

AhR Activation Stabilizes Inflammatory Crosstalk and Promotes Barrier Repair in An Intestinal Co-Culture Model

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Introduction

- Inflammatory bowel disease (IBD) is a growing global health burden driven by two mutually reinforcing defects: a compromised epithelial barrier and dysregulated immune-mediated inflammation.
- The Aryl Hydrocarbon Receptor (AhR) is a ligand-activated transcription factor that coordinates epithelial and immune responses critical for intestinal homeostasis, specifically through barrier maintenance and **resolution of inflammation**.
- In intestinal epithelial cells, AhR activation induces canonical target genes such as CYP1A1, supports tight junction integrity, and dampens NF- κ B and interferon-driven inflammation^{1,2}.
- In immune cells, AhR promotes IL-22 production, which drives epithelial repair, and favors anti-inflammatory macrophage phenotypes with reduced pro-inflammatory cytokine output^{3,4}.
- AhR signaling is reduced in IBD and may contribute to excessive cytokine production, pathogenic responses, and chronic intestinal inflammation⁵.
- To model the dynamic and diverse intestinal environment, we established non-inflamed and cytokine-inflamed Caco-2/macrophage co-cultures that captures both cell-specific responses and their paracrine crosstalk.

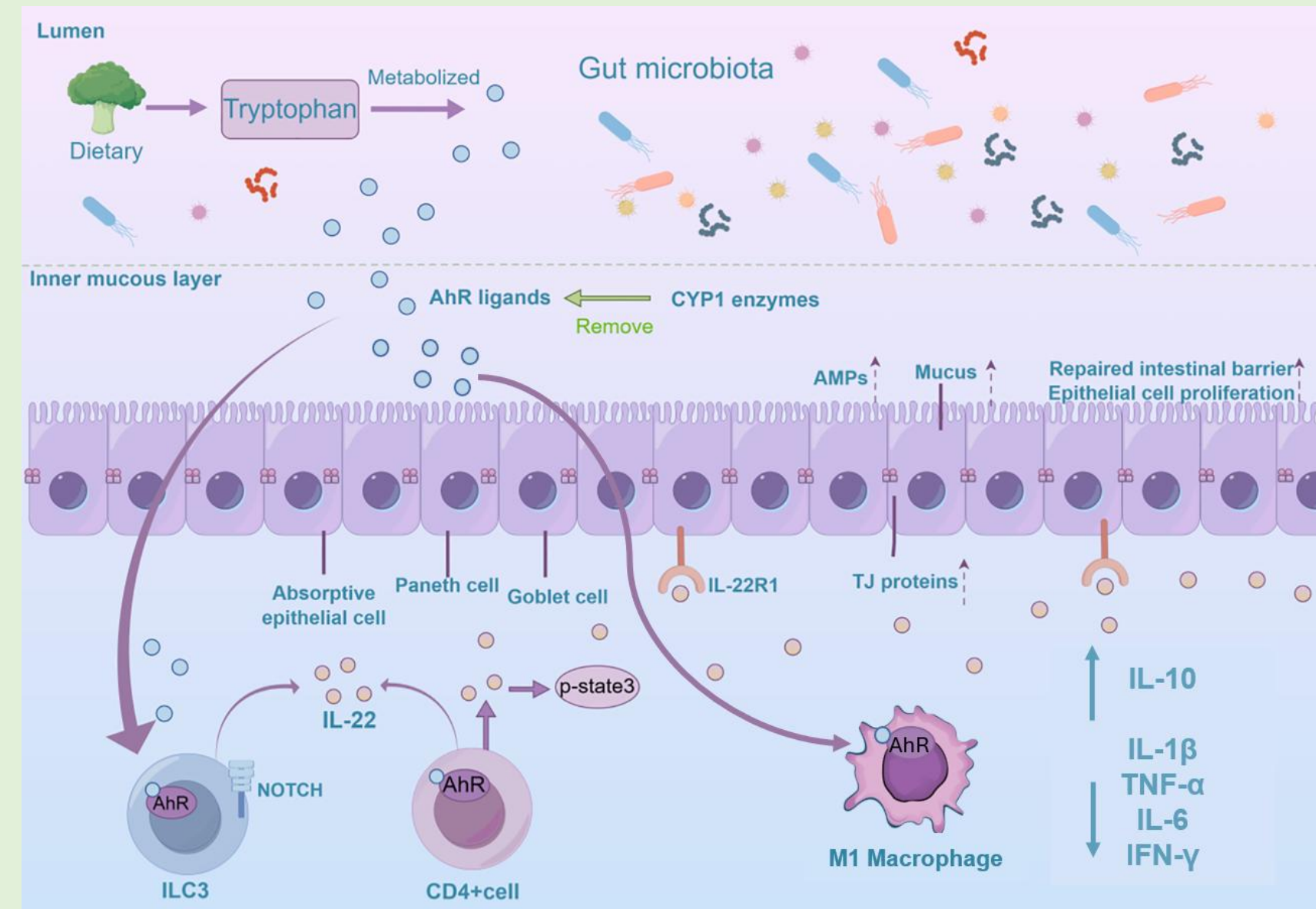


Figure 1. Mechanistic model of AhR signaling in intestinal homeostasis, adapted from Kong et al.⁷

- To investigate the therapeutic potential of AhR modulators in an intestinal context, we evaluated EQ504, a novel small-molecule modulator derived from the endogenous ligand ITE, designed to restore epithelial barrier function and promote inflammatory homeostasis, in non-inflamed and inflamed co-cultures that model the epithelial-immune interface.

Methods

Growth, Differentiation, and Polarization of Cell Types

Caco-2: Caco-2 cells were plated on 12mm, 0.4μm pore polycarbonate membrane inserts and the EVOM instrument was used to record the Transepithelial Electrical Resistance (TEER). The barrier was considered formed when the TEER values were above 600Ω.

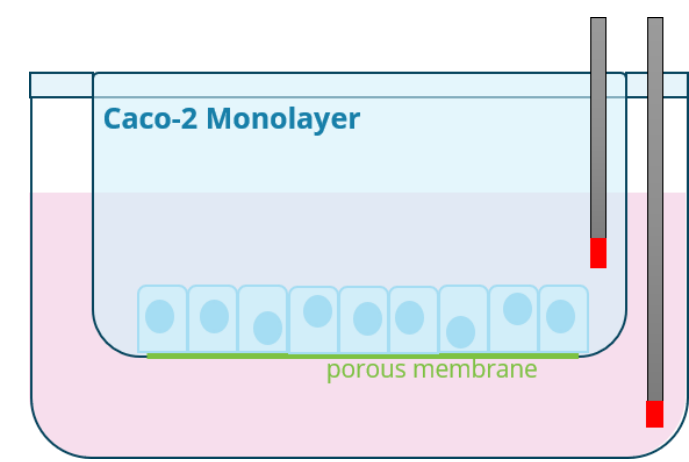


Figure 2. TEER schematic showing Apical and Basolateral sides.

M1 Macrophages: To induce M1 macrophage differentiation, monocytes were isolated from total PBMCs. Monocytes were plated in 12-well TC-treated plates and GM-CSF (20ng/mL) was introduced along with EQ504, Indirubin, and Vehicle control for 4 days. At day 4 there was a media refresh and the addition of LPS, IFN- γ , and IL-6 to further drive polarization. On day 8 there was a media refresh prior to co-culture assembly containing drug only.

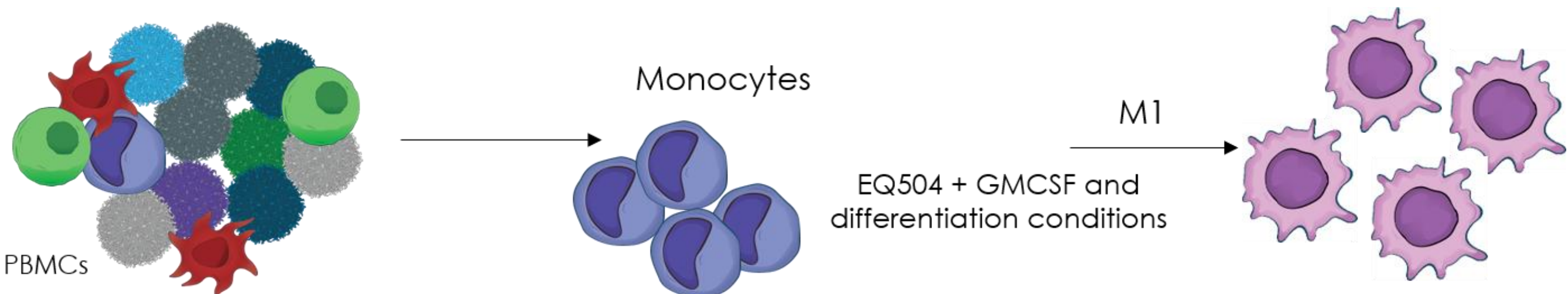


Figure 3. Schematic of the experimental approach to isolate monocytes from PBMCs, and polarize M1 macrophages using GM-CSF, LPS, IFN- γ , and IL-6.

Modeling of Inflammatory vs. Healthy Model

Approximately 24h prior to co-culture assembly, 1/2 of the Caco-2 Transwell plates were refreshed and supplemented with IL-1 β , IFN- γ and TNF- α (25ng/mL each⁶). TEER values were measured to verify barrier disruption (Fig.9B). The other 1/2 were refreshed with standard media.

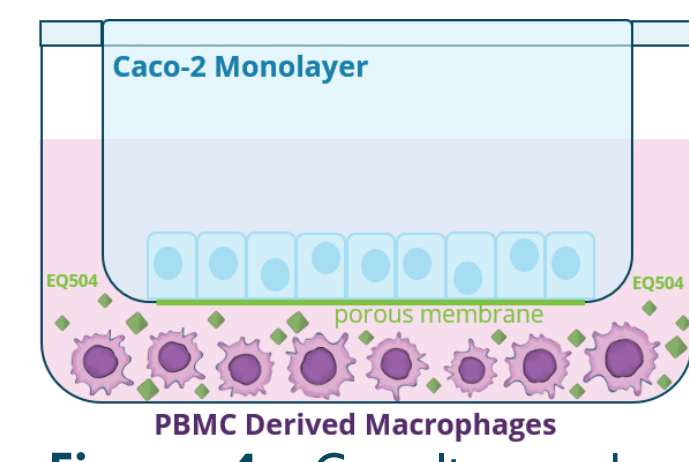


Figure 4. Co-culture schematic showing both cell types.

Co-culture Assembly

The co-culture was assembled on macrophage day 9, 24h post-inflammatory model stimulation. Both cell types were rinsed and refreshed with a 1:1 RPMI and DMEM media mixture and left to rest for an hour. The apical compartments of the Transwell plates were then moved directly into the corresponding wells of the macrophage plates.

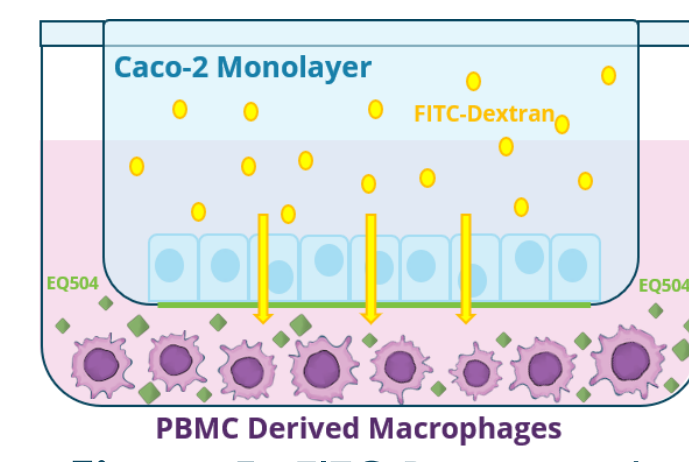


Figure 5. FITC-Dextran schematic displaying the movement of the tracer molecule through the monolayer junctions.

FITC-Dextran

At assembly (0h), FITC-Dextran (1 mg/mL) was added to the apical compartment with readings performed by taking aliquots from the basolateral compartment, diluting 1:5 with PBS in white-walled, clear-bottom 96-well assay plate, and performing a fluorescence readout at 475nm/530nm. The FITC-Dextran assay was repeated at 24h (2 and 24h incubation) and 72h (2h incubation) timepoints.

Gene and Protein Expression

Prior to co-culture assembly supernatant was collected from the macrophages during polarization. During the co-culture supernatant was collected from the basolateral compartment every 24h. The supernatant was then tested using a Meso Scale Discovery (MSD). At 96h each cell type was lysed for gene analysis (SYBR Green qPCR).

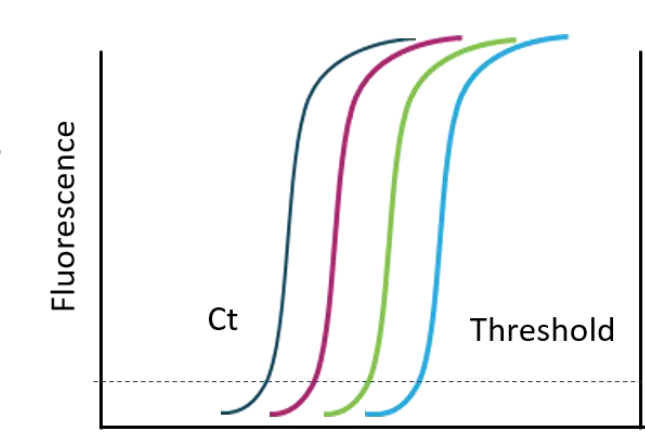


Figure 6. Reverse Transcription quantitative PCR (RT-qPCR)

Statistical Analysis

Co-culture assays were performed in three independent runs: two direct replicates and one with modified conditions (no active AhR modulator in co-culture). Data represent the two replicate runs (mean $\pm$ SD, n = 8 wells/condition). Comparisons to vehicle used one-way ANOVA with Dunnett's multiple comparisons test and Welch's t-test (GraphPad Prism); p < 0.05 was considered significant.

Results

EQ504 Preserved Barrier Function Under Inflammatory Conditions

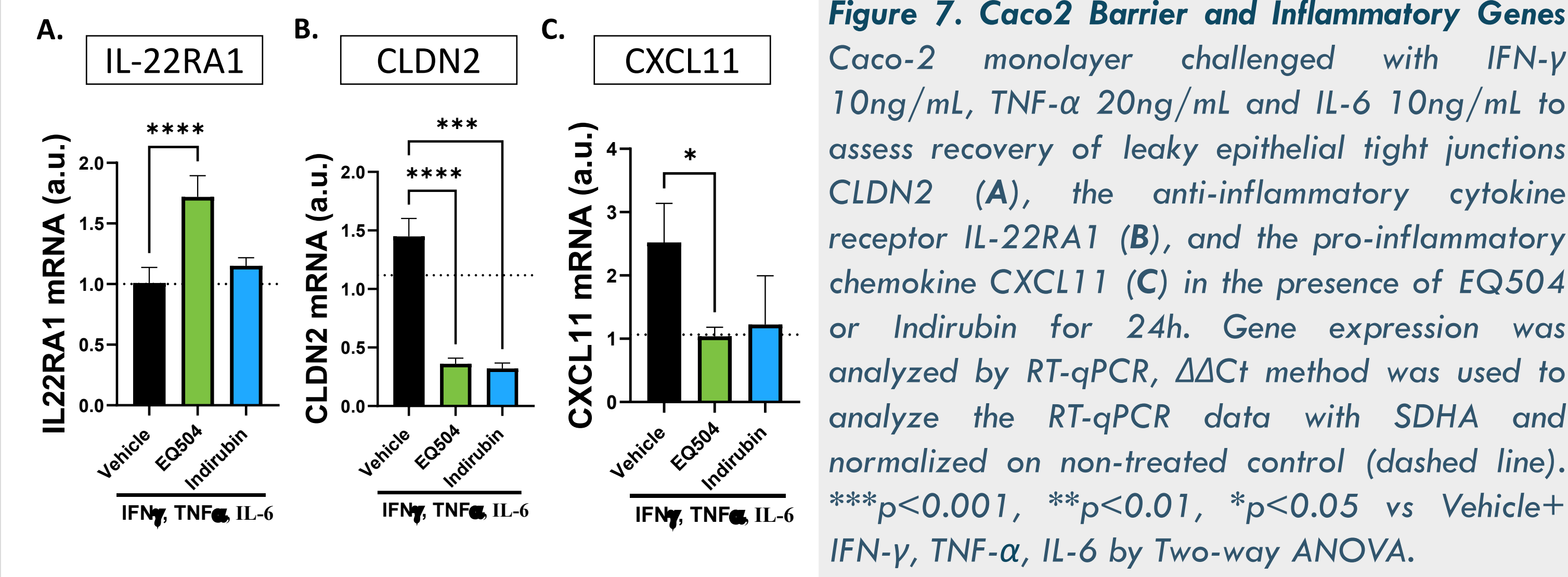


Figure 7. Caco2 Barrier and Inflammatory Genes Caco-2 monolayer challenged with IFN- γ 10ng/mL, TNF- α 20ng/mL and IL-6 10ng/mL to assess recovery of leaky epithelial tight junctions CLDN2 (A), the anti-inflammatory cytokine receptor IL-22RA1 (B), and the pro-inflammatory chemokine CXCL11 (C) in the presence of EQ504 or Indirubin for 24h. Gene expression was analyzed by RT-qPCR, $\Delta\Delta C_t$ method was used to analyze the RT-qPCR data with SDHA and normalized on non-treated control (dashed line). ***p<0.001, **p<0.01, *p<0.05 vs Vehicle+IFN- γ , TNF- α , IL-6 by Two-way ANOVA.

EQ504 Significantly Inhibits Key Pro-inflammatory Cytokine Release in M1 Macrophages

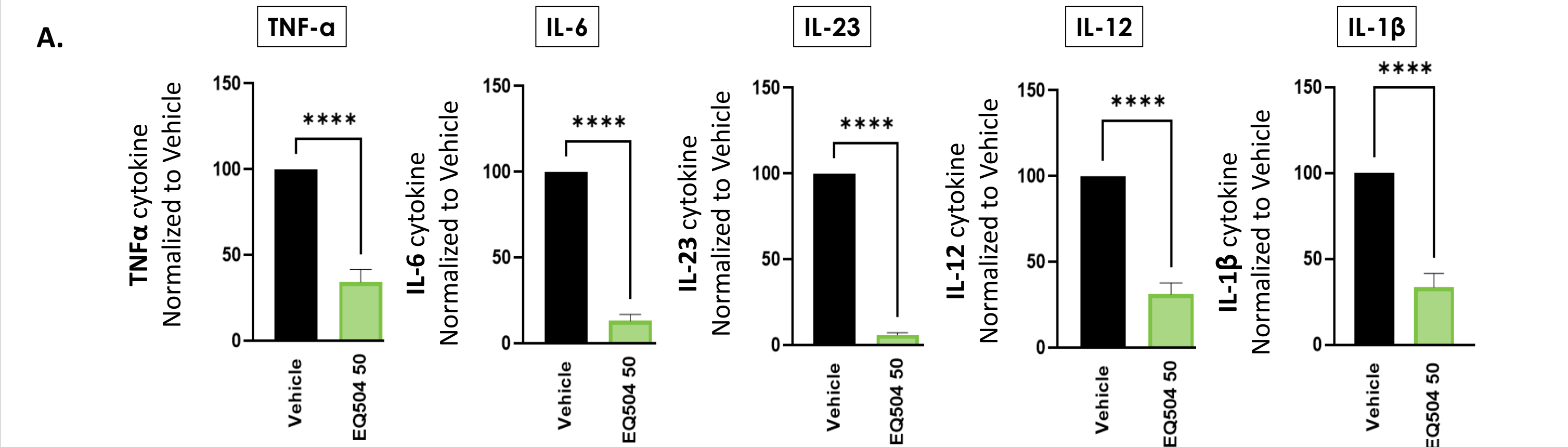


Figure 8. M1 Macrophage Cytokine Secretion Isolated monocytes polarized towards an M1 phenotype with EQ504 for a total of 8 days (A). EQ504 decreased several pro-inflammatory cytokines, thereby attenuating downstream T_H1/T_H17 responses that sustain chronic intestinal inflammation in IBD. ****p<0.0001 by Welch's t-test.

EQ504 Strengthens Barrier Integrity Under Healthy Conditions and Preserves Barrier Integrity Under Inflamed Conditions

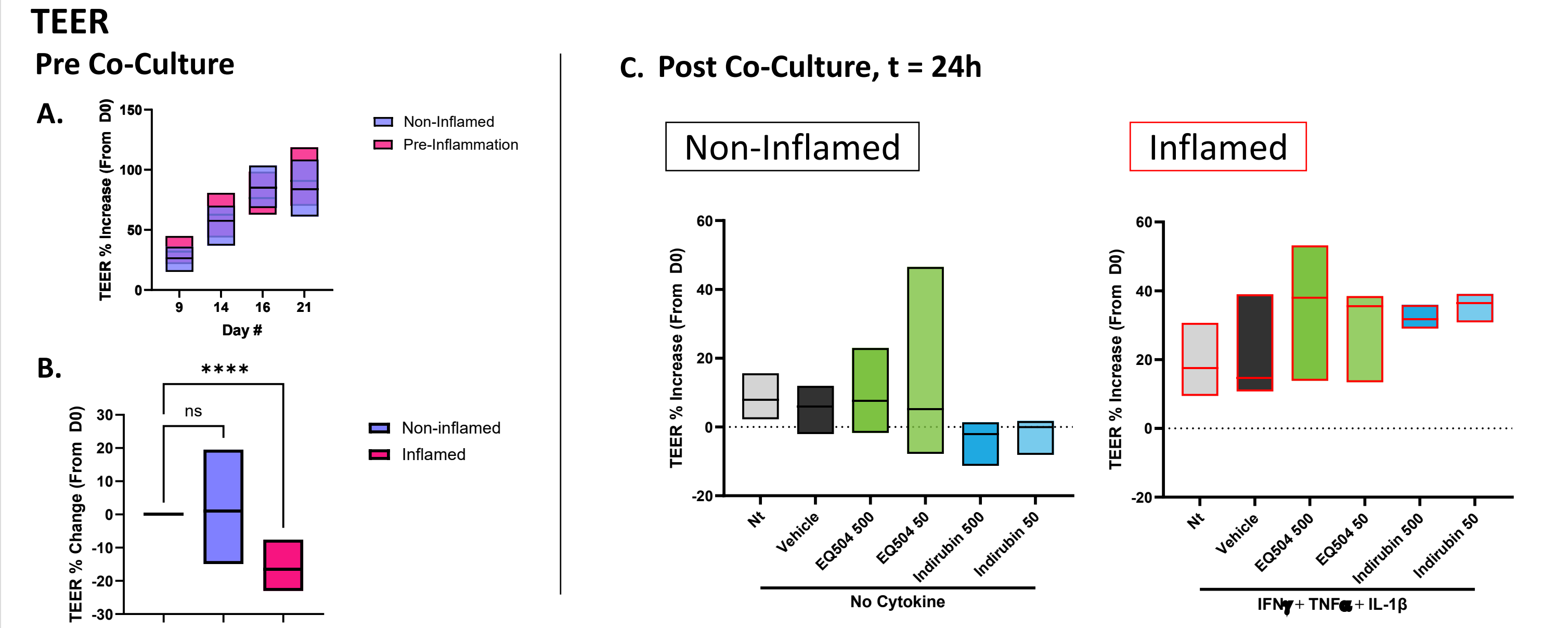


Figure 9. TEER and FITC-Dextran (A) A Caco-2 monolayer was grown for approximately 21 days. (B) Once the barrier was formed, IFN- γ 25ng/mL, TNF- α 25ng/mL and IL-1 β 25ng/mL were added for 24h to create inflamed models and verified by TEER. (C) After co-culture assembly (0h), Caco-2 monolayers were exposed to macrophages and TEER was monitored every 24h. (D) FITC-Dextran normalized concentrations on day 1 at 2 and 24 hours after co-culture assembly. Lower concentrations indicate stronger integrity. ****p<0.0001, *p<0.005 by One-way ANOVA.

EQ504 Confirms Target Engagement Through CYP1A1 Expression in Caco-2 and M1 Macrophages

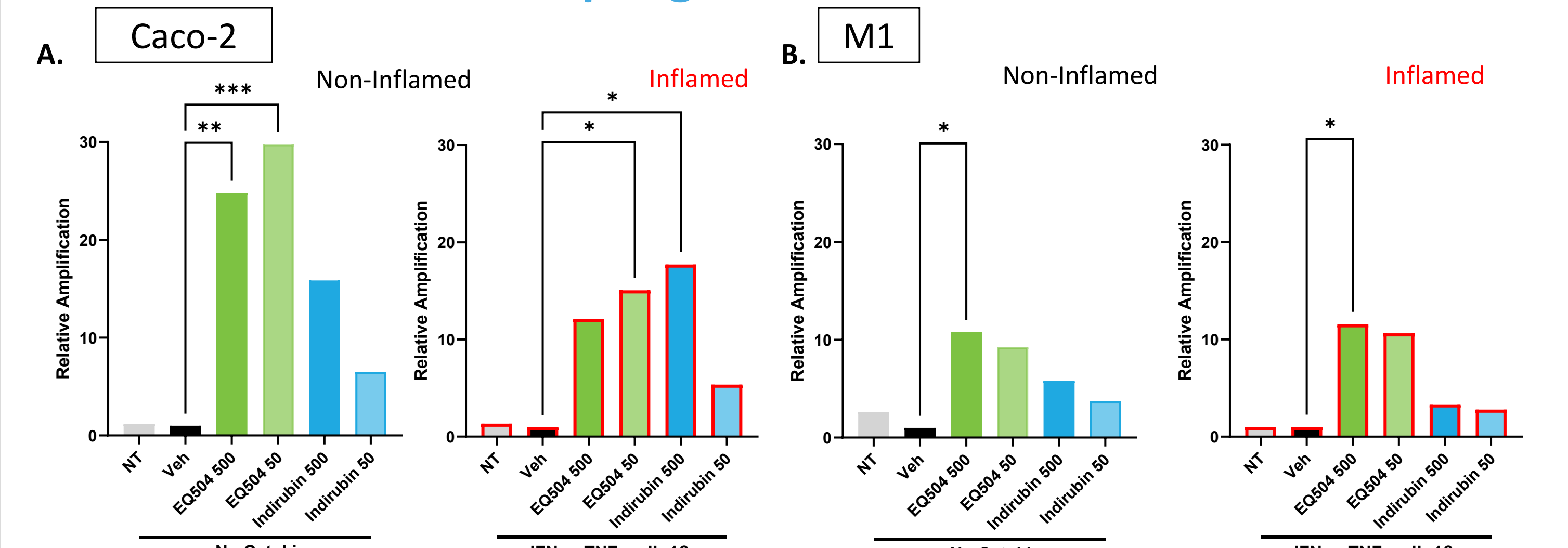


Figure 10. Gene Expression Caco-2 cells were co-cultured with EQ504 or Indirubin treated human-derived M1 macrophages for 4 days. After day 3 both cell types were analyzed for CYP1A1 gene expression by RT-qPCR. Target engagement observed via CYP1A1 in (A) Caco-2 cells and (B) M1 macrophages from both non-inflamed and inflamed models. ***p<0.001, **p<0.001, *p<0.005 by One-way ANOVA.

EQ504 Attenuates Pro-Inflammatory Cytokine and Chemokine Secretion

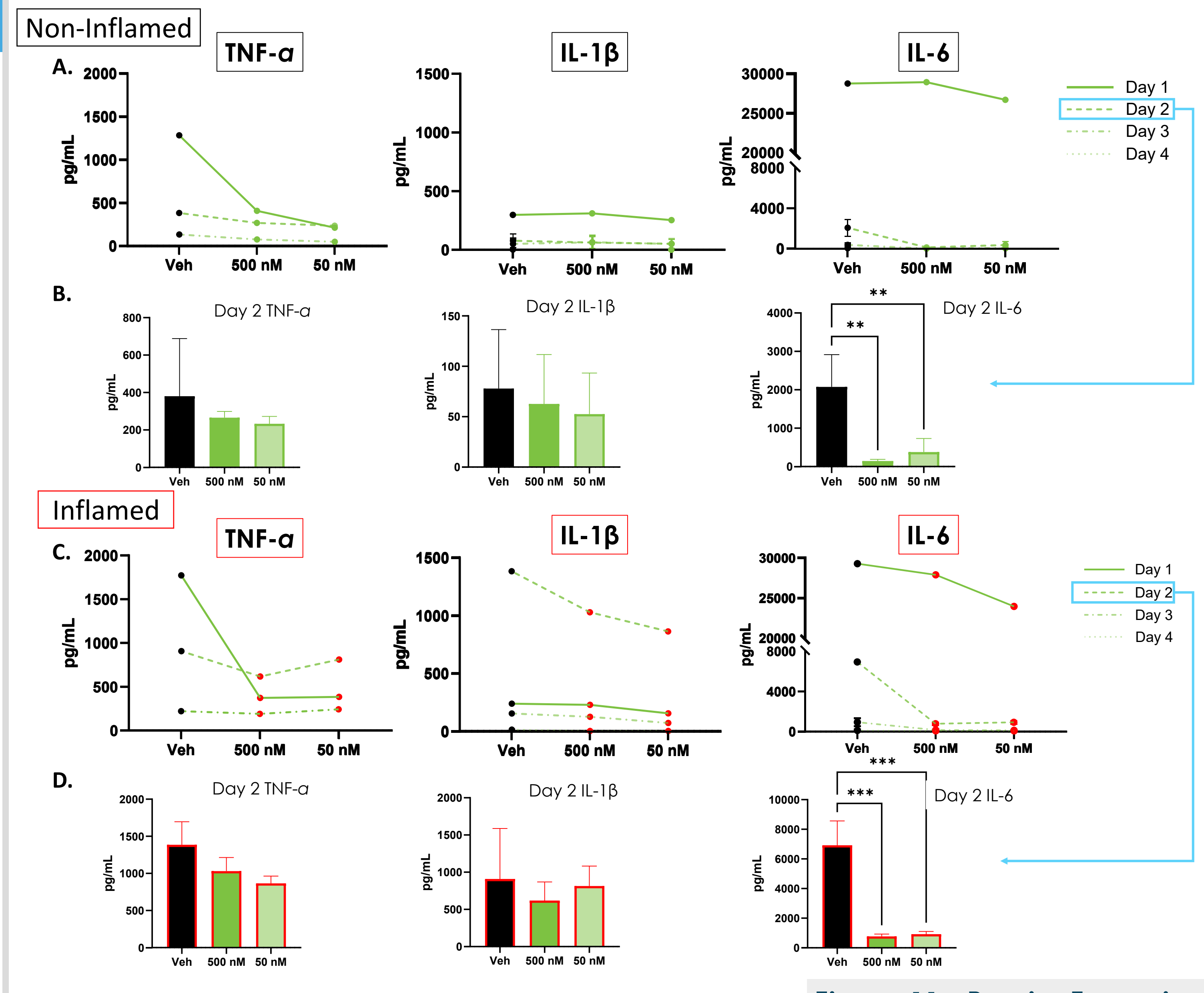


Figure 11. Protein Expression Isolated monocytes were polarized towards an M1 phenotype with EQ504 for a total of 8 days and placed in a co-culture with Caco-2 cells for 4 days. Each day, M1 supernatant was collected. Cytokine detection was measured on an MSD instrument. (A-D) EQ504 decreased several pro-inflammatory cytokines across both non-inflamed and inflamed models. A decrease in TNF- α , IL-1 β , and IL-6 thereby reduces inflammatory M1 outputs. Day 2 results exhibit the most significant differences across non-inflamed (B) and inflamed models (D). (E-H) EQ504 decreased TARC and MCP-1, principle T-cell and macrophage chemoattractants, respectively. At day 2, both showed significant levels of attenuation at both EQ504 concentrations in non-inflamed and inflamed models (H). ****p<0.0001, ***p<0.001, **p<0.001 by One-way ANOVA.

Conclusions

- EQ504 engages AhR in both the epithelial and immune compartments:** EQ504 induced CYP1A1 in a dose-dependent manner in Caco-2 cells and M1 macrophages, under both non-inflamed and inflamed conditions. This confirms target engagement across the co-culture interface.
- EQ504 keeps the barrier intact in non-inflamed conditions and rescues it under inflammation:** Under cytokine-induced inflammation, EQ504 significantly lowered FITC-dextran permeability compared with vehicle (vehicle vs. 50 nM). TEER showed directional improvement in both conditions.
- EQ504 lowers pro-inflammatory cytokine and chemokine secretion by M1 macrophages:** EQ504 reduced basolateral TNF- α , IL-1 β , IL-6, MCP-1 and TARC compared with the vehicles. IL-6 and MCP-1 showed significant reduction, both dropping close to baseline and holding through the time course.
- EQ504 acts on the epithelial-immune interface as a whole:** EQ504 dampened macrophage inflammatory output and supported epithelial barrier function at the same time. That supports AhR as a target in IBD that could address both mucosal inflammation and barrier dysfunction.

These findings demonstrate that EQ504 engages the AhR pathway, improves barrier integrity, and diminishes inflammatory cytokine output in an epithelial-macrophage co-culture model of intestinal inflammation.

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Disclosures

This study was funded by Equillum, Inc. Rosa Closser, Valeria Marrocco, Phoi Tiet, Stephen Connelly, and Jeanette Ampudia are currently employees of Equillum. Jeanette Ampudia is a stockholder of Equillum. Stephen Connelly is currently an employee, stockholder, President and an Officer of Equillum. To request permission or to ask any questions about the poster, please contact IR@equillumbio.com.