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Responses of increasingly complex intestinal epithelium *in vitro* models to bacterial toll-like receptor agonists

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ABSTRACT

The intestine fulfills roles in the uptake of nutrients and water regulation and acts as a gatekeeper for the intestinal microbiome. For the latter, the intestinal gut barrier system is able to respond to a broad range of bacterial antigens, generally through Toll-like receptor (TLR) signaling pathways. To test the capacity of various *in vitro* intestinal models, we studied IL-8 secretion, as a marker of pro-inflammatory response through the TLR pathway, in a Caco-2 monoculture, Caco-2/HT29-MTX di-culture, Caco-2/HT29-MTX/HMVEC-d tri-culture and in a HT29-p monoculture in response to exposure to various TLR agonists. Twenty-one-day-old differentiated cells in Transwells were exposed to Pam3CSK4 (TLR1/2), lipopolysaccharide (TLR4), single-stranded RNA (TLR7/8), Poly(i:C) (TLR3) and flagellin (TLR5) for 24 h. In all systems IL-8 secretion was increased in response to flagellin exposure, with HT29-p cells also responding to Poly(I:C) exposure. All other agonists did not induce an IL-8 response in the tested *in vitro* models, indicating that the specific TLRs are either not present or not functional in these models. This highlights the need for careful selection of *in vitro* models when studying intestinal immune responses and the need for improved *in vitro* models that better recapitulate intestinal immune responses.

1. Introduction

The intestine fulfills many roles such as digestion of food, nutrient uptake, water regulation and it acts as a gatekeeper for the intestinal microbiome (Silverthorn et al., 2016). Specifically, the intestinal epithelium fulfills many of these roles and contains different cell types supporting these different functions of the intestine. *In vitro* systems that can emulate these different functions of the intestinal epithelium are important for the acute toxicological assessment of chemicals. Current intestinal epithelium *in vitro* systems heavily rely on immortalized cell lines like Caco-2. Although Caco-2 cells originate from a large intestinal source, in culture they differentiate into cells with a functionality resembling that of small intestinal enterocytes. Monolayers of differentiated Caco-2 cells are commonly used as a model to assess the potential transport of compounds from the intestine into the systemic blood circulation. This Caco-2 cell-layer model has shown a high predictability towards intestinal transport *in vivo* for certain compounds (Artursson et al., 2001). In addition, this model has also been used to study direct effects of chemicals on the intestinal epithelium and to study interactions between the epithelium and antigens originating from bacteria residing in the human intestine (Sadabad et al., 2015; Tang et al., 1993).

Interactions between commensal bacteria and the intestinal epithelium influence many aspects of the intestinal functionality, including immune responses, metabolism of exogenous substances and barrier integrity (Peterson and Artis, 2014). Interactions of commensal bacteria and pathogenic bacteria with the intestinal epithelium can activate immune responses. Toll-like receptors (TLRs), expressed on intestinal epithelial cells, play a crucial role in the recognition of the different bacteria. TLRs can be activated by pathogen associated molecular patterns (PAMPs), which are structurally conserved molecular components located intracellularly and on the surface of bacteria (Abreu, 2010). Different TLRs recognize specific PAMPs and the various cell types in the human intestinal epithelium display different TLR expression patterns, so not every intestinal epithelial cell type has the same TLRs (Price et al., 2018). Binding of a bacterial antigen to a specific TLR activates an innate immune response. Intracellularly, the TLR signal transduction initiates the myeloid differentiation primary response protein 88 (MyD88) pathway, which leads to the production of pro-inflammatory cytokines such as IL-8 (Takeda and Akira, 2004). The recruitment of neutrophils

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from the blood and lymphatic system caused by the IL-8 secretion initiates the next steps in the intestinal innate immune response. (Newberry and Lorenz, 2005; Rakoff-Nahoum et al., 2004; Säemann et al., 2000).

The TLR1/2 heterodimer recognizes, amongst others, bacterial triacyl lipopeptides, TLR3 recognizes dsRNA, TLR4 recognizes lipopolysaccharides (LPS), TLR5 is well-known for recognizing flagellin, and lastly TLR7 and 8 both recognize ssRNA. The most commonly used *in vitro* intestinal epithelial model, Caco-2 cells, is an obvious choice to develop an intestinal model combining the intestinal barrier with the microbiome. Current knowledge however, indicates the presence of TLR3 and TLR5, but absence of TLR2 and TLR4 in Caco-2 cells (Böcker et al., 2003). The presence of the other TLRs in Caco-2 cells is largely unknown. Absence of TLRs leads to an inability to respond to microbial antigens like LPS. Therefore, to obtain the full range of antigen responsiveness co-culturing of Caco-2 cells with other intestinal cell-types needs to be explored.

HT29-parental (HT29-p) cells were isolated from a colorectal adenocarcinoma and were shown to behave differently depending on the culture method. In glucose containing medium they mostly form a multilayer of undifferentiated intestinal cells. Exposure of the HT29-p cells to methotrexate results in cells with an increased mucus production (the so-called HT29-MTX cells). Therefore these cells, are considered an *in vitro* model for goblet cells (Martínez-Maqueda et al., 2015). Mucus is an important intestinal barrier and energy source for intestinal bacteria and is thus an important feature to enable coculture of intestinal epithelial cells with a microbiome in future experiments (McGuckin et al., 2011; Ottman et al., 2017).

Lastly, vascular endothelial cells have an important function in the intestinal mucosa(Ferrari et al., 2017; Tarnawski et al., 2012), not so much in terms of barrier properties, but mainly for signaling and communication between epithelial cells. HMVEC-d cells are human dermal microvascular cells isolated from small vessels within the skin and are commonly used as an endothelial cell model(Maschmeyer et al., 2015; Schimek et al., 2013). Models combining microvascular cells and Caco-2 cells have previously been used to study interactiuons between the cells and the microbiome(Kim et al., 2016; Shin et al., 2019). They have been shown to express most TLRs except for TLR7, 8 and 10 (Fitzner et al., 2008). It has been shown that antigens, like LPS, can be transported through the intestinal epithelium, so endothelial cells can also play a role in the intestinal immune responses (Akiba et al., 2020). There are multiple studies that show that different intestinal epithelial cells and HMVEC-d cells that show different immune responses and TLR expression patterns compared to Caco-2 cells (Melmed et al., 2003; Uehara et al., 2007). Combining all these different cells into one model may potentially result in a model that is able to respond to a larger variety of bacterial antigens (Angrisano et al., 2010).

We aim to create an *in vitro* gut barrier system that is able to respond to a broad range of bacterial antigens. To do this, we expand on the traditional Caco-2 monolayer cultured in Transwells by the addition of different cell types and we evaluated the contribution of the different cells on the immuno-responsiveness of the model by exposure to a panel of TLR agonists. As an immune response readout we measured the production of IL-8 (Takeda and Akira, 2004). The selected agonists and their targets were Pam3CSK4 (TLR1/2), poly(i:C) (TLR3), lipopolysaccharide (TLR2/4), flagellin (TLR5) and single-stranded RNA (TLR7/8).

2. Materials and methods

2.1. Cell lines

The human intestinal epithelial Caco-2 (ATCC HTB-37) cell line was used from passage 5 to 30 and HT29-p (ATCC HTB38) cells, used from passage 5 to 30, were obtained from ATCC (United Kingdom). The mucus secreting HT29-MTX-E12 (ECACC 12040401) cell line (further referred to as HT29-MTX) was used from passage 5 to 30 and was obtained from the HPA culture collections (Sigma-Aldrich, Germany). The human dermal microvascular cell line HMVEC-d used until passage 10 was obtained from Lonza (Bazel Switzerland). Caco-2, HT29-MTX and HT29-p were cultured in Dulbecco's modified Eagle's medium + GlutaMAX (DMEM+GlutaMAX, Life technologies, Belgium) with 10% FCS (Sigma-Aldrich, The Netherlands), 1% non-essential amino acids (Invitrogen, Breda, The Netherlands) and 1% penicillin/streptomycin (Invitrogen, The Netherlands), further referred to as complete medium. HMVEC-d was grown in Microvascular Endothelial Basal Medium (Sigma-Aldrich, The Netherlands) with Microvascular Endothelial Cell Growth Supplements (Sigma-Aldrich, The Netherlands). The cells were grown in 75 cm^2 flasks (Greiner, The Netherlands) at 37 °C 5% CO_2 in an incubator and passaged by trypsinization at 70–90% confluence.

2.2. TLR agonists

TLR1/2 agonist Pam3CSK4, TLR3 agonist Poly(I:C) high molecular weight, TLR2 and 4 agonist standard LPS from *E. coli* O111:B4, TLR5 agonist flagellin from *Salmonella typhimurium* Ultrapure and TLR8 agonist ssRNA40/LyoVec were obtained from InvivoGen (France).

2.3. Transwell culture

Cells were grown in 12-well Transwell permeable supports, with an 0.4 μm polycarbonate membrane of Corning costar (VWR, the Netherlands). These were coated with a Collagen-1 solution from human fibroblasts (Sigma-Aldrich, The Netherlands) at 10 μg/cm^2 by dissolving the Collagen-1 in 0.25 acetic acid and incubating the solution at 37 °C for at least three hours in the Transwell inserts before removing the liquid. All Transwells were seeded apically at 4×10^4 cells per insert. For the di-culture of Caco-2/HT29-MTX cells were seeded at a 3:1 ratio. Cells were maintained in complete medium for 21 days which is required for the differentiation of Caco-2 cells (Hubatsch et al., 2007), the medium was replaced three times per week.

For the tri-culture model, HMVEC-d cells were seeded on the basolateral side of the Transwells on day 0 before seeding the epithelial cells on the apical side. To do this, the Transwell inserts were inverted and placed on the lid of a 6-well plate placing sterile caps of Eppendorf tubes on the corners to avoid contact between the lid and the drops (Kamelia et al., 2017). HMVEC-d cells were seeded at 2×10^4 cells per Transwell by pipetting a droplet onto the inverted inserts. After putting the lid on top of the caps 3 M micropore tape was used to cover the gap between the lid and the plate. The plates were placed in an incubator at 37 °C and 5% CO_2 for 2 h to allow the cells to attach to the membrane. The Transwells inserts were then inverted back to their original configuration and placed in a 12 wells-plate with HMVEC-d medium in each well. Following, the Caco-2 and HT29-MTX cells were seeded at 4×10^4 cells per insert at a 3:1 ratio. Cells were maintained for three weeks with complete medium apically and HMVEC-d medium basolaterally, medium on both sides was replaced three times per week.

TEER was measured three times per week using a Millicell ERS-2 (Merck Millipore, USA) after refreshing the medium. Washing is needed to remove old medium that might have a changed pH and contain breakdown products that can affect the TEER measurements. The Transwells were allowed to rest for at least three hours between changing the medium and the TEER measurement, as also recommended by (Hubatsch et al., 2007). We also checked paracellular fluorescein translocation after 21 days to further assess the monolayer integrity. Fluorescein was diluted in HBSS without Phenol red at 10 μM and 500 μL was added to the apical compartment. After one hour 150 μL was taken from both compartments. Fluorescence was measured using a spectramax spectrophotometer (Molecular Devices, USA) at 494/512 nm.

2.4. Transwell exposure

After 21 days of growth and differentiation of the cells, the

Transwells were exposed to the TLR agonists for 24 h (Table 1). The final concentrations, LPS (20 μg/mL), Pam3CSK4 (300 ng/mL), Poly(I:C) (20 μg/mL), flagellin (100 ng/mL) or ssRNA (5 μg/mL), were achieved by dissolving stock solutions in complete medium and afterwards added to the apical compartment(Ashtekar et al., 2008; Ivison et al., 2010; Price et al., 2018; Tu et al., 2016). These concentrations are regarded as non-cytotoxic because they are lower than used in previous studies (see Table 2). Reported cytotoxicity following exposure to LPS for instance was in the mg/ml concentration range (Guo et al., 2013). Complete medium without agonists was added to the basolateral compartment. The Transwells were then either processed for confocal microscopy or cells were collected for protein content determination. All experiments were done in technical and biological triplicates.

2.5. IL-8 measurement

The IL-8 concentration in the apical and basolateral medium was measured separately using an ELISA (Enzo life sciences, Belgium) according to the manufacturers protocol. Medium was collected directly after exposure and stored at −80 °C before analysis. The concentration was calculated using a 4-parameter logistic curve. The results are presented as amount secreted (pg) and thus corrected for the different volumes in the apical and basolateral compartments of the Transwells. Statistical significance was analyzed using a one-way ANOVA to compare the effects of the exposures to their relative controls.

2.6. Confocal microscopy

The Transwells were fixated with 4% formaldehyde in PBS for 10 min followed by three wash steps with PBS, permeabilization with 0.25% Triton x-100 and blocking with 1% acetylated-BSA in PBS. Samples were first incubated with ZO-1/TJP1 Antibody Rabbit (polyclonal) - Alexa Fluor 594 (Invitrogen, The Netherlands) at 10 μg/mL for 1 h to stain the tight junctions followed by three washing steps with PBS. Then with Phalloidin Alexa Fluor 488 diluted 1:50 (Life technologies, Belgium) at 6 units/well for 30 min to stain the actin followed by three washing steps with PBS and finally with DRAQ5 (Abcam, United Kingdom) at 10 μM for 30 min to stain the nuclei. The membranes were then cut from the Transwell inserts using a scalpel and tweezers, the mono- and di-cultures were placed on a microscope slide while the tri-culture was placed on a coverslip. SecureSeal Imaging Spacers (Sigma-Aldrich, The Netherlands) were used to avoid crushing of the cells, ProLong Diamond Antifade mountant (life technologies, Belgium) was added to reduce fading. The slides were analyzed using a Leica TCS SP8 laser scanning microscope using an Apochromatic 63×/1.20 water immersion objective with a white light laser and 440 pulsed laser. EX488/EM525 (Phalloidin) EX594/EM617 (ZO-1) and EX594/EM725 (DRAQ5) with pinhole 122.7 μM.

3. Results

3.1. Barrier integrity

To assess monolayer integrity TEER was measured on the same days as the medium was refreshed. During cell differentiation the TEER steadily increased over time, in some cases a slight decline was seen during the last few days. Between day 18 and 20 the final TEER measurement was performed to determine whether the Transwells could be used. Of the studied cell models, the final TEER measurement showed the highest TEER values for the Caco-2 cell-layers, *i.e.* >500 Ω·cm^2 (data not shown). The TEER values were lowest for the HT29-p cells, *i.e.* <100 Ω·cm^2, and TEER values of the Caco-2/HT29-MTX and Caco-2/HT29-MTX/HMVEC-d di-and tri-culture were > 400 Ω·cm^2 (data not shown).

For all models, except HT29-p, only Transwells with a TEER value >300 were used in the experiments (in accordance with (Hubatsch et al., 2007)). For the HT29-p Transwells the lack of TEER has to be taken into account as it indicates that there is no proper barrier so the direction of the response cannot be assessed.

Besides TEER measurements, also fluorescein translocation experiments (Fig. 1) were performed to evaluate the barrier integrity. Fluorescein translocation was measured on day 21, before the exposure. The Caco-2 monoculture showed an average of 3.3% translocation. The Caco-2/HT29-MTX di-culture and the Caco-2/HT29-MTX/HMVEC-d tri-culture showed an average of 2.2% and 3.8% translocation, respectively, and did not significantly differ from the Caco-2 monoculture. However, at 11.9%, the HT29-p monoculture showed a significantly higher translocation than the Caco-2 cells, again indicating improper barrier function in this model.

Table 1
Agonists and their associated TLRs.

Compound	Concentrations used	TLR
Pam3CSK4	300 ng/mL	1/2
Poly(I:C)	20 μg/mL	3
LPS	20 μg/mL	4
Flagellin	100 ng/mL	5
ssRNA	5 μg/mL	7 + 8

3.2. Cellular morphology

We assessed the cellular morphology of the different models using confocal microscopy. Representative images are shown in Fig. 2. The cells on the apical side of the membrane in the Caco-2 monolayer (Fig. 2A) and in the Caco-2/HT29-MTX di-culture (Fig. 2B) strongly expressed the zonula occludens 1 (ZO-1) protein, also known as tight junction protein 1, between the cells. In all three models confluent cell-layers were observed and no overlapping cells could be noted. For the HT29-p model (Fig. 2D and E) we observed overlapping cells indicating that these cells formed a multilayer.

Lastly, the HMVEC-d cells, grown on the basolateral side of the membranes in the tri-culture model, are shown in Fig. 2C. HMVEC-d cells displayed a pronounced actin skeleton and had a wider flattened morphology compared with the intestinal cells. As can be observed from Fig. 2C there were gaps in the cell coverage of the basolateral side of the membrane, indicating that the HMVEC-d cells did not form a confluent monolayer.

3.3. IL-8 measurement

We aim to create an *in vitro* gut barrier system that is able to respond to a broad range of bacterial antigens, and therefore used models with increasing cellular complexity. We firstly determined the baseline secretion of IL-8 of each cell model (mono-, di- and tri- cultures) without the addition of TLR agonists (Fig. 3). Monocultures of Caco-2 cells produced a low amount of IL-8, with values often at or below the limit of detection at 7.8 pg. No differences were observed between apical and basolateral secretion of IL-8. The di-culture model, obtained by the addition of HT29-MTX cells to the model resulted in an increased IL-8 secretion of 38 pg apically and 98 pg basolaterally. The basal IL-8 secretion was further enhanced, and significantly different from the Caco-2 model, by combining Caco-2 with HT29-MTX cells on the apical side of the membrane and growing HMVEC-d cells on the basolateral side of the membrane. In this tri-culture model the amount of IL-8 released was 189 pg IL-8 apically and 336 pg basolaterally. The highest basal overall IL-8 production, and significantly different from the Caco-2 model, was observed in the HT29-p cells, which produced an average of 260 pg apically and 321 pg basolaterally (Supplementary Fig. S1).

In the next step we exposed our four *in vitro* models, *i.e.* the Caco-2 monoculture (Fig. 3), the Caco-2/HT29-MTX di-culture (Fig. 4), the Caco-2/HT29-MTX/HMVEC-d tri-culture (Fig. 5) and the HT29-p

Table 2
Summary of IL-8 responses of Caco-2 HT29-MTX, HT29-p cells from previous studies.

Cells used	Exposure	Concentration (ng/mL)	Exposure time (hour)	TLR target	Barrier integrity	IL-8 response/base level (pg/mL)	Direction of secretion	Comment	Reference
Caco-2	Flagellin	100	3	TLR5	N.T.	191 ± 28.7/None	N.T.	Oxidative stress response induced by H_2O_2 increases the IL-8 excretion	(Ivison et al., 2010)
Caco-2	Flagellin	500.000	16	TLR5	TEER, no effect	160/None	Not reported	Secretion of CCL20 was also increased. *C. difficile* toxin B increased the IL-8 response to flagellin likely by disrupting the tight junctions.	(Yoshino et al., 2013)
Caco-2	Flagellin	1000	2 and 24	TLR5	N.T.	330/100 + 110/100	N.T.	Brucella (flagellated) did not induce an IL-8 response. Response was given as an increase over basal excretion.	(Ferrero et al., 2012)
Caco-2	LPS	100.000	24	TLR4	N.T.	70/25	N.T.	Glutamine decreased the LPS induced IL-8 production. IL-6, TNF-α and IL-10 were below the LOD in all cases.	(Huang et al., 2003)
Caco-2	LPS	100	16	TLR4	N.T.	N.O./100 ± 70	N.T.	An IL-8 response in Caco-2 was observed for IL-1β, but not for IFN-γ, TNF-α and LPS.	(Schuerer-Maly et al., 1994)
Caco-2	LPS	1-10.000	24	TLR4	N.T.	N.O./N.R.	N.T.	The cells showed no TLR4 or 2 mRNA and protein expression.	(Böcker et al., 2003)
Caco-2	LPS	100	12	TLR4	N.T.	N.O./250	N.T.	IL-1β and TNF-α induced an IL-8 response.	(Sang et al., 2005)
Caco-2	LPS	20.000	24	TLR4	TEER, decreased	120/80	Not checked	IL-8 response was attenuated by chitosan nanoparticles. The same trend was seen for the TNF-α, MIF and MCP-1 levels after exposure. Expression of TLR4 was observed (qPCR) in Caco-2 cells.	(Tu et al., 2016)
Caco-2	Pam2CSK4	100	24	TLR2	TEER, effect not checked	140/10	Not checked	Lipoteichoic acid inhibited the response to Pam2CSK4. There was no IL-8 mRNA response to LPS and Poly(I:C).	(Noh et al., 2015)
Caco-2	Pam2CSK4	10.000	24	TLR2/6	TEER, no effect	30 apical 3 basolateral/none	Secretion mostly apical	Apical exposure resulted in higher responses than basolateral exposure. Used the Caco-2 BBE strain.	(Rossi et al., 2013)
Caco-2	Pam3CSK4	50.000	1–24	TLR1/2	Not checked	1200/<50	Not checked	Response to Pam3CSK4 was time dependent between 1 and 24 h. Butyrate attenuated the IL-8 response *via* A20, which blocks the NF-κβ pathway.	(Weng et al., 2007)
Caco-2	Pam3CSK4	20.000	24	TLR1/2	TEER, no effect	7 apical 3 basolateral/none	Secretionmostly apical	Apical exposure resulted in higher responses than basolateral exposure. Used the Caco-2 BBE strain.	(Rossi et al., 2013)
Caco-2	Pam3CSK4	Not reported	24	TLR1/2	N.T.	+70/100	N.T.	Response was noted as increase over basal excretion.	(Ferrero et al., 2012)
Caco-2	Poly(I:C)	10.000	24	TLR3	N.T.	N.O./none	N.T.	8.1% of cells expressed TLR 3 protein. They showed that there was no clear link between TLR expression and immune response.	(Uehara et al., 2007)
Caco-2	ssPolyU	1000	24	TLR8	N.T.	N.O./none	N.T.	They showed that there was no clear link between TLR expression and immune response.	(Uehara et al., 2007)
HT29-MTX	LPS	1-10.000	24	TLR4	N.T.	No/175	N.T.	The cells showed no TLR4 or 2 mRNA expression.	(Böcker et al., 2003)
HT29-MTX	LPS	1-100	12–48	TLR4	N.T.	2800 at highest conc/300	N.T.	Concentration dependent response. The response could be measured between 12 and 48 h after stimulation with a peak at 24 h. LPS also stimulated mucin production.	(Smirnova et al., 2003)
HT29-p	Flagellin	500.000	5	TLR5	TEER, effect not checked	±700/±220	Not reported	Also showed an increase in CCL20. *C. difficile* toxin B increased the IL-8 response to flagellin likely by disrupting the tight junctions.	(Yoshino et al., 2013)
HT29-p	Flagellin	1000	24	TLR5	N.T.	±13.000/<100	N.T.	Brucella (flagellated) did not induce an IL-8 response.	(Ferrero et al., 2012)
HT29-p	LPS	100	16	TLR4	N.T.	±10,000/1400 ± 100	N.T.	Concentration dependent IL-8 response starting at 0.1–100 ng/mL. TNF-α, IFN-γ and IL-1β exposure also induced an IL-8 response.	(Schuerer-Maly et al., 1994)
HT29-p	LPS	1-10.000	24	TLR4	N.T.	1200 at highest conc/100	N.T.	Exposure to butyrate reduced TLR4 mRNA expression and IL-8 excretion in response to LPS. The cells only showed TLR4 expression, not TLR2.	(Böcker et al., 2003)
HT29-p	LPS	100	12	TLR4	N.T.	4500/600	N.T.	IL-1β and TNF-α also induced an IL-8 response. Differentiation of HT29-P using butyrate reduced the IL-8 response and the increase in TLR-4 expression.	(Sang et al., 2005)
HT29-p	Pam3CSK4	Not reported	6	TLR1/2	N.T.	No/<4000	N.T.	TNF-α induced an IL-8 response, IFN-γ did not. Lipoteichoic acid attenuated the response.	(Kim et al., 2012)
HT29-p	Pam3CSK4	Not reported	24	TLR1/2	N.T.	No/N.R.	N.T.	Brucella (flagellated) did not induce an IL-8 response.	(Ferrero et al., 2012)
HT29-p	Poly(I:C)	10.000	24	TLR3	N.T.	±2800/<200	N.T.	19.3% of cells expressed TLR 3 protein. They showed that there was no clear link between TLR expression and immune response.	(Uehara et al., 2007)
HT29-p	ssPolyU	1000	24	TLR8	N.T.	±3600/<200	N.T.	They showed that there was no clear link between TLR expression and immune response.	(Uehara et al., 2007)

Legend: N.O. = Not observed, N.R. = Not reported, N.T. = No Transwell used.

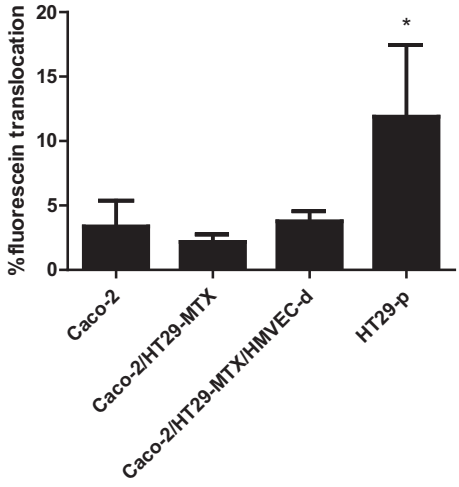

Fig. 1. Percentage of fluorescein translocation from the apical to basolateral side in the different cell systems after one hour of incubation with 10 μM fluorescein. * Statistical significance compared to the Caco-2 monoculture ($p < 0.05\%$).

monoculture (Fig. 6), to five TLR agonists interacting with the TLR1/2 heterodimer, TLR3, 4, 5, 7 or TLR8 (see Table 1). In the Caco-2 monoculture, following exposure to the TLR agonist flagellin, we observed a significant increase in the apical IL-8 concentration ($p < 0.01$) (Fig. 3). No significant responses were observed following exposure to LPS, poly(I:C), Pam3CSK4 and ssRNA. The IL-8 concentrations on the basolateral side were lower as compared to the apical concentrations, but exposure to flagellin also significantly increased the IL-8 concentration basolaterally ($p < 0.05$).

A significant increase in apical secretion of IL-8 was also observed in the Caco-2/HT29-MTX di-culture following exposure to flagellin (Fig. 4; $p < 0.001$), while exposure to LPS, Pam3CSK4, poly(I:C) and ssRNA did not induce an IL-8 response. On the basolateral side none of the compounds induced an increase in IL-8 secretion.

In the Caco-2/HT29-MTX/HMVEC-d tri-culture (Fig. 4) we observed a significant increase in apical IL-8 secretion after exposure to flagellin ($p < 0.001$), with no IL-8 response following exposure to LPS, poly(I:C), Pam3CSK4 and ssRNA. On the basolateral side none of the agonists induced an increase in IL-8 concentration in the medium.

In the HT29-p monoculture (Fig. 5) we observed a significant IL-8 increase both apically and basolaterally following exposure to Poly(I:C) and flagellin ($p < 0.01$). No increase in IL-8 secretion was seen following the other exposures.

3.4. Literature review of present of TLR receptors and IL-8 secretion by intestinal cells in vitro

IL-8 responses of the individual cell types used in this study to TLR agonists have been reported before. No studies using co-culture models were identified by us. However, there are large differences in study design and reported effects. Therefore, these studies have been analyzed and summarized in Table 2. From this overview it becomes clear that the direction (*i.e.* apical or basolateral) of IL-8 secretion has only been studied to a limited extent. The IL-8 responses of Caco-2 cells exposed to LPS are variable, where some studies report IL-8 secretion after LPS exposure while others do not observe this, even when comparable LPS concentrations were used (see Table 2). Pam2CSK4 exposure of Caco-2 cells consistently resulted in an IL-8 secretion at high exposure concentrations (see Table 2). Only very few studies investigated the IL-8 responses of Caco-2 cells to poly(I:C) and ssPolyU.

HT29-MTX cells can respond with IL-8 secretion to LPS exposure as observed in some, but not all, studies (see Table 2), while flagellin triggers the excretion of IL-8 in all studies. No studies were found that exposed HT29-MTX cells to our other TLR agonists.

HT29-p cells have previously been exposed to LPS and in all studies IL-8 secretion was observed (see Table 2), which was the same for flagellin. Pam3CSK4 exposure did not result in IL-8 secretion by HT29-p cells, while only one study showed IL-8 secretion by HT29-p cells following exposure to both poly(I:C) and ssPolyU (see Table 2).

The gene expression of TLRs in the Caco-2, HT29-p and HMVEC-d cells has been studied extensively before. The overview of the literature data is summarized in Table 3. For Caco-2 cells the TLR expression has been studied most extensively. In the case of TLR 1, 3, 5 and 7, the available studies unanimously reported the gene expression of these TLRs. For TLR 2, 4 and 8, the results were contradictory. The latter is likely due to the different read-outs and methods that were used in the expression studies. Most studies used gene expression (qPCR on pooled cells) as a read-out, but some studies (*e.g.* Uehara et al. (2007) and Böcker et al. (2003)) used protein expression (flow cytometry or western blotting) as an additional read-out. Read-out on a protein level showed that 9.6% of the cells expressed TLR 2 and 7.7% of the Caco-2 cells expressed TLR4, but no expression of TLR4 in Caco-2 cells was shown using western blotting as a read-out by Böcker et al. (2003). In HT29-p there is largely a consensus in the literature on the presence of all the TLRs, only for TLR2 some ambiguity is present on the expression of this TLR albeit in both studies rt. PCR was used (Böcker et al., 2003; Kim et al., 2012; See Table 2).The protein expression of TLR2 was determined using flow cytometry and showed that 22.5% of HT29-p cells expressed TLR2(Uehara et al., 2007).For HT29-MTX cells there is very limited data on TLR expression, but it is expected that the TLR expression will not differ from that observed in the parental HT29-p cell line. All 8 TLRs genes are expressed in HMVEC-d cells (Fitzner et al., 2008). For TLR 1–5 expression was present in resting cells and in cells that were activated by exposure to proinflammatory cytokines. Activated cells showed a relatively higher expression of TLR 2 and 4 than observed in the resting cells. For TLR 7 and 8 there was no expression under resting conditions, but both TLRs were expressed in activated cells (Fitzner et al., 2008).

4. Discussion

We aimed to evaluate the potential of *in vitro* intestinal models with increasing cellular complexity to study immunomodulation induced by exogenous factors by measuring their IL-8 responses to a broad range of bacterial antigens. For this, we compared monocultures and combined cultures of different epithelial cell lines, *i.e.* Caco-2 and HT29-p cells, a di-culture of Caco-2 and HT29-MTX cells, and a tri-culture of Caco-2 and HT29-MTX cells with the microvascular cell line HMVEC-d, and evaluated their responses to five TLR agonists, Pam3CSK4, LPS, ssRNA, poly(I:C) and flagellin. These agonists represent model compounds for important TLRs that respond to infection in the human intestine. As no co-culture models studies are available where the following TLR agonist exposure are reported we compare our observations to results obtained from mono-culture models.

A striking difference between all the cell models evaluated in this study is their secretion of IL-8 without immunological stimulation. Caco-2 cells show a relatively low baseline IL-8 secretion while the HT29-p cells and the co- and tri-culture models with HT29-MTX and hMVEC-d cells show a higher baseline secretion. All cell models have been cultured for 21 days, we and others have previously shown that after 21 days of culturing the intestinal cells are fully differentiated as indicated by the activity of alkaline phosphatase (Kulthong et al., 2020). Co-culturing of Caco-2 with HT29-MTX results in the secretion of a mucus layer on top of the cells (Walczak et al., 2015). Mucus is a porous gel-like material with pore sizes reported around 200 nm (for porcine and mouse mucus) (Bajka et al., 2015) and will therefore not act as a size exclusion barrier for the studied agonists. The binding affinity of mucin for bacterial cell wall components like LPS are also reported to be low (Kim and

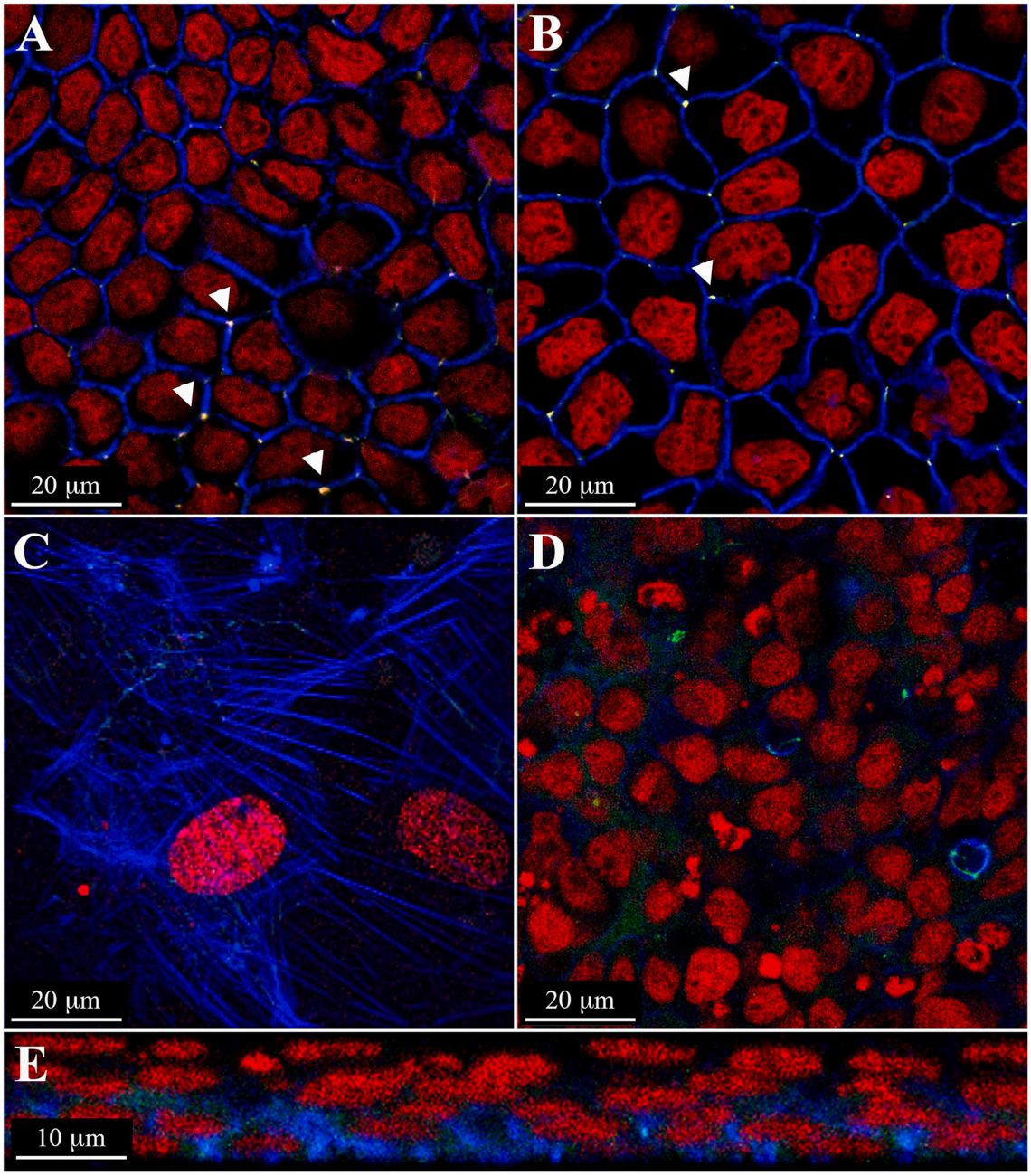

Fig. 2. Confocal images of a horizontal cross-section of the Caco-2 monoculture (A), the Caco-2/HT29-MTX di-culture (B) on the apical side, and of the HMVEC-d cells in the Caco-2/HT29-MTX/HMVEC-d tri-culture on the basolateral side (C). Confocal image of a horizontal cross-section on the apical side (D) and a vertical cross-section (E) of the HT29-p monoculture. Used stainings were for actin (blue C, D and E), DNA (red) and ZO-1 (green, due to the co-localisation of action and Zo-1 the green colour is only visible at the crossings of cells (see arrowheads A and B). Images of individual channels can be found in Supplementary Fig. 2. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Ho, 2010; Smirnova et al., 2003), so taken together mucus will most likely not pose a barrier to the compounds used in this study.

A review of the literature, as presented in Table 2, showed the same general trend, for Caco-2 the baseline IL-8 expression ranged between 10 and 250 pg, for HT29-MTX cells it ranged between 175 and 300 pg and for HT29-p it ranged between 100 and 1400 pg, while the baseline IL-8 secretion by hMVEC-d cells has not been reported. Most studies were not performed in a Transwell system, so no information is available on the direction of IL-8 secretion (*i.e.* apical or basolateral). Besides the difference in intrinsic cell line properties the variety in culture methods makes it difficult to pinpoint a specific factor that contributes to this variation in IL-8 secretion The expression of the TLRs in the individual cell lines has been studied before, as presented in Table 3, showing general consensus on the presence or absence of specific TLR expression in Caco-2 and HT29-p cells. Only for HT29-MTX cells the TLR expression has limitedly been studied. In this study, we focused on the functionality of the models, and thus we studied both the apical and basolateral secretion of IL-8. When the IL-8 secretion was increased following TLR agonist exposure, the direction of the IL-8 secretion was in most cases bilateral. This can be understood from a functional point of view. IL-8 functions as a chemoattractant for neutrophils (Miller and Krangel, 1992), therefore secretion from both sides of the intestinal barrier is needed to guide the neutrophils from the bloodstream to the site of the infection. An extreme situation of this can be seen in ulcerative colitis

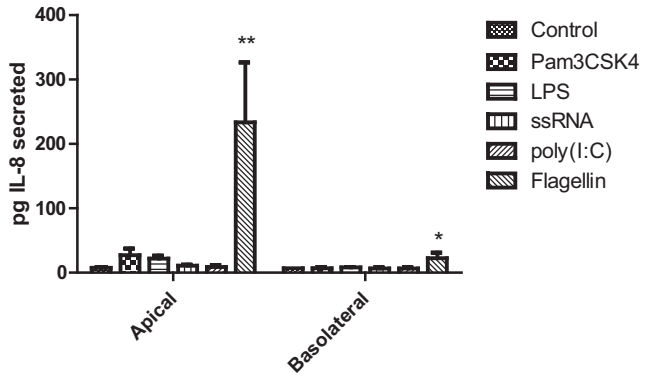

Fig. 3. IL-8 secretion by the Caco-2 monoculture into the apical or basolateral medium after 24 h of exposure to Pam3CSK4 (300 ng/mL), LPS (20 μg/mL), ssRNA (5 μg/mL), poly(I:C) (20 μg/mL), or flagellin (100 ng/mL). Significant differences *versus* the control (no exposure) samples are indicated with * = $p < 0.05$ and ** = $p < 0.01$.

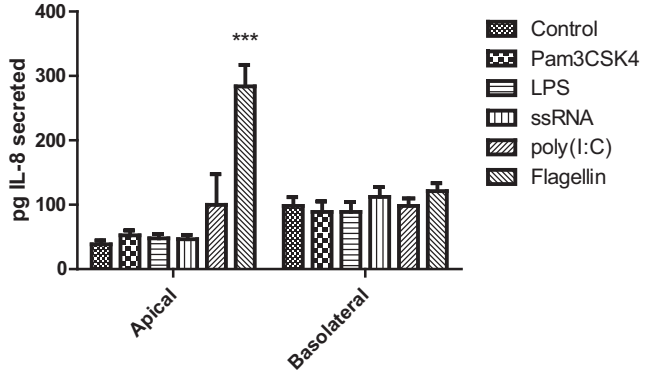

Fig. 4. IL-8 secretion by the Caco-2/HT29-MTX di-culture into the apical or basolateral medium after 24 h of exposure to Pam3CSK4 (300 ng/mL), LPS (20 μg/mL), ssRNA (5 μg/mL), poly(I:C) (20 μg/mL) or flagellin (100 ng/mL). Significant differences *versus* the control (no exposure) samples are indicated with *** = $p < 0.001$.

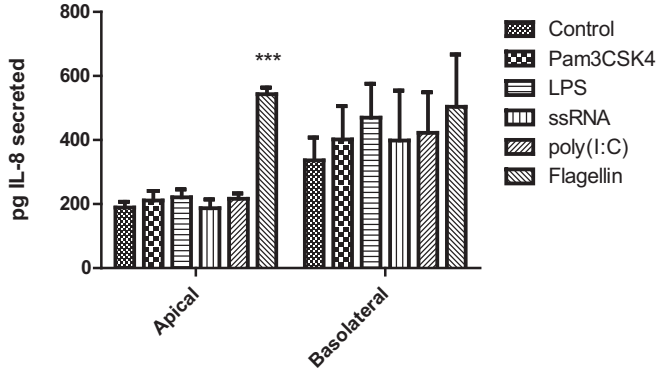

Fig. 5. IL-8 secretion by the Caco-2/HT29-MTX/HMVEC-d tri-culture into the apical or basolateral medium after 24 h of exposure to Pam3CSK4 (300 ng/mL), LPS (20 μg/mL), ssRNA (5 μg/mL), poly(I:C) (20 μg/mL) or flagellin (100 ng/mL). Significant differences *versus* the control (no exposure) samples are indicated with *** = $p < 0.001$.

patients as they show high levels of IL-8 leading to migration of neutrophils to the mucosa on the apical side (Mahida et al., 1992; Mitsuyama et al., 1994). A similar response can be expected in case of a bacterial infection. The intestinal microbiome represents a complex

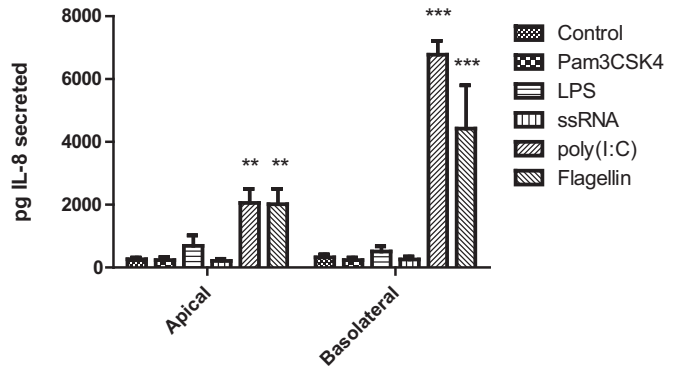

Fig. 6. IL-8 secretion by the HT29-p monoculture into the apical or basolateral medium after 24 h of to Pam3CSK4 (300 ng/mL), LPS (20 μg/mL), ssRNA (5 μg/mL), poly(I:C) (20 μg/mL) or flagellin (100 ng/mL). Significant differences *versus* the control (no exposure) samples are indicated with ** = $p < 0.01$ and *** = $p < 0.001$.

Table 3
Summary of TLR gene expression data from literature in Caco-2, HT29-MTX, HT29-p and HMVEC-d cells.

Cell type	TLR	Gene expression	Reference
Caco-2	1	+	Furrie et al., 2005
	2	+	Furrie et al., 2005; Böcker et al., 2003
	3	+	Furrie et al., 2005; Salaris et al., 2021
	4	+/−	Furrie et al., 2005; Tu et al., 2016; Böcker et al., 2003
	5	+	Devriese et al., 2017
	7	+	Salaris et al., 2021; Yi et al., 2009
	8	+/−	Cammarota et al., 2009; Toki et al., 2009
HT29-MTX	1	N.A.	
	2	–	Böcker et al., 2003
	3	N.A.	
	4	–	Böcker et al., 2003
	5	N.A.	
	7	N.A.	
	8	N.A.	
HT29-p	1	+	Furrie et al., 2005; Graves et al., 2014; Tian et al., 2016
	2	+/−	Kim et al., 2012; Böcker et al., 2003
	3	+	Furrie et al., 2005; Graves et al., 2014; Tian et al., 2016
	4	+	Böcker et al., 2003; Furrie et al., 2005; Sang et al., 2005
	5	+	Graves et al., 2014; Tian et al., 2016
	7	+	Graves et al., 2014; Tian et al., 2016
	8	+	Graves et al., 2014; Tian et al., 2016
HMVEC-d	1–8	+	Fitzner et al., 2008

Legend: N.A. no information available, + TLR expression confirmed, − TLR expression not confirmed; +/− ambiguous results.

system with complex and extensive interactions with the intestinal epithelium. These interactions can be mediated *via* a range of signaling molecules and bacterial metabolites and other bacterial products (Kau et al., 2011; Thaiss et al., 2016). I our study we look at the very first step of interaction between antigens and the intestinal epithelium focusing on the stimulation of IL-8 by well characterized TRL agonists.

Flagellin is a part of the bacterial flagellum, which plays a role in the motility, adhesion and invasion of cells by (certain) bacteria (Haiko and Westerlund-Wikström, 2013). In literature, concentration ranges between 100 and 500,000 ng/mL were used for *in vitro* testing, we used a concentration of 100 ng/mL, as flagellin is known to be a potent immune activator which acts *via* TLR5 (Gewirtz et al., 2001; Murthy et al., 2004). Even though we exposed our cells to a relatively low concentration of flagellin the detected response was as high or higher than that reported in literature for monocultures of Caco-2 and HT29-p cells (Table 2

(Ivison et al., 2010; Yoshino et al., 2013). No human *in vivo* data are available. The only *in vivo* data that are available are derived from a rodent study. Exposure to flagellin in mice resulted in system-wide immune activation, in this study flagellin was administered *via* injection in the bloodstream (Rolli et al., 2010; Strindelius et al., 2004).

Poly(I:C) is a synthetic double stranded RNA used to simulate viral infection (Fortier et al., 2004) and is recognized by TLR3. We observed a significant IL-8 response against Poly(I:C) in the HT29-p culture, but there was no response in the other cell systems. This corroborates with the results described in literature for Caco-2 and HT29-p cells, as Poly(I:C) exposure to HT29-P or Caco-2 cells induced a response in HT29-p cells only. The cause of this difference is unknown as both cell types were shown to express TLR3 protein where Caco-2 and HT29-p cells expressed TLR3 on 19.3% and 8.1% of the total number of cells, respectively (Uehara et al., 2007). The exposure of the di-culture of Caco-2 and HT29-MTX cells to Poly(I:C) did not induce an IL-8 response, which indicates that the HT29-MTX cells differ from their parental cell line in their response to Poly(I:C).

Lipopolysaccharides are part of the outer membrane of gram-negative bacteria and are recognized by TLR4 (Faure et al., 2000; Triantafilou and Triantafilou, 2002). In our studies we did not observe a response to LPS in Caco-2 cells, which is in accordance with most of the existing literature. Reports of responses by Caco-2 cells to LPS vary, some sources report an increase in IL-8 excretion (Huang et al., 2003; Schuerer-Maly et al., 1994), while most sources report a lack of responses attributed to low TLR4 expression (Böcker et al., 2003; Sang et al., 2005). Our di-culture model with HT29-MTX cells also did not show a response to LPS. In the literature, the reported responses of HT29-MTX cells against LPS are variable, likely because various MTX strains of the cell line have been used (Böcker et al., 2003; Smirnova et al., 2003). We did not observe an IL-8 response in HT29-p cells following the LPS exposure, while such a response has been reported before (Sang et al., 2005). Lastly, the inclusion of HMVEC-d cells in our model also did not change the response of the model to LPS. This lack of response can be due a low basolateral LPS concentration upon apical administration of the agonist as monocultures of HMVEC-d have been reported to respond to LPS stimulation by secretion of IL-8 (Arlian et al., 2009) and have been shown to express TLR4 mRNA (Faure et al., 2000).

Pam3CSK4 is a TLR1/2 agonist that has been reported to induce an IL-8 response in Caco-2 cells (Weng et al., 2007), while this response has not been reported for HT29-p cells (Kim et al., 2012). In our results none of the cell lines responded to stimulation with Pam3CSK4. In the previously reported studies far higher Pam3CSK4 concentrations were used, which may explain the difference in results. However, the working concentration of the compound, as recommended by the producer, was between 0.1 and 10 ng/mL. In order for cells to respond to Pam3CSK4 they need to express both TLR1 and TLR2 (Ozinsky et al., 2000). Reports on the expression of TLR1/2 in intestinal cell lines are ambiguous. Some report expression of both TLR1 and 2 in Caco-2 cells (Saguse et al., year), while others report an absence of TLR1 in Caco-2 cells (Melmed et al., 2003). For HT29-p cells the presence of both TLR1 and 2 was reported (Melmed et al., 2003; Saegusa et al., 2004). The observed absence of an IL-8 response in our cell models could be due to the absence of TLR1 and/or 2.

ssRNA is recognized by both TLR7 and TLR8. TLR8 is considered the most important of the two in humans, it is normally present in intracellular compartments like the endosomes (Triantafilou et al., 2005), so activation of the immune response requires endocytosis. Therefore, the ssRNA used for exposure was combined with LyoVec to facilitate its uptake into the cells. The ssRNA40 we used did not elicit a response in any of the models. Very little is reported on the immune responses of intestinal epithelial cells to ssRNA. It has been reported that PolyU, a synthetic ssRNA, stimulates IL-8 excretion in HT29-p cells but not in Caco-2 cells (Uehara et al., 2007). The concentration of PolyU used by Uehara et al. (2007) was five times higher than the one used for ssPolyU to elicit this response, so potentially a synthetic variant like ssPolyU is more potent. In a comparison between IBD and healthy patients immunofluorescence microscopy and immune-EM showed the presence of TLR8 on the luminal epithelial membrane in both groups, which is in contrast to the expected intracellular localization of the receptor that is generally described in literature. They also show that TLR8 mediates IL-8 secretion (Steenholdt et al., 2009). Therefore, the lack of an IL-8 response in our system could indicate the absence of a functional TLR8 in the cells used in this study.

We set out to study which cell model could best be used for future studies focusing on immunomodulation in the human gut. Firstly, we conclude that a di-culture model of Caco-2 and HT29-MTX does not deliver an additional value for this purpose, as both the Caco-2 mono- and di-cultures showed similar responses. The HT29-p monoculture even showed an advantage by additionally responding to Poly(I:C), but does not grow a monolayer. Overall, the monocultures will make interpretation of results easier due to the differences in basal excretion of IL-8 between the cell lines. The addition of the human microvascular cell line HMVEC-d also did not result in a different response pattern but did induce the baseline IL-8 concentrations as well as the IL-8 concentration after exposure to flagellin, thus HMVEC-d cells participate in propagating the immune response, and therefore add to the physiological relevance of the model. Secondly, we conclude that epithelial cell lines cannot capture all the immunomodulatory responses seen in human gut epithelial cells *in vivo*. While the use of cell lines have important practical advantages in the laboratory in terms of reproducibility in comparison with the use of primary cells or stem cell-derived models, exploration of these models needs to be considered in the future. TLR1 through 9, with the exception of TLR10, has for instance been shown to be expressed in freshly isolated intestinal epithelium (Otte et al., 2004). Therefore a careful selection of the *in vitro* model remains needed in future studies and should be based on the research question.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.tiv.2021.105280.

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