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# ENDOTARGET/ FIRST REPORT ON THE EFFECTS OF VARIOUS LPS TYPES ON INTESTINAL EPITHELIUM IN VITRO

WP2, Task 2.3

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## EXECUTIVE SUMMARY

ENDOTARGET explores the relationship between gut microbiota, gut permeability, and systemic endotoxemia with rheumatic diseases (RD) focusing on three most abundant RDs, osteoarthritis (OA), rheumatoid arthritis (RA) and spondylarthritis (SpA). By integrating data from large population cohorts, experimental models (*in vitro* and *in vivo*), and targeted clinical studies, the ENDOTARGET consortium aims to increase understanding of RD pathophysiology and contribute to the development of preventive strategies.

The UH part of WP2 in collaboration with HUS focuses on exploring *in vitro* the proinflammatory burden of immunologically active bacterial compounds, such as lipopolysaccharides (LPS) and bacterial membrane vesicles (BMV), that can cross the epithelial barrier and contribute to systemic endotoxemia. We identified several bacterial species associated with RDs (OA, RA and SpA) based on published scientific studies and proceeded to study the proinflammatory effect of LPSs isolated from the type strains of these species on intestinal epithelium. HT-29 enterocyte cell line was used as the *in vitro* Intestinal epithelial model, as it is known to react to LPS. Furthermore, LPS from previously acquired, anti-inflammatory intestinal isolates, belonging to Bacteroidales order, were explored for their potentially tolerogenic effect *in vitro*. LPS from two commercially available *Escherichia coli* strains were used as positive controls.

Based on the capacity to induce inflammatory response in enterocytes *in vitro*, LPS from five RD-associated species were tested in combination with tolerogenic LPSs. All Bacteroidales LPSs in 10-fold ratio were able to neutralize the IL-8 inducing effect of the proinflammatory LPSs, except for the highly toxic *Proteus* LPS. Pre-treatment of the HT-29 cell monolayer with tolerogenic LPS prior to proinflammatory LPSs was not as effective in attenuation of IL-8 release from HT-29 cells as the direct competition between the LPSs. The capacity of tolerogenic, Bacteroidales LPS to compete with toxic, proinflammatory LPS is a promising basis for further studies in ENDOTARGET WP2 using a triple co-culture of epithelial and immune cells.

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## LIST OF ABBREVIATIONS

ACRONYM	DESCRIPTION
ATCC	American Type Culture Collection
BHI	Brain Heart Infusion
BMV	Bacterial membrane vesicle
DSMZ	German Collection of Microorganisms and Cell Cultures GmbH
FAB	Fastidious anaerobe broth
FBS	Fetal bovine serum

FMT	Fecal microbiota transplantation
GAM	Gifu anaerobic medium
IL-8	Interleukin-8
LPS	Lipopolysaccharide
NEAA	Non-essential amino acids
OA	Osteoarthritis
PEST	Penicillin/Streptomycin
RD	Rheumatic diseases
SE	Systemic endotoxemia
SpA	Spondylarthritis
TEER	Transepithelial electrical resistance
WP	Work package

# 1. INTRODUCTION

The ENDOTARGET project explores the contribution of gut microbiota, intestinal permeability and endotoxemia in the disease onset and activity of three rheumatic diseases (RD), focusing on osteoarthritis (OA), rheumatoid arthritis (RA), and spondylarthritis (SpA). The consortium combines data from large population cohorts, *in vitro* & *in vivo* models, and targeted clinical studies to increase the knowledge on RDs aiming for preventive and therapeutic approaches.

Leakage of immunologically active compounds from intestinal bacteria, such as bacterial cell wall lipopolysaccharides (LPS) and bacterial membrane vesicles (BMV) to the systemic circulation, contributes to chronic inflammation called systemic endotoxemia (SE). This proinflammatory burden is facilitated by unfavorable, altered microbiota composition, increased gut permeability, deficiency in host immune defense and lifestyle factors. As part of ENDOTARGET consortium, UH studies the effect of RD-associated, proinflammatory bacterial LPSs on gut epithelium using *in vitro* cell culture models.

In WP2, we reviewed previously published studies to collect information on potentially RD-linked bacterial species based on available scientific information. The approach to identify RD-associated bacterial species from published, peer-reviewed studies was taken due to the inconclusive results on species-level identification of RD-associated bacteria obtained from the different cohorts in WPI and WP3/Task 3.1 on LPS types found in systemic circulation. This was described as an alternative way of implementation in the research plan. The LPSs from selected RD-associated species and WP2 & WP3-harmonized control species were first tested on the inflammation model. Proinflammatory cytokine interleukin-8 (IL-8) inducing LPSs were selected for further testing with potentially inflammation-neutralizing Bacteroidales-LPSs using competition and IL-8 attenuation assays. The effect of potent, hexa-acylated LPS (control *E. coli* LPS) on epithelial barrier function was also tested using Caco-2 *in vitro* model and transepithelial electrical resistance (TEER).

The results and efforts of WP2 to identify inflammation-inducing RD-associated species, study their LPS for proinflammatory capacity using *in vitro* cell culture models and test competition between tolerogenic Bacteroidales-LPS and RD-associated, potent LPSs, serve as a base for more complex *in vitro* co-culture model

that better mimics the gut environment by combining two enterocyte cell lines, Caco-2 and HT-29, and differentiated THP-1 cell line (macrophages).

## 2. METHODS

### 2.1 HARMONIZED LPS CONTROLS

Three different commercial *E. coli* LPSs were purchased from Merck and tested for their suitability as controls for both, WP2 and WP3. One Bacteroidales LPS (Bacteroidales 1) was selected as a tolerogenic control based on published scientific data (Vatanen et al. 2016). LPS from Bacteroidales 1 was isolated using the in-house method described below.

### 2.2 SELECTION AND GROWTH OF BACTERIAL SPECIES

Type strains of the literature-based, RD-associated bacterial candidates, as well as the *E. coli* control strains, were obtained from Leibniz Institute (DSMZ-German Collection of Microorganisms and Cell Cultures GmbH) or American Type Culture Collection (ATCC). Potentially tolerogenic Bacteroidales strains were fecal isolates from the previous study of UH (Hiippala et al. 2020a). Bacteria were grown in Brain Heart Infusion medium (BHI; Neogen), Gifu anaerobic medium (GAM; Nissui) or Fastidious anaerobe broth (FAB; Neogen) at 37°C for two to five days under anaerobic (Don Whitley anaerobic cabinet) or aerobic conditions.

### 2.3 LPS ISOLATION USING IN-HOUSE METHOD

1 litre of bacterial culture was grown for 48h or longer if needed under anaerobic or aerobic conditions and pelleted by centrifuging 4000rpm for 15min. The cell pellet was washed three times with a single phase Bligh-Dyer with the LPS remaining in the insoluble fraction. The insoluble pellet was resuspended to endotoxin free water using 10–20ml volume depending on the size of the insoluble fraction. LPS concentration was measured with LAL assay (Pierce™ Chromogenic Endotoxin Quant Kit).

### 2.4 HUMAN EPITHELIAL CELL LINES

The human colonic epithelial cell line HT-29 (ACC 299) and Caco-2 (ACC 169) were purchased from the DSMZ. The cell lines were grown under oxic atmosphere with 5% CO<sub>2</sub> at 37°C. Caco-2 cell line was cultivated in RPMI 1640 (Biowest) medium supplemented with 20% heat-inactivated fetal bovine serum (FBS; Gibco), 100 U ml<sup>-1</sup>

1 Penicillin/Streptomycin Solution (PEST; Biozol), non-essential amino acids (1%, NEAA; Gibco), 15 mM HEPES (Gibco) and 2 mM L-glutamine (Gibco). HT-29 was cultivated in McCoy 5A (Biowest) medium containing 10% heat-inactivated FBS and 100 U ml<sup>-1</sup> PEST. The cells were passaged after reaching 80% confluence (every three to four days) using TrypLEExpress (Gibco) and passages 6–20 were used in the experiments. For IL-8 assays, 12.500 HT-29 cells per well were seeded onto 96-well microplate and grown for eight days post-plating. For TEER assay, Caco-2 cells were seeded on 0.4µm pore-sized inserts (Sarstedt) using 25.000 cells per transwell and grown for 14 days. The medium in 96-well plate and inserts (basolateral side) was changed every three to four days and one day before the experiment.

## 2.5 PROINFLAMMATORY CAPACITY OF LPSs

Different concentrations of commercial *E. coli* control LPSs and isolated LPSs were incubated on 8-day post-plating HT-29 cell monolayer for three hours followed by collection of the supernatants (Hiippala et al. 2020b). The level of proinflammatory cytokine IL-8 in the supernatants was measured with ELISA (BD Biosciences) and compared to the medium control. Experiments were repeated two to four times.

## 2.6 LPS COMPETITION AND ATTENUATION ASSAYS

### 2.6.1 Competition assay

Tolerogenic and inflammatory LPSs were incubated with ratio 1:1 (10 ng/ml + 10 ng/ml) or 10:1 (100 ng/ml + 10 ng/ml) on HT-29 cells for three hours. Supernatants were collected and stored at -20°C. IL-8 levels were measured with ELISA and compared to inflammatory LPS (10 ng/ml) control. Experiments were repeated two to four times.

### 2.6.2 Attenuation assay

HT-29 cells were first incubated with the tolerogenic LPS for one hour using two concentrations, 10 ng/ml or 100 ng/ml. After removing tolerogenic LPS from the monolayer, proinflammatory LPS (10 ng/ml) was added onto the cells for three to four hours followed by collection of the supernatants and IL-8 measurements with ELISA. Samples were compared to the inflammatory LPS control (10 ng/ml). Experiments were repeated two to four times.

## 2.7 LPS EFFECT ON CELL MONOLAYER INTEGRITY

The transepithelial electrical resistance (TEER) was measured using EVOM teer meter and STX4 electrode (World Precision Instruments). TEER, a measure of the strength of the Caco-2 monolayer integrity, was carried out before, 24h and 48h after incubating the monolayer with live *E. coli* bacterial cells and *E. coli* LPS. EDTA was used as a negative control.

## 3. RESULTS

### 3.1 COMMON LPS CONTROLS FOR WP2 AND WP3

First, the proinflammatory effect (IL-8 induction) of the control LPSs were tested using the *in vitro* epithelial inflammation cell culture model to establish suitable common controls for different tasks in WP2 and WP3. Human colorectal cell line HT-29 expressing colonocyte morphology has been routinely used in our research group as the epithelial inflammation model. HT-29 cells secrete proinflammatory chemokine IL-8 and do not spontaneously differentiate. *E. coli* 3 LPS was less potent to induce inflammation *in vitro* in enterocytes compared to *E. coli* 1 or 2 LPSs (Figure 1). Both, *E. coli* 1 and *E. coli* 2 LPSs, were chosen as controls in WP2 and WP3 with the latter being more proinflammatory. Bacteroidales 1 LPS was selected as a tolerogenic LPS control with potentially beneficial immunomodulatory characteristics according to previous research (Vatanen et al. 2016, Cell).

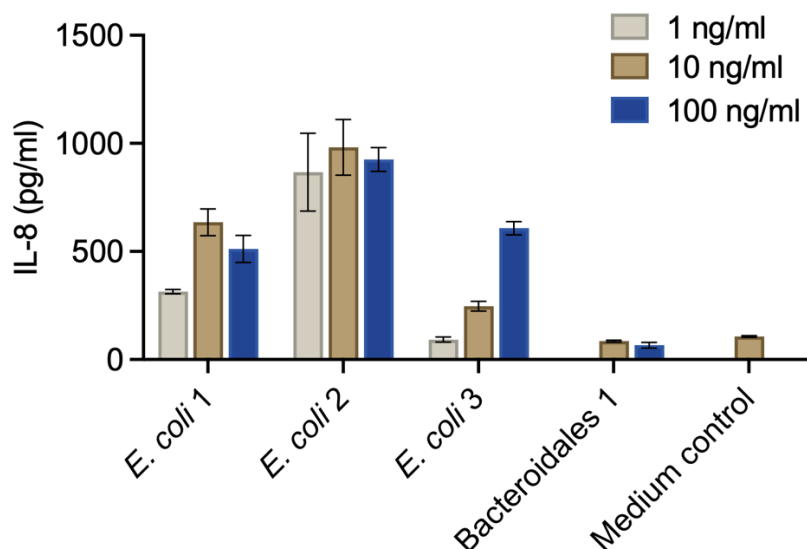


Figure 1. Induction of IL-8 release from HT-29 cells by control LPSs using different concentrations (1 ng/ml, 10 ng/ml and 100 ng/ml LPS). Medium control was included as the background control.

### 3.2 INFLAMMATORY EFFECT OF ISOLATED LPSs

The LPSs from RD-associated species and tolerogenic LPSs as well as the controls studied in WP2 are listed in Table 1. LPSs were isolated using the in-house method from ten different RD-associated, Gram-negative bacteria (type strains). The tolerogenic LPS from anti-inflammatory commensals (Bacteroidales 2-5) were isolated from a healthy fecal microbiota transplantation (FMT) donor in our previous study (Hiippala et al. 2020a).

Table 1. LPSs used in WP2 task 2.3. RD-associated and Bacteroidales LPSs were extracted using the in-house method. Control *E. coli* LPSs were commercially bought.

RD-associated LPS	Tolerogenic LPS	Control LPS
<i>Porphyromonas</i>	Bacteroidales 2	<i>E. coli</i> 1
<i>Proteus</i>	Bacteroidales 3	<i>E. coli</i> 2
<i>Aggregatibacter</i>	Bacteroidales 4	<i>E. coli</i> 3
<i>Prevotella</i> 1	Bacteroidales 5	Bacteroidales 1
<i>Prevotella</i> 2		
<i>Fusobacterium</i>		
<i>Klebsiella</i>		
<i>Phocaiecola</i> 1		
<i>Phocaiecola</i> 2		
<i>Acidaminococcus</i>		

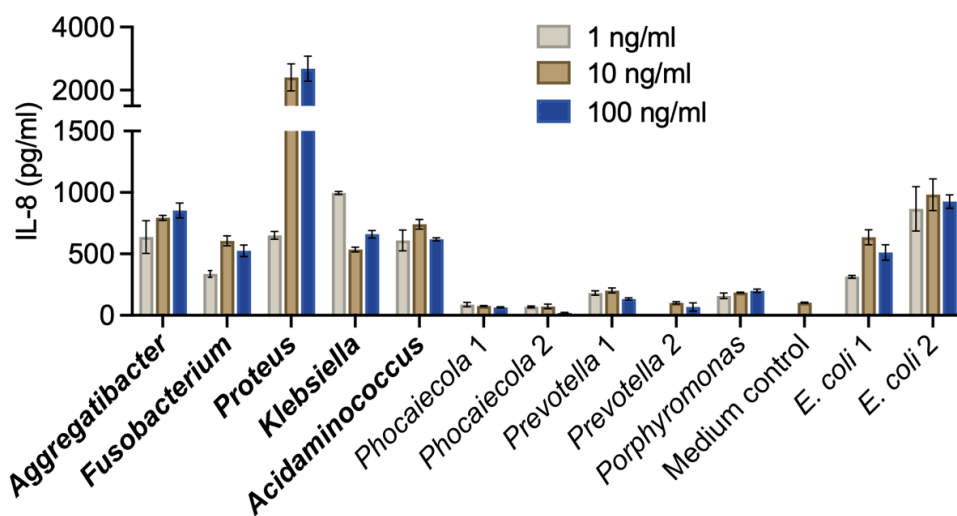
All the LPSs, inflammatory and tolerogenic, were tested in our epithelial inflammation *in vitro* cell culture model to select proinflammatory, IL-8 inducing LPSs for further experiments and to confirm the commensal nature of potentially tolerogenic LPSs (Figure 2).

Five out of ten RD-associated strains induced proinflammatory IL-8 in HT-29 enterocyte cell line at the same level as control *E. coli* LPSs (highlighted in bold in Figure 2A). *Aggregatibacter*, *Proteus* and *Klebsiella* strains belong to Gammaproteobacteria, known to contain many human pathogens. Increased abundance of Gammaproteobacteria with highly proinflammatory cell wall structures, including LPS, is considered as a hallmark of an altered, unbalanced gut microbiota and is associated with intestinal and systemic inflammation (Hou et al. 2022). All five less potent LPSs from RD-associated bacteria (*Phocaiecola* 1 & 2, *Prevotella* 1 & 2 and *Porphyromonas*) only induced IL-8 release at similar level or slightly higher level than that of the medium control. These genera belong to the

Bacteroidales order and can be considered as commensal members of the human gut microbiota. Only *Prevotella* 1 and *Porphyromonas* induced IL-8 in HT-29 cells above the background (medium control ~100 pg/ml), but the induced IL-8 level was low (<200 pg/ml) and the strains were not selected for further testing.

No significant differences were observed in the induction of IL-8 release between potentially tolerogenic LPSs and the medium control (Figure 2B), as expected.

**A**



**B**

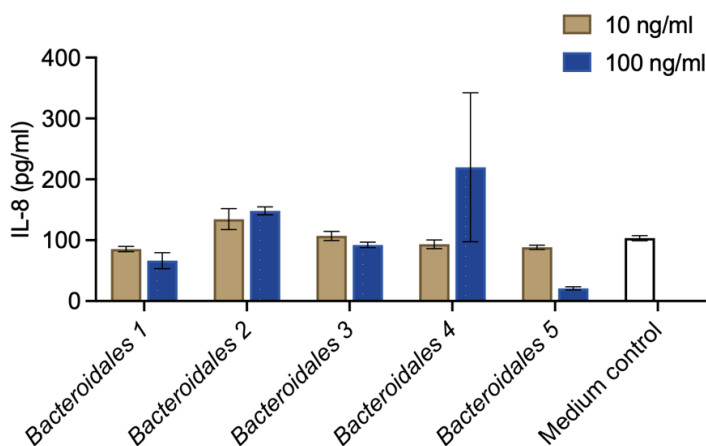


Figure 2. Induction of IL-8 release from HT-29 cells by RD-associated and tolerogenic LPSs. Effect of LPS from RD-associated bacteria (A). LPS concentrations 1 ng/ml, 10 ng/ml and 100 ng/ml were used except for *Proteus* LPS, which was used in 0.1 ng/ml, 1 ng/ml and 10 ng/ml concentrations, respectively due to its high efficacy. McCoy 5A medium and *E. coli* LPS 1 & 2 served as controls. LPSs with proinflammatory capacity from RD-associated strains highlighted in bold. IL-8 induction by tolerogenic LPSs (B). McCoy 5A medium and Bacteroidales 1 served as controls. LPS concentrations 10 ng/ml and 100 ng/ml were used.

### 3.3 PROTECTIVE EFFECT OF TOLEROGENIC LPS IN COMPETITION

After establishing the proinflammatory capacity of the studied RD-associated LPSs, we tested the simultaneous effect of both, proinflammatory and tolerogenic LPS, on HT-29 enterocytes. All tolerogenic LPSs (Bacteroidales 1-5) could neutralize the proinflammatory effect induced by LPS from *E. coli* 1, *E. coli* 2, *Aggregatibacter* and *Fusobacterium* ie. significantly decrease IL-8 release from enterocytes (Figure 3). The Bacteroidales LPSs were used in 1:1 and 1:10 ratio to the proinflammatory LPSs and both ratios were effective. LPS from fecal isolates (Bacteroidales 2-5) showed more consistent tolerogenic effect with 1:1 ratio than the control Bacteroidales 1. The inflammation inducing effect of *Proteus* LPS, even with much lower concentration (0.01 ng/ml), could not be counteracted by most of the tolerogenic LPSs, except for LPS from Bacteroidales 5.

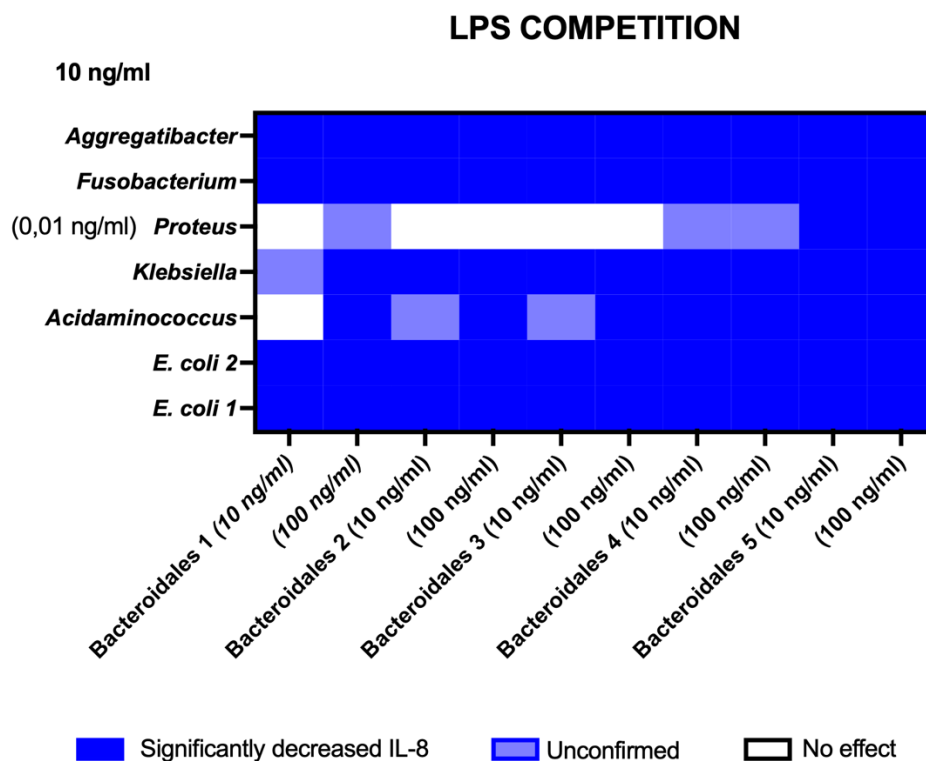


Figure 3. Simultaneous effect (competition) of proinflammatory and tolerogenic LPSs on IL-8 release from HT-29 cells. Different ratios, 1:1 (10 ng/ml and 10 ng/ml) or 1:10 (10 ng/ml and 100 ng/ml) of proinflammatory LPS and tolerogenic LPS, respectively, were used. Due to the toxicity of *Proteus* LPS, only 0.01 ng/ml concentration was tested. Unconfirmed = variable results from 2-3 experiments.

### 3.4 ATTENUATION CAPACITY OF TOLEROGENIC LPSs

After demonstrating the competitive effect of different immunomodulatory LPSs on the epithelium *in vitro*, we measured the IL-8 production in HT-29 cells pretreated with tolerogenic LPS (10 ng/ml or 100 ng/ml) followed by proinflammatory LPS i.e assessed the attenuation capacity of tolerogenic LPSs. When added 10-fold, LPS from tolerogenic control Bacteroidales 1, isolate Bacteroidales 2 and isolate Bacteroidales 4 significantly decreased the IL-8 release induced by LPS from control *E. coli* 1 and 2, *Aggregatibacter*, *Fusobacterium* and *Klebsiella* (Figure 4). Overall, 10-fold higher amount of Bacteroidales LPS (100 ng/ml) seemed more effective in IL-8 attenuation than the equal concentration.

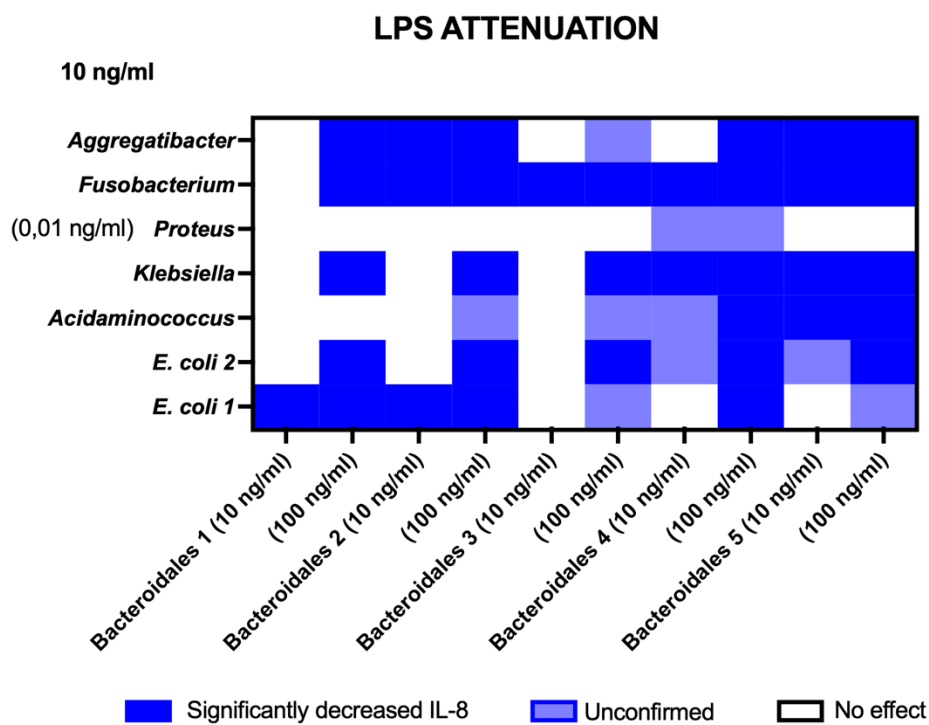


Figure 4. The reducing effect (attenuation) of tolerogenic LPSs on IL-8 release from HT-29 cells. Different ratios of LPS, 1:1 (10 ng/ml and 10 ng/ml) or 1:10 (10 ng/ml and 100 ng/ml) of proinflammatory LPS and tolerogenic LPS, respectively, were used. Due to the toxicity of *Proteus* LPS, only 0.01 ng/ml concentration was tested. Unconfirmed = variable results from 2-3 experiments.

### 3.5 EFFECT OF LPSs ON THE BARRIER INTEGRITY OF CACO-2 MONOLAYER

Caco-2 human intestinal epithelial cells differentiate into polarized enterocytes and are widely used as an *in vitro* cell culture model of the intestinal epithelium. 14 days post-plating, Caco-2 cells are differentiating and forming cell-cell junctions (tight junctions). When cells are grown on a permeable membrane immersed in the cell culture medium (called insert), the electrical resistance of the cell layer can be measured with transepithelial electrical resistance (TEER) reflecting the barrier tightness. Live bacterial cells from either control, *E. coli* 1 or 2, did not influence the barrier integrity of Caco-2 cell monolayer (Figure 5). High concentration (100 ng/ml) of more proinflammatory LPS from control *E. coli* 2 did not decrease the barrier strength of the cell layer. Our results are in line with the previous studies that have shown that Caco-2 cells are unresponsive to LPS stimulation due to defects in TLR4 signaling (Funda et al. 2001), although live cells from other *E. coli* strain K-12 can impair Caco-2 monolayer integrity (Hiippala et al. 2016).

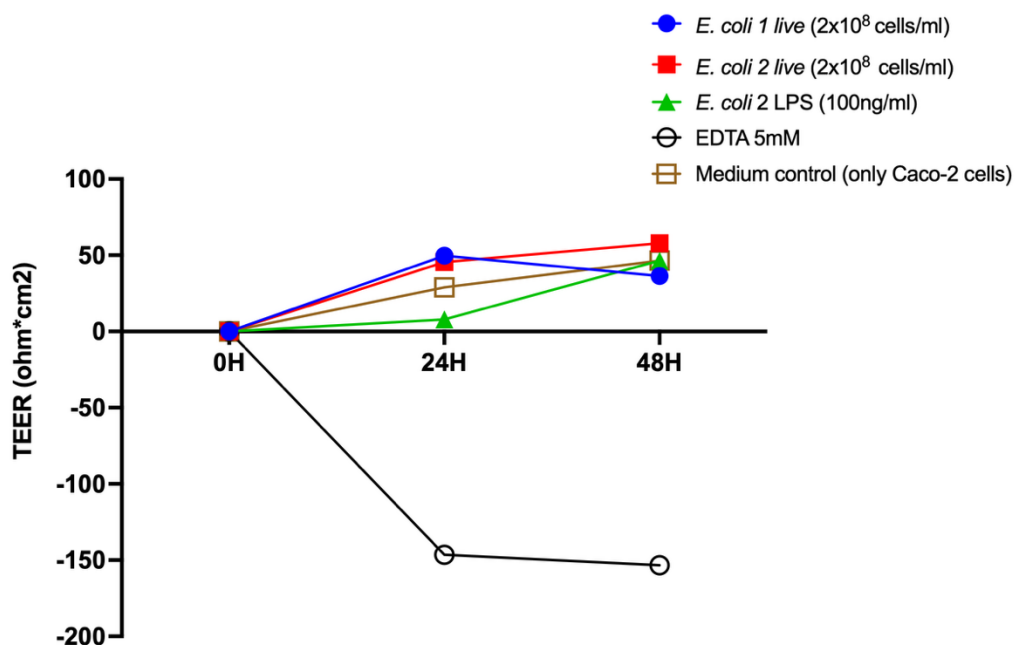


Figure 5. Impact of control *E. coli* live cells and *E. coli* 2 LPS on the epithelial cell line Caco-2 monolayer barrier integrity. The experiment was conducted 14-day post-plating of Caco-2 cells.

## 4. CONCLUSIONS

The work established in WP2 by UH and HUS concerning the *in vitro* cell culture models to study the effect of proinflammatory and tolerogenic LPSs on epithelium is proceeding according to the proposed research plan. We have identified RD-associated species based on scientific literature, explored their inflammation inducing capacity *in vitro* using epithelial cell culture models and tested the protective effect of commensal Bacteroidales LPS in combination with immunologically active RD-linked LPSs.

LPS from several RD-associated bacterial species showed proinflammatory effect on intestinal epithelium *in vitro* and induced IL-8 release from enterocytes. Induction of inflammatory responses in the intestinal epithelium by specific RD-associated species and microbiota dysbiosis in general could be a contributing etiological factor in RD and our results support this hypothesis. On the other hand, commensal species with tolerogenic LPS can counteract the effect of proinflammatory LPS. Our results show that LPS from several Bacteroidales species can effectively decrease the proinflammatory effect on the intestinal epithelium. The neutralizing effect is dependent of the ratio of tolerogenic and proinflammatory LPSs and generally good neutralizing effect was seen, when tolerogenic LPS is present in at least 10-fold amount as compared to the proinflammatory LPS. The neutralizing mechanism of tolerogenic LPS could be due to competition of binding sites or inducing cross-tolerance, but the exact mechanism is yet to be explored.

The results indicate that the characteristics of microbiota dysbiosis in RD (or other diseases) and the relative abundances of proinflammatory and tolerogenic LPSs may contribute to epithelial homeostasis and induction of inflammation on both epithelial and systemic level. The results suggest that analysis of the ratios of Gammaproterobacteria (generally proinflammatory LPS) and Bacteroidales (generally tolerogenic LPS) in overall microbiota profiling could be useful in understanding the implications of disease-associated microbiota imbalance.

The next steps in the *in vitro* studies will assess the immunomodulatory effects of LPSs when they are bound to BMVs, which carry also other cell surface molecules. BMVs present a more natural context of LPS presentation to the gut epithelium as BMVs are highly abundant in the gut and may also cross epithelial barriers (Jones et al. 2020). Also, the reported promising results serve as a base for more complex *in*

*in vitro* co-culture model, which combines two intestinal epithelial cell lines, Caco-2 and HT-29, with immune cells (THP-1 cell line). This triple co-culture model with enterocytes and macrophages models epithelial signal transduction from the intestinal lumen to lamina propria immune cells and facilitates the testing of how different immunomodulatory LPSs affect the epithelium and its integrity in the presence of immune cells, and in turn, how the epithelial cell monolayer interacts with the immune cells via cytokine production during LPS burden.

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