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Interferon gamma upregulates the cytokine receptors IFNGR1 and TNFRSF1A in HT-29-MTX E12 cells

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ABSTRACT

The intestinal mucosa protects the body from physical damage, pathogens, and antigens. However, inflammatory bowel diseases (IBDs) patients suffer from poor mucosal tissue function, including the lack of an effective cellular and/or mucus barrier. We investigated the mucus producing human colonic epithelial cell line HT29-MTX E12 to study its suitability as an *in vitro* model of cell/mucus barrier adaption during IBD. It was found that the proinflammatory cytokine interferon-gamma (IFN-γ), but not tumor necrosis factor-alpha (TNF-α), reduced cell viability. IFN-γ and TNF-α were found to synergize to decrease barrier function, as measured by *trans*-epithelial electric resistance (TER) and molecular flux assays. Cells cultured under an air-liquid interface produced an adherent mucus layer, and under these conditions reduced barrier function was found after cytokine exposure. Furthermore, IFN-γ, but not TNF-α treatment, upregulated the IFN-γ receptor 1 (IFNGR1) and TNF-α receptor super family 1A (TNFRSF1A) subunit mRNA *in vitro*. Co-stimulation resulted in increased mRNA expression of CLDN 2 and 5, two gene known to play a role in epithelial barrier integrity. Analysis of IBD patient samples revealed IFNGR1 and TNFRSF mRNA increased coincidently with guanylate binding protein 1 (GBP1) expression, an indicator of NFkB activity. Lastly, CLDN2 was found at higher levels in IBD patients while HNF4a was suppressed with disease. In conclusion, IFN-γ and TNF-α degrade epithelial/mucus barriers coincident with changes in CLDN gene and cytokine receptor subunit mRNA expression in HT29-MTX E12 cells. These changes largely reflect those observed in IBD patient samples.

1. Introduction

The ability of the intestinal mucosa to form a barrier against luminal antigens and pathogens is of primary importance to host innate immune defense. Failure of this barrier during inflammatory episodes is a core pathological feature of inflammatory bowel diseases (IBDs) such as Crohn's Disease (CD) and Ulcerative Colitis (UC) [1,2]. Importantly, advances in our understanding of the intestinal mucosal barrier reveal that both mucus and cellular epithelial barriers must be maintained to prevent disease [3]. *In vitro* cell culture systems offer an experimentally tractable model for exploring how this dual barrier fails following an inflammatory stimulus.

HT29-MTX E12 cells are an intestinal adenocarcinoma cell line distinct from the HT29 parental strain in that they produce mucus as well as form measurable cellular barrier [4]. However, functional studies detailing the properties of dual mucus/epithelial barrier models after proinflammatory stimulus are limited. Mucus has two essential roles in the intestine; it acts as a lubricant for fecal transit, and forms a barrier to intestinal microbes [5]. The ability to perform these functions is degraded in IBD [6]. HT29-MTX E12 cells produce both secreted and membrane-bound mucins [7]. Importantly, a semi-wet, air-liquid interface (ALI) cell culture environment is needed to produce an adherent mucus layer [8].

Tumor Necrosis Factor Alpha (TNF-α) and Interferon Gamma (IFN-γ) are proinflammatory cytokines commonly found at elevated levels in IBD intestinal tissues [9]. In addition to modifying immune functions, these cytokines also impact epithelial cell barrier capacity and viability in a complex temporal and concentration-dependent manner [10]. The effects of inflammatory stimuli on epithelial cells have been well documented, with cytokine treatment resulting in decreased barriers

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effectiveness [11,12]. This loss is thought to proceed via two mechanisms: apoptosis, and changes in the function of tight junctions structures that seal adjacent cells [10,13]. At the molecular level, the tight junctions seal is semipermeable due to heterogeneous claudin protein expression [13]. The transcription factor Hepatocyte nuclear factor 4a (HNF4a) has been shown to regulate claudin gene expression and has been linked to IBD by genome-wide association studies [14,15]. During inflammation, cytokine-induced changes in epithelial barrier function occur after ligand-receptor binding. IFN-γ and TNF-α cytokines bind to Interferon gamma receptor 1 (IFNGR1), which encodes the ligand-binding alpha subunit, and members of the Tumor necrosis factor receptor superfamily members (TNFRSF), respectively [16,17]. Receptor activation stimulates second messenger activity, altering a number of cellular processes, including gene transcription through the NFκB pathway [16,18]. NFκB regulates a number of inflammatory response genes in epithelial cells, including Guanylate binding protein 1 (GBP1), which functions as a stasis signaling molecule in intestinal epithelial cells [19]. The study below utilizes GBP1 mRNA expression as a positive control indicating cytokine signaling in cells or tissues.

HT29-MTX E12 have been recently employed as a model system to study drug adsorption and host-microbe interactions, frequently in co-culture with the better characterized intestinal epithelial cell line, Caco-2 [20–23]. Given that under physiological circumstances the above processes likely take place in the presence of proinflammatory cytokines, a better understanding of HT29-MTX E12 cytokine responses would aid in the design of more robust invitro disease models. We examined HT29-MTX E12 barrier function under normal growth conditions, following cytokines stimulation, as well as under cell culture conditions that produce a mucus layer. These investigations revealed synergistic effects between TNF-α and IFN-γ that occur coincident with upregulation of IFNGR1 and TNFRSF cytokine receptors. Alterations of these receptors were also found to occur in IBD patients, as IFNGR1 and TNFRSF are overrepresented in tissue-derived cDNA samples that contain correspondingly high levels of the NFκB activity marker GBP1.

2. Methods:

2.1. Cell culture

Caco-2 were purchased from ATCC (ATCC, USA). HT29-MTX E12 cells were obtained through Millipore/Sigma (Merck, USA) via the European Collection of Authenticated Cell Cultures and maintained in DMEM with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, 2 mM L-glutamate and 1% MEM amino acids solution (Cytiva, USA). Prior to cytokine exposure, cells were cultured at 8% CO_2, 37 °C until 80% confluent with media changes every other day. HT29-MTX E12 cells were cultured on 0.4 μm pore Transwell® filter systems (Corning, USA). For standard liquid interface cultures (LI), 250 μL growth media was placed in the top chamber and 500 μL in the bottom. LI cultures treated with cytokine after monolayers achieved TER of ~ 300 Ω•cm^2. ALI conditions were generated by removal of media from the top chamber once the above-mentioned TER was achieved. After transition to ALI, cells were then treated with TNF-α and IFN-γ at 2 ng/ml each for 48 h unless otherwise indicated (bottom chamber, BioVision, USA).

2.2. Alcian blue staining

HT29-MTX E12 cells were grown on a 96-well plate and each cytokine treatment was performed in triplicate for each concentration. After ~14 days of incubation, growth media was removed and cells were stained with an Alcian blue dye for 15 min (Merck, Germany). The cells were rinsed with water twice. Excess water was removed by tapping the plate onto a clean paper towel. The absorbance of each set of cells was then measured using the Biotech Synergy HTX Multi-Mode plate Reader (Agilent, USA) at 595 nm as a measure of mucus and the number of mucus-producing cells present.

2.3. Barrier assay

HT29-MTX E12 cells were grown on 0.4 μm pore Transwell® filter systems and monolayers were monitored for electrical resistance using an epithelial volt-ohmmeter (EVOM/EndOhm; World Precision Instruments, USA). Molecule flux assays utilized 25 μg 4 kDa FITC Dextran in the top chamber. This amount was delivered in 250 μL and 10 μL of PBS 2% FBS in LI and ALI conditions, respectively. Contents of the bottom chamber were assessed after 1 hr. 4 kDa FITC Dextran detection was performed using a BioTek Synergy HTX Multi-Mode plate reader (Agilent, USA).

2.4. mRNA purification and Real-Time Quantitative PCR (RT-qPCR)

HT29-MTX E12 cells treated as above were frozen at −80°C with 1 ml RiboZol RNA extraction reagent (VWR, USA) and processed according to the manufacturer's instructions. The Bio-Rad iScript Reverse Transcription Supermix for RT-qPCR was used to produce cDNA; it was performed with the SsoAdvanced Universal SYBR Green Supermix on a C1000 Touch CFX96 Real-Time System (Bio-Rad Laboratories, USA). Human patient cDNA was obtained from Origene Tissue scan TSC10765-CCRT502 (Origene, USA). Full details concerning these samples can be found at: https://www.origene.com/catalog/tissues/tissuescan/ccrt502/tissuescan-crohnscolitis-cdna-array-ii). Primer sequences can be found in Supplemental Table 1.

2.5. Statistics and meta-analysis

Transcriptomic studies were selected based on data availability and inclusion of UC patients. GEO accession GSE109142, GSE59071, GSE48958, and GSE117993 are summarized in Table 1 after screening for TNFRSF genes and GBP1. Original data, analysis, and project details are available in the primary text. Statistics were performed using GraphPad Prism 9 (GraphPad Software, USA). Biological replicates are indicated, containing at least three technical replicates; error bars are SEM.

3. Results and Discussion

HT29-MTX E12 cells are a valuable system for the study of the colonic barrier, and serve as a model to investigate the function of a dual mucus/cellular barrier. However, the cytokine response of these cells is not well understood (24). To better understand cellular cytokine responses in these cells, HT29-MTX E12s were grown to ~90% confluence and treated with IFN-γ and TNF-α, two cytokines found at high levels in IBD patients and commonly used in *in vitro* cell models of inflammation [12,25]. After 48 h treatment with cytokines, cells were stained with Alcian blue in order to assess mucus production and cell viability. Fig. 1A shows that Alcian blue stained prominent mucus vacuoles within the cells, in addition to a light apical glycocalyx stain. Treatment with low concentrations of IFN-γ (48 h, 2 ng/ml) resulted in a reduction of mucus vacuoles within the monolayer (Fig. 1B). At higher IFN-γ concentrations, low power magnification revealed a stark contrast in Alcian blue staining between untreated control cells (Fig. 1C) and cells treated for 48 h with 100 ng/ml IFN-γ (Fig. 1D). In these IFN-γ treated cultures, an absence of stained vacuoles was again noted, however, the decrease in staining was primarily due to cell loss (Fig. 1C/D). As detailed in Methods above, stained cells are washed prior to imaging, thereby removing dead cells and debris. Therefore, Alcian blue uptake by HT29-MTX E12 cells allowed for the assessment of cell vitality and mucus production by absorbance measurements at 595 nm. The resulting dose response curves are shown in Fig. 1E/F. IFN-γ treatment reduced cell viability in a dose dependent manner as concentrations increased above 1.5 ng/ml (Fig. 1E). Remarkably, TNF-α treatment did not appreciably reduce cell viability under the conditions tested (Fig. 1E). Further studies demonstrated that the addition of TNF-α did not significantly

Table 1
TNFRSF gene family members are commonly upregulated in IBD patients coincident with increased GBP1. Transcriptome *meta*-analysis of IBD patient studies show upregulation of tumor necrosis factor alpha receptor superfamily members (TNFRSF). FC = fold change vs indicated control. UC = ulcerative colitis, IBD = both UC and Crohn's Disease. GEO accession number as indicated.

Gene name	GSE109142		GSE59071		GSE48958		GSE117993		mean FC	SD
	FC UC vs Ctl	p value UC vs Ctl	FC IBD vs Ctl	p value IBD vs Ctl	FC UC vs Ctl	p value UC vs Ctl	FC IBD vs Ctl	p value IBD vs Ctl		
TNFRSF1B	1.64	2.08E−08	1.79	1.12E−06	–	–	1.53	3.51E−09	1.65	0.12
TNFRSF4	6.17	6.37E−39	–	–	–	–	2.24	1.15E−10	4.21	2.27
TNFRSF6B	56.22	<5.34E−43	–	–	–	–	26.14	1.27E−20	41.18	17.36
RTEL1-TNFRSF6B	8.07	<5.34E−43	–	–	–	–	2.28	9.68E−09	5.17	3.34
TNFRSF9	4.11	7.14E−18	3.12	6.76E−10	4.73	2.10E−06	2.78	7.59E−07	3.69	0.83
TNFRSF10B	–	–	1.62	7.78E−09	–	–	–	–	–	–
TNFRSF10C	7.52	1.71E−21	–	–	–	–	5.60	3.39E−14	6.56	1.11
TNFRSF11A	−1.77	1.04E−07	–	–	–	–	–	–	–	–
TNFRSF11B	1.74	6.24E−04	2.89	5.83E−05	2.1	1.09E−02	–	–	2.24	0.53
TNFRSF12A	2.18	5.41E−08	1.92	1.27E−07	–	–	1.76	8.53E−04	1.96	0.19
TNFRSF13B	2.07	1.22E−06	–	–	–	–	–	–	–	–
TNFRSF13C	3.11	1.86E−07	–	–	–	–	–	–	–	–
TNFRSF14	1.51	4.36E−17	–	–	–	–	–	–	–	–
TNFRSF17	2.57	1.62E−19	2.48	3.41E−05	2.91	5.48E−05	1.54	1.18E−04	2.38	0.54
TNFRSF18	5.63	1.79E−42	–	–	–	–	1.84	5.97E−09	3.73	2.19
TNFRSF21	−1.66	1.44E−09	–	–	–	–	–	–	–	–
TNFRSF25	1.58	1.32E−06	–	–	–	–	–	–	–	–
GBP1	5.59	9.75E−27	2.03	5.21E−07	2.22	3.35E−04	3.08	1.08E−10	3.23	1.52
Method	RNA seq		Gene chip		Gene chip		RNA seq			
Patients	pediatric		adult		adult		pediatric			
Undergoing treatment	no		yes		yes		no			

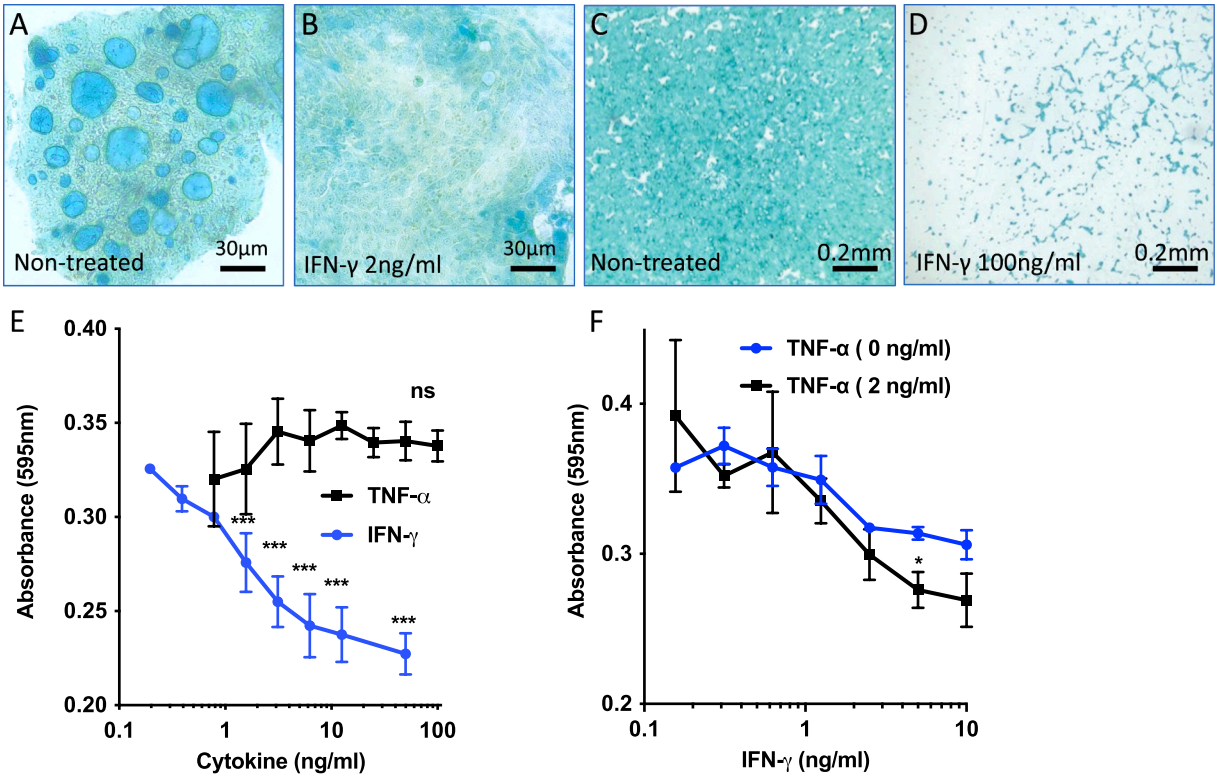

Fig. 1. IFN-γ, but not TNF-α treatment, reduces HT29-MTX E12 epithelial cell viability. **A/B.** HT29-MTX E12 cells stained with Alcian blue to detect acidic mucins (scale bar = 30 µm) showing untreated control and IFN-γ treated cells (48 h with 2 ng/ml). **C/D.** Alcian blue staining reveals reduced cell numbers after treatment. Low magnification images (scale bar = 0.2 mm) showing untreated control and IFN-γ treated cells (48 h with 100 ng/ml). **E.** Alcian blue stained HT29-MTX E12 cells assessed for absorbance at 595 nm after 48 h treatment at indicated doses of IFN-γ or TNF-α; n = 3–4. *** = $p < 0.001$ ANOVA Dunnett post-test vs untreated control, ns = not significant. **F**. Dose response curve of IFN-γ treated cells with or without 2 ng/ml TNF-αafter 48 h. * = $p < 0.05$; n = 3–4.

alter IFN-γ-induced changes to cell viability below IFN-γ concentrations of 5 ng/ml (Fig. 1F). Together, these studies demonstrate a simple, novel, and effective methodology for assessing cytokine responses in HT29-MTX E12 cells.

The epithelial cellular barrier is comprised of a cell monolayer joined together by tight junction protein structures (13). Alterations in cell barrier effectiveness can result from either increased cell death or changes in the composition of tight junction [10,11]. The experiments detailed in Fig. 1 allowed us to study barrier adaptation at sub-lethal concentration of cytokine (2 ng/ml for 48 h), thereby allowing for the investigation of cellular factors that regulate barrier function independent of apoptosis. Fig. 2A shows a schematic of the Transwell® system that was used to assess barrier function across a suspended semi-permeable membrane. Growth media is present in both the top and bottom chamber; conditions forming a liquid interface (LI). The barrier integrity of HT29-MTX E12 cells grown in this fashion were monitored for Trans-epithelial resistance (TER) as a measure of barrier integrity (Fig. 2B). Cells were then treated with cytokines as indicated and barrier function was assessed following 48 h of treatment. As shown in Fig. 2B, both IFN-γ and TNF-α treatments suppressed barrier function as indicated by lowered TER. Additionally, cotreatment of IFN-γ and TNF-α resulted in further suppression of TER to values below those found with either individual treatment alone (Fig. 2B).

In addition to the regulation of ion flow, tight junction structures form a barrier against the paracellular passage of small molecules, termed the leak pathway (26]. To determine how HT29-MTX E12 cells adapt to cytokine exposure, 4 kDa FITC Dextran was added to the apical surface of the Transwell® cell culture system and flux was monitored by sampling the bottom chamber after between 1 and 2 h following addition of the tracer. As shown in Fig. 2C, 48 hr treatment with IFN-γ, but not TNF-α, resulted in an increase in 4 kDa FITC dextran flux. However, in concordance with our findings in Fig. 2B, cotreatment of IFN-γ and TNF-α produced a dramatic increase in molecular flux. As a control, unseeded wells were assessed with 4 kDa FITC dextran to determine maximal rate of flux (max, Fig. 2C). Together, these findings demonstrate the both IFN-γ and TNF-α depress barrier function and that cotreatment elicits a synergistic suppression.

HT29-MTX E12 cells produce an adherent mucus layer in addition to a polarized epithelial layer, and recent studies have used HT29-MTX E12 cells to model colonic mucosal tissue [4,8,26,27]. However, studies evaluating dual mucus-epithelial barrier adaptation to cytokines are limited [24]. To address this knowledge gap we altered cell culture

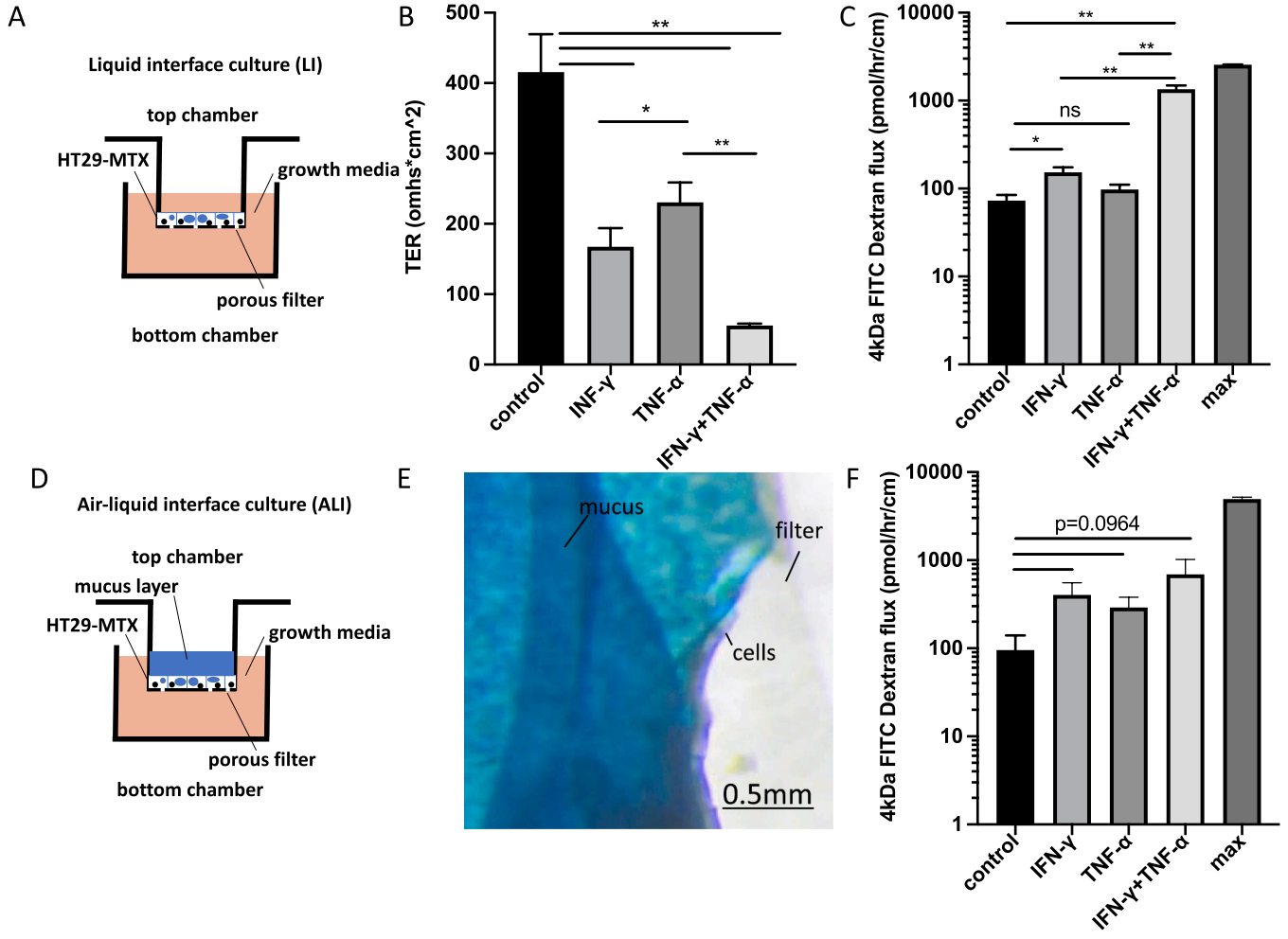

Fig. 2. IFN-γ and TNF-α synergize to decrease epithelial barrier function in HT29-MTX cells. **A.** Schematic showing liquid interface (LI) growth conditions on Transwell® filters. **B.** Assessment of ion flux by *trans*-epithelial electrical resistance (TER) in HT29-MTX E12 cells in the presence of IFN-γ, TNF-α or both cytokines together (each at 2 ng/ml for 48 h). **C.** Assessment of the ability of HT29-MTX E12 cells to restrict the passage of small molecules (4 kDa FITC dextran) in the presence of IFN-γ, TNF-α or both cytokines together (each at 2 ng/ml for 48 h); n = 5–6, ns, not significant, * = $p < 0.05$, ** = $p < 0.01$ by one-way ANOVA, Tukey post-test. Max equal maximum flux rate across empty filters (max); n = 12. **D.** Schematic demonstrating air-liquid interface (ALI) growth conditions that allow for adherent mucus formation. **E.** Whole-mount Alcian Blue stained cells after ALI growth conditions showing mucus layer. **F.** Assessment of the epithelial/mucus dual barrier to restrict the passage of small molecules (4 kDa FITC dextran), in the presence of IFN-γ and/or TNF-α cytokine (each at 2 ng/ml for 48 h); n = 5–6. p value by matched ANOVA, Holm-Sidek post-test.

procedures to provide an air-liquid interface (ALI), previously described as a semi-wet culture method [8]. In short, HT29-MTX E12 cells were grown on Transwell® filters until confluent and the TER had reached ~ 300 Ohms*cm^2 (approximately 2 weeks). Growth media was then removed from the apical chamber, stimulating the accumulation of an apical mucus layer (Fig. 2D). To visualize the mucus layer, filters were excised and stained with Alcian blue. The image in Fig. 2E shows a stained filter with the mucus layer folded backwards to reveal the cells underneath. After one week in ALI conditions, cytokines were added to the bottom chamber, and barrier function was assessed following 48 h of treatment as in Fig. 2 (Fig. 2F). TER could not be evaluated in these cultures due to the lack of fluid in the apical chamber. Therefore, dextran flux was investigated by the addition of 4 kDa FITC dextran to the apical surface. After 1–2 h, the media in the bottom chamber was assayed for the presence of FITC dextran by fluorescence spectrometry (Fig. 2F). Similar to our findings in Fig. 2C, cytokine treatment increased barrier permeability. This was observed for both IFN-γ and TNF-α individually, as well as for co-stimulation of IFN-γ and TNF-α together, exhibiting trends that indicate co-stimulation results in reduced barrier integrity. Together these data show that IFN-γ and TNF-α decrease HT29-MTX E12 barrier function in both LI and ALI interface cultures.

IBDs are caused by complex pathologies where multiple cytokines likely act simultaneously on epithelial cells to inhibit epithelial barrier function [28,29]. Previous studies in Caco-2 cells demonstrated that IFN-γ and TNF-α cotreatment reduce barrier function and upregulate TNF cytokine receptor expression [30,31]. To better understand our findings that IFN-γ and TNF-α cotreatment depresses barrier function to a greater extent than either cytokine alone, we examined the mRNA expression of cytokine receptor subunits after cotreatment (Fig. 3 A/B). Expression of Interferon gamma receptor 1 (IFNGR1), which encodes the ligand binding alpha subunit and Tumor necrosis factor receptor superfamily member 1A (TNFRSF1A) were examined by qPCR in HT29-MTX E12 cells 48 h after cotreatment with IFN-γ and TNF-α. As shown in Fig. 3A/B, both IFNGR1 and TNFRSF1A mRNA were upregulated after cytokine treatment. In contrast, no change in receptor mRNA was observed in Caco-2 cells at these cytokine concentrations, indicating greater cytokine sensitivity in HT29-MTX E12 cells. Given the functional enhancement of IFN-γ and TNF-α cotreatment, receptor mRNA expression was assessed after treatment with IFN-γ or TNF-α alone. As seen in Fig. 3C, IFN-γ exposure induced expression of both IFNGR1 and TNFRSF1A whereas TNF-α stimulation had no discernable effect on receptor mRNA. As a control to assess the level of cytokine stimulation on cellular transcription, we monitored guanylate binding protein 1 (GBP1), an interferon stimulated gene [32]. As shown in Fig. 3D, TNF-α did not upregulate GBP1 in HT29-MTX E12 cells at these cytokine concentrations, whereas IFN-γ or cotreatment resulted in large increases in GBP1 mRNA quantity. We conclude that IFN-γ/TNF-α reduction in barrier function is coincident with increases in cytokine receptor mRNA and increased expression GBP1.

The above data indicate reduced barrier loss in HT29-MTX cells after co-treatment with IFN-γ and TNF-α. Previous studies have shown that cytokine treatment alters the mRNA expression of genes involved in regulating barrier tightness [12]. In order to better characterize HT29-MTX cells cytokine responses with respect to barrier function, the mRNA levels of the transcription factor HNF4a, an IBD-linked gene, was investigated. HNF4a mRNA can be produced from promoters P1 and/or P2, resulting in isoforms with distinct N-termini (Fig. 4A). Previous studies have shown that P2 is spatial restricted to the crypt proliferative zone and increases colitis susceptibility in mice [33]. Using RT-qPCR, we demonstrate that HNF4a is expressed in HT29-MTX cells primarily as the P2 isoform (Fig. 4B). Treatment with IFN-γ and TNF-α did not significantly alter HNF4a levels, however we note that P1 levels trended higher after treatment in all tests. HNF4a is known to regulate CLDN gene expression. Claudin proteins compose the transcellular component of tight junctions and are vital for paracellular ion/antigen regulation [10]. We therefore investigated HT29-MTX cells to assess CLDN mRNA expression, limiting our analysis to the most abundant colonic CLDN gene family members. The CLDN family members detected are indicated in Fig. 4C; CLDN8 and 10 were assessed but not detected. Following cytokine treatment CLDN2 and CLDN5 were found to be unregulated relative to non-treated control (Fig. 4C). Enhanced CLDN2 and CLDN5 expression is associate with "leaky" tissues *in vivo*, and is consistent with our findings that HT29-MTX cells exhibit reduced barrier function.

IFN-γ and TNF-α are commonly found expressed at high levels in the intestinal tissue in IBD patients [9]. Given our above findings that IFN-γ and TNF-α increase cytokine receptor mRNA levels, IBD patient samples were examined for relative mRNA levels of IFNGR1 and TNFRSF1A (Figure 5). RT-qPCR was performed using cDNA libraries form 47 patient samples. Patient sample arrays contained cDNA from control, UC, and CD patients collected from rectum, colon, or small intestine. RT-qPCR was performed using primers for IFNGR1 and TNFRSF1A, as well as GBP1 mRNA levels, which were assessed as an indicator of NFκB stimulation. As shown in Figure 5A, a robust positive correlation was discovered between IFNGR1 and GBP1 levels in all samples (p =

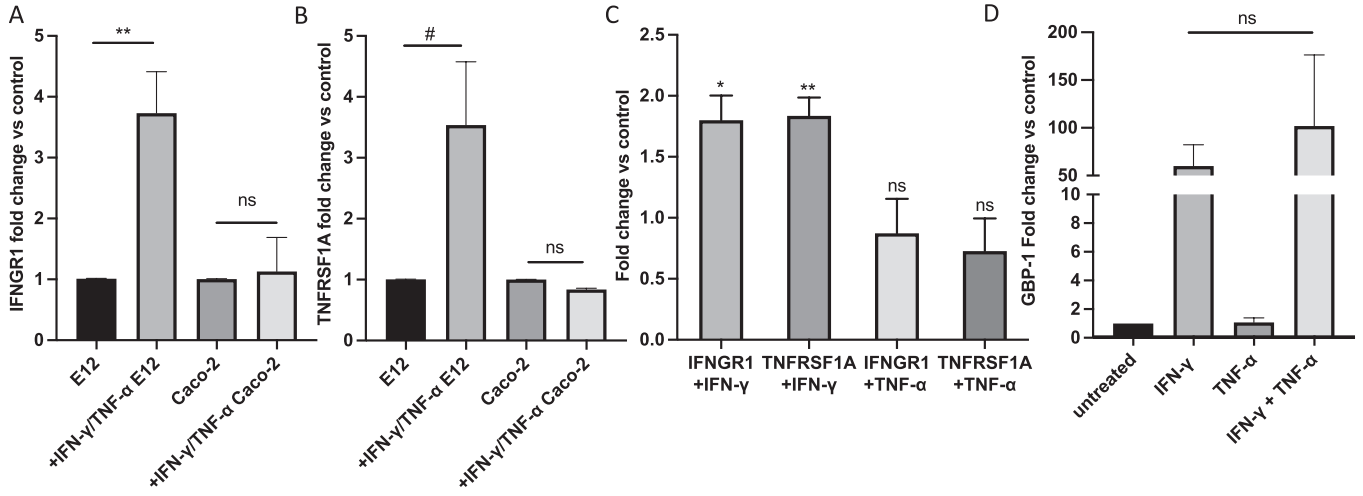

Fig. 3. IFN-γ exposure results in increased mRNA expression of IFNGR1 and TNFRSF1A cytokine receptors. **A.** HT29-MTX E12 (E12) and Caco-2 cells treated for 48 h with both IFN-γ and TNF-α (2 ng/ml each). Relative levels of IFNGR1 mRNA were determined by RT-qPCR. ** = $p < 0.01$; n = 2–4. **B.** TNFRSF1A mRNA is upregulated in HT29-MTX E12 cells but not in Caco-2 cells after cytokine treatment. # = $p < 0.1$; n = 2–4. **C.** IFN-γ, but not TNF-α, increase cytokine receptor mRNA expression. * = $p < 0.05$, ** = $p < 0.01$; n = 4. **D.** GBP1, an NFκB responsive gene, increases mRNA levels after IFN-γ treatment. Ns = not significant; n = 3–8. one-way ANOVA, Dunnett post-test for all comparisons.

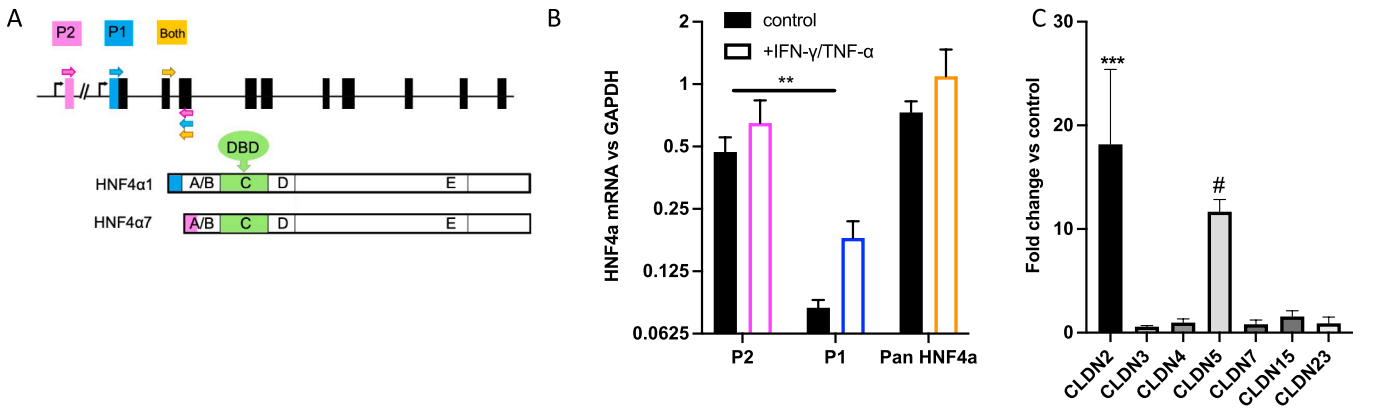

Fig. 4. HNF4a and CLDN mRNA in HT29-MTX E12 cells after IFN-γ and TNF-α stimulation. **A.** Schematic showing PCR primer placement along the HNF4a gene. Exons are depicted as vertical bars and colored arrows indicate approximate primer locations. Protein schematic illustrating protein domains in each isoform (protein domains as indicated, DBD = DNA binding domain). **B.** HT29-MTX E12 (E12) cells treated for 48 h with both IFN-γ and TNF-α (2 ng/ml each) and assayed by RT-qPCR for HNF4a isoform mRNA. Primers used are as indicated. One way ANOVA, P2 vs P1 $p < 0.01$, all other comparisons are not significant. **C.** HT29-MTX E12 (E12) cells treated for 48 h with both IFN-γ and TNF-α (2 ng/ml each). Relative levels of CLDN mRNA were determined by RT-qPCR. By one way ANOVA vs. CLDN3 n = 3–4. *** = $p < 0.001$, # = $p < 0.1$.

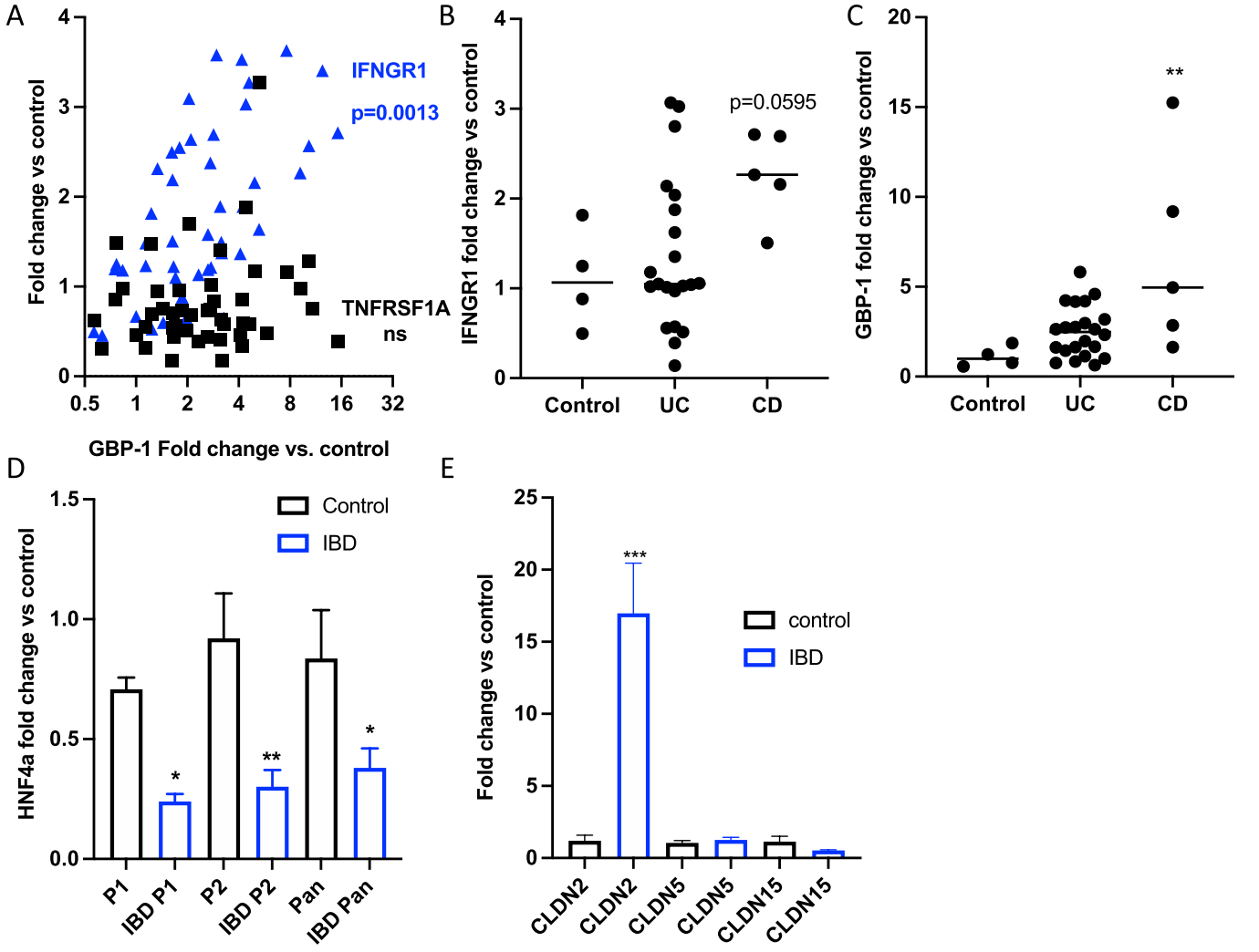

Fig. 5. INFGR1 mRNA expression correlates with GBP1 in human UC and CD colon tissue samples. **A.** Human patient cDNA from histologically normal (n = 7), UC (n = 27) and CD (n = 14) biopsies screened by RT-qPCR for IFNGR1, TNFRSF1A, and GBP1 (as an indicator of cellular NFκB response). The resulting GBP1 values were correlated with IFNGR1 and TNFRSF1A mRNA levels normalized to control colon patient samples. Pearson correlation, $p = 0.0013$, $r = 0.4683$. ns = not significant. **B.** Human colon samples assessed for IFNGR1 mRNA. **C.** Human colon samples assessed for GBP1 mRNA. ** = $p < 0.01$. **D.** Human patient cDNA from histologically normal colon samples (n = 4), or IBD (UC (n = 27) and CD (n = 5)) biopsies screened by RT-qPCR for HNF4a isoforms. **=$p < 0.01$, *=$p < 0.05$. **E.** Biopsies screened by RT-qPCR for CLND 2, 5 or 15. *** = $p < 0.001$. one-way ANOVA, Dunnett post-test for all comparisons.

0.0013). This was not the case for TNFRSF1A, which did not demonstrate a relationship with GBP1 levels. A significant increase in IFNGR1 mRNA was observed in an analysis that include only colon tissues (Fig. 5B). Additionally, these same samples also expressed an increased level of GBP1 mRNA (Fig. 5C). IBD patient samples were then examined for the occurrence of HNF4a isoforms and CLDN genes (Fig. 5D/E). Unlike HT29-MTX cells, IBD patient samples exhibited lowered levels of HNF4a isoforms in comparison to histologically normal colon controls (Fig. 5D). Examination of CLDN 2, 5 and 15 revealed a dramatic upregulation of CLDN2 only, when compared to controls. Importantly, claudin 2 is known to form a cation channel in epithelial tissues [13]. Together, the findings demonstrate general concurrence between IBD samples and HT29-MTX responses to cytokine exposure, with the notable exception of reduced HNF4a levels.

We next sought to investigate the discrepancy between our *in vitro* and *in vivo* findings with respect to TNFRSF1A, which is upregulated in HT29-MTX E12 cells but not in IBD samples. TNF receptors are composed of a large superfamily of 29 genes [34]. To assess the possibility that alternative TNFRSF genes were upregulated in IBD, we performed a meta-analysis of available transcriptomic studies (Table 1). Four studies were selected that analyzed rectal or colon biopsies were taken from healthy controls and IBD patients, encompassing both pediatric and adult IBD patients. Indeed, TNFRSF gene family members are commonly upregulated in IBD patients compared to controls, and this is coincident with increased levels of GBP1. Interestingly, TNFRSF1A was not reported in these studies, and not all upregulated receptors were found in all studies. These differences in detection may be attributable to the cellular heterogeneity in patient biopsies as well as our comparison to a clonal epithelial cell line. Combined with our previous data, these findings demonstrate increased IFNGR1 and TNFRSF receptor levels in IBD tissues that also express high levels of GBP1.

IBDs are conditions of globally increasing incidence with an accelerating expected global health burden [35]. Our findings will allow for further investigation of mucosal barrier function in an *in vitro* model of mucus/cellular barrier during exposure to proinflammatory cytokines. Furthermore, future studies can focus on cellular contributions to mucus function, as barrier adaption was observed at cytokine concentrations below levels shown to increase apoptosis. Our studies demonstrate that HT29-MTX E12 cells respond to cytokines in a similar manner to the well-studied epithelia cell line Caco-2. As in Caco-2 cells, IFN-γ and TNF-α cooperate to alter HT29-MTX E12 barrier properties, likely through the upregulation of cytokine receptors. However, this occurs at lower cytokine concentrations than are commonly used in Caco-2 studies (IFN-γ 2 ng/ml vs. 10 ng/ml [30,36]. Therefore, studies of cytokine stimulation in mixed Caco-2/HT29-MTX E12 should consider the complication of divergent responses when utilizing these two cell lines simultaneously. Additionally, we find that both *in vitro* and *in vivo* cytokine receptors are upregulated during inflammatory stimulus, indicating that HT29-MTX E12 may be an effective model system for the study of epithelial cytokine exposure. Interestingly, while CLDN2 levels are altered both *in vivo* and *in vitro*, HNF4a expression levels are not suppressed in HT29-MTX cells after inflammatory stimulation. Given that HT29-MTX cells are cancer cells, incongruities between transcription factors in patient samples and in tumor-derived cell culture models are to be expected. However, further studies will be required to better understand these phenomena. In conclusion, our finds show synergy between IFN-γ and TNF-α coincident with upregulation of cytokine receptors and CLDN2. These findings add to our understanding of cellular cytokine response in this system. Furthermore, we demonstrate similarities between HT29-MTX E12 cytokine responses and those found in IBD; a correspondence indicative of a robust *in vitro* disease model.

CRediT authorship contribution statement

Brandon Johnson: Formal analysis, Writing – original draft, Writing – review & editing. **Paulina Panek:** Formal analysis, Writing – original draft, Writing – review & editing. **Andy Yu:** Writing – review & editing. **Elizabeth Fischer:** Writing – review & editing. **Marli Koba:** Writing – review & editing. **Daniel Mendoza Hermosillo:** Writing – review & editing. **Christopher T. Capaldo:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Christopher Capaldo reports financial support was provided by Hawaii Pacific University.

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References

[1] G. Roda, S. Chien Ng, P.G. Kotze, M. Argollo, R. Panaccione, A. Spinelli, A. Kaser, L. Peyrin-Biroulet, S. Danese, Crohn's disease, Nat. Rev. Dis. Primers. 6 (1) (2020), https://doi.org/10.1038/s41572-020-0156-2.
[2] T. Kobayashi, B. Siegmund, C. Le Berre, S.C. Wei, M. Ferrante, B.o. Shen, C. N. Bernstein, S. Danese, L. Peyrin-Biroulet, T. Hibi, Ulcerative colitis, Nat. Rev. Dis. Primers 6 (1) (2020), https://doi.org/10.1038/s41572-020-0205-x.
[3] C.T. Capaldo, D.N. Powell, D. Kalman, Layered defense: how mucus and tight junctions seal the intestinal barrier, J Mol Med (Berl). 95 (9) (2017) 927–934.
[4] T. Lesuffleur, A. Barbat, E. Dussaulx, A. Zweibaum, Growth adaptation to methotrexate of HT-29 human colon carcinoma cells is associated with their ability to differentiate into columnar absorptive and mucus-secreting cells, Cancer Res. 50 (19) (1990) 6334–6343.
[5] P. Paone, P.D. Cani, Mucus barrier, mucins and gut microbiota: the expected slimy partners? Gut 69 (12) (2020) 2232–2243.
[6] A. Swidsinski, V. Loening-Baucke, F. Theissig, H. Engelhardt, S. Bengmark, S. Koch, H. Lochs, Y. Dorffel, Comparative study of the intestinal mucus barrier in normal and inflamed colon, Gut 56 (3) (2007) 343–350.
[7] G. Huet, I. Kim, C. de Bolos, J.M. Lo-Guidice, O. Moreau, B. Hemon, C. Richet, P. Delannoy, F.X. Real, P. Degand, Characterization of mucins and proteoglycans synthesized by a mucin-secreting HT-29 cell subpopulation, J. Cell Sci. 108 (3) (1995) 1275–1285.
[8] N. Navabi, M.A. McGuckin, S.K. Lindén, B. Foligne, Gastrointestinal cell lines form polarized epithelia with an adherent mucus layer when cultured in semi-wet interfaces with mechanical stimulation, PLoS ONE 8 (7) (2013) e68761.
[9] M.F. Neurath, Cytokines in inflammatory bowel disease, Nat. Rev. Immunol. 14 (5) (2014) 329–342.
[10] A. Buckley, J.R. Turner, Cell Biology of Tight Junction Barrier Regulation and Mucosal Disease, Cold Spring Harb Perspect Biol. 10 (1) (2018) a029314, https://doi.org/10.1101/cshperspect.a029314.
[11] J.D. Schulzke, S. Ploeger, M. Amasheh, A. Fromm, S. Zeissig, H. Troeger, J. Richter, C. Bojarski, M. Schumann, M. Fromm, Epithelial tight junctions in intestinal inflammation, Ann. N. Y. Acad. Sci. 1165 (1) (2009) 294–300.
[12] C.T. Capaldo, A. Nusrat, Cytokine regulation of tight junctions, BBA 1788 (4) (2009) 864–871.
[13] C. Zihni, C. Mills, K. Matter, M.S. Balda, Tight junctions: from simple barriers to multifunctional molecular gates, Nat. Rev. Mol. Cell Biol. 17 (9) (2016) 564–580.
[14] A.E. Farkas, R.S. Hilgarth, C.T. Capaldo, C. Gerner-Smidt, D.R. Powell, P. M. Vertino, et al., HNF4alpha regulates claudin-7 protein expression during intestinal epithelial differentiation, Am. J. Pathol. 185 (8) (2015) 2206–2218.
[15] Consortium UIG, J.C. Barrett, J.C. Lee, C.W. Lees, N.J. Prescott, C.A. Anderson, et al., Genome-wide association study of ulcerative colitis identifies three new susceptibility loci, including the HNF4A region, Nat. Genet. 41 (12) (2009) 1330–1334.
[16] P. Gough, I.A. Myles, Tumor Necrosis Factor Receptors: Pleiotropic Signaling Complexes and Their Differential Effects, Front. Immunol. 11 (2020), 585880.
[17] M.A. Farrar, R.D. Schreiber, The molecular cell biology of interferon-gamma and its receptor, Annu. Rev. Immunol. 11 (1) (1993) 571–611.
[18] W.M. Schneider, M.D. Chevillotte, C.M. Rice, Interferon-stimulated genes: a complex web of host defenses, Annu. Rev. Immunol. 32 (1) (2014) 513–545.

[19] N. Britzen-Laurent, C. Herrmann, E. Naschberger, R.S. Croner, M. Sturzl, Pathophysiological role of guanylate-binding proteins in gastrointestinal diseases, World J. Gastroenterol. 22 (28) (2016) 6434–6443.
[20] A. Ferraretto, M. Bottani, P. De Luca, L. Cornaghi, F. Arnaboldi, M. Maggioni, A. Fiorilli, E. Donetti, Morphofunctional properties of a differentiated Caco2/HT-29 co-culture as an in vitro model of human intestinal epithelium, Biosci. Rep. 38 (2) (2018), https://doi.org/10.1042/BSR20171497.
[21] G. Ozkan, T. Kostka, T. Esatbeyoglu, E. Capanoglu, Effects of Lipid-Based Encapsulation on the Bioaccessibility and Bioavailability of Phenolic Compounds, Molecules 25 (23) (2020) 5545, https://doi.org/10.3390/molecules25235545.
[22] A. Strugari, M. Stan, S. Gharbia, A. Hermenean, A. Dinischiotu, Characterization of Nanoparticle Intestinal Transport Using an In Vitro Co-Culture Model, Nanomaterials (Basel) 9 (1) (2018) 5, https://doi.org/10.3390/nano9010005.
[23] C.M. Feather, J.M. Hawdon, J.C. March, Ancylostoma ceylanicum infective third-stage larvae are activated by co-culture with HT-29-MTX intestinal epithelial cells, Parasit Vectors. 10 (1) (2017) 606.
[24] I. Schoultz, A.V. Keita, The Intestinal Barrier and Current Techniques for the Assessment of Gut Permeability, Cells 9 (8) (2020).
[25] M. Yu, Q. Wang, Y. Ma, L. Li, K. Yu, Z. Zhang, G. Chen, X. Li, W. Xiao, P. Xu, H. Yang, Aryl Hydrocarbon Receptor Activation Modulates Intestinal Epithelial Barrier Function by Maintaining Tight Junction Integrity, Int J Biol Sci. 14 (1) (2018) 69–77.
[26] L.e. Shen, C.R. Weber, D.R. Raleigh, D. Yu, J.R. Turner, Tight junction pore and leak pathways: a dynamic duo, Annu. Rev. Physiol. 73 (1) (2011) 283–309.
[27] C. Reuter, M. Alzheimer, H. Walles, T.A. Oelschlaeger, An adherent mucus layer attenuates the genotoxic effect of colibactin, Cell. Microbiol. 20 (2) (2018) e12812, https://doi.org/10.1111/cmi.v20.210.1111/cmi.12812.
[28] M. Mahapatro, L. Erkert, C. Becker, Cytokine-Mediated Crosstalk between Immune Cells and Epithelial Cells in the Gut, Cells. 10 (1) (2021) 111, https://doi.org/10.3390/cells10010111.
[29] F. Sanchez-Munoz, A. Dominguez-Lopez, J.K. Yamamoto-Furusho, Role of cytokines in inflammatory bowel disease, World J. Gastroenterol. 14 (27) (2008) 4280–4288.
[30] F. Wang, W.V. Graham, Y. Wang, E.D. Witkowski, B.T. Schwarz, J.R. Turner, Interferon-gamma and tumor necrosis factor-alpha synergize to induce intestinal epithelial barrier dysfunction by up-regulating myosin light chain kinase expression, Am. J. Pathol. 166 (2) (2005) 409–419.
[31] F. Wang, B.T. Schwarz, W.V. Graham, Y. Wang, L. Su, D.R. Clayburgh, et al., IFN-gamma-induced TNFR2 expression is required for TNF-dependent intestinal epithelial barrier dysfunction, Gastroenterology 131 (4) (2006) 1153–1163.
[32] Y.S. Cheng, R.J. Colonno, F.H. Yin, Interferon induction of fibroblast proteins with guanylate binding activity, J. Biol. Chem. 258 (12) (1983) 7746–7750.
[33] K. Chellappa, P. Deol, J.R. Evans, L.M. Vuong, G. Chen, N. Briancon, et al., Opposing roles of nuclear receptor HNF4alpha isoforms in colitis and colitis-associated colon cancer, Elife. 5 (2016).
[34] B.B. Aggarwal, S.C. Gupta, J.H. Kim, Historical perspectives on tumor necrosis factor and its superfamily: 25 years later, a golden journey, Blood 119 (3) (2012) 651–665.
[35] G.G. Kaplan, J.W. Windsor, The four epidemiological stages in the global evolution of inflammatory bowel disease, Nat. Rev. Gastroenterol. Hepatol. 18 (1) (2021) 56–66.
[36] M. Cao, P. Wang, C. Sun, W. He, F. Wang, Amelioration of IFN-gamma and TNF-alpha-induced intestinal epithelial barrier dysfunction by berberine via suppression of MLCK-MLC phosphorylation signaling pathway, PLoS One 8 (5) (2013) e61944.