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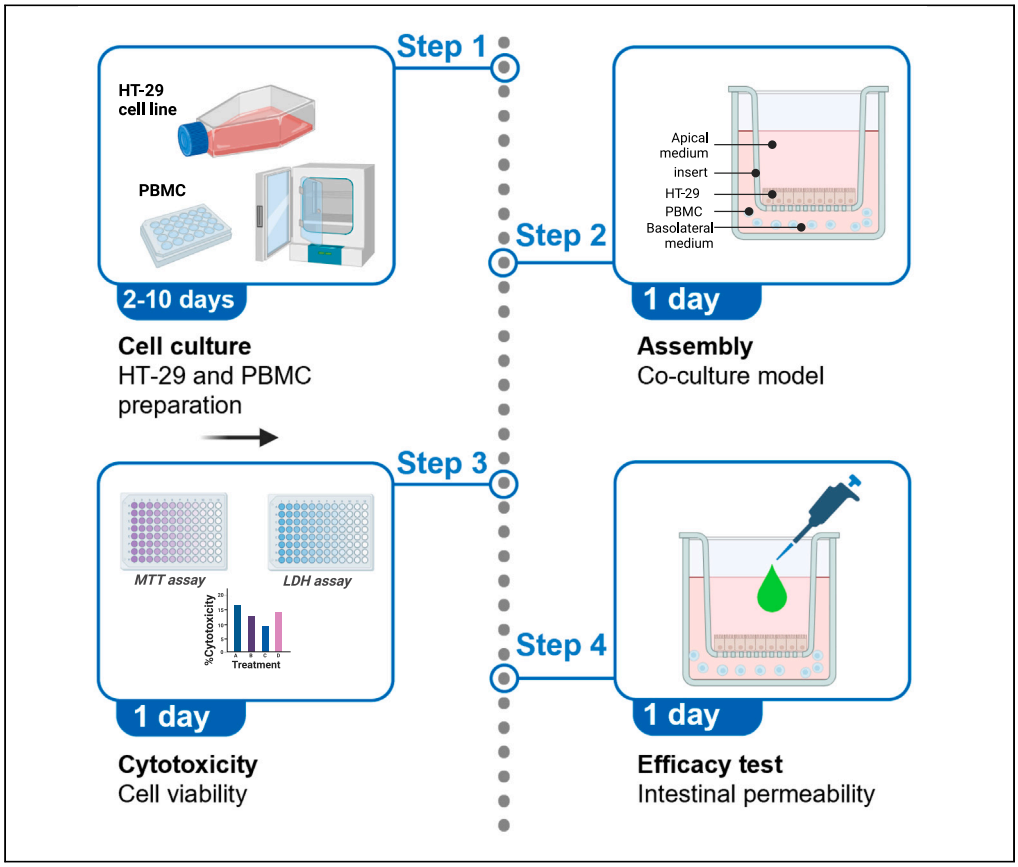
Protocol for the preclinical evaluation of gut barrier function and immune interaction in an HT-29/PBMC co-culture model



Here, we present a protocol for the pre-clinical evaluation of gut barrier function and immune interaction in an HT-29/PBMC co-culture model. We describe steps for culturing HT-29 cells and peripheral blood mononuclear cells (PBMCs), assembling the co-culture model, and testing cell viability. We then detail procedures for performing efficacy tests through stress challenges and barrier permeability assays.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Steps to culture HT-29 cells to 70%–80% confluence before co-culturing

Isolate and prepare PBMCs for co-culture in RPMI 1640 medium with 10% FBS

Assemble co-culture by placing HT-29 cells on a Transwell insert above PBMCs

Evaluate cell viability and gut barrier function via cytotoxicity and permeability assays

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Protocol for the preclinical evaluation of gut barrier function and immune interaction in an HT-29/PBMC co-culture model

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SUMMARY

Here, we present a protocol for the pre-clinical evaluation of gut barrier function and immune interaction in an HT-29/PBMC co-culture model. We describe steps for culturing HT-29 cells and peripheral blood mononuclear cells (PBMCs), assembling the co-culture model, and testing cell viability. We then detail procedures for performing efficacy tests through stress challenges and barrier permeability assays.

For complete details on the use and execution of this protocol, please refer to Abot et al.[1]

BEFORE YOU BEGIN

Overview

In recent decades, various models have been developed to study the functions and metabolism of the intestinal epithelium under pro-inflammatory conditions. While animal models enable the study of whole-organism physiology, they have significant limitations due to species differences and the difficulty in extrapolating results to humans. Consequently, *in vitro* models using human-derived intestinal epithelial cells (IECs) have become widely used in bioavailability and toxicology studies within the food and pharmaceutical fields. These models offer the advantage of reducing the reliance on animal experimentation and provide a simple, reproducible method to study the molecular mechanisms of therapeutic compounds.

The human colon cell line HT-29, traditionally used to study human colon cancers, is currently drawing considerable attention in studies focused on food digestion and bioavailability owing to its capacity to express characteristics of mature intestinal cells. External and internal signals shape these cells to better interact with the gut ecosystem, thus contributing to intestinal physiology. The human colon carcinoma Caco-2 cells are also used as model for the human intestinal epithelium. Compared to HT-29 cells, Caco-2 cells require 21 days of proliferation to form a confluent monolayer with tight

junctions. Additionally, HT-29 cells exhibit a heterogeneous morphology, displaying both epithelial-like and goblet cell-like characteristics. The integrity of the intestinal epithelial barrier is a critical component of gut immune tolerance and can be regulated by multiple factors. Numerous studies describe the close interactions between peripheral blood mononuclear cells (PBMCs) and intestinal epithelium, which significantly contribute to the intestinal barrier function. Disruption of this interaction results in altered secretion of pro- and anti-inflammatory cytokines. Moreover, drugs or bioactive compounds with anti-inflammatory effects, such as butyrate or 1α,25-dihydroxyvitamin D3, may help restore functional communication between these two cell types.

To investigate the impact of bioactive compounds on the modulation of the effector response by IECs, we employed a Transwell co-culture model. In this setup, IECs are typically grown as a monolayer on permeable supports, forming a polarized epithelial barrier that mimics the intestinal epithelium. PBMCs from healthy donors are added to the basolateral compartment of the culture system. This model allows for the examination of the complex interactions between intestinal epithelial cells and immune cells, providing a valuable tool for studying the modulation of gut barrier functions and immune responses by bioactive compounds.[2–6]

The coculture protocol can be adapted to use THP-1 cells instead of PBMCs, offering significant advantages in studying intestinal epithelial-immune cells interactions. This modification reduces inter-individual variability and provides a more controlled experimental environment. Moreover, THP-1 cells can be differentiated into specific macrophage phenotypes (M0, M1, M2) allowing for precise investigation of phenotype-specific interactions with intestinal epithelial cells enabling detailed study of epithelial-macrophage crosstalk in mucosal immunity.

Institutional permissions

Informed consent was obtained from all subjects involved in the study. Human cells were obtained from STEMCELL Technologies with the authorization #IE-2020-1126 and approved by the "WIRB-Copernicus Group (WCG) Institutional Review Board" ethic comity.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Critical commercial assays		
Cell proliferation kit (MTT)	Merck	11465007001
Cytotoxicity detection kit (LDH)	Merck	11644793001
Experimental models: Cell lines		
HT-29 cells	Merck	85061109
PBMCs	STEMCELL Technologies	70025.3
Other		
DMEM	Life Technologies - Gibco	11584516
RPMI 1640	Life Technologies - Gibco	11534446
Fetal bovine serum	Merck	F7524
Penicillin-Streptomycin	Merck	P4333
DPBS	Merck	L0615
L-glutamine	Merck	G7513
Insert membrane, PET, transparent, 0.4 μm pore size, flat base	Sarstedt	83.3932.041
Trypsin-EDTA	Merck	SM-2003-C
FITC dextran 4 kDa	Merck	46944
Triton X-100	Merck	T9284
24 wells plate for culture	VWR	734-2325
Hank's balanced salt solution	Merck	H6648

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Plastic suitable for culture	Sarstedt	70.3050.255 / 86.1254.001 / 72.695.201
96 wells black plate	Dominique Dutscher	655076
96 wells plate sterile	VWR	734-2327
96 wells plate non sterile	VWR	43001-0119
T75 culture flask	Sarstedt	83.3911.002
Microscope camera C-B5 and software Optikaproview	Optika	85258100
Plastic cell counting slides Kovas Slide	Dominique Dutscher	050126
Plate shaker	Grant-Bio	PMS-1000i
Microplate reader FLUOstar Omega	BMG	415-4347
Water bath 5L	VWR	462-0556P
CO_2 humidified incubator CellXpert C170	Eppendorf	6734000011

STEP-BY-STEP METHOD DETAILS

Cell culture (HT-29 and PBMCs)

Timing: 7–10 days for HT-29; 2–3 days for PBMCs

This step involves preparing and culturing HT-29 cells and PBMCs.

1. HT-29 cell preparation for culture.
   a. Prepare complete cell culture media for HT-29 cells: Dulbecco's Modified Eagle Medium (DMEM) with 10% heat-inactivated Fetal Bovine Serum (FBS) and 1% Penicillin-Streptomycin (P/S).

   Note: Complete culture medium can be kept in refrigerator for 4 days as DMEM used contains stabilized L-alanyl-L-glutamine dipeptide.

   Note: Complete culture medium is heating up to 37°C in the water bath before use.

   b. Thaw the vial (approximatively 5 million of cells) by gentle agitation in a 37°C water bath for 2 min. Remove and decontaminate the vial by dipping in 70% ethanol. Transfer the vial contents to a centrifuge tube containing 5 mL complete culture medium and spin at room temperature at approximately 200 × *g* for 5 min.
   c. Resuspend cell pellet with 10 mL of complete culture medium and dispense into a 75 cm^2 culture flask.

   Note: Perform repeated pipetting to effectively dissociate the cells.

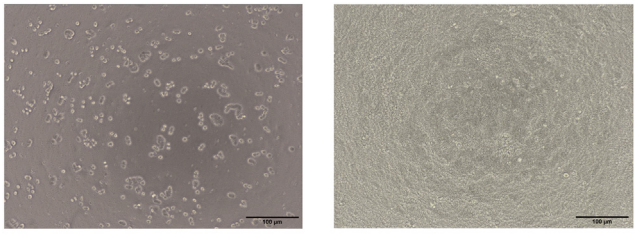

Figure 1. Growth of HT-29 cells in culture
Images show HT-29 cells observed under light microscopy (10x magnification) at two different growth stages. Scale bar represents 100 µm. On the left: Day +1 post-seeding with individual cells visible, scattered across the culture surface. On the right: Day +7 post-seeding where cells have proliferated to reach confluence, forming a dense monolayer ready for use in subsequent experiments.

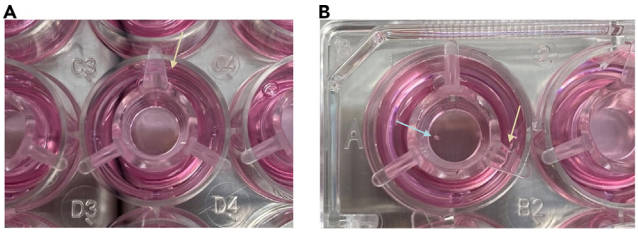

Figure 2. Correct and incorrect positioning of inserts in a cell culture plate

(A) Properly positioned insert. A yellow arrow indicates the correct placement of the insert's orientation tab at the top. (B) Improperly positioned insert. The yellow arrow shows the misaligned orientation tab. The blue arrow indicates an air bubble beneath the insert, which may interfere with cellular monolayer formation. Correct positioning of inserts is crucial for ensuring consistent cell growth and experimental reproducibility in barrier model studies.

Note: It is suggested that, prior to the addition of the vial contents, the culture vessel containing the complete culture medium be placed into a 5% CO_2 humidified incubator for at least 15 min to allow the medium to reach its normal pH (7.0 to 7.6).

d. The day after (once the cells reach 70%–80% confluence), remove and discard culture medium of the 75 cm^2 flask.
e. Rinse the cell layer with DPBS at 37°C to remove all traces of serum which contains trypsin inhibitors.
f. Add 2 mL of 0.25% Trypsin-EDTA solution to detach the cells for 5 min in a 37°C cell culture incubator.
g. Add 8 mL of complete culture medium and count them (i.e., KOVA slide).
h. Proceed to the seeding of HT-29 cells at 100 000 cells/cm^2 in 200 μL of complete culture medium in 96 well plate (experimental condition assessed in triplicate).
i. Change medium every day. Gently aspirate the medium from the wells and add 200 μL of complete medium.

⚠ CRITICAL: This step is very important to maintain a high glucose concentration in cell culture medium.

j. Once the cells reach 70%–80% confluence (approximatively 1 or 2 days), trypsinize using 0.25% Trypsin-EDTA solution to detach the cells for 5 min in a 37°C cell culture incubator.
k. Fill basolateral compartment of 24 well plate with 500 μL of complete HT-29 cell medium. Seed HT-29 cells at 100 000 cells/cm^2 onto insert in 200 μL of complete HT-29 cell medium.
l. Replace the apical medium daily. Use sterile forceps to securely hold the insert at eye level, grasping it by the side rather than the arm to avoid damaging the fragile structure.

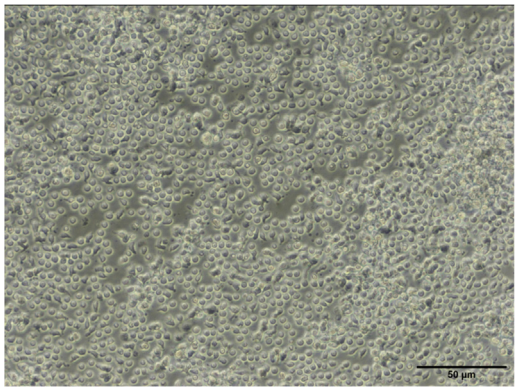

Figure 3. Peripheral Blood Mononuclear Cells (PBMCs) in culture

Light microscopy image of freshly isolated PBMCs observed at day+1 post-isolation. The cells were imaged using bright-field microscopy at 20x magnification. The scale bar represents 50 μm. The image shows a heterogeneous population of PBMCs, primarily consisting of lymphocytes and monocytes, distributed across the field of view. The circular morphology of the cells and their uniform size distribution are characteristic of freshly isolated PBMCs.

m. Using 200 μL pipette remove carefully the medium in the apical chamber without touching cell layer. Add quickly 200 μL of new complete culture medium.
n. Replace the basolateral medium every 2–3 days. Take a new 24 well plate and add 500 μL of complete culture medium. Transfer the insert onto the appropriate well.

⚠ CRITICAL: Check if no bubble is trapped under the well. Full confluency is attending after 5 or 7 days, depending on the vial (see Figure 1, right) until confluence.

⚠ CRITICAL: Growth times to form an airtight cell monolayer may differ according to laboratory culture conditions and the number of cell passages. Avoid high passage (up to 20) rates, as cells tend to form small clusters.

⚠ CRITICAL: Check that the incubator shelf is level to guarantee cell monolayer homogeneity (see Figure 1 left).

⚠ CRITICAL: Check that no bubble is trapped under the insert to guarantee no disruptions in the cell monolayer (see Figure 2B).

2. PBMCs preparation for culture.
   a. Prepare complete cell culture media for PBMC cells: RPMI 1640 medium with 10% heat-inactivated FBS, 2 mM L-alanyl-glutamine, and 1% Penicillin-Streptomycin (P/S). Complete culture medium is heating up to 37°C in the water bath before use.
   b. Thaw the vial (number depending on the manufacturer) by gentle agitation in a 37°C water bath for 1 min. Remove and decontaminate the vial by dipping in 70% ethanol. Transfer the vial contents to a centrifuge tube containing 5 mL complete culture medium and spin at room temperature at approximately 200 × *g* for 5 min.
   c. Resuspend cell pellet with the required volume of complete culture medium in order to have 2×10^6 cells/mL. Plate 500 μL of resuspended PBMC within wells of a 24-well plate and incubate at 37°C in a 5% CO_2 humidified incubator for 24 h.
   d. Following 24 h incubation (see Figure 3), replace half of the medium by carefully removing and discarding half the volume ensuring not to aspirate any cells by aspirating the medium from the surface. Then, add 250 μL of fresh complete culture medium to each well.

⚠ CRITICAL: Use very gentle aspiration with a pipette to avoid aspirating non-adherent PBMCs.

Note: PBMCs are primary cells derived from a single donor. To account for inter-individual variability, it is essential to work with cells from at least five different donors.

Assembly of Co-culture model

Timing: 24 h

The assembly of the co-culture model involves treating HT-29 cells, following a 24-h PBMC seeding. The appropriate concentrations of TNF-α and NaBut are applied. Critical steps include ensuring the insert is level and removing air bubbles to maintain communication between the intestinal epithelial cells and PBMCs.

Note: Following 24 h post PBMCs seeding, treat HT-29 cells as shown in Figure 4 and Methods video S1. The integrity of the intestinal epithelial barrier is crucial for gut immune tolerance and is regulated by multiple factors. Numerous studies have shown that TNF-α disrupts tight junction expression and the structure of the intestinal mucosa.[7] Drugs or bioactive

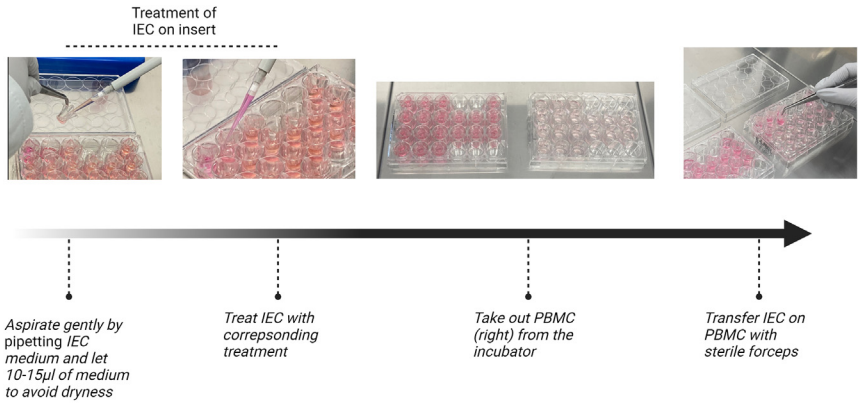

Figure 4. Schematic representation of the co-culture assembly process for HT-29 cells on inserts with PBMCs in the basolateral compartment

The timeline illustrates the key steps in the assembly of an intestinal epithelial barrier model using HT-29 cells and peripheral blood mononuclear cells (PBMCs). The process is as follows: Gentle aspiration of IECs (Intestinal Epithelial Cells) medium from the insert, leaving a small volume to maintain cell hydration. Application of the designated treatment to the IECs on the insert. Retrieval of PBMCs (shown on the right) from the incubator. Transfer of IECs onto the PBMC layer using sterile forceps to handle the insert. This procedure ensures proper assembly of the co-culture system, allowing for the study of epithelial-immune cell interactions in a controlled *in vitro* environment.

compounds with anti-inflammatory effects, such as butyrate (NaBut) or 1α,25-dihydroxyvitamin D3, may help restore the integrity of the intestinal barrier.[8]

Note: TNF-α and NaBut concentrations were validated in the cytotoxicity section: 200 ng/mL and 5 mM for 24 h, respectively. When a compound of interest is tested, the concentrations are chosen because of a cytotoxicity study conducted on HT-29 cells (see cytotoxicity section). This phase can be assessed during the HT-29 growth period (see section 4 e).

3. Assemble the co-culture model.
   a. Prepare all treatments needed in 2 mL sterile reaction tube, diluted in warmed complete culture medium: Vehicle (sterile water, same volume as used for TNF-α and NaBut), TNF-α and NaBut.

   Note: Perform the treatments in small volumes (i.e. 10 µL to add per well) and use the appropriate solvent as vehicle (sterile water, DMSO, etc.).

   b. Carefully remove medium and replace with the corresponding treatment in 200 µL final volume, as it is describing in 1.f.

   ⚠ CRITICAL: Cells are sensitive to dryness. Add the treatment solution as fast as possible.

4. Co-culture HT-29 cells with PBMCs by placing the inserts with HT-29 cells into wells containing PBMCs (0.5 mL at 2×10^6 cells/mL).

   ⚠ CRITICAL: Check that the insert is level to guarantee cell monolayer homogeneity.

   ⚠ CRITICAL: Bubbles could be trapped under insert when transferring insert on PBMC. Remove them by gentle shaking of the insert to ensure correct communication between IEC (apical side) and PBMC (basolateral side).

5. Maintain co-culture for 24 h at 37°C in a 5% CO_2 incubator.

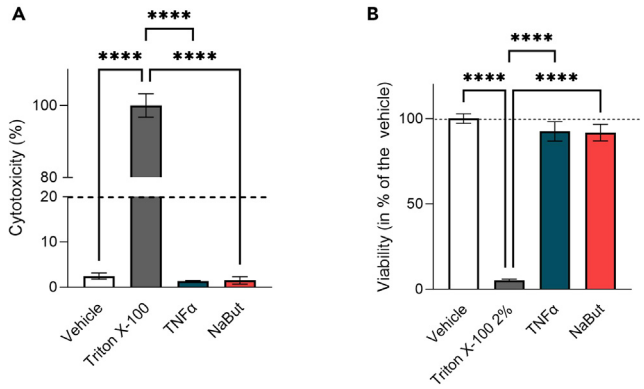

Figure 5. Cytotoxicity and viability assessment of HT-29 cells exposed to TNF-α and sodium butyrate

(A) LDH release assay measuring cytotoxicity. Cells were treated with vehicle (control), Triton X-100 (2%, positive control for maximum LDH release), TNF-α, or sodium butyrate (NaBut). Results are expressed as percentage of cytotoxicity relative to the maximum LDH release.

(B) MTT assay assessing cell viability. Cells were exposed to the same conditions as in (A). Results are presented as a percentage of viability relative to the vehicle control. Data are shown as mean ± SEM. The associated *p*-values were obtained using 1-way ANOVA followed by Bonferroni's post-hoc test. Statistical significance is indicated by asterisks (****$p < 0.0001$) compared to the vehicle control or between indicated groups. Triton X-100 induced significant cytotoxicity and reduced viability as expected. TNFα and sodium butyrate treatments did not cause significant cytotoxicity and maintained high cell viability, suggesting their potential use for non-cytotoxic cellular modulation in intestinal epithelial models.

Cytotoxicity assay

Timing: 2 days

The cytotoxicity assay is essential for determining non-toxic working concentrations of test compounds in HT-29 cells, which is critical for further permeability experiments. Over two days, HT-29 cells are treated with varying concentrations of test compounds for 24 h. LDH and MTT assays are performed to evaluate cytotoxicity and cell viability, respectively (Figures 5A and 5B). The LDH assay measures LDH release from damaged cells, while the MTT assay assesses cell metabolic activity. Triton X-100 is used as a positive control to ensure full cell lysis. Results are expressed as percentages of cytotoxicity and viability.

Note: This step is crucial and allow to evaluate potential cytotoxicity to perform the permeability experiment with appropriate working concentrations, i.e. the maximal concentration without cytotoxic effect in HT-29 cells.

Note: This step can be carried out during the HT-29 growth period when this cell type is being prepared (see section 1i).

6. To evaluate the toxicity of the test compounds on IEC, treat cells with different concentrations of the respective test compounds for 24 h.
   a. Prepare complete cell culture media for HT-29 cells: Dulbecco's Modified Eagle Medium (DMEM) with 10% heat-inactivated Fetal Bovine Serum (FBS) and 1% Penicillin-Streptomycin (P/S).

Note: Complete culture medium can be kept in refrigerator for 4 days as DMEM used contains stabilized L-alanyl-L-glutamine dipeptide.

Note: Complete culture medium is heating up to 37°C in the water bath before use.

b. Thaw the vial (approximatively 5 million of cells) by gentle agitation in a 37°C water bath for 2 min. Remove and decontaminate the vial by dipping in 70% ethanol.
c. Transfer the vial contents to a centrifuge tube containing 5 mL complete culture medium and spin at room temperature at approximately 200 × *g* for 5 min.
d. Resuspend cell pellet with 10 mL of complete culture medium and dispense into a 75 cm^2 culture flask.

Note: Perform repeated pipetting to effectively dissociate the cells.

Note: It is suggested that, prior to the addition of the vial contents, the culture vessel containing the complete culture medium be placed into a 5% CO_2 humidified incubator for at least 15 min to allow the medium to reach its normal pH (7.0 to 7.6).

e. The day after (once the cells reach 70%–80% confluence), remove and discard culture medium of the 75 cm^2 flask.
f. Rinse the cell layer with DPBS at 37°C to remove all traces of serum which contains trypsin inhibitors.
g. Add 2 mL of 0.25% Trypsin-EDTA solution to detach the cells for 5 min in a 37°C cell culture incubator.
h. Add 8 mL of complete culture medium and count them (i.e., KOVA slide).
i. Proceed to the seeding of HT-29 cells at 100 000 cells/cm^2 in 200 μL of complete culture medium in 96 well plate (experimental condition assessed in triplicate).

Note: Keep a 75 cm^2 flask with HT-29 cells containing 5–10 million of cells in 10 mL of complete culture medium to proceed to the permeability experiment (next step after cytotoxicity).

j. After 24 h, perform the treatments with tested compounds. Prepare all treatments needed in 2 mL sterile reaction tube, diluted in warmed complete culture medium: Vehicle (sterile water, same volume as used for TNF-α and NaBut), TNF-α, NaBut and Triton X-100 2%.

Note: Triton X-100 is added directly into the well (2 μL) 20 min before the cytotoxicity assay to ensure the fully lysis, providing a positive control and baseline for maximum cytotoxicity.

Note: Perform the treatments in small volumes (i.e. 10 μL to add per well) and use the appropriate solvent as vehicle (sterile water, DMSO, etc.).

Note: Perform the treatments in 10 μL per well and use the appropriate vehicle as control (sterile water, DMSO, etc.).

7. LDH assay.
   a. Collect 100 μL of the supernatant at the end of the treatment period (24 h) and transfer in classic 96 wells plate. Keep it in the refrigerator while preparing LDH mixture to ensure representative results.

Note: Keep the plate with cells and remaining supernatant (100 μL) at 37°C in the humidified 5% CO_2 incubator.

   b. Prepare the LDH reaction mixture with the catalyst (Cytotoxicity detection kit, Merck: buffer 1) and the dye solution (Cytotoxicity detection kit, Merck: buffer 2).

Note: For 100 tests, shortly before use, mix 250 μL of reconstituted buffer 1 with 11.25 mL of buffer 2. Keep in the dark. Mixture solution must be used as soon as it is prepared.

c. Take out the 96 well plate from the refrigerator and add 100 μL of reaction mixture per well according to the manufacturer instructions and let it at room temperature (20°C–22°C) for 30 min in the dark with gentle shaking (370 rpm) using a plate shaker. https://www.sigmaaldrich.com/FR/fr/product/roche/11644793001?srsltid=AfmBOoqLAEd0Gal9cD6j5PJdSX1HeoeyC2bFQrX1Y0JBSgaCeOdadnKJ.
d. Read the plate at 490 nm.
e. Percentage of cytotoxicity is calculated as follow:

$$\%\ Cytotoxicity\ =\ \frac{(Experimental\ LDH\ release\ -\ Spontaneous\ LDH\ release)}{(Maximum\ LDH\ release\ -\ Spontaneaous\ LDH\ release)} \times 100$$

Note: Using the mean of triplicate:
Experimental LDH release is LDH activity measured from cells treated with test compound.
Spontaneous LDH release is LDH activity measured from vehicle treated cells.
Maximum LDH release is LDH activity measured from cells lysed by Triton X-100.

8. MTT cell proliferation assay.
   a. Add 10 μL MTT labeling reagent (dilution 1:10) directly on the remaining supernatant from the last step (100 μL) containing the cells and incubate 3 h at 37°C in a humidified 5% CO_2 incubator.

   Note: As MTT results is depending on metabolic activity of cells, incubation time would be adapted if timing treatment is more than 24 h.

   b. Add 100 μL Solubilization buffer and incubate overnight (minimum 9 h) at 37°C in a humidified 5% CO_2 incubator.
   c. Read the plate at 550 nm.
   d. Percentage of viability is calculated as follow:

$$\%\ Viability\ =\ \frac{(Experimental\ absorbance)}{(Vehicle\ absorbance)} \times 100$$

Note: Using the mean of triplicate:
Experimental absorbance is the absorbance value measured from cells treated with test compound.
Vehicle absorbance is the absorbance value measured from vehicle treated cells.

Note: The activity of the lactate dehydrogenase (LDH) released by damaged cells was quantified using the LDH assay. The viability of IEC was also evaluated by measuring the formazan produced by metabolic active cells using the MTT cell proliferation assay. The combination of these two cell viability tests are essential for preclinical *in vitro* testing as they provide complementary measurements of cell viability and cytotoxicity, respectively, allowing for a comprehensive assessment of a compound's effects on cellular health allowed to test the working concentrations of tested compounds for further preclinical testing.

Assessment of intestinal permeability

Timing: 1 day

The assessment of intestinal permeability involves preparing a FITC-dextran solution, which must be protected from light. A stock solution is created, diluted to working concentrations, and filtered for use in permeability experiments. Cells are gently washed and treated with FITC-dextran, followed by incubation for 1 h at 37°C. After incubation, fluorescence measurements are taken using a black

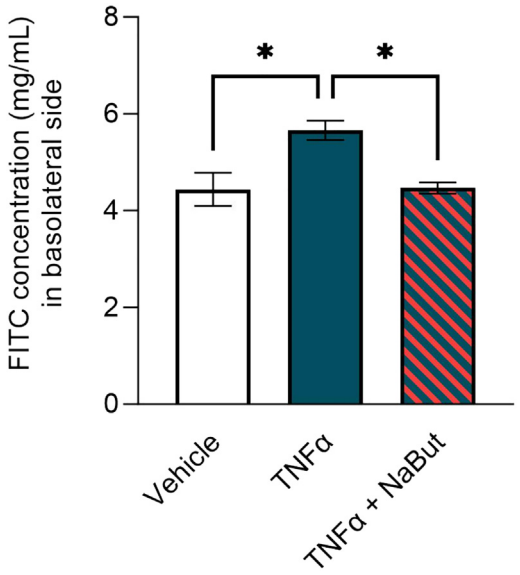

Figure 6. Effect of TNF-α and Sodium Butyrate (NaBut) on FITC-Dextran (4 kDa) Permeability

The bar graph shows the concentration of FITC-Dextran (4 kDa) on the basolateral side under three different conditions: Vehicle (control), TNF-α treatment, and combined TNF-α + NaBut treatment. The y-axis represents the FITC concentration in mg/mL. The associated *p*-values were obtained using 1-way ANOVA followed by Bonferroni's post-hoc test. The results indicate that TNF-α significantly increases the permeability of FITC-Dextran compared to the vehicle control, as shown by the elevated concentration of FITC on the basolateral side ($p < 0.05$, indicated by *). However, the addition of NaBut alongside TNF-α treatment significantly reduces the permeability compared to TNF-α treatment alone ($p < 0.05$, indicated by *). These findings suggest that NaBut mitigates the TNF-α induced increase in permeability, highlighting its potential protective effect against TNF-α mediated barrier dysfunction. Data are presented as mean ± SEM (Standard Error of the Mean).

plate and excitation at 485 nm, emission at 520 nm. A standard curve is generated to determine the FITC concentration, which reflects the permeability of the intestinal barrier (Figure 6).

9. FITC dextran preparation.

   Warning: FITC dextran must be protected from the light.

   a. Prepare a stock solution at 25 mg/mL in warm HBSS pre-warmed to 37°C. Aliquot and store at −20°C for 3 months.

   ⚠ CRITICAL: Do not use a stock solution that is more than 3 months old. It could be freeze/thaw no more than 5 times.

   b. Prepare the working FITC solution at 1 mg/mL with warm HBSS pre-warmed to 37°C. 200 µL of the working solution is needed per well condition and 1 mL is needed to prepare the standard.
   c. Sterile filter the solution at 0.22 µm.
   d. Prepare the FITC standard with HBSS; use the filtered FITC solution at 1 mg/mL to make a dilution series (dilute 1:2 for 7 dilutions – HBSS is the zero standard).
   e. Prepare the working FITC solution at 1 mg/mL with HBSS pre-warmed to 37°C. 200 µL of the working solution is needed per well condition and 1 mL is needed to prepare the standard.
   f. Sterile filter the solution at 0.22 µm.
   g. Prepare the FITC standard with HBSS; use the filtered FITC solution at 1 mg/mL to make a dilution series (dilute 1:2 for 7 dilutions – HBSS is the zero standard).

10. Permeability experimentation.

a. Prepare a new 24 well plate for washing by adding 500 μL of HBSS pre-warmed to 37 °C S per well.
b. Carefully remove the medium from the apical chamber of the insert (let a residual volume 10–15 μL to avoid dryness) and add 200 μL of HBSS pre-warmed to 37°C for a gentle wash.

⚠ CRITICAL: Cells are sensitive to dryness. Let approximately 10–15 μL (representing by a halo around the insert) in the insert.

c. Transfer the insert in the plate containing 200 μL HBSS pre-warmed to 37°C as 9a for washing. Let it equilibrate for 20 min at 37°C in a 5% CO_2 incubator.
d. Prepare a new 24 well plate for the permeability experiment by adding 500 μL of HBSS pre-warmed to 37°C per well.
e. Carefully remove the HBSS solution from the apical chamber of the insert and add 200 μL of the 1 mg/mL FITC solution. Protect from the light.
f. Transfer the insert to the plate for the permeability experiment. Incubate for 1 h at 37°C in a 5% CO_2 incubator.

11. Fluorescence measurement.
a. Take a 96 well black plate for fluorescence measurement. Perform the following steps in the dark.
b. Add in duplicate 100 μL of the FITC dilution series standard per well.
c. Collect, in duplicate, 100 μL of HBSS basolateral supernatant from the plate for the permeability experiment and add it to the 96 well black plate.
d. Collect 100 μL of basolateral supernatant from the insert and put in the dark plate for florescence measurement.
e. Read the fluorescence in the plate using excitation at 485 nm and emission at 520 nm.
f. To determine the FITC concentration (mg/mL), a standard curve was generated using known concentrations of FITC-dextran.

Note: The standard curve was fitted using a non-linear regression model in GraphPad Prism.

Note: The fluorescence values of the samples were then interpolated on the standard curve to obtain the corresponding FITC concentrations.

EXPECTED OUTCOMES

This preclinical co-culture model allows for the assessment of gut barrier function, cytotoxicity, and cell viability under inflammatory conditions. HT-29 cells will form a monolayer on inserts, simulating the intestinal epithelial barrier, while PBMCs in the basolateral compartment will provide immune cell interactions. The protocol includes viability assays (MTT and LDH) and permeability tests (FITC-dextran flux) to evaluate the integrity and function of the epithelial barrier. Additionally, gene expression analysis (interleukins, biomarkers involved in antioxidative defenses…) and cytokine measurements (IL-8) will provide insights into the inflammatory and oxidative responses to treatment compounds.

LIMITATIONS

This protocol is optimized for HT-29 cells and PBMCs. Variations in cell lines, culture conditions, and inflammatory stimuli may affect the outcomes. Additionally, this *in vitro* model does not fully replicate the complexity of the gut environment, including the presence of microbiota and other immune cell types.

TROUBLESHOOTING

Problem 1

Cells are forming clusters.

Potential solution

Culturing HT-29 cells long term in tissue culture might lead them to form cell clusters (step 1). We recommend avoiding high passage rates (up to 20).

Problem 2

Cell monolayer is not homogenic and the cells are not covering the entire insert membrane (step 1).

Potential solution

Check if the incubator shelf is level using a spirit level.

Problem 3

In the permeability experiment, we observe cell detachment in insert (step 9).

Potential solution

Culture the cells in insert carefully. Avoid placing tips directly in contact with the insert. Do not remove the entire volume of HBSS, you can let 10 μL.

Problem 4

Strong color reaction in the LDH or MTT measurements (step 6 or step 7 respectively).

Potential solution

Protect samples from the light during the incubation. If the strong color is only in control wells, dilute them with medium or DPBS as there are many cells in the well.

Problem 5

Low fluorescence in the permeability measurement.

Potential solution

Protect samples and standard from the light during the experimentation. Do not re-use an aliquot of stock solution. Do not use a stock solution that is more than 3 months old. You may use an empty insert to compare your results. It should be at least twice as concentrated as the control value.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Pr. Claude Knauf (claude.knauf@inserm.fr).

Technical contact

Further information and requests for technical issues should be directed to and will be fulfilled by the technical contact, Dr. Anne Abot (anne.abot@enterosys.com).

Materials availability

This research did not produce any novel reagents.

Data and code availability

This study did not generate any new datasets or code.

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AUTHOR CONTRIBUTIONS

G.A.: formal analysis, investigation, supervision, and writing – review and editing. L.C.: formal analysis and investigation. N.P.: formal analysis and investigation. O.P.: formal analysis, investigation, supervision, and writing – review and editing.

P.D.C.: conceptualization, validation, writing – original draft, and writing – review and editing. C.K.: conceptualization, validation, writing – original draft, and writing – review and editing. A.A.: conceptualization, formal analysis, investigation, project administration, supervision, validation, writing – original draft, and writing – review and editing.

DECLARATION OF INTERESTS

P.D.C. was co-founder of The Akkermansia Company SA. P.D.C. and C.K. co-founded Enterosys SAS. G.A., L.C., N.P., O.P., and A.A. are employees at Enterosys.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2024.103416.

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