction of experimental colonic inflammation by DSS[34] and by *C rodentium* infection, the later simulating human enteropathogenic *Escherichia coli* infection and associated large-intestinal inflammation.[16] Both models are known to physically and functionally impair the intestinal barrier and corroborate some histopathologic features of human IBD.

We began by investigating the potential of putrescine to exacerbate intestinal barrier function before the onset of overt intestinal inflammation, tissue damage, and systemic manifestations. To this aim, mice were exposed to DSS for 5 days, when no significant weight loss was yet observed (Supplementary Figure 8*D* and *E*). Compared with vehicle-treated mice, putrescine-administered mice featured enhanced gut permeability, as indicated by an increased systemic fluorescein isothiocyanate-dextran influx (Figure 6*C*), a higher electrical short-circuit current (Figure 6*D*), and a lower colonic TEER (Figure 6*E*).

As another readout of local gut leakiness, we detected increased amounts of microbial pattern recognition receptor ligands in the mesenteric lymph nodes in putrescine-treated mice (Figure 6*F*) and to a lesser extent in the spleen or liver (Supplementary Figure 8*F* and *G*). During the inflammatory stage of DSS-induced colitis, putrescine-treated mice featured enhanced disease severity compared with vehicle-treated mice, despite comparable intake of DSS during treatment (Supplementary Figure 9*A* and *B*). The increased putrescine-induced disease burden manifested as exacerbated weight reduction, significantly lower survival, enhanced colonoscopy severity score, decreased colon

Figure 6. Disruptive effect of putrescine ex vivo and in mice with DSS-induced colitis. (*A*) Representative histology images and (*B*) quantification of ZO1 intensity in colon tissues cultured with control medium or putrescine (1:300, 33.2 mmol/L). Wild-type mice with oral gavage of putrescine or vehicle control (phosphate buffered saline [PBS]) were administered with 2% DSS in the drinking water for (*C–F*) 5 days or (*G–K*) 7 days (n = 8–10 mice per group). (*C*) Fluorescein isothiocyanate–dextran levels recovered from the serum 3 hours after oral gavage (80 mg/mL fluorescein isothiocyanate-dextran), (*D*) Ussing chamber recording of short circuit current (Isc) and (*E*) TEER across colon epithelial layer, and (*F*) pattern recognition receptor stimulation by lymph node extracts from mice on day 5 after DSS administration. (*G*) Weight loss, (*H*) survival, (*I*) measurement of colon length, (*J*) representative histology images, and (*K*) pathology scoring on day 12 after DSS treatment. Ctrl, control medium; PUT, putrescine; TAU, taurine. Data are presented as the mean ± SD (*error bars*). *$P < .05$; **$P < .01$; ***$P < .001$; ns, not significant.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F
G
spleen
CFU/organ (spleen)
ns
H
liver
CFU/g liver
I
mLN
CFU/organ (lymph node)
J
Isc(uA)
K
TEER (Ω.cm²)

length, and aggravated histopathologic severity (Supplementary Figure 9*C–J*).

We next assessed whether supplementation of a newly discovered TJ stabilizer could rescue the disruptive effect of putrescine in vivo. To this aim, mice were coadministered with 3% taurine in drinking water and putrescine by oral gavage for 10 days, followed by DSS treatment. The susceptibility to DSS colitis in putrescine-treated mice could be partially but significantly ameliorated by coadministration of taurine, characterized by less weight loss (Figure 6*G*), lower mortality rate (Figure 6*H*), increased colon length (Figure 6*I*), and improved histopathologic severity (Figure 6*J* and *K*).

Likewise, putrescine treatment of mice infected with a bioluminescent variant of *C rodentium* exacerbated disease, as noted by higher bacterial burden in the colon and systemic sites, more disruptive intestinal barrier function, and increased levels of inflammatory cytokines (Supplementary Results and Supplementary Figures 10 and 11). The addition of taurine partially but significantly restricted the susceptibility to *C rodentium* infection in putrescine-treated mice, characterized by lower bacterial shedding in feces (Figure 7*A*) and lower bacterial bioluminescence (Figure 7*B* and *C*). Taurine supplementation also ameliorated putrescine-induced colonic bacterial attachment (Figure 7*D–F*) and systemic dissemination to spleen, liver, and mesenteric lymph nodes (Figure 7*G–I*), accompanied by significantly lower electrical current recording and higher TEER noted in intestinal epithelial cells under this stabilizing treatment (Figure 7*J* and *K*).

To assess the effects of putrescine and taurine treatment on sorted mice colonic epithelial cells positive for epithelial cell adhesion molecule, we performed RNA sequencing analysis on these cells, showing a possible rescuing effect of taurine in the oxidative stress and cellular contractility caused by putrescine (Supplementary Results and Supplementary Figure 12). Taken together, our ex vivo and in vivo data validated that while the novel TJ-disrupting metabolite putrescine contributes to impaired intestinal barrier function, aggravates gut leakiness, and subsequently disease susceptibility during colonic autoinflammation and infection, cosupplementation with the stabilizer taurine may enable significant reversal of this disruptive effect.

Discussion

We demonstrate that a variety of compounds derived from the host, microbiota, or from screening libraries can modulate the structural integrity of TJs and FAs in intestinal epithelial cells, thereby affecting their barrier functionality. Using cultured CaCo-2 cells as a screening system and TJ morphology as our primary readout, we have identified multiple secreted molecules (including inflammatory cytokines) and microbial-associated metabolites that disrupt the integrity of TJs. This structural effect was manifested by increased TJ tortuosity (type I disruptive effect) or by gross perturbation of TJ topology throughout the epithelial monolayer (type II disruptive effect).

One of the characteristic structural features of both type I and type II effects was the radical disarray of the intercellular apical junctional complex. This complex consists of an apical belt-like structure, known as the "occludens," or "tight junction," which controls the apical-basal polarity of the cells as well as the transepithelial permeability,[35,36] and a closely associated subapical belt, known as "adherens junction," which is a robust, actin-rich, cadherin-based cell-cell adhesion that is believed to mechanically stabilize the adjacent TJ.[37,38] As shown here, the 2 junctions in control cells are indeed closely associated (Figure 2*D*), yet both modes of disruption lead to major disengagement of the 2 junctions. The molecular "targets" of the various disruptors and how the disengagement of the junctional components leads to the loss of barrier integrity remains to be determined in future studies. Moreover, our functional validation experiments confirmed that these structural effects are accompanied by a considerable decrease of TEER and an increase in dextran permeability in the treated cells (Figure 4*C* and *D*) in both in vitro and in vivo experiments.

Our findings bear several implications. First, we demonstrate that intestinal barrier function is a highly regulated and dynamic process that is influenced by multiple environmental players from both host and microbiota origin, which include the junction modulators discovered here as well as yet to be discovered physiological, pathologic, or pharmacologic effectors of epithelial intercellular adhesions. Such critical gate-watcher regulation, which may have critical consequences on the host's antipathogen protection[39] as well as its metabolic and immune homeostasis,[3,40] must react to a combination of signals that integrate to accurately reflect a changing microenvironment. Using our high-throughput screening system, we demonstrate that these assorted signals come from both the host and microbiota, highlighting further the concerted mucosal behavior of the prokaryotic and eukaryotic components of the holobiont.[41]

Second, our study provides proof-of-concept to new prototypes of gut epithelial cell molecular regulation of TJ and FA integrity and their biochemical basis. Indeed, a cross talk between the apical junctional complex of epithelial cells (including the TJs and AJ) and FAs was demonstrated in

Figure 7. Restoration of the disruptive effect of putrescine by taurine supplementation in mice with *C rodentium* infection. Wild-type mice treated with phosphate-buffered saline (PBS), putrescine (PUT), or putrescine plus taurine (TAU) were infected with *C rodentium* (results were pooled from 3 independent experiments). (*A*) Colony-forming units (CFUs) recovered from stool, (*B*) abdominal bioluminescence quantification, and (*C*) imaging during the postinfection course. (*D*) CFUs recovered from colonic tissue, (*E*) ex vivo colonic bioluminescence quantification, and (*F*) imaging and on day 7 after infection. CFUs recovered from (*G*) spleens, (*H*) livers, and (*I*) lymph nodes on day 7 after infection. (*J*) Ussing chamber recording of short circuit current (Isc) and (*K*) TEER across the colon epithelial layer on day 7 after infection. Data are presented as the mean ± SD (error bars). **P* < .05; ***P* < .01; ****P* < .001; ns, not significant.

multiple systems, suggesting that increased contractility generated by the actomyosin system stimulates FA formation and growth and, at the same time, has a detrimental effect on cell-cell junctions.[42,43] The results described here, indicating that nearly all TJ disruptors augmented FA formation (Figure 1) whereas most stabilizers exerted the opposite effect, strongly support these findings and indicate that the interadhesion cross talk may be important for maintaining the gut homeostasis.

We further show here that mechanical forces play an important role in TJ/FA regulation. Thus, selected prominent disruptors (eg, histamine, putrescine; Figure 5*C*) stimulated cell contractility, and a subset of epithelial stabilizers prevented this effect, as measured by traction force microscopy. In addition, a classical actomyosin contractility blocker, blebbistatin, blocks the effect of all tested disruptors (Figure 5*A*). This is also in line with previous reports suggesting that the activation of myosin II by myosin light-chain kinase correlates with increased transepithelial permeability in the gut.[44,45] The disruptive effect of excessive contractility was further confirmed, showing that the phosphatase inhibitor calyculin, which enhances actomyosin contractility (Supplementary Figure 7*A*), acts as a strong and rapidly acting disruptor, whose activity can be blocked by pretreatment with different stabilizers (Supplementary Figure 7*B*). The differential (largely inverse) effects of disruptors on the apical adhesions (cell-cell) and basal adhesions (cell-matrix) can bear relevance to intestinal epithelial biology. Naturally, TJ instability can play key and direct roles in the penetration of lumenal microbiota across the epithelium and in initiating an inflammatory response, and adhesion to the underlying matrix plays a major role in reinforcing the gut wall integrity.

Third, our study highlights the potential physiological importance of this complex regulatory network on mammalian physiology and microbiota-associated disease. Our results support the paradigm that an aberrant combination of "disrupting signals" or loss of "stabilizing signals" from the host and its microbiota, coupled with genetic susceptibility for such barrier instability, may drive the system toward pathological intestinal barrier function in a variety of disorders, such as IBD,[46] cardiometabolic disease,[47] infectious mucosal disorders,[13] and even mental illness.[48] In the face of genetic susceptibility, such a common-denominator intestinal barrier function–disrupting initiating event may drive an aberrant contact between foreign elements in the form of food- and microbiota-associated molecules and the mucosal immune arm, triggering a perpetuated and self-amplifying immune response that contributes to disease development and further augments barrier dysfunction.

Importantly, our results suggest that many of the new barrier stabilizers discovered in this study may dominantly act when coadministered with disruptors in restoring barrier integrity. As such, the stabilizers increase the apparent coherence of TJs in untreated cells, and more importantly, they effectively block the disruptor effects, irrespective of their molecular origin or expected mode of action. For example, the TJ disruption by histamine can be specifically inhibited by the H_2 receptor blocker ranitidine, yet it can also be inhibited by several other stabilizers, which are not expected to have any effect on histamine receptor signaling (Figures 1 and 3). This apparent dominance of TJ stabilizers' effect over disruptors is unique for the physiological and pathologic gut molecular ecosystem and suggests that a combination of stabilizing molecules and even drugs may enable this chain of events to be contained upstream to uncontrollable inflammation as a means of ameliorating microbiota- and food antigen-associated disease.

Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of Gastroenterology at www.gastrojournal.org, and at https://doi.org/10.1053/j.gastro.2020.07.003.

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Conflicts of interest
The authors disclose the following: E.E. is a consultant to DayTwo and BiomX. None of the topics related to this work involve these or other commercial entities. None of the other authors have any financial or nonfinancial competing interest.

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