## 2. Materials and Methods

### *2.12. Organoid-derived monolayer culture*

Human small intestinal organoids were obtained from Dr. Jason's laboratory at the University of Michigan. Organoids were expanded as Matrigel dome cultures using growth factor-reduced Matrigel (Cat#356231; Corning) and IntestiCult™ Organoid Growth Medium (OGM). The complete OGM consisted of IntestiCult™ OGM Human Basal Medium (Cat#100-0190, STEMCELL Technologies) and IntestiCult™ Organoid Supplement (Cat#100-0191, STEMCELL Technologies), prepared according to the manufacturer's recommendations. Y-27632 (Cat#S1049; Selleck Chemicals) and Primocin (Cat#ant-pm-2; InvivoGen) were added where indicated. Organoids were passaged every 7–8 days by mechanical disruption followed by incubation in Gentle Cell Dissociation Reagent (Cat#100-0485, STEMCELL Technologies) for 5–10 min at room temperature. Pelleted fragments were washed briefly with DMEM/F-12 medium (Cat#36254, STEMCELL Technologies), resuspended in Matrigel, and overlaid with pre-warmed culture medium. Cultures were maintained at 37°C and 5% $CO_2$ in a humidified incubator, with medium replacement every 2–3 days. For monolayer generation, organoids were dissociated into single cells using a previously reported protocol with minor modifications [58]. Organoids were sequentially treated with Gentle Cell Dissociation Reagent and 0.05% Trypsin-EDTA (Cat#07910, STEMCELL Technologies). Trypsinization was stopped with DMEM/F-12 medium containing 10% fetal bovine serum (FBS). The resulting single-cell suspension was seeded onto Matrigel-coated HTS Transwell® 96-well inserts (0.4 μm pore size, Cat#7369, Corning). Cells were maintained in IntestiCult™ Organoid Differentiation Medium (ODM; Cat#100-0214, STEMCELL Technologies), and medium was replaced every other day. Barrier development was followed by transepithelial electrical resistance (TEER) measurements.

### *2.13. TEER measurements*

TEER was measured with the EVOM™ Auto TEER Measurement System (EVA-MT-03-01, World Precision Instruments). Final TEER values were obtained by multiplying the raw resistance readings by the membrane surface area of the Transwell insert.

### *2.14. Permeability assay*

Paracellular permeability was measured using 4 kDa FITC-dextran (Cat#46944-500MG-F, Sigma-Aldrich, USA). After PBS washing, 0.25 mM FITC-dextran was placed in the apical chamber, and ODM was added to the basolateral chamber. Monolayers were incubated for 1 h at 37°C with 5% $CO_2$, after which basolateral medium was collected. Fluorescence intensity was quantified using a SpectraMax iD5 plate reader (Molecular Devices). Values were corrected for ODM autofluorescence and normalized to the vehicle control.

### *2.15. Sample and cytokine treatment*

Monolayers were exposed to test samples diluted 1:100 in ODM for 24 h before cytokine challenge. Cytotoxicity was evaluated with the CellTiter-Glo® 3D Cell Viability Assay (Cat#G9682, Promega) following the manufacturer's protocol. After pretreatment, inflammatory barrier disruption was induced by adding recombinant human IFN-γ (Cat#300-02-20UG, Lot#091927; PeproTech) and recombinant human TNF-α (Cat#210-TA, Lot#DDHB0421121; R&D Systems), each at 40 ng/mL, for an additional 48 h. Monolayers were then processed for barrier function assays and molecular analyses.

*2.16. Immunofluorescence microscopy*

Monolayers were fixed in 4% paraformaldehyde for 10 min and permeabilized with 0.3% Triton X-100 in PBS for 20 min at room temperature. Cells were blocked for 1 h with 5% BSA in PBS. Primary antibodies against ZO-1 (Cat#33-9100, Lot#WL337900; Thermo Fisher Scientific), claudin-2 (Cat#51-6100, Lot#YG373730A; Invitrogen), villin (Cat#sc-58897, Lot#11624; Santa Cruz Biotechnology), MUC2 (Cat#MA5-12345, Lot#XC3543824; Invitrogen), and E-cadherin (Cat#3195, Lot#15; Cell Signaling Technology) were prepared in 2.5% BSA in PBS and applied overnight at 4°C. Following three PBS washes, samples were incubated for 1 h at room temperature in the dark with Alexa Fluor-conjugated secondary antibodies: Alexa Fluor™ 594 goat anti-mouse IgG (H+L) (Cat#A11037, Lot#3174388, Thermo Fisher Scientific), Alexa Fluor™ Plus 488 goat anti-rabbit IgG (H+L) (Cat#A32731, Lot#XA339480, Thermo Fisher Scientific), Alexa Fluor™ Plus 594 goat anti-mouse IgG (H+L) (Cat#A32742, Lot#WG328516), or Alexa Fluor™ Plus 488 goat anti-mouse IgG (H+L) (Cat#A32723, Lot#ZB388196, Thermo Fisher Scientific). Nuclei were counterstained with DAPI, and images were captured using a Leica DMI6000B microscope.

*2.17. Analysis of tight junction–associated genes*

Total RNA was extracted with the RNeasy Kit (QIAGEN), and cDNA was generated using the iScript cDNA Synthesis Kit (Bio-Rad) according to the manufacturers' protocols. Quantitative real-time PCR was performed on a Bio-Rad CFX96 Real-Time PCR Detection System. Tight junction-associated transcripts were analyzed using the RT² Profiler™ PCR Array Human Tight Junctions (Cat#330231, QIAGEN) with RT² SYBR® Green qPCR Mastermix (Cat#330502, QIAGEN). Melting curve analysis was used to verify PCR specificity, and GAPDH served as the internal reference gene.

*2.18. Immunoblotting*

For immunoblotting, treated samples were lysed in RIPA buffer containing protease inhibitors. Protein concentrations were measured using Pierce™ Dilution-Free™ BSA Protein Standards (Cat#186882, Lot#AB397429; Thermo Fisher Scientific). Equal protein amounts were combined with Bio-Rad sample buffer, heat-denatured, separated on Mini-PROTEAN TGX 4–20% precast gels (Cat#4561094, Bio-Rad), and transferred to low-fluorescence PVDF membranes (Cat#1620260, Bio-Rad) using the Trans-Blot Turbo Transfer System (Bio-Rad). Membranes were blocked with Intercept® TBS Blocking Buffer (Cat#927-60001, LI-COR Biosciences) for 1.5 h at room temperature and incubated with primary antibodies overnight at 4°C. After TBST washing, membranes were incubated with IRDye® secondary antibodies, including IRDye® 800CW goat anti-rabbit IgG (Cat#925-32211, Lot#D30110-01) or IRDye® 680RD goat anti-mouse IgG (Cat#925-68070, Lot#D302074; LI-COR Biosciences). Fluorescent signals were acquired using the Odyssey CLx Imaging System (LI-COR Biosciences) and quantified with Image Studio software.

*2.19. Statistical analysis for in vitro experiments*

Data were analyzed using GraphPad Prism 7 software (GraphPad Software, USA). Group differences were assessed by one-way analysis of variance (ANOVA). Statistical significance was defined as $p < 0.01$, and data are shown as mean ± SEM.