Establishment of an *in vitro* model for the simulation of inflammatory bowel disease as an alternative to animal experimentation

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“It always seems impossible until it’s done” – Nelson Mandela

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I. Summary

This thesis focuses on the development of an *in vitro* co-culture assay to enable the screening of novel APIs for the treatment of inflammatory bowel disease (IBD) without the need of animal experimentation. The assay simulates both, the epithelial barrier and immune response in IBD on a cellular level. In healthy conditions, the co-culture shows an intact epithelial barrier defined by a low release of the analysed pro-inflammatory cytokines, while induction of the inflammatory state leads to a loss of barrier function resulting in an increased release of the pro-inflammatory cytokines. Moreover, the predictivity of the assay was evaluated according to the OECD Guidance Document for Describing Non-Guideline In Vitro Test Methods by the application of commonly administered APIs related to the severity of IBD based on the determination of the *in vitro-in vivo* correlation (IVIVC). Furthermore, API combination therapy and treatment with the natural product EGCG were performed to simulate clinically relevant treatment and to address the increasing interest in complementary therapy. Both treatment strategies led to a restoring of the epithelial barrier and to a reduction of pro-inflammatory cytokine release. In conclusion, the established *in vitro* assay offers the potential to evaluate the cytotoxicity and efficacy of novel APIs for the treatment of IBD *in vitro*. In the future, optimization and validation are essential to improve the predictivity and to achieve regulatory approval.

II. Zusammenfassung

Diese Arbeit befasst sich mit der Entwicklung eines *in vitro* Ko-Kultur Modells, um neue Wirkstoffe für die Behandlung von chronisch-entzündlichen Darmerkrankungen (CED) ohne die Verwendung von Tierversuchen zu identifizieren. Das Modell simuliert die epitheliale Barriere und die zelluläre Immunantwort bei CED. Im gesunden Zustand zeigt das Modell eine intakte epitheliale Barriere und eine geringe bis keine Ausschüttung von pro-entzündlichen Zytokinen, während das Auslösen einer Entzündung zu einem Verlust der Barrierefunktion und einer gesteigerten Zytokinausschüttung führt. Des Weiteren wurde die Vorhersagekraft basierend auf dem OECD *Guidance Document for Describing Non-Guideline In Vitro Test Methods* durch die Anwendung von etablierten Wirkstoffen bei unterschiedlichen CED Schweregraden evaluiert und eine Korrelation zur *in vivo* Situation berechnet. Zusätzlich wurde die Behandlung mit Wirkstoffkombinationen aus anti-inflammatorischen Wirkstoffen und Immunsuppressivum sowie dem Naturstoff EGCG durchgeführt. Beide Behandlungen führten zu einer Wiederherstellung der Barrierefunktion und zu einer Reduzierung der entzündlichen Zytokinausschüttung. Somit bietet die etablierte Ko-Kultur das Potential die Zytotoxizität und die Wirksamkeit von neuen Wirkstoffen für die Behandlung von CED zu untersuchen. Die Optimierung und Validierung des Modells sind in Zukunft essenziell, um die Vorhersagekraft weiter zu steigern und eine regulatorische Akzeptanz zu erreichen.

III. List of Abbreviations

3R	Replacement, Reduction and Refinement
5-ASA	5-aminosalicylic acid
6-MP	6-mercaptopurine
ADA	Anti-drug antibody
AOP	Adverse outcome pathway
APC	Antigen-presenting cell
API	Active pharmaceutical ingredient
AMP	Antimicrobial peptide
API	Active pharmaceutical ingredient
BM	Bone marrow
CD	Crohn's disease
DAI	Disease activity index
DC	Dendritic cell
DNBS	2,4-Dinitrobenzene-sulfonic acid
DSS	Dextran sulfate sodium
dTHP-1	Differentiated THP-1
DTT	Dual targeted therapy
ECM	Extracellular matrix
EGCG	Epigallocatechin-3-gallate
ELISA	Enzyme-linked immunosorbent assay
FBS	Fetal bovine serum
FDA	Food and Drug Administration
GCA	GCCP acceptance criteria
GCCP	Good Cell Culture and Tissue Culture Practice
GI	Gastrointestinal

GIVIMP	Guidance Document on Good In Vitro Method Practices
GTE	Green tea extract
IBD	Inflammatory bowel disease
IC	Indeterminate colitis
IEBC	Intestinal Explant Barrier Chip
IEC	Intestinal epithelial cell
IL	Interleukin
ILC	Innate lymphoid cell
INF	Interferon
IOMC	Inter-Organisation Programme for the Sound Management of Chemicals
IV	Intravenous
IVIVC	*In vitro-in vivo* correlation
JAK	Janus kinase
JAM	Junction adhesion molecule
KE	Key event
Lab	Laboratory
LADME	Liberation, absorption, distribution, metabolism and excretion
LOR	Loss of response
LPS	Lipopolysaccharides
MARVEL	Myelin and lymphocyte and related proteins for vesicle trafficking and membrane link
M-cells	Microfold cells
MDM	Monocyte-derived macrophages
MLCK	Myosin light-chain kinase
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

MTX	Methotrexate
NCCIH	National Center for Complementary and Integrative Health
NF-κB	Nuclear factor 'kappa-light-chain-enhancer' of activated B-cells
NKT cells	Natural killer T cells
NLR	Nod-like receptor
NOD2	Nucleotide-binding oligomerization domain-containing protein 2
OECD	Organisation for Economic Co-operation and Development
PBMCs	Peripheral blood mononuclear cells
PD	Pharmacodynamic
P-glycoprotein	Permeability-glycoprotein
PK	Pharmacokinetic
PRR	Pattern recognition receptor
PMA	Phorbol-12-myristate-13-acetate
QA	Quality assurance
QC	Quality control
QM	Quality management
SOP	Standard operating procedure
STAT	Signal transducer and activator of transcription
TAMP	Tight junction-associated MARVEL proteins
TC	Trough concentration
T/CAM	Traditional/complementary and alternative medicine
TDM	Therapeutic drug monitoring
TEER	Transepithelial electrical resistance
TER	Transepithelial resistance
TGF	Transforming growth factor

Th cell	T helper cell
TJ	Tight junction
TLR	Toll-like receptor
TNBS	Trinitrobenzene-sulfonic acid
TNF-α	Tumour necrosis factor-alpha
UC	Ulcerative colitis
USA	United States of America
WHO	World Health Organisation

1. Introduction

1.1 The need for inflammatory gastrointestinal *in vitro* models

The occurrence of inflammatory bowel disease (IBD) as multifactorial gastrointestinal (GI) disease can be connected to numerous risk factors and is affecting a high number of people worldwide [1,2]. As state of the art, the efficacy and safety of novel therapeutic approaches in research or in preclinical studies is investigated through the usage of animal models [3]. For the simulation of IBD, various animal models are available as presented in the section '1.5 IBD-related animal models'. Nowadays, the ethical concern regarding animal experimentation is increasingly addressed based on the 3R (Replacement, Reduction and Refinement) principle of Burch and Russel [4], anchored in the European Directive 2010/63 [5]. On December 29th 2022, the Food and Drug Administration (FDA) Modernization Act 2.0 was signed into law allowing for alternatives to animal experimentation in preclinical studies [6].

However, despite the ethical question, the main drawback of using animal models is the transfer of animal model-based data to the human *in vivo* situation due to species differences [7]. To overcome this drawback and further limitations such as lack of reproducibility and to address the ethical concern of animal experimentation [8], various *ex vivo* and *in vitro* models simulating IBD were developed (see section '1.6 Alternatives to animal experimentation'). Although the presented models are simulating key events (KE) such as immune response or disturbance of the epithelial barrier of the IBD pathophysiology, as described in the section '1.3 Pathophysiology of IBD', the question of predictivity of those models is rarely discussed in literature. For the evaluation of predictivity, the determination of the *in vitro-in vivo* correlation (IVIVC) is essential [9]. Possible approaches to investigate the IVIVC of *in vitro* models simulating IBD are described in the section '1.7 Evaluation of *in vitro* models' including the application of commonly administered active pharmaceutical ingredients (APIs) for the treatment of IBD (see section '1.4 Treatment options for IBD') to prove the simulation of the *in vivo* treatment effect. To develop a suitable *in vitro* assay, the understanding of IBD and its pathophysiology is an important first step and is presented in the following.

1.2 Global incidence and prevalence of inflammatory bowel disease

IBD describes different chronic inflammations of the GI-tract. The disease can be divided into the subtypes Crohn's disease (CD), ulcerative colitis (UC) and indeterminate colitis (IC) with inflammation pattern that cannot be clearly assigned to CD or UC [10]. The clinical course is characterized by phases of acute inflammation and spontaneous or drug-induced remission [10].

Patients suffer from numerous symptoms such as fatigue, abdominal pain and diarrhoea [11], affecting their quality of life [12]. Initially, the distribution of IBD was predominantly observed in westernized countries [13] while nowadays cases of IBD are recorded worldwide [14]. The pathology of IBD is connected to an interaction of environmental and genetic risk factors, however their causal relation is not fully discovered yet [1]. Genetic screening of the human genome led to the conclusion that some gene variants such as mutations in the nucleotide-binding oligomerization domain-containing protein 2 (NOD2) gene cause susceptibility for IBD [15,16]. However, not only the genetic predisposition but numerous environmental factors such as westernized high fat diet, the long-time intake of oral contraceptive pills and nonsteroidal anti-inflammatory drugs, air pollution and also psychological stress or sleep disturbances increase the risk for the development of IBD [17,18].

Kaplan *et al.* classified the evolution of IBD in four epidemiological stages: emergence of IBD, acceleration in incidence, compounding prevalence and prevalence equilibrium [19]. Figure 1 depicts the distinct stages of IBD evolution based on the degree of industrialization. Developing countries are allocated to the emergence state, in which sporadic cases of IBD are reported. Newly industrialized countries are characterized by an acceleration in incidence (rate of new cases) and a lower rate of prevalence (total number of cases). Longitudinal cohort studies documented the increase of CD and UC. The longest continuous study for CD performed in Cardiff (Wales) starting in 1932 showed the increased incidence over the years and the following stabilizing of incidence in 2003 to 7.0 cases per 100,000 persons [19,20]. Similar results were observed in a cohort study for CD and UC in Olmsted County (Minnesota) from 1940 to 2010 resulting in an incidence rate of CD of 10.7 and for UC of 12.2 cases per 100,000 persons [21,22]. Since the twentieth century, the western world reached the stage of compounding prevalence characterized by a stable incidence and a linear rising of prevalence owing to previous decades with high incidence and low mortality [19]. In 2019, approximately 4.9 million IBD patients were recorded worldwide with the highest number of cases in China (66.9 per 100,000 persons) and the United States of America (USA) (245.3 per 100,000 persons) [2]. In the future, the transition of the western world to the prevalence equilibrium is a conceivable scenario based on the ageing of the IBD population and perhaps the unexpected mortality by virtue of the mortality caused by the COVID-19 pandemic that counteract the incidence of IBD [19]. Notwithstanding, the prevalence of IBD, especially of the western world, these days reached its peak, which implies a high number of people suffering from IBD.

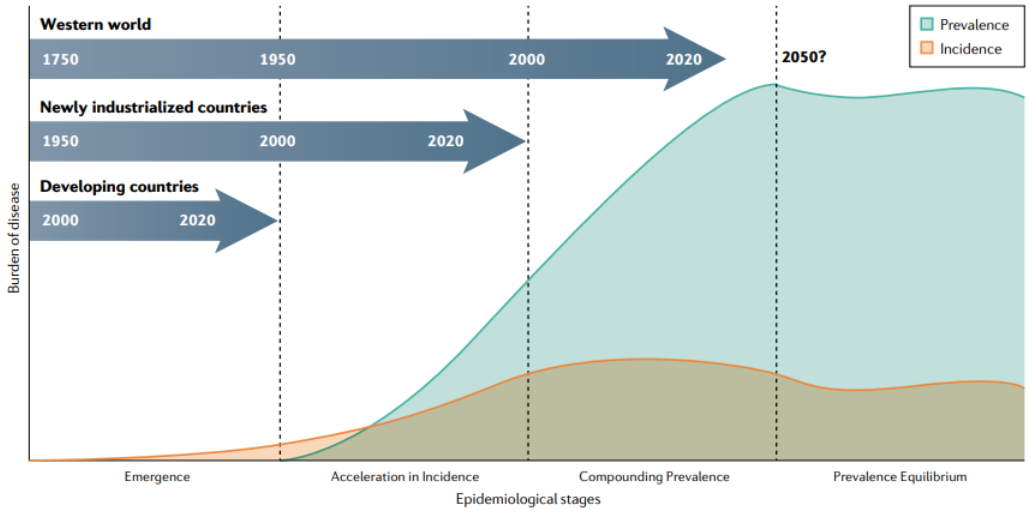

Figure 1. Classification of IBD evolution in four epidemiological stages by Kaplan *et al.* The developing countries can be assigned to the emergence state with an increasing incidence. The newly industrialized countries are in the stage of acceleration in incidence, while the western world is characterized by compounding prevalence. In the future, a prevalence equilibrium in the western world is conceivable due to the ageing IBD population and possibly due to the unexpected mortality caused by the COVID-19 pandemic. [19] *Reprinted from: Kaplan et al. 'The four epidemiological stages in the global evolution of inflammatory bowel disease', copyright © 2020, Nature Reviews Gastroenterology & Hepatology, license number: 5507061288125.*

1.3 Pathophysiology of IBD

In the pathogenesis of IBD, the combination of environmental factors, genetic disposition, the immune system and the predominant composition of the microbiome lead to the disturbance of the intestinal mucosa [23]. In the clinical course, UC affects most frequently the mucosa of the colon spreading continuously to the rectum. In phases of inflammation, the surface of the mucosa exhibits haemorrhage and the formation of ulcers [24]. CD is defined by discontinuous transmural affection of different GI-segments, mostly the terminal ileum, the proximal colon and the descending and sigmoid colon as well as the rectum. The size of the inflamed patches is variable and interrupted by segments of healthy tissue (skip areas). In acute inflammation phases, fistulas, inflammatory lesions, such as erosions or ulcers, and abscesses penetrating through multiple layers of the tissue can occur [24]. These inflammations of the GI-tract are mediated by an inappropriate activation of innate and adaptive immune responses and the loss of tolerance to the dysbiotic enteric commensal bacteria [25,26]. Patients that suffer from CD or UC show a decreased diversity of the microbiome [27]. Notably, the bacteria *Firmicutes* and *Bacteroidetes* are depleted while *Actinobacteria* and most prominent *Proteobacteria* such as *E. coli* are enriched in comparison to the healthy gut which reflects the dysbiosis in IBD [28,29]. Moreover, recent metabolomic studies with multi-omic technologies and metagenomic analyses represent the depletion and enrichment of various metabolites and species in IBD patients that are hypothesized to have either anti-inflammatory or pro-inflammatory effects

[29,30]. Additionally, patients show a reduced mucus production (especially in UC) by goblet cells as well as a reduced defensins (cationic host defence peptides) content of the inner mucus layer that lead to bacterial penetration through the mucus layers to the epithelium [31,32].

Furthermore, the integrity of the epithelial barrier plays a key role in the development of IBD. Different types of intestinal epithelial cells (IECs) such as enterocytes, enteroendocrine cells, microfold cells (M-cells), Paneth cells or goblet cells maintain the natural homeostasis of the intestine by secretion of antimicrobial peptides (AMPs) such as defensins [33,34]. The IECs possess numerous pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs) and Nod-like receptors (NLRs) to sense pathogens and activate inflammatory pathways and to regulate the release of defensins to restore the homeostasis in the gut [26,33]. Besides the steady state between cell proliferation and cell shedding [35], the epithelial barrier is strongly connected with tight junction (TJ) proteins resulting in a semipermeable barrier for water, ions and small solutes and a defensive network preventing the entry of pathogens to the lamina propria [36]. Patients with IBD show an abnormally increased paracellular permeability of the epithelial barrier [37], reduction of defective defensin production and a decrease in the number of goblet cells that secrete mucins as well as an altered composition of the mucus layer [34].

The increased permeability of the epithelial barrier is connected to a disturbance of the IEC proliferation and differentiation due to alternations of the TJ structure [34,37,38]. The TJ network consists, amongst others, of the junction adhesion molecule (JAM) and the tight junction-associated MARVEL (myelin and lymphocyte and related proteins for vesicle trafficking and membrane link) proteins abbreviated as TAMP. The TAMP family includes for instance the barrier forming transmembrane protein occludin, MarvelD3 and tricellulin [39], which is the contact point for up to three IECs [40]. The family of either barrier- or pore-forming claudin proteins is the major modulator of TJ permeability [39]. Patients that suffer from either UC or CD show an increased expression of the pore-forming claudin-2 related to an increased flux of cations and water into the gut lumen. Hereby, the increased release of pro-inflammatory cytokines such as TNF-α, IL-13 and IL-6 is connected to the increased claudin-2 level [38,41–43]. Moreover, the elevated release of TNF-α led to a downregulation and re-localization of the barrier-forming claudin-5 and claudin-8 to the sub-membrane TJ regions and into the endosomes [42]. In cell studies, the re-localization of occludin and an increased expression of the myosin light-chain kinase (MLCK) causing an enhanced permeability was observed after the stimulation with IL-1β [44]. Furthermore, not only the re-localization but downregulation of occludin and barrier-forming claudin expression is reported in literature [41,42,45]. Figure

2 summarizes the consequences of epithelial barrier disturbance in IBD. In healthy condition, occludin and barrier-forming claudins such as claudin-3, -4, -5, -8 lead to a strong epithelial barrier with a low paracellular permeability. Inflammation of the epithelial barrier, caused for instance by the pro-inflammatory cytokines TNF-α and IL-13, results on one hand in an increased expression of the channel-forming claudin-2 (pore-pathway) and the shift of barrier-forming claudins to subareas of the TJ-network or into endosomes. On the other hand, the expression of occludin and barrier-forming claudins (leak-pathway) decreases. As a consequence, the barrier turns leaky and water, small solutes and macromolecules are able to cross the epithelium [34].

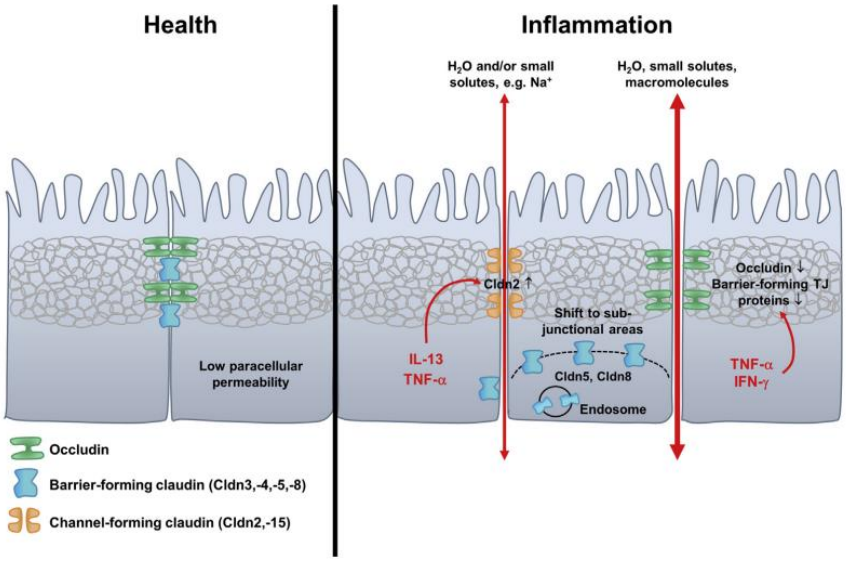

Figure 2. Comparison of barrier integrity in healthy condition and inflamed condition in IBD by Martini *et al*. In healthy condition, the barrier is characterized by a high integrity and a low paracellular permeability. Inflammation is caused by the stimulation with pro-inflammatory cytokines such as IL-13, TNF-α and Interferon (INF)-γ that are overexpressed in IBD. This leads to the increased expression of the channel-forming claudin-2 (pore-pathway) and decreased expression of barrier-forming claudins and occludin (leaky-pathway). Additionally, barrier-forming claudins are shifted to subareas of the TJ-network or into endosomes. As a consequence, the epithelial barrier turns leaky and allows the crossing of water, small solutes and macromolecules [34]. *Reprinted from: Martini et al. 'Mend Your Fences, The Epithelial Barrier and its Relationship With Mucosal Immunity in Inflammatory Bowel Disease', copyright © 2017 The Authors, Cell Mol Gastroenterol Hepatol., open access CC BY-NC-ND license: http://creativecommons.org/licenses/by-nc-nd/4.0/.*

The disbalance of the epithelial barrier and the increased paracellular permeability as consequence lead to the entering of pathogens and commensal bacteria to the lamina propria [32] and to the infiltration of leukocytes [23]. This induces the overactivation of the innate and adaptive immune system characterized by excessive cytokine expression [46,47]. In healthy state, the balance of the released pro- and anti-inflammatory cytokines maintains the homeostasis between the IEC barrier, the immune system and the microbiome [47]. In IBD, the entering of commensal bacteria and pathogens induces an inflammatory cascade of signalling pathways as depicted in Figure 3.

The activation of dendritic cells (DC) and macrophages as antigen-presenting cells (APC) of the innate mucosal immune system induce the release of pro-inflammatory cytokines such as IL-6, IL-12, IL-18, IL-23, IL-1β and TNF-α and the activation of naïve T helper 0 cells (Th0 cells) of the adaptive immune system [47,48]. Subsequently, the release of the pro-inflammatory cytokines IL-12, IL-27, IL-4, IL-6, IL-23 and transforming growth factor β (TGF-β) by the activated Th0 cells causes the differentiation into various Th cell phenotypes like Th1 (in CD), Th2 (in UC), Th17 (in CD and UC) and Th9 (in UC) that are respectively characterized by specific cytokine release pattern [47–49]. The secretion of IL-12 by activated macrophages stimulates a signalling cascade involving innate lymphoid cells (ILC) 3 that release IL-1β, which activates ILC1 and the release of cytokines that are connected to the immune phenotype of CD [47,48]. Beside the Th cell response, the entering of bacteria results in the activation of Natural killer T (NKT) cells and the secretion of IL-13 which leads to the differentiation into the Th2 cell phenotype. Additionally, the production of AMP by Panet cells is decreased in IBD leading to a reduced activation of regulatory T (T_{reg}) cells and therefore to a reduced release of anti-inflammatory cytokines such as IL-10 and to an imbalance of the gut microbiota [47,49,50]. As consequence, the integrity of the intestinal barrier is decreased while the permeability is increased leading to the characteristic chronic inflammations in IBD.

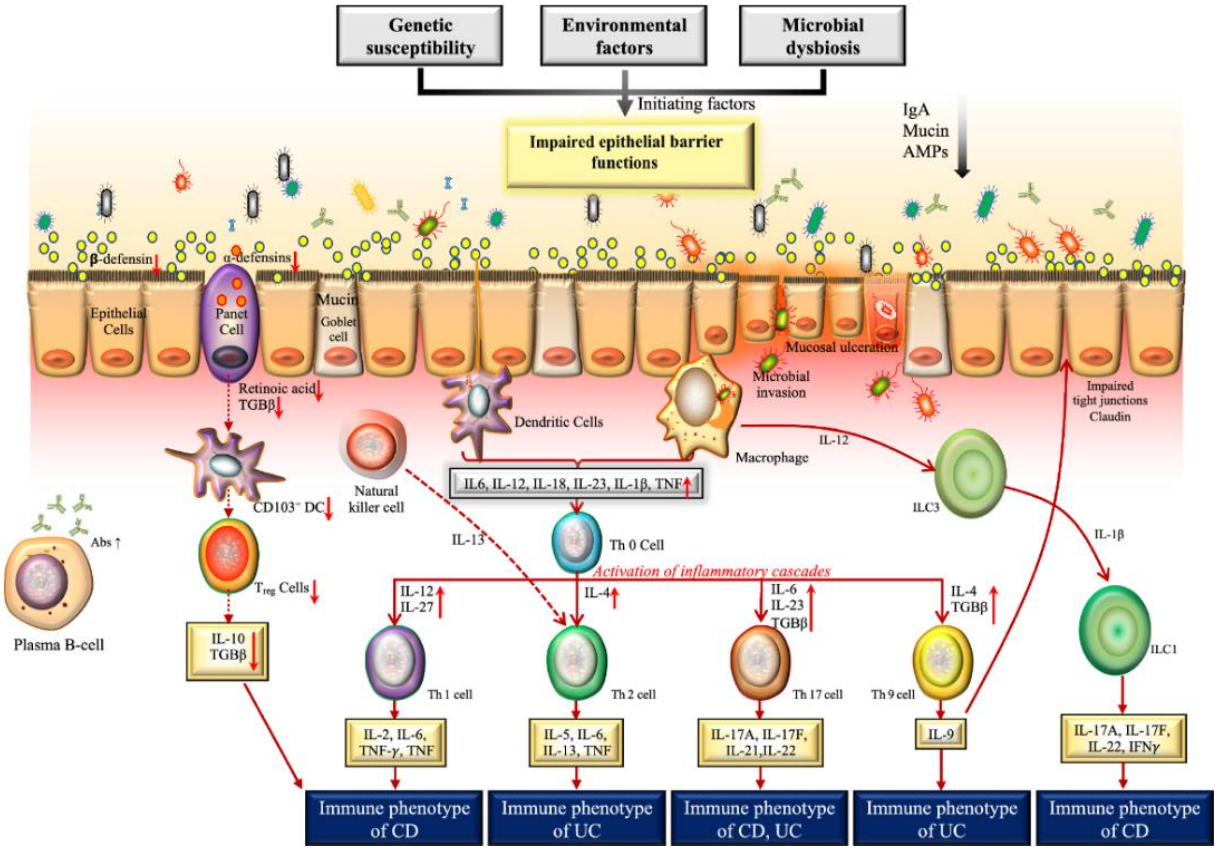

Figure 3. Overview of the activation of the innate and adaptive immune system in IBD.
The entering of commensal bacteria and pathogens to the lamina propria leads to the activation of dendritic cells (DC) and macrophages and therefore to the release of numerous pro-inflammatory cytokines. This induces the differentiation of naïve T helper (Th0) cells into Th1, Th2, Th17 or Th9 cells that are respectively characterized by specific cytokine release pattern. Furthermore, natural killer T (NKT) cells are activated and differentiate into Th2 cells via the secretion of IL-13. The production of antimicrobial products (AMP) by Panet cells is decreased which leads to a diminished activation of regulatory T cells (T_{reg}) and therefore to a reduction of anti-inflammatory cytokine release [47]. *Reprinted from: Kumar et al. `Integrating omics for a better understanding of Inflammatory Bowel Disease: a step towards personalized medicine´; copyright © 2019 The Authors, J Transl Med., open access CC BY 4.0 license: http://creativecommons.org/licenses/by/4.0/.*

1.4 Treatment options for IBD

1.4.1 Active pharmaceutical ingredients for IBD therapy

The therapeutic approach for IBD focuses on the treatment of the acute inflammatory phases and the prolonging of remission phases. Based on the severity level of the disease different classes of APIs are applied resulting in a gradual therapy, either in a step-up or a top-down approach, that can be depicted in a ‘treatment pyramid’ (Figure 4) [51,52]. As described in the ECCO Guidelines for the medical treatment of UC and CD, the severity of the disease is decisive for the selection of pharmaceuticals. In mild to moderately active inflammations (level 1) antibiotics or 5-aminosalicylic acid (5-ASA) derivates are applied to induce and maintain (in UC) remission phases by intervening with the nuclear factor 'kappa-light-chain-enhancer' of activated B-cells (NF-κB) pathway resulting in a decreased release of pro-inflammatory cytokines and inflammatory mediators [53–55]. In moderate to severe cases of the disease (level

2), the application of systemic steroids such as prednisolone is recommended which strongly inhibits the NF-κB and therefore induces and maintains phases of remission [53,54,56]. For the treatment of severe inflammations and the maintaining of remission phases, immunosuppressants are applied to diminish the overall immune response in steroid-dependent IBD [53,54]. In severe cases where the patient does not react adequately to the treatment with conventional therapy, biologics such as anti-TNF-α agents like for instance the monoclonal antibodies infliximab and adalimumab or small molecules like Janus kinase (JAK) inhibitors are utilized [53,54,57]. If the reaction to medical therapy is not sufficient, surgery of the affected regions remains as a non-medicinal approach. [58,59].

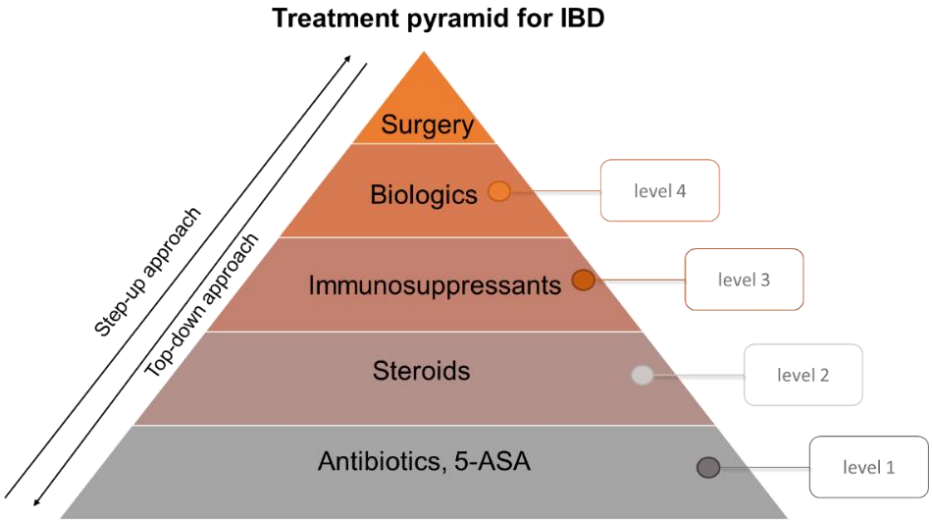

Figure 4. Overview of the step-up or top-down approach for the treatment of IBD depicted as treatment pyramid. In mild to moderate cases (level 1), the intestinal inflammations are treated with antibiotics or 5-aminosalicylic acid (5-ASA) derivates. For the treatment of moderate to severe inflammations (level 2), steroids are utilized. Immunosuppressants diminish the overall immune response and are applied in severe courses. When the response to the treatment is not adequate (level 4), alternative therapies in form of biologics are performed. Surgery of inflamed regions of the GI-tract remains as final option. *Reprinted from: Schnur et al. 'Inflammatory bowel disease addressed by Caco-2 and monocyte-derived macrophages: an opportunity for an in vitro drug screening assay', copyright © 2022 The Authors, in vitro models, open access CC BY 4.0 license: http://creativecommons.org/licenses/by/4.0/.*

1.4.2 Combination therapy in IBD

The medical treatment of IBD according to a step-up or top-down approach is well established, however, the efficacy of single drugs might be limited in some proportions of patients or the efficacy diminishes over time. Therefore, combination therapy is an option to utilize additive or synergistic effect of drugs to improve the treatment or to address the variability of patients [60]. A limited response or loss of response (LOR) of a significant proportion of patients to anti-TNF-α agents such as infliximab was reported [61]. Reasons for this might be the expression of anti-drug antibodies (ADAs) that decrease the efficacy of the drug [61,62]. The combination of the TNF-α agent infliximab with immunosuppressants showed a decreased ADA development and an improved infliximab pharmacokinetic [63,64]. Further combinations

that are discussed in literature are the combination of 5-ASA derivates with immunosuppressants, which might reduce the risk of colon rectal cancer [65] and the application of corticosteroids to immunosuppressed patients [66]. In first clinical trials, the dual targeted therapy (DTT), meaning the applications of biologics and/or small molecules, is tested as future strategy for the treatment of inflammatory phases in IBD [60].

1.4.3 Natural products for IBD prevention or alternative treatment

Besides the conventional medical treatment with single APIs or combination therapy, the usage of natural products as traditional/complementary and alternative medicine (T/CAM) is increasing nowadays. Considerable reasons for this are the potential prevention of diseases and the management of existing chronic diseases [67]. The implementing of T/CAMs into the conventional medicine is recently focused in numerous future strategies like the National Center for Complementary and Integrative Health (NCCIH) Strategic Plan FY 2021–2025 [68] or the World Health Organisation (WHO) Traditional Medicine Strategy 2014–2023 [69]. In IBD, mostly herbal therapies are utilized by patients to complement the conventional therapy [70]. Numerous natural products such as resveratrol, curcumin, boswellic acid, triptolide and Epigallocatechin-3 gallate (EGCG) are reported to provide anti-inflammatory, anti-oxidant or even anti-cancerogenic effects [71]. The green tea extract (GTE) containing EGCG restored the colon architecture, decreased the disease activity index (DAI) and the level of pro-inflammatory markers such as TNF-α in plasma and tissue of IBD-related animal models [72–74]. However, the investigation of the EGCG effect in human-based models and clinical trials is still ongoing [75].

1.5 IBD-related animal models

Animal models are commonly used to investigate the safety and efficacy of potential novel drug candidates or therapies and are mandatory for preclinical studies [3]. In regard to IBD, numerous animal models are available and regulatory approved [76]. For instance, chemically-induced models like the prominent Dextran sulfate sodium (DSS) or the 2,4-Dinitrobenzene-sulfonic acid (DNBS)/ Trinitrobenzene-sulfonic acid (TNBS) models are utilized. The advantages of these models are the simple and fast induction of the IBD-characteristic inflammations several days after administration of the respective chemical compound and the controllability of the inflammation severity controlled by the frequency and concentration of the administered chemical [77–80]. Additional animal models besides the spontaneous (inbred) mediated models [81,82] are immune-mediated/adoptive transfer [83–85] or genetically

engineered models like transgenic or cytokine-knockout models that are applied to investigate especially the effects on the immune response in IBD [86–90].

Although animal models are generally carefully selected in terms of similarity of the pathophysiology of the disease to the human condition, there is a lack of comprehensive validation of these models [8]. This leads to challenges for data reproducibility which is influenced by the inflammation severity and development time of the disease, the genetic background of the used strain, the gender and age of the laboratory animals and environmental factors [78,91]. Furthermore, the caused suffering from symptoms, pain and distress during the animal experimentation led to an unknown effect on experimental data and is ethically questionable [92]. Overall, the transfer of animal model-based data to the human condition remains as hurdle due to physiological differences between species [7].

In regard to the aforementioned complications, animal experimentation itself is increasingly connected to the 3R principle of Burch and Russel [4] which is anchored in the European Directive 2010/63 [5]. Recently, the Food and Drug Administration (FDA) Modernization Act 2.0 was signed into law on December 29th in 2022 which allows for alternative methods to animal testing in the development of novel medicines [6].

1.6 Alternatives to animal experimentation

1.6.1 *Ex vivo* models

Ex vivo models for the simulation of IBD can be used as alternatives to animal testing when intestinal tissue biopsies from humans or animal tissue that accrue as waste product of the meat industry are deployed. One prominent example of an *ex vivo* system is the Ussing chamber which allows the evaluation of barrier integrity via transepithelial resistance (TER) measurements and the investigation of the intestinal permeability [93–95]. However, the main disadvantages of the Ussing chamber system are the short-time viability of the tissue (from two up to five hours), the complex handling and the low throughput [93,96].

To improve the handling and the throughput, the InTESTine^{TM} model was developed by the Netherlands Organization of Applied Scientific Research. The model consists, comparable to the Ussing chamber, of two compartments: apical and basolateral with a horizontally inserted patch of fresh tissue derived from porcine or human in-between [97]. The set-up can be easily integrated in standard 6 or 24 well-plates and allows for instance the investigation of absorption or permeability of drugs as well as GI processes and host-microbiome interactions [97,98].

Although the throughput could be significantly increased and the handling was simplified comparable to the Ussing Chamber, the viability of the tissue remains as main challenge [97].

Therefore, Amirabadi *et al.* developed the Intestinal Explant Barrier Chip (IEBC) which connects tissue explants with a microphysiological system to enable experiments for up to 24 hours of incubation [99]. In general, microfluidic gut-on chip-models can be used to combine tissue sections of patients with microphysiology for the investigation of the pathophysiology of IBD [100]. However, the low throughput, the complex handling and accordingly the reproducibility and the potential influence of the chip material are the drawbacks of such systems [101].

Additionally, von Erlach *et al.* developed a robotically handled high-throughput system to successfully investigate the absorption of oral formulations with porcine tissue explants that showed a prolonged viability depending on the media and the presence of the stromal layer [102]. Although the model enables the investigation of drug absorption of the GI-tract, the pathophysiology of the disease is missing.

Recently, it is reported that biopsies from patients enable the generation of patient tissue-derived organoids for the investigation of the pathophysiology of IBD and the evaluation of new treatment options [103–106]. Considering the fact, that patient-derived organoids are not well established yet, the maintaining of the inflamed phenotype under culture conditions remains as a present challenge for their usage [103,105,106].

1.6.2 In vitro models

Models to investigate the epithelial barrier function

In the past decades, an increasing number of *in vitro* models for the simulation of IBD were developed to overcome the limitations of animal experiments and to study specific mechanisms [107]. Starting with epithelial monolayers of intestinal cell lines, such as the human colon cancer-derived cell line Caco-2, the cytokine release of the inflamed epithelial barrier and the efficacy of potential anti-inflammatory compounds as well as the absorption of APIs, especially transported via the paracellular route, can be investigated [108–112]. Another epithelial intestinal cell line used for studies addressing IBD, is the colon cancer-derived HT-29 cell line, which can differentiate in the absence of glucose or by the addition of inducers into enterocytes. HT-29 cells release enzymes and cytokines similar to Caco-2, however the HT-29 cell line is able to differentiate into goblet-like cells that produce mucus [113–116]. Although this is an advantage of this cell line, HT-29 cells show an impaired glucose metabolism characterized by

a high consumption of glucose, high rates of lactose production and only moderate rates of glycogen accumulation [117–119]. The combination of Caco-2 cells and HT-29 cells in Caco-2/HT-29 methotrexate (MTX) co-cultures results in a model that provides enterocytes with a brush border phenotype expressing microvilli on the surface and goblet cells that secrete mucus. However, the ratio between those two cell lines is crucial for a homogenous mucus production [120]. Dosh *et al.* further improved the morphology of a Caco-2/HT-29 MTX co-culture by cultivating the cells on a hydrogel scaffold under dynamic conditions to simulate the extracellular matrix (ECM) improving cell differentiation [121]. A third widely used intestinal colon cancer-derived cell line is the T84 cell line that can differentiate into crypt-like cells by the addition of transforming growth factors [107,122,123].

An approach to align the physiological situation of the epithelial cells more with the *in vivo* situation might be the generation of organoids which allow the investigation of epithelial cell activity or the addition of ECM-simulating scaffolds in general [124]. However, in comparison to monolayers, organoids are more expensive and the reproductivity and the investigation of the epithelial barrier integrity are difficult [125].

Recently, numerous gut-on-chip models, where epithelial cells like Caco-2 are cultivated in a microphysiological microfluidic system, were developed [126]. The continuous exchange of media by pumps leading to constant nutrient and oxygen supply and removal of waste products simulates the intestinal microenvironment more accurately [127,128]. With a continuous flow, epithelial cells are able to differentiate into a villi-like phenotype characterized by tightly connected cells with villi-structures and brush borders that represent the epithelial barrier [126,129]. Despite the potential of gut-on-chip models, the high complexity, the low throughput, the potential interaction with chip material and the challenge of reproducibility owing to the complex handling are current limitations [101].

The *in vitro* models representing the epithelial barrier in IBD enable the investigation of cellular processes and signalling pathways on a molecular level, however IBD pathophysiology involves not only the disturbance of the epithelial barrier, but the overactivation of the immune system. Therefore, the combination of IECs with immunological components is more closely connected to the *in vivo* pathophysiology.

Co-culture models including immune cells

Based on the pathophysiology of IBD, immune cells like macrophages or dendritic cells show an upregulated activation and release of pro-inflammatory mediators such as cytokines that affect the integrity of the epithelial barrier [47]. To address the interaction between the epithelial and immune cells, several co-culture systems were developed. Commonly used immune cells with human origin are for instance the human leukemic cell line THP-1, which can be differentiated into macrophages (differentiated THP-1 (dTHP-1)) by the stimulation with phorbol-12-myristate-13-acetate (PMA) [130] and can release pro-inflammatory mediators such as numerous cytokines after induction of inflammation [131–133]. Due to their origin, the cell line provides a stable genetic background which leads to a high reproducibility in experiments [134]. Nevertheless, while co-culturing with IECs like Caco-2, barrier breakdown in form of decreased transepithelial electrical resistance (TEER) values and less organization of the epithelial barrier in healthy condition (without application of an inflammatory stimulus) occurred after 24 to 48 hours in various Caco-2/dTHP-1 models [135–137]. Kämpfer *et al.* established a more stable Caco-2/dTHP-1 co-culture using a Transwell system after adjusting the time for the PMA differentiation of the THP-1 cells and the ratio between Caco-2 cells and THP-1 macrophages. Although the increased stability enables the investigation of the barrier integrity in inflamed conditions, pre-priming of both Caco-2 cells and dTHP-1 with INF-γ and INF- γ + lipopolysaccharides (LPS) respectively was necessary to obtain a significant and stable inflamed state [138]. However, there is recent literature about the evaluation of potential anti-inflammatory natural compounds using the Caco-2/dTHP-1 co-culture for the limited time of 24 hours [139–141] Weber *et al*. investigated the effect of potential natural anti-inflammatory compounds on a Caco-2/HT-29 MTX-E12/dTHP-1 triple co-culture for 48 hours [142].

While the aforementioned co-cultures were composed out of IECs in the apical and dTHP-1 cells in the basolateral compartment, there are (triple) co-cultures that are inverted with IECs cultivated on a Transwell insert facing the basolateral compartment [143,144]. Moreover, numerous 3D co-cultures including an ECM-simulating scaffold to improve cell differentiation were established [124]. For instance, Susewind *et al.* utilized an inflammable 3D co-culture with dTHP-1 and the dendritic cell line MUTZ-3 embedded in a collagen scaffold with Caco-2 cells seeded on top to investigate the safety of nanomaterials [145]. The model was further optimised by Hartwig *et al.* resulting in the ‘leaky gut model’, where the disturbed epithelial barrier was simulated by a not fully confluent Caco-2 layer to investigate siRNA-loaded nanocarriers [146]. Leonard *et al.* initially developed this 3D co-culture model using primary

monocyte-derived macrophages (MDM) instead of dTHP-1 cells and evaluated the model by applying the corticosteroid budesonide as free drug or encapsuled in nanoparticles or liposomes [147,148]. MDM can be differentiated out of human peripheral blood mononuclear cells (PBMCs) isolated from buffy coat obtained from blood donations and can be utilized as immune component in *in vitro* models [149]. Despite the fact that MDM are more closely connected to the *in vivo* situation, the primary origin from different donors leads to an increased variability of the amount of released cytokines [150].

A 3D bioengineered co-culture model was recently developed by Roh *et al.* utilizing primary human colon organoids generated from large intestine biopsies cultivated as inner layer and MDM cultivated as outer layer of a spongy biomaterial scaffold to investigate macrophage migration to the epithelium at inflammatory conditions [151]. Furthermore, 3D co-cultures consisting of IECs and immune cells such as MDM and dTHP-1 cultivated on gut-on-chip scaffolds were reported in literature [152,153]. Both approaches provides an increased simulation of the *in vivo* situation, however the reproducibility owing to the complex handling and the low throughput are remaining challenges [101,125].

1.7 Evaluation of *in vitro* models

Despite the wide range of numerous *in vitro* models simulating IBD, the predictivity of these approaches for the *in vivo* situation is the key factor for their potential to be accepted by authorities to reduce or replace animal experimentation. Therefore, the evaluation of the IVIVC is an important step to prove the applicability of the respective *in vitro* model [9]. In literature, the evaluation of the predictivity of *in vitro* models is rarely performed or presented. For the aforementioned models, the similarity to the human *in vivo* situation is often put into focus, however, the question if more complex models such as 3D models show not only an increased similarity, but simultaneously a higher IVIVC related to the prediction of drug effects remains unanswered. Although the anti-inflammatory effect of (natural) compounds is often investigated, valid human data as comparison is generally missing. Some *in vitro* models are using APIs as control, for instance the co-culture of Lenoard *et al.* applied the corticosteroid budesonide as free drug as comparison to their budesonide liposomes and nanoparticles [148]. The usage of APIs that are commonly applied during the treatment of IBD in patients might be a good starting point for the investigation of IVIVC. However, the application of different API classes might be necessary to improve the evaluation of the predictivity.

Considering regulatory approval, the evaluation of *in vitro* models based on official guidelines is crucial. The Organisation for Economic Co-operation and Development (OECD) provides a guidance document named ‘Guidance Document for Describing Non-Guideline *In Vitro* Test Methods’ in the context of their Inter-Organisation Programme for the Sound Management of Chemicals (IOMC). The Guidance Document outlines the necessity of evaluation and description of *in vitro*-models to facilitate the assessment of the data quality, the interpretation of results and the potential to utilize the models in regulatory testing [154]. It is focusing on the investigation of the safety of chemicals by defining an adverse outcome pathway (AOP) framework, where the stressor/active compound is connected including causally linked critical events to the response in humans [155,156]. Despite the evaluation of the safety of chemicals which is the initial aim of the guideline, it is additionally suitable for the evaluation of the *in vitro* assay since high quality and standardized operations are crucial for IVIVC.

This thesis focuses on the development of a stable and inflammable co-culture assay consisting of Caco-2 cells representing the epithelial barrier in IBD and the immune cells MDM as alternative to the dTHP-1 cells due to their reported possible negative effects on the integrity of the Caco-2 barrier [135–137]. For the evaluation of predictivity, four different classes of APIs commonly applied in IBD therapy are utilized. Additionally, the *in vitro* assay was evaluated according to the OECD guideline ‘Guidance Document for Describing Non-Guideline *In Vitro* Test Methods’.

2 Aims of the thesis

Due to the need of gastrointestinal *in vitro* models (see section 1.1), the first aim of the thesis is the development of a predictive cell-based co-culture simulating IBD as alternative to animal experimentation. To reduce complexity and simultaneously improve reproducibility and throughput, the *in vitro* assay should consist of colon epithelial cells and immune cells. The first step was the establishment of a combination of both cell types that simulates healthy GI-tract functions such as a stable epithelial barrier and a low release of pro-inflammatory cytokines. The inflamed state was induced by the application of lipopolysaccharides (LPS) as inflammation stimulus. The inflamed *in vitro* assay should simulate the two major KEs of the IBD pathophysiology: the disturbance of the epithelial barrier visible by a decrease in the transepithelial resistance (TEER) and the immune response characterized by the release of IBD-related pro-inflammatory cytokines.

Although various IBD-related *ex vivo* and *in vitro* models were developed based on the 3R principle to overcome the limitations of animal models (see section '1.5 IBD-related animal models'), the predictivity of those *in vitro* models is rarely investigated in literature. As the determination of IVIVC is an essential step of the development of a predictive *in vitro* assay, the second aim of the thesis was the evaluation of the *in vitro* assay. Therefore, four different classes of APIs commonly used in the treatment of IBD in patients, representing different levels of severity as depicted in the 'treatment pyramid' (Figure 4, section '1.4 Treatment options for IBD') were applied to the co-culture. Additionally, an evaluation according to the OECD guideline 'Guidance Document for Describing Non-Guideline *In Vitro* Test Methods' [154] (see '1.7 Evaluation of *in vitro* models') was performed.

Finally, the combination of APIs and the potential anti-inflammatory effect of the natural compound Epigallocatechin-3-gallate (EGCG) was investigated as third aim to address clinical relevance and the increasing interest in T/CAM to complement the conventional therapy of IBD (see section '1.4.2 Combination therapy' and '1.4.3 Natural products'). The effects of both, the API combinations and EGCG, were compared to the effect of the four APIs used for the evaluation of the predictivity of the *in vitro* assay.

Based on the aforementioned aims of the thesis, the following acceptance criteria were defined for the *in vitro* assay:

- the co-culture provides stable TEER values indicating intact epithelial barrier integrity and a low release of IBD-related pro-inflammatory cytokines by the macrophages in the healthy un-stimulated state.
- inflammation with pro-inflammatory substances is possible and leads to a significant decrease in TEER values and increase in the pro-inflammatory cytokine level released by the macrophages.
- treatment of the inflamed co-culture leads to an increased TEER value compared to inflammation control and to a reduced release of pro-inflammatory cytokines by the macrophages ideally related to the severity level that is represented by the respective class of APIs according to the IBD treatment pyramid. The evaluation using an AOP framework proves that the model meets important KE of the human response.
- investigation of combination therapy and of potential anti-inflammatory natural compounds is possible in order to apply the co-culture for the investigation of novel treatment options.

3 Methodology

The first part of the development of the inflammable co-culture system combining the two major KEs: the epithelial barrier and the immune system in a healthy state and inflamed state, was the co-cultivation of Caco-2 in the apical compartment and MDM in the basolateral compartment of a Transwell system. Figure 5 depicts the principle of the established *in vitro* model to provide an overview of the experimental procedures including the readouts utilized for the evaluation of the model itself and of the treatment efficacy. All applied methods are described in the material and methods sections of the respective publications (see 4.1 Establishment of an *in vitro* co-culture model simulating Inflammatory Bowel Disease: original publication and 4.3 Investigation of the natural product EGCG as potential complementary therapy: original publication).

After the set up of the co-culture, LPS was applied in a first step to the basolateral compartment to induce the inflammatory state. LPS as outer membrane component of gram-negative bacteria binds to the TLR4 of the MDM and induces an inflammatory response of the *in vitro* assay leading to the release of pro-inflammatory cytokines by the MDM and a decrease of the barrier integrity of the Caco-2 cells that was triggered by the cytokine release. In a second step, APIs from the IBD treatment pyramid, including four different classes of drugs commonly applied in IBD management that are related to the severity level of the disease (ranking from level 1: minor inflammation up to level 4: severe inflammation), were applied to the basolateral compartment to investigate the IVIVC of the model. Namely, 5-aminosalicylic acid (5-ASA), the corticosteroid prednisolone, the immunosuppressant 6-mercaptopurine (6-MP) and the biological TNF-α antagonist infliximab were selected as a representative treatment. Subsequently, combination therapy was simulated by the application of three different drug combinations (5-ASA + 6-MP, prednisolone + 6-MP and infliximab + 6-MP). Finally, the natural product EGCG was applied to the inflamed model for investigation of the potential of EGCG as T/CAM for the treatment of IBD.

The efficacy of the respective treatment option was evaluated focusing on the integrity of the epithelial barrier and on the cytokine release of the immune cells. TEER measurements were performed to monitor the barrier integrity in the healthy, inflamed, and treated state of the model. Focusing on the immune cells, the release of the pro-inflammatory cytokines TNF-α, IL-6 and IL-8 and of the anti-inflammatory cytokine IL-10 was determined using enzyme-linked immunosorbent assays (ELISA).

Additionally, the cell viability of the Caco-2 cells and the MDM was determined performing 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays either with the exposure to different concentrations of the respective API or the API combination or directly after the exposure to EGCG at the end of the experiment to exclude cytotoxic effects.

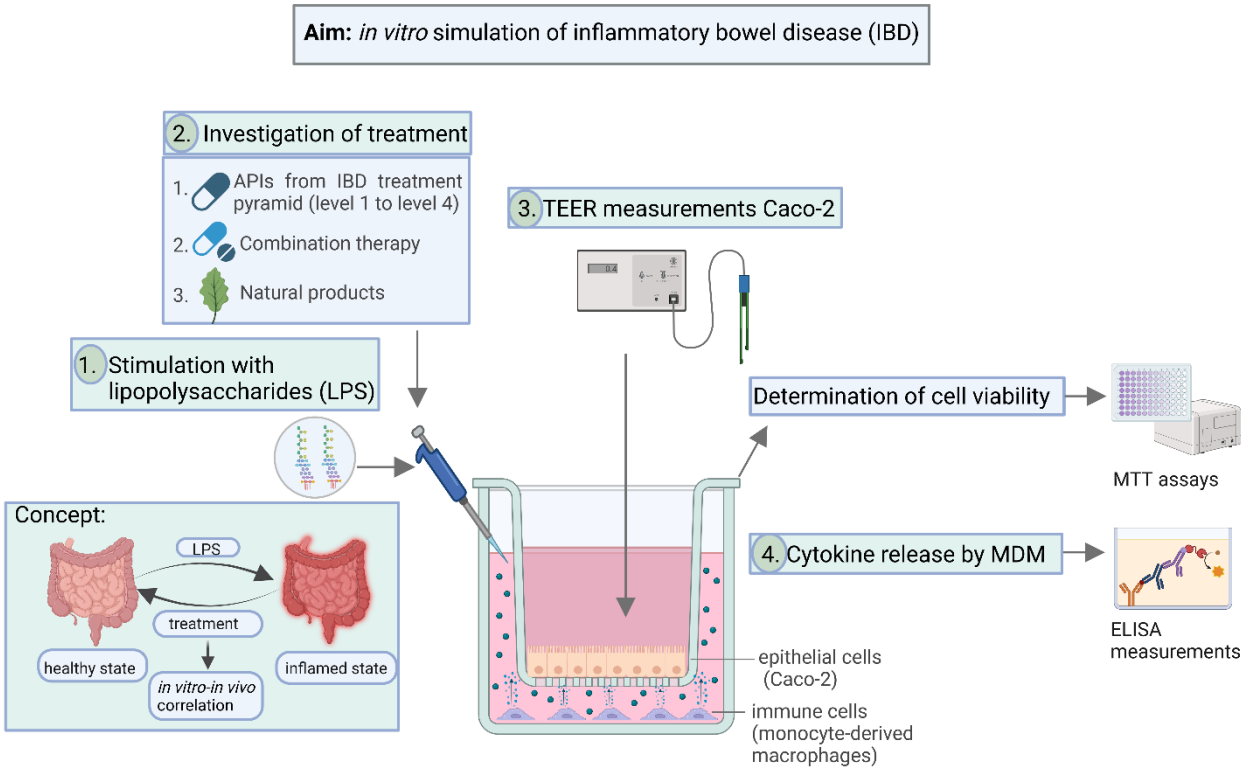

Figure 5. Overview of the *in vitro* co-culture principle and the readouts utilized for the evaluation of the model itself and of the *in vitro-in vivo* correlation.
The aim is the establishment of a cell-based co-culture simulating inflammatory bowel disease (IBD) in the healthy and inflamed state to investigate potential treatment options for IBD. The co-culture consists of Caco-2 representing the epithelial barrier and of monocyte-derived macrophages (MDM) representing the immune system. In a first step, LPS is applied to the basolateral compartment to induce the inflammatory state, leading to the release of pro-inflammatory cytokines by the MDM. Subsequently, the release of pro-inflammatory cytokines affects the barrier integrity of the Caco-2 cells. In a second step, different treatment options are applied in the basolateral compartment of the inflamed co-culture. First, active pharmaceutical ingredients (APIs) from the IBD treatment pyramid including four different drugs that are commonly utilized for the treatment of IBD related to the severity level of the disease (level 1: minor to level 4: severe inflammation) were applied. Second, combination therapy was simulated by the application of combined APIs. Third, the potential of the natural product EGCG as complementary medicine was evaluated. The effect on the barrier integrity was monitored by performing transepithelial electrical resistance (TEER) measurements and the cytokine release of the pro-inflammatory TNF-α, IL-6 and IL-8 and the anti-inflammatory IL-10 was analysed using ELISA. The cell viability of the Caco-2 and the MDM was determined either before the experiments or after the exposure to EGCG. The figure was created with BioRender.com.

4 Results

4.1 Establishment of an *in vitro* co-culture model simulating Inflammatory Bowel Disease: original publication

Title of original publication: Inflammatory bowel disease addressed by Caco-2 and monocyte-derived macrophages: an opportunity for an in vitro drug screening assay

Authors: Sabrina Schnur, Vanessa Wahl, Julia K. Metz, Jessica Gillmann, Fabian Hans, Katharina Rotermund, Ralf-Kilian Zäh, Dietmar A. Brück, Marc Schneider, Marius Hittinger

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Author contribution (described using CRediT taxonomy, see: https://credit.niso.org/): Sabrina Schnur performed the conceptualization (together with Marius Hittinger and Julia K. Metz), the design of methodology, formal data analysis, practical investigation, writing-original draft preparation, visualization and project administration. Julia K. Metz, Vanessa Wahl, Jessica Gillmann, Katharina Rotermund, Ralf-Kilian Zäh and Dietmar A. Brück assisted the methodology and the writing-review and editing. Vanessa Wahl supported the practical investigation. Marc Schneider and Marius Hittinger were responsible for the supervision, funding acquisition and supported the writing-review and editing.

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ORIGINAL RESEARCH

Inflammatory bowel disease addressed by Caco-2 and monocyte-derived macrophages: an opportunity for an in vitro drug screening assay

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Abstract

Inflammatory bowel disease (IBD) is a widespread disease, affecting a growing demographic. The treatment of chronic inflammation located in the GI-tract is dependent on the severity; therefore, the IBD treatment pyramid is commonly applied. Animal experimentation plays a key role for novel IBD drug development; nevertheless, it is ethically questionable and limited in its throughput. Reliable and valid in vitro assays offer the opportunity to overcome these limitations.

We combined Caco-2 with monocyte-derived macrophages and exposed them to known drugs, targeting an in vitro-in vivo correlation (IVIVC) with a focus on the severity level and its related drug candidate. This co-culture assay addresses namely the intestinal barrier and the immune response in IBD. The drug efficacy was analyzed by an LPS-inflammation of the co-culture and drug exposure according to the IBD treatment pyramid. Efficacy was defined as the range between LPS control (0%) and untreated co-culture (100%) independent of the investigated read-out (TEER, P_{app}, cytokine release: IL-6, IL-8, IL-10, TNF-α). The release of IL-6, IL-8, and TNF-α was identified as an appropriate readout for a fast drug screening ("yes–no response"). TEER showed a remarkable IVIVC correlation to the human treatment pyramid (5-ASA, Prednisolone, 6-mercaptopurine, and infliximab) with an R^2 of 0.68. Similar to the description of an adverse outcome pathway (AOP) framework, we advocate establishing an "Efficacy Outcome Pathways (EOPs)" framework for drug efficacy assays. The in vitro assay offers an easy and scalable method for IBD drug screening with a focus on human data, which requires further validation.

Keywords Chronic inflammation of GI-tract · Cell-based co-culture · IVIVC · Drug testing · Efficacy outcome pathways

Introduction

Inflammatory bowel disease (IBD) describes a group of chronic inflammations of the gastrointestinal (GI)-tract including the two main types Crohn's disease (CD) and ulcerative colitis (UC). With a maximal annual incidence of 20.2 per 100,000 people for CD (North America) and 24.3 per 100,000 people for UC (Europa), the disease diminishes the health and quality of life of many patients [1]. IBD is characterized by remission and acute inflammation phases, whereby, the CD is commonly affecting the ileum (small intestine) and the colon in patches through multiple layers of the tissue [2], while UC occurs as a continuous inflammation of the innermost layers (mucosa and submucosa) in the colon spreading to the rectum [3]. As the incidence for CD and UC increases, the industrial life style seems to be a prominent factor causing the occurrence of IBD [1]. However, the causes of chronic inflammations of the GI have not yet been fully identified, but multiple factors seem to have an impact [4]. Gaps in knowledge of IBD itself, the absence of a fully curative therapy and the lack of novel drug substances for an effective treatment have led to numerous animal experiments in basic research, and, above all, they

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are needed to fulfill safety and efficacy testing during drug development. In order to replace animal testing, an understanding of molecular mechanisms (especially the intestinal barrier) is crucial for developing an effective treatment for IBD patients. Therefore, the development and a consistent definition of an IBD in vitro assay according to the "Guidance Document For Describing Non-Guideline In Vitro Test Methods" [5] is the aim of this study. To fully investigate assay development, the main biological mechanism involved in inflammation and treatment states are important.

The intestinal barrier consists of different components, which build a strong semipermeable barrier that allows absorption of nutrients and small solutes on the one hand, but on the other hand, prevents the entering of pathogens. Primarily, the mucus layer produced and secreted by the epithelium covers the small intestine discontinuously and the colon continuously, forming the first barrier component. Secondly, the epithelium built by a cell layer that is strongly connected by tight junction (TJ) proteins is responsible for absorptive processes and simultaneously the production of mucus. Finally, cells of the immune system like monocytes/macrophages and lymphoid cells (e.g., T- and B-cells) are located in the submucosa in a "tolerant state," ready to be activated when pathogens have crossed the mucosal and the epithelial barrier [6]. In healthy conditions, the intestinal barrier tolerates the surrounding microbiome consisting of bacteria, fungi, and viruses while the mucosa is mostly sterile and not contaminated with bacteria. In cases of CD and UC, a high bacterial contamination of the whole mucus layer and the mucosa can be observed [7]. Additionally, a "leakage" of the epithelial layer caused by the disturbance of the TJ network is reported [8]. This leads to the persistent activation of the adaptive immune system including the secretion of pro-inflammatory cytokines like tumor necrosis factor-alpha (TNF-α), which consequently sustains inflammatory conditions [9].

Human treatment options for IBD

Presently, the cure for IBD is unknown; consequently, the therapeutic approach consists of treating the acute inflammatory phases, avoiding accompanying complications, and prolonging the remission phases. Fundamentally, the treatment is targeting the activated adaptive immune system during the acute inflammation phase of IBD. The kind of treatment applied is strongly connected to the severity of the disease [10, 11]. According to the severity level, a gradual increase in the strength of the drug treatment is widely used as depicted in the treatment pyramid (Fig. 1) [12].

Mild disease courses (level 1) are typically treated with antibiotics or 5-aminosalicylic acid (5-ASA) derivates like mesalazine [13], which intervene with the NF-κB pathway, resulting in reduced release of pro-inflammatory cytokines

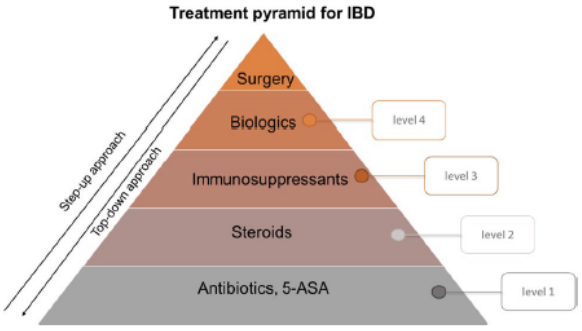


Fig. 1 Treatment pyramid for (IBD) as step-up or top-down approach (adapted figure according to Aloi et al. [12]). Inflammatory phases with mild inflammations of the intestine are treated with antibiotics or 5-ASA derivates (level 1). Increasing severity corresponds to the usage of steroids (level 2). In severe cases, immunosuppressants (level 3) are chosen. Biologics (level 4) are used prior to surgery being considered

[14]. In mild to moderate cases (level 2), steroids like budesonide or prednisolone, that act in a similar manner on the release of pro-inflammatory cytokines by strongly inhibiting the NF-κB pathway, are applied [10, 11, 15]. For the treatment of moderate to severe disease progression (level 3), immunosuppressants like azathioprine and 6-mercaptopurine are utilized. These diminish the immune response of the body to certain antigens, especially to prolong the remission phases in steroid-dependent states. In severe cases (level 4), in which the aforementioned drugs were insufficient, biologics such as the TNF-α antibody Infliximab are deployed [10, 11]. If the efficiency of medication is limited, surgery to remove the affected regions remains as a final option [16, 17].

Based on the treatment-pyramid known drugs are able to test the prediction for the human in vivo situation of disease-related in vitro models. Due to the high rate of strong adverse effects caused by the currently administrated drugs [18], the need for new therapeutic strategies is evident. Therefore, novel drugs like JAK-inhibitors and additional antibodies have been intensively investigated [19], which are typically tested in IBD-related animal models as explained below.

IBD-related animal models

Animal IBD models are used for the investigation of the safety and efficacy of new potential drugs in preclinical studies. Advantages of such models can be especially for chemically induced models, for instance, the simplicity in terms of inducing the inflammation, the rapidity (disease occurrence after several days after chemical administration), and controllability, as the concentration and frequency of chemical

substance administration correlates with the severity of the inflammation [20–22]. In general, animal models allow for the investigation of complex physiological and biochemical interactions and modifications of the genome affecting the immune system [23, 24].

However, there are various challenges when using IBD-related animal models. One critical aspect of using chemically induced IBD models is that immune cells such as T- and B-cells are not required to induce the inflammation as occurs in humans. IBD induced by uncontrolled immune responses can be investigated with genetically engineered animals like IL-10 knockout mice [25]. Although, it is also reported that the gene depletion in different strains leads to variations in inflammation severity [26]. Crucially, the gastrointestinal physiology differs between different species [27]. However, not only is the genetic background of the used strain important to study the inflammation, many other factors, for instance, the age or gender of the animals and environmental factors influence the severity and the development time of the disease after the induction, resulting in a challenge to represent the clinical course of the disease [22]. Additionally, the induced symptoms like rectal bleeding or diarrhea cause pain, suffering, and distress for the animals which has an unknown effect on the results of the experiments [24].

Nowadays, the implementation of animal experiments is strongly connected to the ethical 3R (replacement, reduction, and refinement) principle enshrined in the European directive 2010/63. This is widely supported, for instance, by the UK-based scientific organisation NC3Rs, with the aim to replace, or at least reduce, animal experiments and to improve animal welfare [28, 29]. Consequently, reliable and validated in vitro assays as an alternative to animal experimentation are in high demand. Moreover, in vitro assays provide the potential to increase throughput for more experimental data in a reduced amount of time with the additional benefit of cost savings [24].

Development of in vitro assay for drug screening

The purpose of the in vitro assay described here is the screening of drugs connected with human-relevant output. Based on the previous studies from co-cultures including macrophages and intestinal cells [30–36], we aimed for a co-culture which is able to address both the intestinal barrier and the immune response in IBD. As it is reported in the literature, several challenges after combining the epithelial and immune cells such as a decrease of barrier integrity when mimicking the healthy state without any stimulation [37–39] or the recovering of the inflamed barrier without any treatment or pre-stimulation of macrophages [31] occurred. In this study, we successfully developed a Caco-2/human monocyte-derived macrophages (MDM) co-culture that is able to mimic a stable healthy state and showed a significant and lasting decrease in barrier integrity measured by transepithelial electrical resistance (TEER) after stimulation with lipopolysaccharides (LPS). Therefore, this co-culture enables the evaluation of drug efficacy in vitro and provides significant benefits over previously described models. We investigated the effect of pharmaceuticals with known human efficacy according to the treatment pyramid (Fig. 1) to determine the in vitro-in vivo correlation (IVIVC) for different readouts of the assay. Finally, the in vitro assay was evaluated based on the OECD guideline "Guidance Document For Describing Non-Guideline In Vitro Test Methods" [5] to further assess the relevance of the in vitro assay and to discuss the validation that is needed to at least reduce animal experimentation in the future.

Material and methods

The development and characterization of the co-culture were both performed by a stepwise approach. Firstly, the effects of TNF- on Caco-2 barrier properties were determined by TEER as a basis for further experimentation. Secondly, the immune response of lipopolysaccharides (LPS)-stimulated MDM was measured by analyzing the release of the cytokines tumor necrosis factor-alpha (TNF)- α interleukin (IL)-6, IL-8, and IL-10 with an enzyme-linked immunosorbent assay (ELISA). Supernatant from LPS stimulated and not stimulated MDM was then collected and added to the basolateral compartment of Caco-2 cells cultivated in a transwell system. Afterwards, the final co-culture model combining Caco-2 cells in the apical compartment and MDM in the basolateral compartment of a transwell plate was set up. The co-culture was stimulated with LPS and four different drugs, one for each severity level (according to the treatment pyramid) commonly applied to IBD patients. The co-culture system was evaluated using the parameters TEER, P_{app}, and the release of TNF- α, IL-6, IL-8, and IL-10. Cytotoxicity of the drug substances was tested for Caco-2 and MDM via MTT assays.

Cell culture

Cultivation of Caco-2 cells

The human colon epithelial cell line *Caco-2 HTB 37* was obtained from ATCC (American Type Culture Collection, RRID:CVCL_0025). The cells were cultivated in MEM (Minimal Essential Media, Gibco, Thermo Fisher Scientific, USA) supplemented with 20% FBS (Fetal Bovine

Serum Supreme, South America origin, PAN-Biotech, Germany), 1% 100 × MEM-NEAA (MEM Non-Essential Amino Acids Solution, Gibco, Thermo Fisher Scientific, USA), 1% 100 mM Na-Pyruvate (Sodium Pyruvate, Gibco, Thermo Fisher Scientific, USA), and 1% Pen/Strep (Penicillin (10.000 units/mL)/Streptomycin (10.000 µg/mL), Gibco, Thermo Fisher Scientific, USA). The cells were maintained at 37 °C in a constant humid environment with 5% CO_2 and were used up to passage number 50.

Isolation of MDM from buffy coat

For the isolation of primary MDM human blood, obtained from the Blutspendenzentrale Saar-Pfalz GmbH (Germany), was used. The blood was first diluted 1:1 with 1 × DPBS (Dulbecco's phosphate-buffered saline, Gibco, Thermo Fisher Scientific, USA). Subsequently, the diluted blood was slowly layered above Ficoll-Paque (GE Healthcare, UK) and then centrifuged (20 min, 750 g without deceleration at room temperature (RT)) in order to break down the blood into its components. The buffy coat phase was then collected and washed twice with 1 × DPBS followed by centrifugation (7 min, 750 g at RT). The cell pellet was reconstituted in 20 mL RPMI-1640 (Gibco, Thermo Fisher Scientific, USA) supplemented with 10% hS (human Serum, heat inactivated (from male AB clotted whole blood), USA origin, Sigma-Aldrich, Merck Millipore, USA), 1% 100 × MEM-NEAA, 1% 100 mM Na-Pyruvate and 1% Pen/Strep (Penicillin (10.000 units/mL)/Streptomycin (10.000 µg/mL)). Finally, 50 ng/mL GM-CSF (Granulocyte–macrophage–colony-stimulating factor, Gibco, Thermo Fisher Scientific, USA) was added to the cell suspension, and the cells were incubated at 37 °C in a constant humid environment with 5% CO_2. The medium was changed after 24 h, and the macrophages were used after 72 h for the experiments.

Inflammation of monolayers

Inflammation of Caco-2 monolayer by TNF-α

1×10^5 cells per well were seeded in the apical compartment of a transwell system (1.12 cm^2 growth area, 12 mm transwell with 0.4 µm pore polyester membrane inserts, 12-well plate, Corning, USA). The apical and the basolateral compartment received 0.5 mL and 1.5 mL culture medium, respectively. Caco-2 cells were cultivated until a stable epithelial barrier (TEER > 500 Ω*cm^2) was formed. The inflammation of the Caco-2 monolayer was induced by adding TNF-α (Invitrogen, Thermo Fisher Scientific, USA) in different concentrations (1 ng/mL, 2 ng/mL, 10 ng/mL, 20 ng/mL, 40 ng/mL and 60 ng/mL) to the apical and the basolateral compartment. During the experiment, the cells were maintained at 37 °C in a constant humid environment with 5% CO_2. The effect of the stimulation on the barrier properties was monitored by TEER measurements every 24 h for 72 h.

Inflammation of macrophages by LPS

MDM were inflamed with LPS (lipopolysaccharides from *E. coli*, Sigma-Aldrich, Merck Millipore, USA). For this, 0.3×10^6 cells/well were seeded in a 12-well plate (Greiner Bio-One International GmbH, Austria). Each well was filled with 1.5 mL MDM medium (RPMI-1640, 10% hS, 1% 100 × NEAA, 1% 100 mM Na-Pyruvate, 1% P/S). After 24 h of incubation, the macrophages were washed twice with MDM medium. Finally, 200 ng/mL LPS was added to the cells. Subsequently, the cells were incubated for 24 h at 37 °C in a constant humid environment with 5% CO_2. The cytokine release for TNF-α, IL-6, IL-8, and IL-10 was measured by ELISA (see "Analytical methods" section "ELISA measurements").

Co-culture experiments

Stimulation with MDM supernatant

First, the supernatant of non-stimulated MDM and of MDM stimulated with LPS was used to investigate the proof of concept of the co-culture. For this, 1×10^5 Caco-2 cells/well were seeded in the apical compartment of a transwell system (1.12 cm^2 growth area, 12 mm transwell with 0.4 µm pore polyester membrane inserts, 12-well plate, Corning, USA). The apical and the basolateral compartment received 0.5 mL and 1.5 mL culture medium, respectively. The cells were cultivated until a stable epithelial barrier (TEER > 500 Ω*cm^2) was formed. The supernatant from the macrophages was collected, and 1.5 mL/well was added to the basolateral compartment of the transwell plate with Caco-2 cells in the apical compartment. MDM medium served as control group. Cells were incubated at 37 °C in a constant humid environment with 5% CO_2. The effect of the MDM supernatants on the barrier properties was observed with TEER-measurements every 24 h for 48 h.

Set-up of the MDM/Caco-2 co-culture

0.3×10^5 cells/well were seeded in the apical compartment of a transwell system (0.33 cm^2 growth area, 6.5 mm transwell with 0.4 µm pore polyester membrane inserts, 24-well plate, Corning, USA). The apical and the basolateral compartment received 0.2 mL and 0.8 mL culture medium, respectively. The cells were cultivated until a stable epithelial barrier (TEER > 500 Ω*cm^2) was formed.

The MDM were removed separately from the flask using 0.05% Trypsin–EDTA (Gibco, Thermo Fisher Scientific, USA). 0.15×10^6 MDM/well were then seeded in the basolateral compartment of the transwell plate (Corning, USA). Each well was filled with 0.8 mL MDM medium (RPMI-1640, 10% FBS, 1% 100 × NEAA, 1% 100 mM Na-Pyruvate). After 24 h of incubation, the macrophages were washed twice with medium. Finally, the transwell inserts containing the Caco-2 cells were set above the macrophages located in the basolateral compartment. The co-culture was maintained at 37 °C in a constant humid environment with 5% CO_2. The barrier properties of the co-culture were monitored by performing TEER-measurements after 4 h, 24 h, and 48 h.

Inflammation of co-culture

To induce an inflammation of the co-culture, LPS was added basolaterally to each well (final concentration per well: 200 ng/mL) directly after the co-culture was initiated. TEER-measurements were carried out after 4 h, 24 h, and 48 h.

Proof of concept study for efficacy verification of commonly applied drugs for IBD patients

For the treatment of the inflamed cell state, the four different drugs: the 5-amino salicylic acid derivate mesalazine (Cayman Chemical Company, USA), the corticosteroid prednisolone (TCI Deutschland GmbH, Germany), the immunosuppressant 6-mercaptopurine (TCI Deutschland GmbH, Germany), and the TNF-α antibody infliximab (Merck Millipore, USA) were selected, which are commonly used for the treatment of IBD. After applying the respective drug to the in vitro model, the treatment efficacy of each drug was investigated. A drug concentration of 200 µg/mL was chosen empirically as a high dose with non-cytotoxic effects. The dose is expected to be much higher than the in vivo exposure and is sufficient for in vitro proof of concept investigations. Dose findings in vitro with a transfer to in vivo data are a future challenge.

Treatment of MDM

After isolating MDM from buffy coat as described in Sect. Cell culture, the cells were seeded on a 12-well plate (Greiner Bio-One International GmbH, Austria). For this, the MDM were removed from the flask using 0.05% Trypsin–EDTA (Gibco, Thermo Fisher Scientific, USA). 0.15×10^6 MDM/well were then seeded on the 12-well plate. Each well was filled with 1.5 mL MDM medium (RPMI-1640, 10% FBS, 1% 100 × NEAA, 1% 100 mM Na-Pyruvate). After 24 h of incubation, the macrophages were washed twice with the medium. Subsequently, MDM were exposed to the selected drug suspensions in a concentration of 200 µg/mL (volume: 1.5 mL). Immediately afterwards, LPS were applied to all wells (final concentration 200 ng/mL) except for the medium control. LPS-stimulated MDM that were not treated with drugs were used as positive control. The cells were maintained at 37 °C in a constant humid environment with 5% CO_2. After 24 h, the supernatants of the cells were collected and the cytokine release was analysed by ELISA (see "Analytical methods" section "ELISA measurements").

Treatment of the MDM/Caco-2 co-culture

After setting up the co-culture, the selected drugs were applied in a final concentration of 200 µg/mL to each basolateral compartment to the macrophages. MDM medium (RPMI-1640, 10% FBS, 1% 100 × NEAA, 1% 100 mM Na-Pyruvate, 1% P/S) was used as a negative control (no inflammation). Immediately afterwards, LPS in a final concentration of 200 ng/mL were added to all wells in the basolateral compartment except for the negative control (medium control). Macrophages that were stimulated with LPS but not treated served as positive control (inflammation). The co-culture was maintained at 37 °C in a constant humid environment with 5% CO_2. Treatment efficacy was investigated by doing TEER measurements after 4 h, 24 h, and 48 h followed by a transport study with sodium fluorescein. The cytokine release of the MDM was investigated by performing ELISA (see "Analytical methods" section "ELISA measurements").

Analytical methods

MTT assay

The cell viability after exposure to the selected drugs was investigated by MTT assays as described by Scherließ et al. [40]. In brief, the cells (4×10^4 Caco-2 cells or 2.72×10^4 MDM) were seeded in a 96-well plate (Greiner Bio-One International GmbH, Austria) using a volume of 200 µL/well. The cells were incubated for 24 h at 37 °C in a constant humid environment. One day before the experiment, the dilutions of the drugs were prepared. For Mesalazine (Cayman Chemical Company, USA), prednisolone (TCI Deutschland GmbH, Germany) and 6-Mercaptopurin (TCI Deutschland GmbH, Germany) concentrations from 0.005 mg/mL up to 5 mg/mL and for infliximab (Merck Millipore, USA) from 0.005 mg/mL up to 2 mg/mL were chosen. The dilutions were prepared in 1 × HBSS (Hanks' Balanced Salt Solution, Gibco, Thermo Fisher Scientific, USA). To remove medium residues, the cells were washed twice with 200

µL HBSS, respectively. Afterwards, 200 µL of the respective drug concentration was applied to the cells followed by an incubation on a shaker at 37 °C and 35 rpm for 4 h. Onefold-concentrated HBSS (100% viability) and Triton-X-100 (AppliChem GmbH, Germany) (0% viability) were used as controls. The supernatant was then disposed, and the cells were washed once with 1 × HBSS. Subsequently, the MTT reagent ((3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, Agros Organics, Thermo Fisher Scientific, USA) was added in a concentration of 0.5 mg/mL per well, and the cells were incubated again on a shaker for 4 h at 37 °C and 35 rpm, protected from light. The MTT reagent was then removed and 100 µL DMSO was added to the wells, respectively, incubated for 15 min on a shaker at 37 °C and 35 rpm, again protected from light. Finally, the absorbance was measured at 550 nm with the Synergy2 plate reader (BioTek Instruments GmbH, USA). The viability of the cells was determined by the following formula:

$$Viability\ [\%] = \frac{absorbance_{test\ formulation} - absorbance_{TritonX-100}}{absorbance_{HBSS} - absorbance_{TritonX-100}} * 100 \quad (1)$$

Formula 1 describes the calculation of cell viability in %.

TEER measurements

The TEER-values of the Caco-2 monolayer were measured with the epithelial voltohmmeter EVOM2 (World Precision Instruments, USA) together with the STX2 Chopstick Electrode Set (World Precision Instruments, USA). First, the electrodes were cleaned with 70% isopropanol, dried, and then placed in the apical or basolateral compartment, respectively.

Transport studies

The transport studies were performed using sodium fluorescein as a marker substance. For this, medium of Caco-2 cells was replaced by 1 × HBSS (Gibco, Thermo Fisher Scientific, USA) and the cells were incubated for 30 min at 37 °C. Subsequently, the initial TEER values were measured before 10 µg/mL sodium fluorescein (final concentration apical); solution was applied to the cells in each well in the apical compartment. The experiment was performed at 37 °C under constant shaking (30 rpm) and light protection. Samples were taken from the basolateral compartment after 0 min, 20 min, 40 min, 60 min, 90 min, and 120 min and from the apical compartment after 0 min and 120 min. To ensure that the epithelial barrier was not damaged during the experiment, the TEER values of the cells were measured at the end of the experiment. The absorbance of the samples was analysed at 528 nm with the Synergy2 plate reader (BioTek Instruments GmbH, USA). Finally, the apparent permeability coefficient (P_{app}) values were determined by the following formula:

$$Papp = \frac{\Delta q}{\Delta t} * \frac{1}{c_0(apical)} * \frac{1}{A_{Transwell}} \quad (2)$$

Formula 2 describes the calculation of the apparent permeability coefficient P_{app} (cm/s). $\Delta q/\Delta t$ is the mass transport over time [µg/s], c_0 is the initial concentration of sodium fluorescein [µg/mL], and $A_{\text{Transwell}}$ is the area of the transwell membrane (1.12 cm^2).

ELISA measurements

For the investigation of the cytokine release of the macrophages in stimulated or non-stimulated conditions, the Human Uncoated ELISA Kits for TNF-α, IL-6, IL-8, and IL-10 from Thermo Fisher Scientific, USA were used. The assay was performed according to the manufacturer's protocol. Briefly, 96-well plates (Corning, USA) were coated with a 100 µL/well capture antibody. The plates were incubated at 4 °C overnight. Each well was then washed three times with 200 µL/well wash buffer, composed of 1 × DPBS containing 0.05% polysorbate-20 (Merck Millipore, USA) before the wells were blocked with 100 µL/well 1 × ELISA spot diluent for 1 h at room temperature. After the incubation, the wells were washed once with 200 µL/well wash buffer. The samples (100 µL/well) were first centrifuged for 5 min at 0.8 g and were then applied to the wells in the following dilutions: 1:1 and 1:100 for TNF-α samples, 1:100 and 1:1000 for IL-8 samples, and undiluted and 1:100 for IL-6 samples. The standards thus provided were used to obtain a calibration curve for each tested cytokine. The plates were again incubated at 4 °C overnight. The next day, each well was washed five times and 100 µL/well detection antibody was added followed by an incubation for 1 h at room temperature. Subsequently, the wells were washed again five times and then 100 µL/well Avidin-horseradish peroxidase (HRP) was applied, followed by an incubation for 30 min at room temperature. After washing six times, 100 µL/well 3, 3′, 5, 5'-tetramethylbenzidine (TMB)-substrate was added and the wells were incubated for 15 min at room temperature. Finally, 100 µL/well stop solution (1 M ortho-phosphoric acid, VWR International, USA) was used to stop the enzymatic reaction. The samples were measured at 450 nm and 570 nm with the Synergy2 plate reader.

ZO-1 staining of Caco-2 monolayer

To visualize the barrier-forming protein zonula occludens (ZO)-1, the Caco-2 cells were fixed and stained as described in the following. First, Caco-2 cells were washed with 0.5 mL 1 × DBPS (Gibco, Thermo Fisher Scientific, USA) apical

and 1.5 mL 1×DPBS basolateral. The cells were fixed with ice-cold methanol (VWR International, USA). 0.5 mL was added apical and 1.5 mL basolateral for 5 min at 4 °C. Washing with 1×DPBS was repeated three times. Afterwards, a blocking step with 0.5 mL 1% BSA (Thermo Fisher Scientific, USA) in the apical compartment was done for 30 min at 4 °C. Subsequently, 1 µL ZO-1 primary antibody in a final concentration of 2 µg/mL was added to each well filled with 0.5 mL 1% BSA, respectively. The cells were incubated overnight at 4 °C, protected from light. After that, the cells were washed three times with 1×DPBS with an incubation time of 5 min at each step at RT. Then, 1 µL Alexa Fluor 488 goat anti rabbit IgG (Thermo Fisher Scientific, USA) in a final concentration of 2 µg/mL was added to 0.5 mL 1% BSA, followed by an incubation over night at 4 °C, protected from light. Afterwards, the cells were washed twice with 0.5 mL DPBS with an incubation time of 5 min at RT for each washing step. The nuclei of the Caco-2 cells were stained with 1 µL 4′, 6-diamidino-2-phenylindole (DAPI) (Thermo Fisher Scientific, USA) in a final concentration of 2 µg/mL. Each well was filled with 0.5 mL 1×DPBS followed by an incubation for 15 min at RT. Finally, the cells were mounted with mounting medium (Sigma-Aldrich, Merck, USA) and were imaged via confocal laser scanning microscopy (LSM710, Zeiss, Oberkochen, Germany).

Statistical analysis

The statistics were performed with the program OriginPro 2021. As different numbers (n) of independent replicates were performed, the data is presented as the mean ± standard deviation (SD). For the statistical analysis, one-way analysis of variance (ANOVA) and the post hoc Bonferroni test were used. A two-sampled t-test was applied whenever two groups were compared.

Results

Inflammation of monolayers

Inflammation of Caco-2 monolayer by TNF-α

Due to the expression of tight junction proteins (see Supplementary information, ZO-1 staining Caco-2 monolayer Fig. S1), the Caco-2 cells formed a stable epithelial barrier (TEER > 500 Ω*cm^2). After the application of TNF-α in different concentrations, a dose-dependent decrease of the TEER values (see Supplementary information, Fig. S2) was measured, indicating a loss of barrier integrity. The maximal effect could be observed after stimulation with 10 ng/mL TNF-α. Higher concentrations of TNF-α up to 60 ng/mL did not result in a higher decrease of TEER values.

Inflammation of macrophages by LPS

The stimulation of MDM with 200 ng/mL LPS led to an increased release of the cytokines TNF-α, IL-6, and IL-8 compared to the medium control (see Supplementary information, Fig. S3), which demonstrates a successfully induced inflammation of the macrophages. As the MDM were freshly isolated from the human buffy coat for each experiment, the cytokine release of the MDM showed an increased variance between different donors. However, a clear difference in the measured cytokine profile of the non-inflamed and inflamed condition was nevertheless observable.

Set-up and LPS stimulation of MDM/Caco-2 co-culture

The set-up of the MDM/Caco-2 co-culture was first assessed by only applying the supernatant from non-stimulated or with LPS-stimulated macrophages in the basolateral compartment of the Caco-2 cells. The TEER measurement over 48 h showed a slight decrease in TEER after the application of non-stimulated MDM supernatant compared to the medium control. The supernatant from stimulated macrophages led to a significant decrease to 38% of the initial TEER value (Fig. 2A). The combination of MDM and Caco-2 in a co-culture system was characterized by a stable TEER for the control group without any stimulation and a significant drop in TEER to 21% after 48 h of LPS stimulation (Fig. 2B).

Treatment of the co-culture

The MDM/Caco-2 co-culture was treated with four different drugs, which correlate with the treatment-pyramid for IBD (Fig. 1, section "Human treatment options for IBD"). Depending on the severity level, different types of drugs are commonly applied to the patients to cure acute inflammations and to maintain remission phases of the disease. As a first step in treating the MDM/Caco-2 co-culture, the viability of the two cell types after incubation with the active ingredients was investigated by MTT assays to exclude cytotoxic effects. Concentrations up to 2 mg/mL or 10 mg/mL, depending on the drug, can be applied to the cells without decreasing the cell viability (see Supplementary information, Figs. S4 and S5).

Effect on barrier integrity (Readout: TEER, P_{app})

The drugs' effect on the integrity of the barrier was investigated with TEER measurements for 48 h followed by transport studies with sodium fluorescein. The immunosuppressant 6-MP has no significant effect on TEER compared to

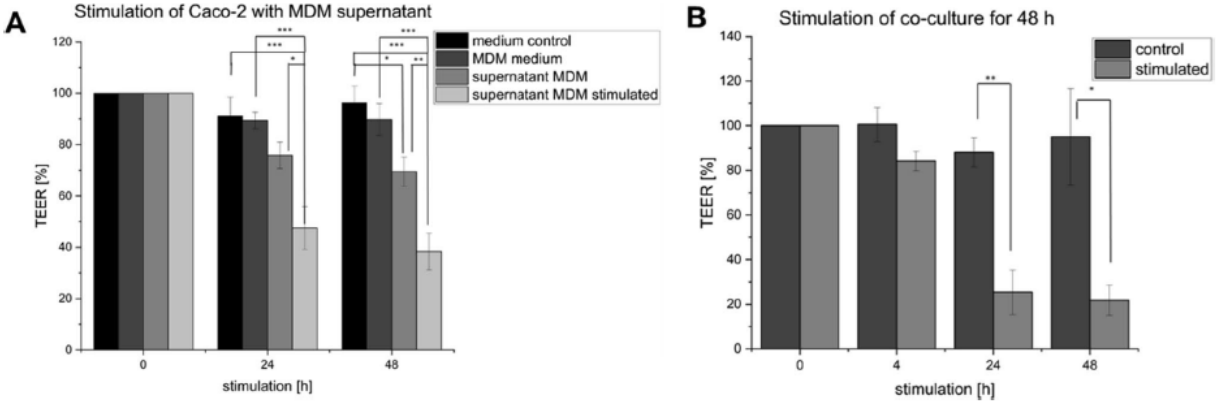


Fig. 2 Effect on barrier properties investigated by TEER measurements after incubation with different stimuli. The Caco-2 cells stimulated with the supernatant of LPS-stimulated MDM resulted in a significant decrease of TEER to 38% of the initial value while the stimulation with non-stimulated MDM supernatant showed only a slight decrease. Results are represented as mean ± SD for in summary $n = 9$ wells for each group performed in 3 biological replicates (**A**). In the co-culture system, stable TEER values could be obtained for the combination of Caco-2 and MDM without any stimulation (= medium control), which indicates an intact epithelial barrier. The LPS-stimulation of the MDM in the basolateral compartment induced a significant decrease in TEER. Results are represented as mean ± SD and for in summary $n = 18$ wells for each group performed in three biological replicates (**B**). $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$ indicate significant difference

the medium control, unlike on the LPS control after 24 h (Fig. 3). The treatment with the TNF-α antibody infliximab resulted in a stable TEER of 92% after 48 h compared to the initial value and showed a significant difference to the LPS control where a decrease in TEER to 48% could be observed. The corticosteroid prednisolone reduced the LPS-induced TEER decrease to 82% of the initial value. The slightest effect could be measured for the treatment with the 5-ASA derivate mesalazine, where the LPS-induced TEER decrease was stabilized to 67% of the initial value after 48 h.

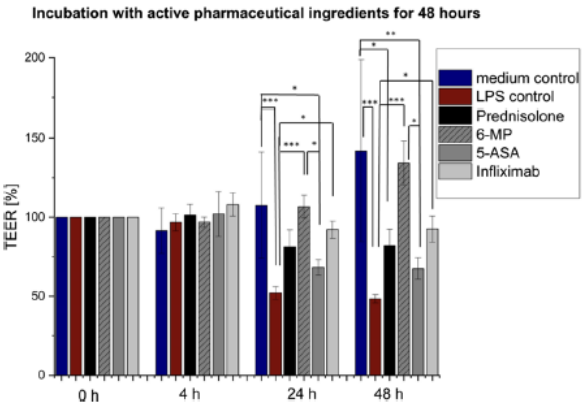


Fig. 3 Investigation of drug efficacy for 5-ASA, prednisolone, 6-MP, and infliximab on the barrier integrity of the MDM/Caco-2 co-culture observed by TEER measurements for 48 h. The strongest stabilization effect on the barrier integrity could be reached after the treatment with the immunosuppressant 6-MP (no difference to medium control), whereas TNF-α antibody infliximab maintained 92% of TEER. The Caco-2 cells affected with 5-ASA and prednisolone showed still higher TEER values in comparison to the LPS control. Results are represented as mean ± SD for in summary $n = 10$ wells for each group performed in five biological replicates with $n = 2$ wells. $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$ indicate significant difference

Transport studies with sodium fluorescein that were performed to investigate the permeability of the epithelial barrier yielded P_{app} values in an interval from 9.70×10^{-8} cm/s to 1.11×10^{-6} cm/s with no visible differences for the respective groups (see Supplementary information, Fig. S6), indicating no increased permeability of the epithelial barrier.

Effect on cytokine release of MDM

The release of the cytokines TNF-α, IL-6, IL-8, and IL-10 by the MDM in the co-culture system after 48 h of stimulation and drug treatment was analysed by ELISA. No level of TNF-α was observed after the treatment with the TNF-α antibody infliximab (Fig. 4A). The supernatant contained 3786 pg/mL and 5191 pg/mL of TNF-α after prednisolone and 6-MP treatment, which showed only a slight effect on the TNF-α release in comparison to the LPS control, which had a TNF-α content of 6741 pg/mL. The treatment with 5-ASA resulted in a reduced cytokine release to 1362 pg/mL. Figure 4B presents the results for the IL-6 release, only prednisolone reduced the IL-6 release significantly to 104 pg/mL compared to the LPS control containing 151 pg/mL IL-6. For the IL-8 release a significant decrease could be measured after the treatment with 5-ASA (28,190 pg/mL), prednisolone (20,048 pg/mL), and

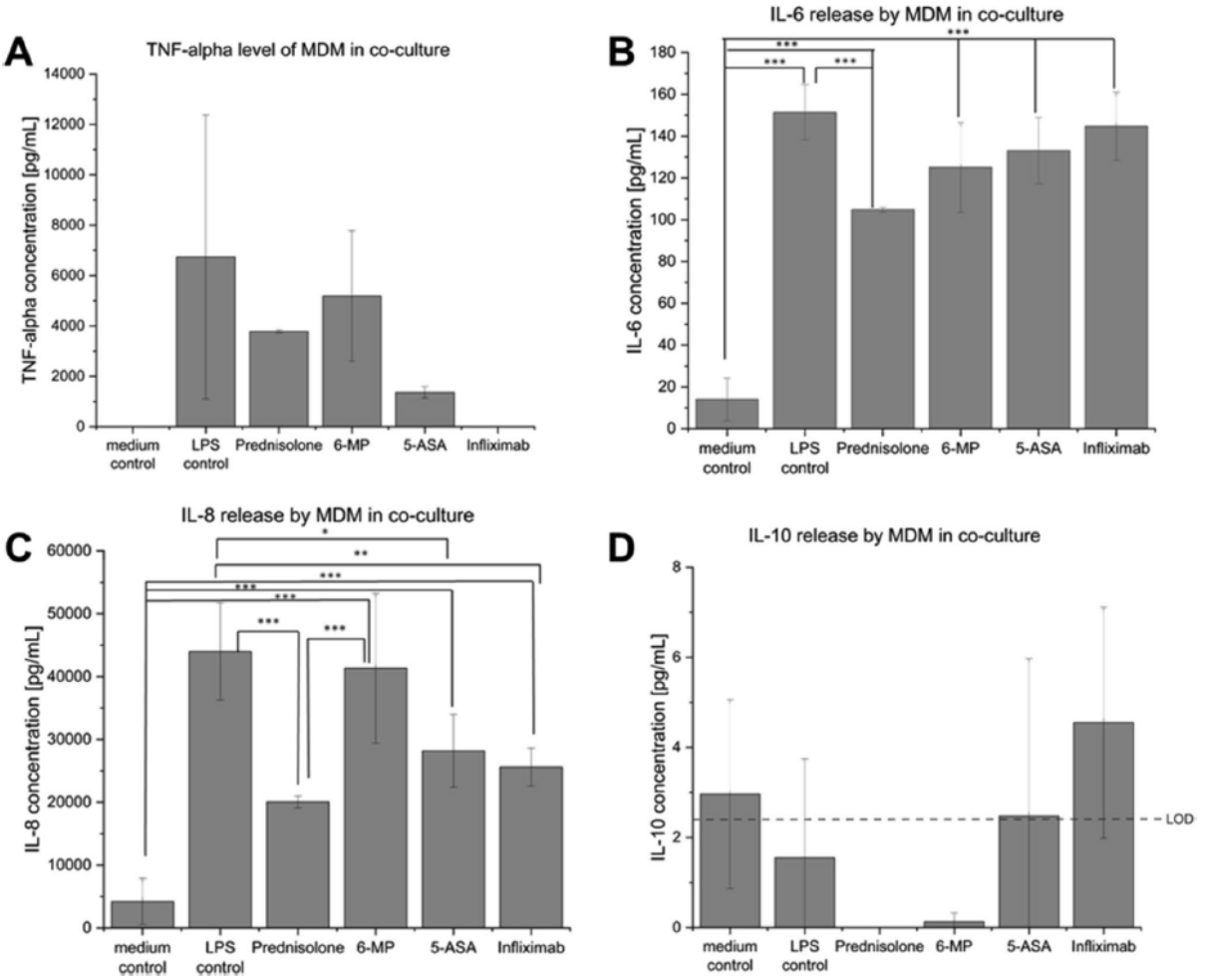


Fig. 4 Cytokine release by MDM in the co-culture system after 48 h after the treatment of 5-ASA, prednisolone, 6-MP, and infliximab. **A** TNF-α: as a TNF-α antibody infliximab inhibited the TNF-α release by the macrophages. Prednisolone and 6-MP only had a slight effect on the release compared to the LPS control, whereas 5-ASA had a stronger effect. **B** IL-6: the release of IL-6 could be significantly reduced in comparison to the LPS control by the treatment with prednisolone, whereas 5-ASA, 6-MP, and infliximab only showed a small effect. **C** IL-8: a significant decrease of the secreted IL-8 was achieved after the treatment with 5-ASA, prednisolone and infliximab. **D** IL-10: the overall IL-10 content was low in comparison to the pro-inflammatory cytokine release. The highest release of IL-10 was observed after the treatment with infliximab. *LOD* = limit of detection. Results are represented as mean ± SD for in summary $n = 6$ wells for each group performed in three biological replicates. $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$ indicate significant difference

infliximab (25,603 pg/mL) compared to the LPS control (44,024 pg/mL) (Fig. 4C). The investigation of the anti-inflammatory cytokine IL-10 showed almost no release with the highest measurable values of 2.96 pg/mL for the medium control and of 4.55 pg/mL for the treatment with infliximab (Fig. 4D).

Evaluation of readout parameter with focus on drug efficacy

To evaluate the proof of concept for the in vitro drug efficacy for the above-mentioned drugs, based on the known efficacy in IBD patients, the measured values in the co-culture model for each readout: TEER, TNF-α, IL-6, and IL-8 were calculated in percent relative to the medium control. No inflammation is indicated by 100% drug efficacy (medium control) and a complete inflamed state by 0% drug efficacy (LPS stimulation). Concerning the read-out P_{app} no influence of stimulation or drug treatment (see Supplementary information, Fig. S6) was visible, and for IL-10 the release was very low (maximum of 4.55 pg/mL, Fig. 4D), so they were not included in the drug efficacy evaluation. The results displayed in Fig. 5 are plotted against the severity levels 1 to 4 of the disease as represented by the selected drugs. TEER,

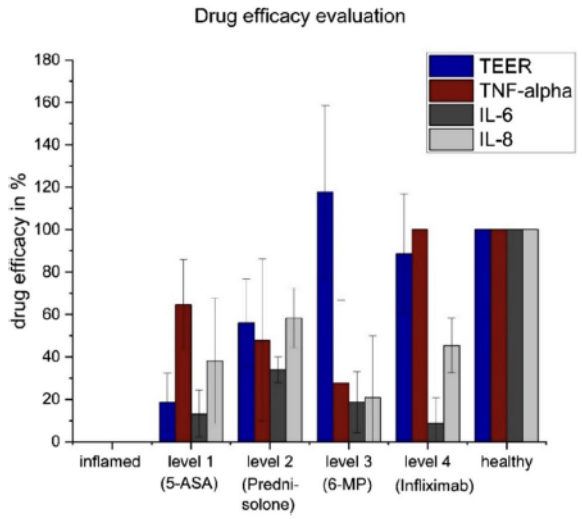


Fig. 5 Assessment of in vitro drug efficacy for the readouts TEER, TNF-α, IL-6, and IL-8. The drug efficacy was calculated in percent relative to the medium control (healthy, 100% drug efficacy) and the LPS control (inflamed, 0% drug efficacy) against the severity level represented by the respective drugs. The readouts TEER, TNF-α, IL-6, and IL-8 were used for the evaluation of drug efficacy with the co-culture model. Results are represented as mean ± SD for in summary $n = 10$ wells for each group performed in five biological replicates for the TEER measurements and for in summary $n = 6$ wells for each group performed in three biological replicates for the cytokines

TNF-α, IL-6, and IL-8 all produced positive responses and are thus qualified as potential readouts for an IVIVC which will be investigated in the next sections.

Correlation with severity level

Readout: cytokine release

The calculated minimal and maximal drug efficacy for the readouts TNF-α, IL-6, and IL-8 are summarized in Table 1. The drug efficacy depended on the type of drug and differed in the analysed cytokine releases. In terms of TNF-α, the minimal drug efficacy of at least 28% was observed for the immunosuppressant 6-MP, while the TNF-α-antibody infliximab showed an efficacy of 100%. A mean efficacy of 60 ± 44% for the tested drugs was calculated. The treatment resulted in a minimal drug efficacy for IL-6 of 9% for infliximab and a maximum of 34% for prednisolone. The calculated mean drug efficacy for IL-6 was 19 ± 50%. For the reduction of IL-8 release, prednisolone was the most effective drug with an efficacy of 68%, and 6-MP was the least effective drug with an efficacy of 21%. The mean drug efficacy for IL-8 was 41 ± 19%. Based on these observations, the cytokine release, especially for TNF-α, is a responsive readout for the drug treatment and offers the possibility to investigate "yes–no"-responses from the co-culture model to test the efficacy of drugs.

Readout: TEER

The plot against the increasing severity level represented by the respective drug for the readout TEER is shown in Fig. 6. The treatment response increases from 19% for 5-ASA (level 1) to 56% for prednisolone (level 2) and up to a 118% response for 6-MP (level 3). The alternative biological drug infliximab (level 4) showed a slight decrease in response to 89% in comparison to 6-MP (level 3). The correlation of TEER with the severity level of the disease resulted in a R^2 of 0.68. Based on this, a possible chance for IVIVC, additional to the yes–no response for drug efficacy, is conceivable, when TEER as a readout is investigated.

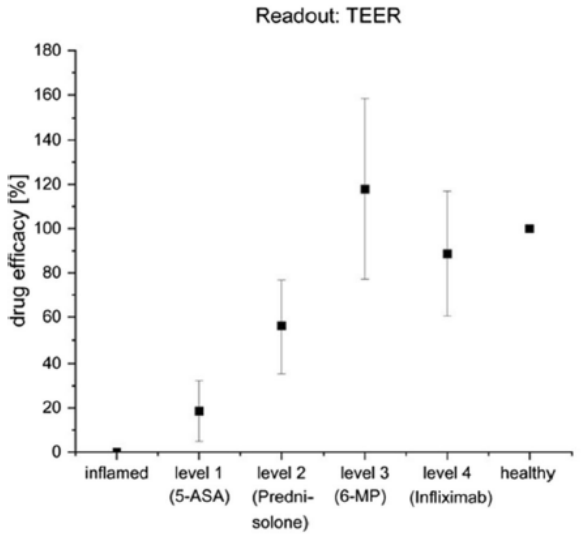
Discussion

In vitro assay development

The in vitro assay was developed following a stepwise approach. Firstly, the suitability of the human colorectal adenocarcinoma cell line Caco-2 was investigated in terms of its change in barrier properties after stimulation with the pro-inflammatory cytokine TNF-α in different concentrations. The stimulation with 10 ng/mL TNF-α showed the maximum TEER decrease, which is in accordance with previously reported data by Ma et al. [41]. The advantage of working with Caco-2 cells is their good availability and culture constancy, as well as their ability to form a stable tight junctions network measurable via TEER, the spontaneous

Table 1 Summary of the calculated minimal, maximal, and mean drug efficacy for TNF-α, IL-6, and IL-8. The analysis of the cytokine release allows a "yes–no" response from the co-culture for the drug efficacy, which depends on the applied drug and the analysed cytokine. Mean drug efficacy was calculated as mean ± SD for the different drugs out of in summary $n = 6$ wells for each group performed in three biological replicates

	TNF-α	IL-6	IL-8
Minimal drug efficacy	28% (6-MP)	9% (infliximab)	21% (6-MP)
Maximal drug efficacy	100% (infliximab)	34% (prednisolone)	58% (prednisolone)
Mean drug efficacy	60 ± 44%	19 ± 50%	41 ± 19%



	TEER
minimal drug efficacy	19% (5-ASA)
maximal drug efficacy	118% (6-MP)
mean drug efficacy	70 ± 53%
R^2	0.68

Fig. 6 Plot of calculated responses in % for the drug efficacy investigated by TEER measurements against the severity level represented by the respective drugs. The response increases from ASA (level 1) to prednisolone (level 2) up to 6-MP (level 3). Infliximab (level 4) had a slight decrease in response in comparison to 6-MP (level 3). The correlation of TEER with the severity level of the disease resulted in a R^2 of 0.68. Results are represented as mean ± SD for in summary $n = 10$ wells for each group performed in five biological replicates

differentiation into polarized enterocytes, and the good correlation to the human tissue especially in terms of permeability and drug absorption [42–44]. Additionally, as expected, the effect of stimulation with TNF-α, a key mediator in inflammatory bowel disease [45, 46], resulted in a disturbance of the tight junctions network. Therefore, the reduction of barrier properties similar to IBD has been regularly reported [8, 47, 48]. However, the Caco-2 monolayer alone is not able to mimic the complex cell interactions in vivo, especially the important role of immune cells in IBD [19].

Secondly, the macrophages play a central role in the innate immune system of the intestine by accepting the natural microbiome, being able to react quickly to environmental changes by adapting their phenotype, and fighting entering pathogens [49, 50]. Therefore, in this study, MDM were isolated from human blood and characterized in terms of cytokine release in an inflammatory state, induced by LPS stimulation. For the purpose of differentiation, GM-CSF was used which stimulates the differentiation of monocytes to macrophages designated as M1-cells [49]. Human GM-CSF macrophages provide the ability to release high levels of Th1-cytokines (e.g., IL-12 and IL-23) and other pro-inflammatory cytokines such as TNF-α and IL-6. Besides, they release very low to undetectable levels of IL-10 [49] [51], which could be verified in this study by measuring no IL-10 release of the MDM, indicating the M1-type. While in the non-stimulated state of MDM, no levels of TNF-α and IL-6 and only a very low release of IL-8 was measured in the co-culture, the LPS stimulation led to an increased release of the previously mentioned cytokines, which is typical for GM-CSF macrophages [51] [52]. Despite the donor dependency observed in the experiments, the human origin of MDM and the high release of pro-inflammatory cytokines offer a chance for an increased IVIVC.

Similar to the in vivo situation, in which the MDM are located directly under the epithelium [50], the cells were seeded in the basolateral compartment of a transwell system and combined with the Caco-2 cells grown in the apical compartment. The combination of intestinal epithelial cells and macrophages was addressed in various in vitro models that allow studying the cell interaction of epithelial and immune cells in healthy and inflamed conditions [30–32, 53]. However, challenges in combining differentiated macrophages with epithelial cells in a co-culture system, affecting the barrier integrity without any further stimulation, were also reported [37–39]. In this study, the Caco-2 cells accepted the medium of MDM measurable as stable TEER values (Section "Set-up and LPS stimulation of MDM/Caco-2 co-culture", Fig. 2A). Even in the presence of the MDM in the basolateral compartment, the barrier properties of the Caco-2 cells remained stable over the tested time period of 48 h (Section "Set-up and LPS stimulation of MDM/Caco-2 co-culture", Fig. 2B). The stimulation with supernatant of LPS-stimulated MDM led to significant decreases in TEER values after 24 h indicating the presence of inflammatory processes (Fig. 2A). In our co-culture set-up, the inducing of inflammation with LPS resulted in a significant TEER drop of 52% after 48 h (Fig. 2B). The four drugs, mesalazine, prednisolone, 6-mercaptopurin, and infliximab, which, in that order, correspond to the severity levels 1 to 4 as presented in the treatment pyramid (Fig. 1), were applied as proof of concept for evaluating the drug efficacy. The treatment with mesalazine recovered the epithelial barrier to 67% of the initial TEER value, prednisolone to 82%, 6-mercaptopurine fully recovered the barrier, and infliximab did so to 92% of the initial TEER value. The treatment with mesalazine, prednisolone and infliximab also significantly decreased the IL-8 release of the immune cells in comparison to the LPS control.

The efficacy testing of drugs using in vitro models mimicking IBD was previously reported. For instance, Leonard et al. developed a 3D-co-culture model out of human blood-derived macrophages, dendritic, and intestinal epithelial cells embedded in a collagen layer for drug screening addressing the treatment of IBD. The IL-1β induced

inflammation led to a drop in TEER of 10 to 20% after 48 h. The treatment with different budesonide (nano)formulations (except of liposomal formulation) recovered the intestinal barrier and free budesonide and a PLGA-budesonide formulation decreased the IL-8 release [54]. The effect of siRNA-based nano-medicine was tested with the "leaky gut" model that consists of Caco-2 cells, THP-1 macrophages, and MUTZ-3 cells, developed by Hartwig et al. The negatively affected intestinal barrier in IBD was mimicked by performing the treatment experiments just before the Caco-2 cells reached confluency resulting in TEER values below 200 Ω*cm^2. The LPS stimulation of the leaky model showed comparable higher release of the pro-inflammatory cytokines IL-8 and TNF-α [35], which was also observed in our experiments. The comparable strong TEER decrease in the inflammatory state, that was successfully obtained with our model, allows for drug efficacy screening of different drugs with variable strongness and therefore for IVIVC evaluation, which is highly necessary when developing an in vitro assay according to the 3R principle.

Evaluation of in vitro assay readout

Data obtained from assay development have to be discussed in the context of the "Guidance Document For Describing Non-Guideline In Vitro Test Methods" in order to evaluate the possibility for an IVIVC [5]. Therefore, the in vitro model was described based on the idea of the AOP framework, where tested compounds and their (toxic) in vivo response are presented. The AOP framework is described by the OECD as an analytical construct that combines a series of successive events on different compartments of the organism that are causally linked, focused on the critical steps [55, 56]. The starting point of the AOP framework is the molecular initial event (MIE), such as the initial effect of the interaction of a stressor/active compound with the biological target. The process continues with a series of essential biological activities or pathways and ends with the final adverse outcome (AO) in the organism. Key events (KE) express the measurable essential changes or reactions on a cellular or physiological level [55, 56]. In this study, the efficacy of IBD-related drugs was evaluated; consequently, the active compound here is an active pharmaceutical ingredient (API). The in vivo effect of the APIs on macromolecular interactions, cellular, organ, organism, and population responses was therefore defined as "Efficacy Outcome Pathway (EOP)." In contrast to this, the stressor LPS, which is used to induce the inflammation, is described as a typical AOP. The readouts of the in vitro assay are clearly located on the cellular response level (KE)(Fig. 7).

The first MIE, described along essential steps of the pathway through the AOP framework, is the LPS-binding to toll-like receptor 4 (TLR-4) which activates a signal transduction via two principal signalling pathways (MyD88-or TRIF-mediated) that results in NF-κB activation which further leads to induction of TNF-α-mRNA [57–59]. As a consequence, TNF-α enhances the myosin light chain kinase (MLCK) expression, which invokes a hyperphosphorylation of the myosin light chain (MLC) followed by an enhanced myosin-actin contractility [60]. Besides, the activation of the NF-κB pathway changes the expression level of tight junctions proteins such as claudin and occludin proteins [61, 62]. As a result, the TJ protein network is disturbed and the permeability of the epithelial barrier is increased [60] [62].

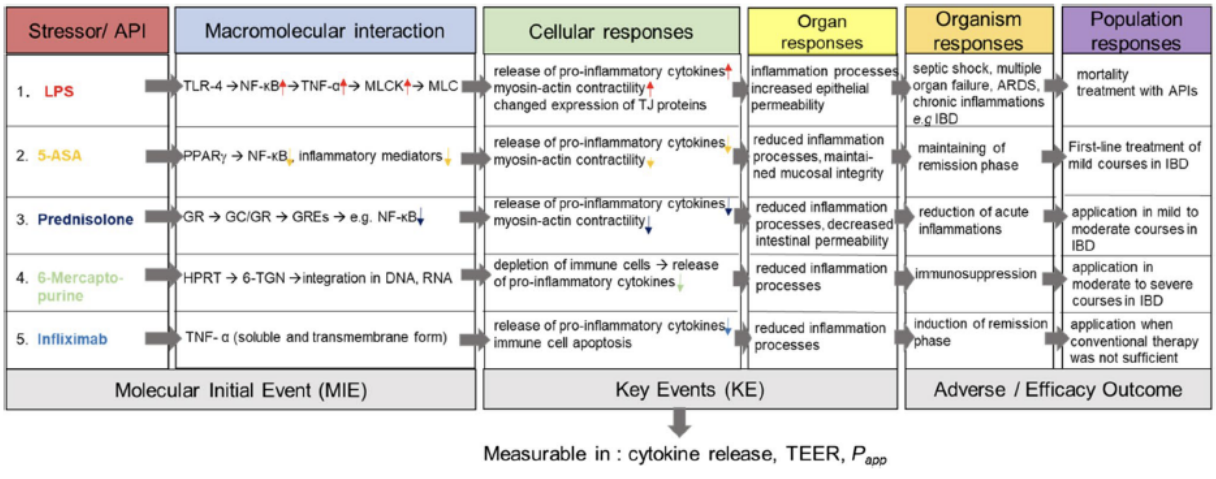


Fig. 7 Tested compounds and their in vivo response presented as adverse outcome pathway (AOP) for LPS and efficacy outcome pathway (EOP) for the APIs. Starting with the molecular initial event (MIE), the binding to the biological target, followed by a series of essential biological events, including the measurable key events (KE) cytokine release, TEER, P_{app} and with the adverse outcome (AO)/efficacy outcome (EO) that represents the endpoint

The organism response consists of septic shock, multiple organ failure, acute respiratory distress syndrome (ARDS), and inflammatory diseases like IBD [63–65].

One important KE is the release of not only TNF-α, but numerous pro-inflammatory cytokines such as IL-6 or IL-1 and different cytokines from human MDM as LPS response [58, 66]. In the in vitro assay, the release of the pro-inflammatory cytokines TNF-α, IL-6, and IL-8 was measured by performing ELISA measurements. A significant increase in the release of the respective cytokines could be observed after LPS stimulation of the co-culture systems, which indicates the sensitivity of the in vitro assay for this KE. Moreover, a significant decrease of TEER to 48% of the initial value was measured within 24 h of LPS stimulation, which shows the negative effect on the barrier integrity. The calculated P_{app} values from the transport studies with the marker molecule sodium fluorescein indicated no difference between the stimulated and non-stimulated groups. The Caco-2 permeability assay is established as a "gold standard" when it comes to the prediction of human intestinal absorption because of its similarities (e.g., transporter, efflux pumps) to the intestinal epithelium [67] [68]. However, it is also reported that the Caco-2 cells form less permeable tight junctions than in vivo, which results in a low permeability for molecules that are transported mainly over the paracellular route [69] [70], which could be described as the main transport route for the small hydrophilic drug sodium fluorescein [71]. Additionally, the GI epithelium is classified as "leaky," when TEER values of 50 to 100 $\Omega*cm^2$ are obtained [72], which indicates that the measured TEER values in this paper for the LPS control (200–350 $\Omega*cm^2$) were still too high to meet the KE of increased permeability, despite the significant decrease in comparison to the medium control (> 600 $\Omega*cm^2$). As a conclusion, transport studies with sodium fluorescein in the co-culture system showed that the P_{app} is not a predictive readout for the presented in vitro assay.

The second MIE is the first-line treatment drug for IBD recommended in "Guidelines for the management of inflammatory bowel disease in adults" mesalzine, a 5-aminosalicylic acid derivate (5-ASA), that is applied to cure mild inflammations (level 1) and to prolong the remission phase of the disease [13]. Mesalazine acts locally on the colonic mucosa and initiates various anti-inflammatory processes. It has been discovered that the main mode of action is the activation of the peroxisome proliferator activated receptor (PPAR) regulates the production of pro-inflammatory cytokines such as TNF-α, IL-6, IL-8, and IL-1, reduces the NF-κB activity, regulates the synthesis of prostaglandins and leukotrienes, and maintains the mucosal integrity [14, 73, 74]. The treatment of the LPS-induced inflammations of the in vitro assay resulted in a slight decrease of the TNF-α, IL-6, and IL8 release. The TEER values were increased to around 68% compared to the LPS control (48%) of the initial values. Therefore, the in vitro assay seems to be sensitive to the KE of mesalazine treatment concerning the readouts cytokine release and TEER.

The glucocorticoid (GC) prednisolone (severity level 2) binds to the glucocorticoid receptor (GR) (MIE 3) present in the cytoplasm. Based on the GC/GR interaction, which leads to conformational changes, the complex is translocated to the nucleus. The complex binds to DNA at specific glucocorticoid-responsive elements (GREs) and regulates the stimulation and suppression of numerous gene transcriptions such as the synthesis of pro-inflammatory cytokines and of the transcription factor NF-κB [15, 75, 76]. Moreover, prednisolone is known to decrease intestinal permeability [15, 77]. The treatment of the LPS-induced inflammation with prednisolone in the in vitro assay led to a decrease of TNF-α, IL-6, and IL-8 and increases the TEER values in comparison to the LPS control to around 82% of the initial values.

In moderate to severe disease progression in steroid-dependent IBD, the immunosuppressant 6-mercaptorpurine (6-MP) is administered to maintain the remission phase (level 3), as it is recommended in the European Crohn's and Colitis Organisation (ECCO) Guidelines [10, 11]. In the intestinal mucosa and the liver, the prodrug 6-MP gets activated by the enzyme hypoxanthine phosphoribosyltransferase (HPRT) and is metabolized to 6-thioguanine nucleotides (6-TGNs) that act as purine analogues in DNA and RNA (MIE 4) [78]. This leads to an interference in numerous biological processes including the activation and apoptosis of immune cells, which results in immunosuppression [79–82]. The in vitro activation of 6-MP and azathioprine and the building of different thiopurine metabolites has recently been investigated by Genova et al., using the virus-immortalized human healthy colon cell line HCEC [83]. The treatment with 6-MP of the LPS-induced in vitro assay did not lead to a decrease of cytokine release for TNF-α, IL-6, and IL-8, which might indicate that the release of additional inflammatory mediators needs to be further investigated or longer incubation times and higher concentrations could be tested. Nevertheless, a strong barrier stabilizing effect of 6-MP (to 134% of initial value) on the LPS-inflamed MDM/Caco-2 co-culture was observed with TEER measurements.

The chimeric monoclonal TNF-α antibody infliximab is recommended by the ECCO in moderate to severe cases of IBD to induce clinical remission, when conventional therapy is unsuccessful (level 4) [10, 11]. Infliximab binds to the soluble and transmembrane form of TNF-α (MIE5), which results in the loss of bioactivity for soluble TNF-α in blood serum. It also induces apoptosis of immune cells such as T-cells and monocytes, resulting in a decrease of pro-inflammatory cytokine and chemokine production [84–86]. The findings of this investigation showed that after treatment of the LPS-induced inflamed co-culture with infliximab

for 48 h, a significant decrease in the measured cytokine levels of TNF-α and IL-8, but not for IL-6 occured. The LPS-induced decrease in barrier integrity was treated successfully, which was indicated by TEER values of 92% after 48 h in comparison to the LPS control (48%). Accordingly, the effect of infliximab was detectable in the present in vitro assay with the readouts cytokine release (TNF-α, IL-8) and TEER, representing KE.

The calculation of drug efficacy for the readout parameter "cytokine release" showed that the present in vitro assay is able to simulate the effect for three out of four tested drugs on the release of the pro-inflammatory cytokines TNF-α, IL-6, and IL-8, whereas the effect of infliximab was limited to TNF-α and IL-8. As a result, there is a chance to identify active drug candidates with this readout (yes–no response), but the IVIVC might be poor as there are only macrophages present in the model. However, the predictivity of the in vitro assay might be improved by measuring further pro-inflammatory cytokines (e.g., IL-1 or IL-23). Regarding the readout TEER, the correlation of drug efficacy measured in the assay with the severity level of the disease (treatment pyramid) an R^2 of 0.68 was recorded. This indicates a possible IVIVC for the readout TEER, which needs to be further investigated and verified by evaluating the efficacy of more drug candidates. As in vitro assay, a higher throughput is possible compared to animal experiments: one 12-well plate can be used for testing four drug candidates plus medium and LPS control (two wells, respectively), giving the possibility to repeat to the desired *n* of experiments. Performing one experiment took approximately 2 weeks (including cell seeding, MDM isolation stimulation, set-up of co-culture, inflammation treatment, TEER and cytokine release measurements, transport and ELISA studies). The acceptance criteria are a stable epithelial barrier of Caco-2 in the co-culture set-up (measured by TEER) and a significant decrease in LPS-induced epithelial integrity which is required to remain stable over the test period to ensure that a potential effect of drugs is measurable. For the proof of concept of the presented in vitro assay, FBS was used as a medium supplement to cultivate the cells. In the future characterization and validation of the assay, the FBS should be replaced by defined supplements to further reduce the pain and suffering of animals.

The in vitro assay represents different KE which can be compared to the KE observable in animal experiments. To compare the predictability of the in vitro assay to in vivo experiments, a comparison with the readouts obtained from IBD-related animal models was performed (see Supplementary information, Tables S1 and S2). One important readout for the commonly applied animal models such as the chemically IBD induced dextran sulfate sodium (DSS) model or the 2,4-dinitrobenzene-sulfonic acid (DNBS)/trinitrobenzene-sulfonic acid (TNBS) model is the disease activity index (DAI). Employing this score system, the symptoms of the diseased animals, for instance, rectal bleeding, weight loss, diarrhea, or anaemia, are rated according to their severity and are compared to the treated group [22, 87]. Epithelial erosions and ulcerations, crypt abscess, infiltration of immune cells, and tumour formation (for TNBS/DBNS models) are rated with the histology injury score (HIS) [21, 22]. These organism responses cannot be simulated with in vitro models. In contrast, the activation of inflammatory pathways, as investigated in animal models, is able to be analysed with the in vitro model by measuring pro-inflammatory cytokine release. Additionally, the assessment of the barrier function in vivo gives the potential to be addressed by TEER measurements in vitro. Consequently, the in vitro assay represents two important KE: cytokine profile and barrier integrity, which might link the in vivo situation represented by animal models.

In recent years, in vitro co-culture models have regularly been applied to test the anti-inflammatory properties of compounds, for instance, budesonide nanoformulations, cinnamon extracts, or natural marine organism products with readouts such as TEER, cytokine release, m-RNA level, and NF-κB activity [54, 88, 89]. Dooley et al. further studied the correlation of gene expression between IBD tissue samples and Caco-2 cells, which were treated with IBD drugs to identify potential biomarkers [90]. However, it is very important to evaluate the IVIVC of the individual readouts to optimize the predictability of the in vitro models in order to finally have the opportunity of validation and standardization regarding "Good Cell and Tissue Culture Practice (GCCP)" and to ideally achieve regulatory application.

There are numerous requirements that need to be considered when standardizing in vitro cell models to achieve a high reproducibility and predictivity. First, crucial factors that affect the cell culture system need to be identified. Therefore, the origin of the cell line and its phenotype/genotype, the purity, and culture stability should be confirmed. Furthermore, the influence of reagents such as medium additives and antibiotics and the environment of the cells needs to be investigated [91]. In literature, it is reported that the composition of medium, passage number, culture time, and the culturing system itself are important factors for the cultivation of Caco-2 cells, which have an impact on the differentiation and permeability of the cell layer [92]. Moreover, for the Caco-2 cell line, different sub-populations are reported, which differ in morphology [93]. Natoli et al. additionally identified that the confluency has also an influence on the polarization and homogeneity of the cells [94]. MDM are known to have a heterogeneous phenotype. Microbial or environmental factors can lead to variations in their phenotype [95]. In addition, the stimuli utilized for differentiation such as bacterial agents, cytokines or chemokines [96], or colony stimulating factors [97] have an impact on the differentiation either in the M1 or M2 type, which emphasizes

the need for identity control. Based on these observations, the definition of culture condition and of the culture protocols for both the Caco-2 cells and MDM are essential for standardizing the in vitro co-culture. Besides that, the MDM show a donor dependency, which is particularly noticeable in the wide variations in TNF-α release [98] and were also present in our experiments. This characteristic requires the setting of acceptance criteria in the standardization process that need to be fulfilled by the MDM used for the co-culture experiments [5].

Secondly, the implementation of a quality management (QM), consisting of quality assurance (QA) and quality control (QC), is required to ensure consistent integrity, validity, and reproducibility [91]. The way of implementation and the exact standard operation procedures (SOPs) depend on the laboratory and the research project. However, the "Guidance Document on Good In Vitro Method Practices (GIVIMP)" already describes required key aspects such as quality control of materials used, the laboratory environment and equipment, defining acceptance criteria as well as ensuring the competence of the experimenter [99]. In the next step, the documentation system should be focused to track the materials and methods that are used for performing the in vitro assay. Moreover, risk assessment is necessary to develop measures to protect the environment and individuals from potential hazards. Furthermore, the compliance with regulations and laws has to be investigated and adequate education and training for the persons, that perform the cell experiments, must be provided [91]. In conclusion, the standardization process is a future challenge that should be addressed to ensure high reproducibility and predictivity of the in vitro assay.

Conclusion

The development of an in vitro assay for the screening of novel IBD-related drugs with a relevant human output formed the core focus. The readouts cytokine release, TEER, and P_{app} of sodium fluorescein were investigated in terms of their predictability; pharmaceuticals with known human efficacy in IBD were used as controls. The assay was evaluated in the context of the "Guidance Document For Describing Non-Guideline In Vitro Test Methods." Leading to the creation of an AOP/EOP framework to describe the KE relevance for the in vitro assay. The readout P_{app} calculated from transport studies with sodium fluorescein could not simulate the KE; in contrast, the assay successfully represented the KE of LPS stimulation regarding cytokine release and TEER. Likewise, the readout TEER showed a comparably high IVIVC of $R^2 = 0.68$ calculated by drug efficacy related to the severity level represented by the evaluated drugs. The present in vitro assay provides a good platform for additional investigations. Further drug candidates need to be tested to improve the reliability of the in vitro assay and also standardizing of the in vitro assay is fundamentally to open up the opportunity to reduce animal experiments in drug testing.

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Data availability The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Conflict of interest The authors declare no competing interests.

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4.2Evaluation of API combination

After the successful establishment of the *in vitro* co-culture model simulating IBD and the evaluation of its predictivity based on the efficacy of commonly applied APIs [52] (see section 4.1), the combination therapy was focused as second step. Therefore, three pairs of API combinations: 5-ASA + 6-MP, prednisolone + 6-MP and infliximab + 6-MP were selected that are applied in medical treatment to inhibit acute GI-inflammations and to prolong remission phases.

The co-culture set-up, inflammation and investigation of the readouts were performed as described in Schnur *et al.* [52]. Analogously to the treatment with the APIs of the IBD treatment pyramid, the inflamed co-culture was treated with the API combinations. Every API was applied in a concentration of 200 µg/mL (total: 400 µg/mL APIs). The integrity of the Caco-2 barrier was investigated by TEER measurements after 4 hours, 24 hours and 48 hours of treatment followed by the analysis of the released cytokines by the MDM via ELISA. Additionally, MTT assays, as described by Scherließ *et al.* [157], were performed to exclude cytotoxic effects of the API combinations for the Caco-2 cells and the MDM. Depending on the API combinations, concentrations up to 1 mg/mL and 3 mg/mL showed no cytotoxic effects (see supplementary information). Therefore, the selected concentration of 200 µg/mL for each API selected according to the concentration used for the single API treatment can be applied to the cells. In the following, the effect of the API treatment on the barrier integrity of the co-culture is presented.

4.2.1 Effect on barrier integrity

Figure 6 depicts the measured TEER values in %. After 4 hours of incubation, the inflamed co-culture showed a decrease in barrier integrity that increased over time. In contrast, the treatment of the LPS-inflamed co-culture with the three API combinations successfully prevented the decrease in TEER values and even led to an increase in TEER values after 48 hours to 111.81 ± 19.84% for 5-ASA + 6-MP; 129.16 ± 22.64% for prednisolone + 6-MP and 114.77 ± 13.93% for infliximab + 6-MP.

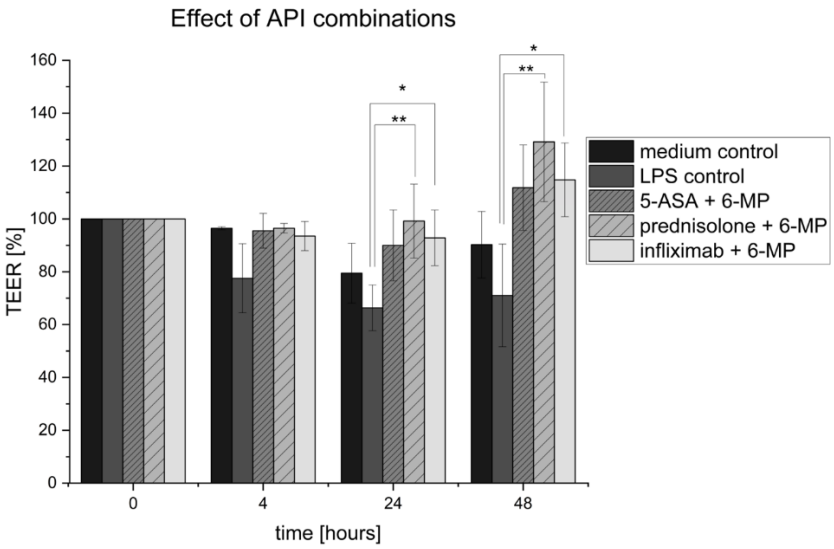

Figure 6. Effect on TEER of the LPS-inflamed co-culture with the API combinations 5-ASA + 6-MP, prednisolone + 6-MP and infliximab + 6-MP. All API combinations stabilized the epithelial barrier and led to an increase of TEER values over the treatment time. Results are represented as mean ± SD for in summary n=6 wells for each group performed in three biological replicates. *$p < 0.05$ and **$p< 0.01$ indicate significant difference.

As presented and discussed in Schnur *et al.* [52], the single APIs 5-ASA, prednisolone, 6-MP and infliximab showed a stabilization of the barrier integrity based on their mode of action and their efficacy correlated with the IBD treatment pyramid. According to the results, the combination of APIs resulted in an increased effect, where the highest TEER values were measured after the treatment with prednisolone + 6-MP (Table 1).

Table 1. Comparison of the efficacy of the single APIs and API combinations for the treatment of the LPS-inflamed co-culture determined by TEER measurements after 48 hours. Every API was applied in a concentration of 200 µg/mL. The API combinations showed an improvement of the TEER values compared to the single APIs.

API	**TEER after 48 hours**	**API combinations**	**TEER after 48 hours**
5-ASA	67.48 ± 6.79%	**5-ASA + 6-MP**	111.81 ± 19.84%
prednisolone	81.92 ± 10.31%	**prednisolone + 6-MP**	129.16 ± 22.64%
6-MP	134.33 ± 13.88%	-	-
infliximab	92.41 ± 8.17%	**infliximab + 6-MP**	114.77 ± 13.93%

4.2.2 Effect on cytokine release

Besides the effect of the API combinations on the barrier integrity of the epithelial cells in the co-culture system, the release of the pro-inflammatory cytokines TNF-α, IL-6 and IL-8 and the anti-inflammatory cytokine IL-10 by the MDM of the co-culture system was determined. Figure 7 A-D presents the results for the respective cytokine release by the LPS-inflamed MDM after treatment with the API combinations. The data for the pro-inflammatory cytokines was

normalized to the LPS control that represents the state of 100% inflammation, the data for IL-10 as anti-inflammatory cytokine was normalized to the medium control representing the healthy state (no inflammation). Regarding the TNF-α release, the stimulation with all of the API combinations showed a significant decrease of TNF-α in comparison to the LPS control. The API combination prednisolone + 6-MP led to a significant decrease of the IL-6 release, whereas no significant effect on the IL-8 release was observed for all API combinations. The treatment showed no effect on the release of the anti-inflammatory cytokine IL-10.

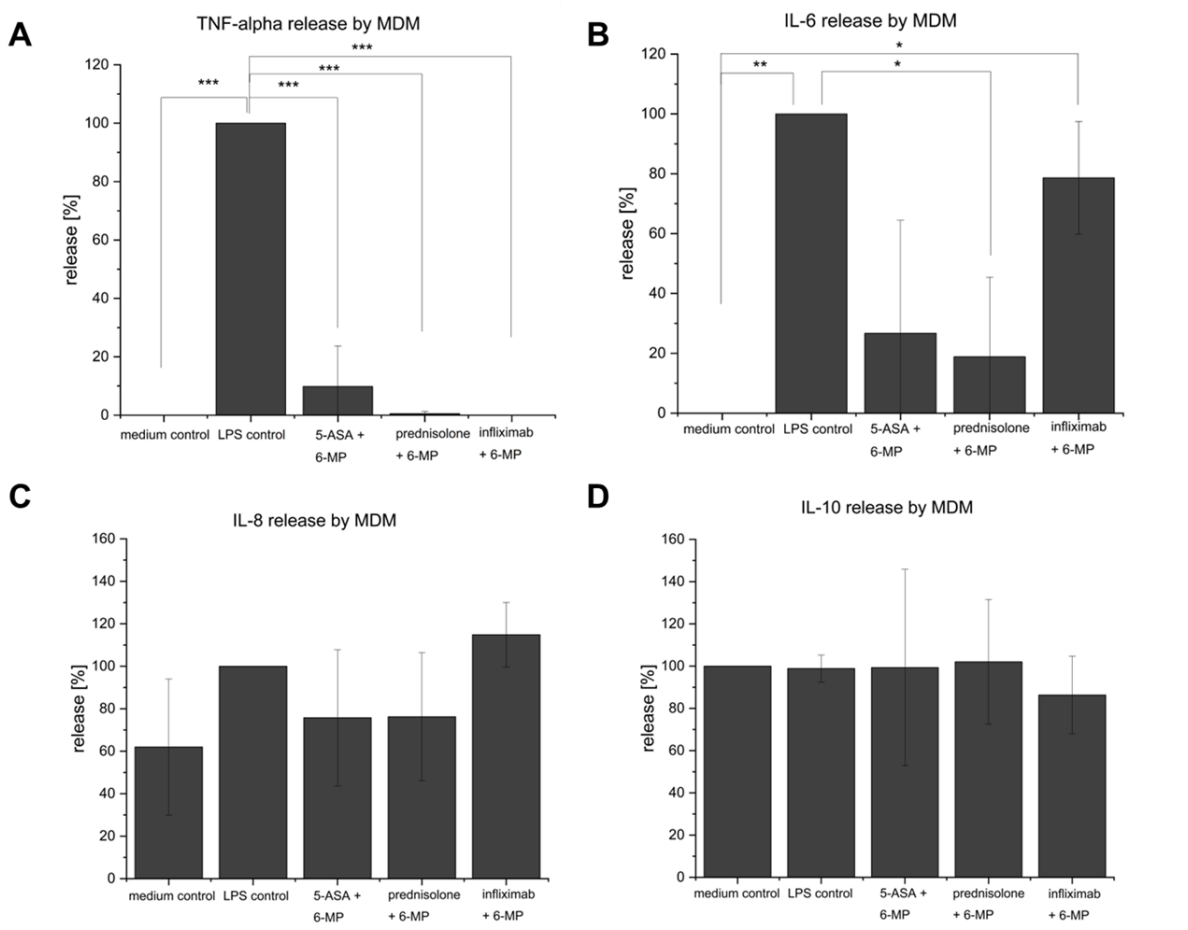

Figure 7. Cytokine release by MDM after 48 hours treatment of the LPS-inflamed co-culture with the API combinations 5-ASA + 6-MP, prednisolone + 6-MP and infliximab + 6-MP. **(A) TNF-α**: All API combinations decreased the release of TNF-α significantly compared to the LPS-control (100% inflammation). **(B) IL-6**: The API combination prednisolone + 6-MP reduced the IL-6 release significantly in comparison to the LPS control **(C) IL-8**: No significant decrease in the IL-8 release was observed after the treatment with the mentioned API combinations. **(D) IL-10:** The release of the anti-inflammatory cytokine IL-10 was not affected by the treatment with the API combinations compared to the medium control (100% healthy). Results are represented as mean ± SD for in summary n=6 wells for each group. Samples out of three biological replicates were utilized. *$p < 0.05$; **$p < 0.01$ and ***$p < 0.001$ indicate significant difference.

To evaluate the results obtained, table 2 compares the single API treatment with the API combinations. The application of the API combinations led to a clear decrease of the TNF-α release by the MDM. Concerning the IL-6 release, only prednisolone applied as single API and

the API combinations prednisolone + 6-MP showed a significant decrease in comparison to the LPS control. All applied single APIs except of 6-MP decreased the release of IL-8, whereas no significant effect was measured after the treatment with the respective API combination. The release of IL-10 was not affected neither by the applied single APIs nor by the API combinations.

Table 2. Comparison of the treatment effect of single APIs and API combinations on the release of the pro-inflammatory cytokines TNF-α, IL-6 and IL-8. All APIs were applied in a concentration of 200 µg/mL. ↓, ↓↓ and ↓↓↓ represent a significant difference of $p< 0.5$, $p< 0.01$ and $p< 0.001$.

API	TNF-α	IL-6	IL-8	API combination	TNF-α	IL-6	IL-8
5-ASA	-	-	↓	**5-ASA + 6-MP**	↓↓↓	-	-
prednisolone	-	↓↓	↓↓↓	**prednisolone + 6-MP**	↓↓↓	↓	-
6-MP	-	-	-	**-**	-	-	-
infliximab	↓	-	↓↓	**infliximab + 6-MP**	↓↓↓	-	-

4.3 Investigation of the natural product EGCG as potential complementary therapy: original publication

Title of original publication: The Potential of Epigallocatechin-3-gallate (EGCG) as Complementary Medicine for the Treatment of Inflammatory Bowel Disease

Authors: Sabrina Schnur, Fabian Hans, Annika Dehne, Janina Osti, Malte-Ole Schneemann, Marc Schneider, Marius Hittinger

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Author contribution (described using CRediT taxonomy, see: https://credit.niso.org/): Sabrina Schnur performed the conceptualization (together with Marius Hittinger), the design of methodology, formal data analysis, practical investigation, writing-original draft preparation, visualization and project administration. Annika Dehne, Janina Osti, Fabian Hans and Malte-Ole Schneemann assisted the methodology and the writing-review and editing. Marc Schneider and Marius Hittinger were responsible for the supervision, funding acquisition and supported the writing-review and editing.

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The Potential of Epigallocatechin-3-gallate (EGCG) as Complementary Medicine for the Treatment of Inflammatory Bowel Disease

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Abstract: Complementary and alternative medicine has the potential to enrich conventional therapy to improve the treatment of various diseases. Patients that suffer from inflammatory bowel disease, which requires a constant need for medication, have to deal with the adverse effects of repeated application. Natural products such as Epigallocatechin-3-gallate (EGCG) possess the potential to improve symptoms of inflammatory diseases. We investigated the efficacy of EGCG on an inflamed co-culture model simulating IBD and compared it to the efficacies of four commonly applied active pharmaceutical ingredients. EGCG (200 µg/mL) strongly stabilized the TEER value of the inflamed epithelial barrier to 165.7 ± 4.6% after 4 h. Moreover, the full barrier integrity was maintained even after 48 h. This corresponds to the immunosuppressant 6-Mercaptopurin and the biological drug Infliximab. The EGCG treatment significantly decreased the release of the pro-inflammatory cytokines IL-6 (to 0%) and IL-8 (to 14.2%), similar to the effect of the corticosteroid Prednisolone. Therefore, EGCG has a high potential to be deployed as complementary medicine in IBD. In future studies, the improvement of EGCG stability is a key factor in increasing the bioavailability in vivo and fully harnessing the health-improving effects of EGCG.

Keywords: intestinal barrier integrity; transepithelial electrical resistance; pro-inflammatory cytokines; monocyte-derived macrophages; natural product; green tea; antioxidant

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1. Introduction

The interest in traditional, complementary, and alternative medicine (T/CAM) has increased over the past decades [1]. Here, "traditional medicine" describes indigenous health traditions, and the term "complementary medicine" is defined as a combination of a non-mainstream approach with conventional medicine, such as nutritional supplements (e.g., natural products) or manual therapies. "Alternative medicine" refers to the usage of these approaches instead of conventional medicine [2,3]. The National Center for Complementary and Integrative Health (NCCIH) focuses on the integration of evidence-based complementary medicine in conventional therapy, as recently addressed in the NCCIH Strategic Plan FY 2021–2025 [4]. Furthermore, the World Health Organisation (WHO) states in their WHO Traditional Medicine Strategy 2014–2023 that the development of norms and standards based on reliable data for T/CAM to safely integrate qualified and effective therapies in the health systems of the WHO Member States [5]. Thus, it is evident that reliable and meaningful data for potential T/CAM therapies are required. One considerable motivation of the general public to utilize T/CAM in their daily life is the potential prevention and management of chronic, often lifestyle-related, diseases [1]. A prominent example is patients that suffer from Inflammatory Bowel Disease

(IBD) [6,7]. IBD is defined by chronic inflammations of the gastrointestinal (GI) tract that are subdivided into two main types of the disease: crohn's disease (CD) and ulcerative colitis (UC) [8]. Numerous patients complement their conventional treatment of the symptoms with T/CAM applications, mostly herbal therapies [6]. In this context, Epigallocatechin-3-gallate (EGCG) is a promising natural product for complementary therapy of chronic inflammations [9].

EGCG is the most abundant polyphenol in green tea extract (GTE) and a strong antioxidant, which is reported to provide numerous health benefits, such as anti-inflammatory, anti-bacterial, and anti-cancerogenic effects [10]. With respect to IBD, the efficacy of EGCG was shown by using various animal models mimicking UC or CD [11–15]. For instance, EGCG significantly improved disrupted colon architecture and leukocyte infiltration and reduced the level of the pro-inflammatory cytokine tumor necrosis factor-alpha (TNF-α) in a dinitrobenzene sulphonic acid (DNBS) rat model. Moreover, disease-related symptoms such as diarrhea and weight loss were improved [11]. The efficacy of EGCG for the treatment of IBD was further evaluated by comparison with the active pharmaceutical ingredient (API) sulfasalazine that was applied to treat an acetic acid-induced colitis rat model or a dextran sodium sulfate (DSS) mouse model. Analogous effects for EGCG and sulfasalazine were observed, such as a decrease in the disease activity index (DAI) and histological score, as well as the reduction of TNF-α, INF-γ, and the nuclear factor-κBp65 (NF- κBp65) serum levels [12,13]. Recently published studies underline the potential of EGCG for the treatment of IBD in animal studies [14,15]. However, the transfer of the EGCG data to the human condition remains an evident challenge [16]. One reason is the low bioavailability and stability of EGCG at physiological conditions due to its chemical structure [16–18] and physiological metabolic biotransformation [19–21]. The biotransformation, such as methylation or glucuronidation of EGCG, is reported to be highly dependent on the tissue and the species [19]. Further open questions that need to be addressed are the dose-finding as well as the time and frequency of administration [16]. The most prominent challenge is the translation of the EGCG in vitro data to the human in vivo situation to achieve a non-toxic, effective dose [22]. Although several clinical trials were performed and are still ongoing, EGCG has not been approved for medical usage yet and showed in some clinical trials a rather poor efficacy combined with a low plasma concentration [10,16,22]. Therefore, the investigation of EGCG efficacy using human-based, reliable, and predictive in vitro models that simulate different tissues and diseases for pre-clinical assessment are required to improve the limitations of EGCG.

In this study, we focused on the investigation of the potential of EGCG for the treatment of IBD, simulated by a recently established human-cell-based inflammable co-culture to mimic the application of EGCG to humans. The co-culture consists of epithelial cells and primary immune cells to address the role of both: the intestinal barrier and the immune cells in IBD, including donor dependency on the immune system. In our previous work, we evaluated the predictivity of our in vitro model by the treatment of the induced inflammation with commonly applied active pharmaceutical ingredients (APIs) related to the severity level of the disease [23]. We identified the transepithelial electrical resistance (TEER) as the most predictive readout, which facilitates the investigation of the epithelial barrier integrity in IBD. Additionally, the mode of action-dependent effect on the reduction of pro-inflammatory cytokines can be analyzed [23]. We aimed for the comparison of the EGCG efficacy with the efficacies of commonly applied APIs in IBD related to four different severity levels: from minor inflammations up to major inflammations. The investigation of EGCG on the same in vitro model in an equipotent concentration enables both: the assessment of the general efficacy and the evaluation of the efficacy level (minor up to strong) on two disease-related key compartments: the intestinal barrier and the immune system. The results might augment the database for T/CAM integrating EGCG as a promising complementary medicine. The presented in vitro model provides the possibility to investigate improved formulations of EGCG for the treatment of IBD with respect to stability enhancement or increased bioavailability to address limitations in vivo. In our

experimental setup, we tested EGCG at the two concentrations 2 µg/mL and 200 µg/mL. EGCG was applied to the immune cells located in the basolateral compartment after the co-culture was inflamed. The integrity of the epithelial barrier was determined over 48 h by TEER measurements. In addition, the effect of EGCG on the immune cells was investigated by analysis of the cytokine release. The cell viability of the epithelial and immune cells was determined to investigate possible cytotoxic effects during the treatment. In a next step, the effect of EGCG on the non-inflamed intestinal Caco-2 barrier was analyzed in more detail. Finally, we compared our results for EGCG with the efficacies of the APIs currently used in the treatment of IBD.

2. Results

2.1. EGCG Treatment of the Inflamed Co-Culture

The potential anti-inflammatory effect of EGCG of the two concentrations, 200 µg/mL and 2 µg/mL, was investigated. EGCG was applied to the lipopolysaccharides (LPS) stimulated monocyte-derived macrophages (MDM) in the basolateral compartment of the co-culture setup. Figure 1 shows the effect on the barrier properties of the Caco-2 cells located in the apical compartment determined by TEER measurements after 4 h, 24 h, and 48 h. The application of 2 µg/mL EGCG showed no measurable positive effect on the TEER values and was equal to the LPS control. However, the treatment with EGCG in the concentration of 200 µg/mL resulted in a significant increase in the TEER values up to 165.7 ± 4.6% after 4 h in comparison to the LPS-inflamed co-culture (91.6 ± 9.1%) and even to the co-culture without any stimulation (healthy state). After 24 h and 48 h, the increase in TEER in comparison to the LPS-inflamed group was still significant; however, it was decreasing over time and not as high as the TEER value measured after 4 h. Nevertheless, the EGCG treatment led to a regain of healthy barrier properties by restoring the TEER values to the level of the healthy co-culture.

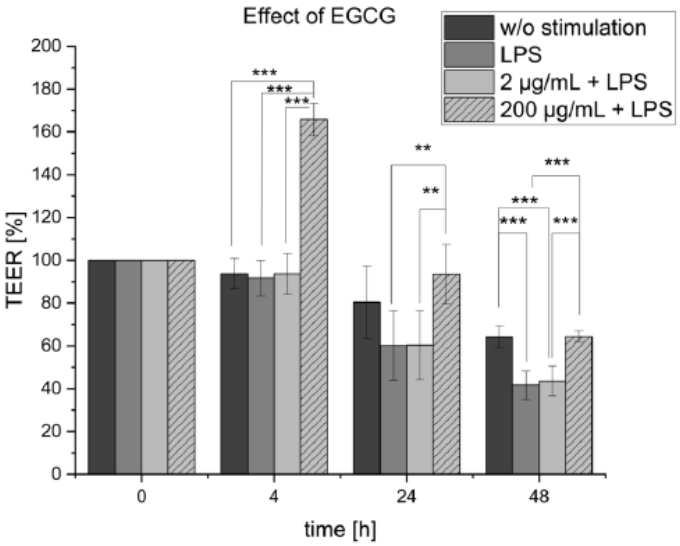

Figure 1. Effect of 2 µg/mL and 200 µg/mL Epigallocatechin-3-gallate (EGCG) over time on the lipopolysaccharides (LPS)-inflamed co-culture determined by transepithelial electrical resistance (TEER) measurements of the Caco-2 cells located in the apical compartment. The co-culture without any stimulation served as negative control (no inflammation = healthy state), and the LPS-inflamed co-culture was utilized as a positive control (inflammation = disease state). Results are displayed as mean ± standard deviation for in summary n = 9 wells for each group based on three biological replicates. The *p*-values ** $p < 0.01$, and *** $p < 0.001$ imply significant differences.

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2.2. Effect of EGCG on Cytokine Release of MDM

Following the stimulation and treatment of the co-culture, the cytokine release from the MDM located in the basolateral compartment was investigated (Figure 2). In the healthy state, no TNF-α was secreted; whereas the stimulation with LPS resulted in a TNF-α release of 2086.9 ± 118.42 pg/mL. The treatment of the inflamed co-culture with 2 µg/mL EGCG and 200 µg/mL EGCG led to no significant changes (1212.3 ± 899.95 pg/mL for 2 µg/mL and 1767.7 ± 1207.94 pg/mL for 200 µg/mL EGCG) of the TNF-α release compared to the inflammation control. A significant increase in the release after LPS stimulation (to 626.5 ± 380.36 pg/mL) was additionally observed for IL-6. Similar to the TNF-α release, no effect on the IL-6 release after the treatment with 2 µg/mL EGCG (588 ± 431.54 pg/mL) was measurable; however, there was a significant change to the release of IL-6, which was undetected after the treatment with 200 µg/mL EGCG. The stimulation with LPS led to a significant increase of the IL-8 release (to 14,831.9 ± 1702.29 pg/mL) in comparison to the medium control (1994.7 ± 488.43 pg/mL). While the treatment of the LPS-inflamed co-culture with 2 µg/mL EGCG (9778.6 ± 5566.65 pg/mL) had no effect on the IL-8 release, a significant effect for 200 µg/mL EGCG (decrease to 2100.6 ± 384.19 pg/mL) was observed. For the anti-inflammatory cytokine IL-10, only a lower level of around 60 pg/mL was measured for all groups.

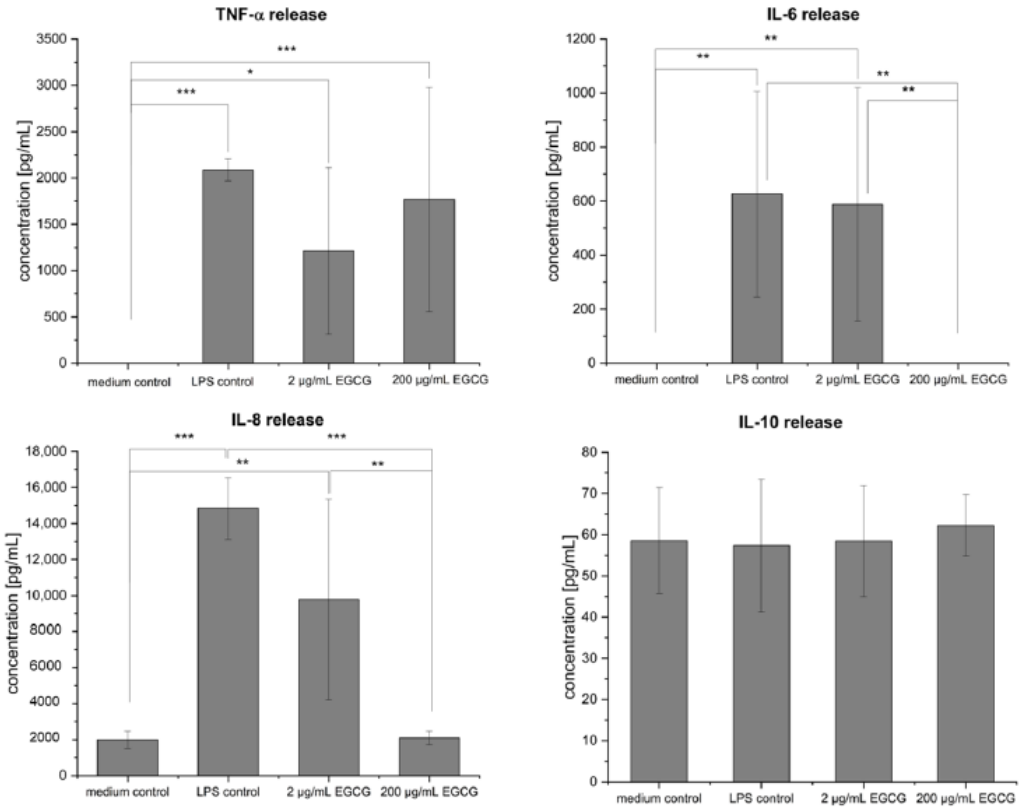

Figure 2. Effect of LPS stimulation, 2 µg/mL, and 200 µg/mL EGCG treatment on the cytokines released by the MDM in the co-culture setup in comparison to the healthy state (medium control). The stimulation with LPS led to an increase in the tumor necrosis factor-alpha (TNF-α), Interleukin (IL)-6, and IL-8 release. No significant effect of the treatment with 2 µg/mL EGCG was observed. After the treatment with 200 µg/mL EGCG, no IL-6 release was detectable, and a significant decrease in the IL-8 release was achieved. Results are displayed as mean ± standard deviation for in summary n = 9 wells for each group (except for LPS (TNF-α) n = 6 wells). Samples out of three biological replicates were utilized. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ imply a significant difference.

2.3. Cell Viability of the Co-Culture Setup after EGCG Treatment

The cell viability of the co-culture setup was addressed after performing the EGCG treatment of the inflamed co-culture. MTT assays were performed separately for the Caco-2 cells located in the apical compartment and the MDM located in the basolateral compartment of the co-culture setup. EGCG showed no cytotoxic effect on the Caco-2 cells, as the cell viability did not decrease compared to the medium control (Figure 3). For the MDM, EGCG in the concentration of 2 µg/mL resulted in no decrease in cell viability, while the concentration of 200 µg/mL led to a decrease in cell viability of the MDM to 57 ± 5.1%.

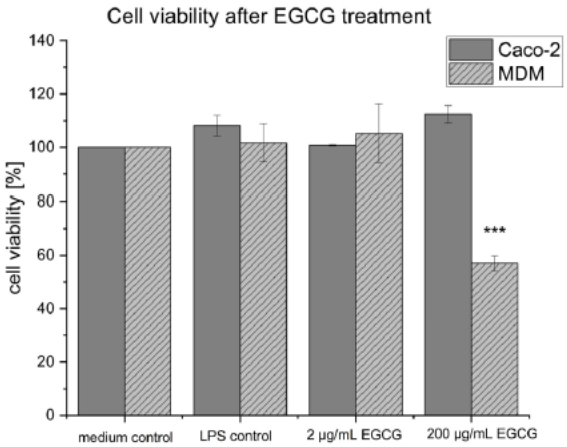

Figure 3. Cell viability of the Caco-2 cells and MDM after LPS stimulation and treatment with 2 µg/mL and 200 µg/mL EGCG compared to the medium control. Cell viability was determined by MTT assays at the end of the co-culture experiments. No cytotoxic effect was observed for the concentration of 2 µg/mL EGCG. A significant decrease in the cell viability compared to all other groups was measured for the MDM after the treatment with 200 µg/mL EGCG while the Caco-2 cells maintained full cell viability. Results are displayed as mean ± standard deviation for in summary $n = 6$ wells for each group based on two biological replicates. The p-value *** $p < 0.001$ imply significant differences.

2.4. Effect of EGCG on Caco-2 Monolayer

The EGCG treatment in the concentration of 200 µg/mL showed a significant increase in the Caco-2 barrier integrity of the co-culture setup, and additionally, an effect on the cytokine release of the MDM was observed. To further analyze the effect of EGCG on the in vitro setup, the effect of EGCG only on the Caco-2 monolayer and without an inflammation stimulus was investigated. Figure 4 depicts the TEER values that were measured for 48 h after the stimulation with 200 µg/mL EGCG either in the basolateral or apical compartment. After 4 h of stimulation, a strong increase in the TEER values to 133.3 ± 10.2% of the basolateral treated cells in comparison to the medium control (96.8 ± 3.6%) was determined. This effect was still prominent after 24 h, with a decreased value of 109.3 ± 5.8% which was further reduced after 48 h (88.1 ± 5.0%). In contrast, the cells apically stimulated with EGCG showed no significant increase in TEER (102.3 ± 3.4%) in comparison to the medium control. The TEER value decreased slightly to 90.7 ± 2.5% after 48 h.

2.5. Comparison of EGCG's Efficacy with IBD-Related Active Pharmaceutical Ingredients

In our previous work, we investigated the effect of commonly applied APIs related to the severity of the disease (from level 1 = minor inflammation to level 4 = major inflammation) using the same co-culture system and the same concentration of 200 µg/mL [23]. Table 1 compares the efficacy of EGCG with our results for the four APIs: 5-aminosalicylic acid (5-ASA, severity level 1), the corticosteroid Prednisolone (severity level 2), the im-

munosuppressant 6-Mercaptopurine (6-MP, severity level 3), and the monoclonal antibody Infliximab (severity level 4). EGCG showed barrier stabilizing effects after 24 h, similar to the immunosuppressant 6-Mercaptopurine (6-MP) and the biological drug Infliximab. Based on the effect of EGCG after 4 h of incubation, the result fits the maximal effect that was measured for 6-MP after 48 h. Referenced to the decrease in pro-inflammatory cytokine release of IL-6 and IL-8, EGCG corresponds to the effect of the corticosteroid Prednisolone.

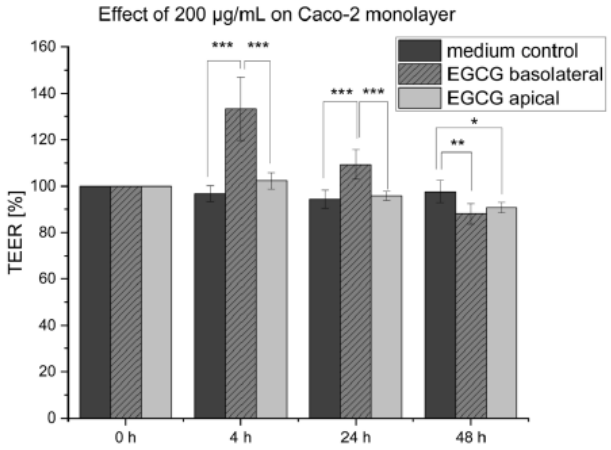

Figure 4. Effect of the stimulation of the Caco-2 monolayer with 200 µg/mL EGGC for 48 h either in the basolateral or apical compartment. The TEER value of the basolateral stimulated group increased significantly after 4 h of incubation. This effect was still measurable after 24 h; however, the TEER value decreased in comparison to the 4 h measurement. After 48 h, the TEER values of the basolateral stimulated Caco-2, and in addition, the apical-stimulated Caco-2 decreased in comparison to the medium control. Results are represented as mean ± SD for in summary $n = 9$ wells for each group performed in three biological replicates. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ indicate significant difference.

Table 1. Comparison of the efficacy of EGCG and IBD-related APIs that are utilized depending on the severity level of the disease (level 1 = minor inflammation up to level 4 = major inflammation). All compounds were applied in a concentration of 200 µg/mL to the co-culture system. ↓, ↓↓ and ↓↓↓ represent a significant difference of $p < 0.5$, $p < 0.01$, and $p < 0.001$. Abbreviations: 5-ASA = 5-aminosalicylic acid; 6-MP = 6-Mercaptopurine.

API	TEER in % after 24 h	TNF-α	IL-6	IL-8
5-ASA (level 1)	68.23 ± 7.2%	-	-	↓
Prednisolone (level 2)	81.29 ± 13.1%	-	↓↓	↓↓↓
6-MP (level 3)	106.73 ± 6.7% maximal effect after 48 h (134.33 ± 10.3%)	-	-	-
Infliximab (level 4)	92.2 ± 6.2%	↓	-	↓↓
EGCG	93.47 ± 14.8% maximal effect after 4 h (165.72 ± 4.6%)	-	↓↓	↓↓↓

3. Discussion

The effect of EGCG on the TEER values of the Caco-2 cells was investigated over 48 h. Two conditions were tested: the stimulation of the Caco-2 monolayer with 200 µg/mL EGCG in the apical or basolateral compartment and the investigation of EGCG as an anti-inflammatory compound for the treatment of the LPS-inflamed co-culture at the concentrations 2 µg/mL and 200 µg/mL. Both experiments showed a comparable response. The TEER values of the Caco-2 cells increased significantly for the basolateral stimulation

of the monolayer (to 133.3 ± 10.2% after 4 h) and considerably elevated for the co-culture treatment with 200 µg/mL EGCG (to 165.7 ± 4.6% after 4 h). Hereafter, the effect decreased over time; however, the treated co-culture still regained the full barrier integrity after 48 h compared to the LPS and the medium control. Compared to the efficacies of APIs related to the severity level of IBD that we investigated in our previous study [23], the barrier stabilizing effect corresponds to the immunosuppressant 6-Mercaptorpurine (maximal effect: 134.33 ± 10.3% after 48 h) and the monoclonal antibody Infliximab (92.2 ± 6.2% after 24 h), representing level 3 and level 4 of severity. In the literature, Ran et al. and Oz et al. both observed analogous effects of EGCG and sulfasalazine in IBD mouse models [12,13]. However, there is a lack of data for the application of EGCG with IBD-related APIs. The results of our study confirm that EGCG has the potential to be a complementary medicine for IBD patients and should be further investigated. The decreasing effect of EGCG can be correlated with the auto-oxidation of EGCG in the cell culture medium. In the literature, the investigation of EGCG stability in 200 mM phosphate buffered saline at 37 °C showed the degradation into EGCG auto-oxidation products (EAOPs). After a mere 4 h of incubation, EGCG was undetected by HPLC analysis, whereas the content of the EGCG component gallic acid (GA) increased [17]. Therefore, the enhancement of the stability of EGCG and the prevention of auto-oxidation might play a key role in the application of EGCG as complementary medicine in vivo.

Focusing on the barrier stabilizing effect in more detail, Watson et al. observed the prevention of epithelial barrier dysfunction induced by INF-γ stimulation of the epithelial cell line T84 either by pre-treatment with 100 µM EGCG or simultaneous treatment with 100 µM EGCG and stimulation with 20 ng/mL INF-γ [24]. This underlines the positive effect of EGCG on the barrier integrity that was observed in our study using 200 µg/mL (approximately 0.44 mM) EGCG. Additionally, a strong effect on TEER values of Caco-2 cells as monolayer was observed by Amasheh et al. after the application of 200 µmol/L of quercetin, a further polyphenol and nutrition factor. The barrier integrity already increased in their experiments after a few hours of incubation, comparable to our EGCG experiments, while the highest TEER value of 157 ± 4% was measured after 48 h. Following that, the effect decreased; however, it was still higher for the 72 h measurement than for the control group [25]. In a previous study, Amasheh et al. showed that the increase in TEER after the treatment with quercetin may be connected to an increase in the barrier-forming tight junction (TJ) protein claudin-4 expression and the assembling of claudin 4 in TJ domains and subdomains [26]. The alternation of the TJ network is also a conceivable effect of EGCG on barrier integrity. The restoration of the TJ-proteins zonula occludens-1 (ZO-1), occludin, and claudin-1 after an intestinal injury induced by cyclophosphamide after EGCG treatment was recently shown in mice [27]. Different IBD-related animal studies prove the positive effect of EGCG on DAI, histological damage, and intestinal permeability [11–13,28,29]. Furthermore, the potential of EGCG-rich Polyphenol E was investigated in a human pilot study, where UC patients received this medication as complementary medicine to their conventional drugs compared to a placebo group. A total of 66.7% of the patients in the EGCG-rich Polyphenol E group responded to treatment. Moreover, every patient that responded to the complementary treatment showed an improvement in their endoscopic score [30]. Despite that, the full mechanisms behind the barrier-stabilizing effect of EGCG have not been fully investigated yet.

EGCG has not only the potential to improve barrier integrity, furthermore it is reported to have immunomodulating properties [10]. In our in vitro assay, we aimed for the simulation of IBD, and therefore, we integrated MDM as a component of the immune system, which enables the investigation of pro-inflammatory cytokines. We observed no significant effect for the treatment with 2 µg/mL EGCG, although we detected a significant decrease in the release of IL-8 back to the level of the healthy co-culture after the treatment with 200 µg/mL EGCG. Additionally, IL-6 was undetected after the treatment with 200 µg/mL EGCG. The release of the anti-inflammatory cytokine IL-10 was not significantly affected, and for TNF-α, no significant change was measured. However, especially for the TNF-α

measurements, a high standard derivation was present based on the donor dependency of the MDM. Nevertheless, the human origin and the potential to release high amounts of different cytokines induced by LPS stimulation [31] are advantageous for simulating IBD. In comparison to the APIs for the treatment of IBD, EGCG showed a similar decrease in the release of IL-6 (to 0%) and IL-8 (to 14.2%) to the glucocorticoid (GC) Prednisolone (level 2 of severity). The mechanism of action of Prednisolone includes the binding to the glucocorticoid receptor (GR) followed by an interaction of the GC with the GR, leading to changes in the conformation and the translocation from the cytoplasm to the nucleus. Subsequently, the GC/GR complex binds to specific glucocorticoid-responsive elements (GREs), which stimulate and suppress gene transcriptions, including the synthesis of NF-κB and pro-inflammatory cytokines [32–34]. The activation of NF- κB and its translocation into the nucleus play a central role in IBD [35]. The mode of action of EGCG has not been fully investigated yet; however, effects on different pathways were identified in previous studies [36]. For instance, Yang et al. showed that EGCG successfully inhibited the IκB kinase complex activity and, therefore, the activation of NF- κB in vitro [37]. The blocking of NF- κB activation leads to a reduced release of numerous pro-inflammatory cytokines [35]. Shin et al. showed that the treatment of inflamed human mast cells-1 (HMC-1) with 100 µM EGCG resulted in a decrease in the TNF-α, IL-6, and IL-8 level by the attenuation of NF-κB and the extracellular signal-regulated kinase (ERK) [38]. Additionally, these effects on the release of pro-inflammatory cytokines have been proven by numerous animal studies [11–13]. In regard to human-derived data, LPS-stimulated $CD14^+$ macrophages alone mixed with the T cell subpopulation $CD4^+$ $CD45^+$ RO, isolated from the peripheral blood of IBD patients, showed a decrease in pro-inflammatory cytokine production as well as a significantly induced apoptosis of the mentioned cell populations after 24 h of treatment with 5 µg/mL EGCG [39]. Correlating with these results, we observed in the co-culture system a significant decrease in the MDM viability to 57 ± 5.1% after 48 h of treatment, while the Caco-2 cells were not affected. However, to investigate the cell viability in more detail and to increase the reliability, additional methods such as the adenylate kinase assay might be considered.

One key factor for high efficacy and bioavailability in vivo is the stability of EGCG. The auto-oxidation in vitro is reported as lower when higher initial concentrations of EGCG were applied [17,40,41]. Additionally, several factors that have an influence were identified, such as storage conditions, pH, temperature, or the level of serum albumin in the blood plasma [16,18]. In human blood plasma, the peak concentration for an oral dose of EGGC (2 mg/kg) taken after overnight fasting was reached after 1 to 2 h and was decreased constantly to undetectable amounts after 24 h. The calculated elimination half-life of EGCG was 3.4 ± 0.3 h [42]. In our in vitro experiments, we observed the most prominent effect of EGCG after 4 h of treatment. Further open questions are the dose-finding and the time and frequency of administration [16]. Based on our results, EGCG should be administered daily. However, the transfer of dosages from in vitro to in vivo remains a significant hurdle. It is reported that the oral application in vivo of an equivalent dosage of 10 µM to 100 µM in vitro in the form of 2-3 cups of green tea led to a plasma concentration of only 0.1 to 0.6 µM [43]. This underlines the need for increasing the bioavailability of EGCG in vivo. An improvement of the plasma level in humans was obtained by the intake of EGCG without caffeine yet in combination with the antioxidant ascorbic acid and omega-3 fatty acids derived from salmon after an overnight fasting period [16]. Additionally, formulation strategies to attenuate the auto-oxidation processes might be a conceivable way to improve bioavailability. For instance, the stabilization of EGCG by encapsulation in chitosan nanoparticles resulted in enhanced intestinal absorption in mice and, therefore, higher bioavailability [44]. Recently, Wang et al. improved the storage stability by forming an inclusion complex of EGCG and γ-cyclodextrin while combining the therapeutical potential of both compounds [15]. Parallel to the susceptibility for auto-oxidation, it is shown that tea catechins similar to EGCG undergo biotransformation such as methylation,

glucuronidation, sulfation, and ring-fission metabolism depending on the tissue and the species, leading to catechin metabolites with unknown biological activities [19].

The understanding of the metabolic mechanisms and the investigation of the bioavailability, efficacy, and safety of the metabolites are further key factors in evaluating the potential of natural products such as tea catechins. The here presented in vitro model provides the possibility to test the efficacy and cytotoxicity of improved EGCG formulation as well as identified natural metabolites related to IBD. A key factor for the usage of EGCG is the further assessment of the current bottlenecks, such as dose-finding, investigation of biotransformation, and bioavailability in vivo to benefit from the full potential of EGCG as complementary medicine. On this occasion, the application as complementary medicine is not only limited to IBD but can be beneficial for various diseases with uncontrolled immune activation, such as multiple sclerosis, psoriasis, and rheumatoid arthritis [9]. The amelioration of inflammatory processes and immune responses, as well as oxidative stress by natural dietary polyphenols, could assist in the prevention of carcinogenesis for colorectal cancer [45], which is known as the long-term complication of IBD [46]. EGCG itself is reported as an epigenetic regulator for cancer and a strong chemoprotective compound that interferes with different cancer signaling pathways [21,47]. Furthermore, the potential of EGCG for the treatment of respiratory diseases such as acute respiratory distress symptom (ARDS) or COVID-19 due to its antioxidant, anti-fibrotic properties and the ability to attenuate the production of various inflammatory mediators is discussed in the recent literature [48,49].

4. Materials and Methods

4.1. Cell Culture Experiments

4.1.1. Co-Culture Setup

The effect of EGCG as CAM for the treatment of intestinal inflammations was tested on an epithelium (Caco-2)/immune cell (monocyte-derived macrophages (MDM)) co-culture, as described in our previous work [23]. In brief, the Caco-2 cells were seeded in the apical compartment of Transwell® inserts (12-well plate, 12 mm Transwell® with polyester membrane inserts, 0.4 µm pore size, 1.12 cm^2 growth area, Corning, Glendale, AZ, USA) until an intact epithelial barrier with TEER values > 500 Ω* cm^2 was established. Primary monocytes were isolated from human blood, obtained from the Blutspendezentrale Saar-Pfalz GmbH (Saarbrücken, Germany), and differentiated by the addition of granulocyte-macrophage colony-stimulating factor (GM-CSF, Gibco, Thermo Fisher Scientific, Waltham, MA, USA) into MDM. After 24 h of differentiation, the MDM were washed, and a fresh cell medium with GM-CSF was added. Subsequently, the MDM were incubated for 72 h and then seeded in the basolateral compartment of a 12-well plate. The MDM were grown in the well plate for 24 h, washed, and the Transwell®-inserts with the Caco-2 cells were carefully placed above the macrophages. The barrier integrity of the co-culture was investigated by TEER measurements (see Section 4.2.1 TEER measurements). All cells, single monolayers, and the co-culture setup were maintained at a constant humidity with 5% CO_2 at 37 °C.

4.1.2. Inflammation of Co-Culture

To simulate the inflammatory state of IBD, the co-culture was inflamed by using Lipopolysaccharides (LPS) from *E. coli* (Sigma-Aldrich, Merck Millipore, Darmstadt, Germany) in a final concentration of 200 ng/mL for each well (12-well plate, 12 mm Transwell® with polyester membrane inserts, 0.4 µm pore size, 1.12 cm^2 growth area, Corning, Glendale, AZ, USA). The LPS was added to the basolateral compartment directly after starting the co-culture in all wells except for the medium control, which served as negative control (no inflammation; healthy state).

4.1.3. ECGC Treatment of Inflamed Co-Culture

EGCG (Viktoria Apotheke, Saarbrücken, Germany) was applied to the inflamed co-culture in two concentrations, 200 µg/mL and 2 µg/mL, on the inflamed co-culture to

investigate a potential anti-inflammatory effect. EGCG was added to the basolateral compartment containing the MDM directly after the LPS stimulation, except for the wells with the medium control and the LPS control, which was defined as a positive control (no treatment). The TEER values were measured after 4 h, 24 h, and 48 h of incubation to analyze the EGCG effect on the epithelial barrier properties. After 48 h of incubation, the supernatant of the basolateral compartment was collected, and the release of different cytokines was measured by enzyme-linked immunosorbent assay (ELISA).

4.1.4. Investigation of Cell Viability of the Co-Culture Setup after EGCG Treatment

The cell viability after the incubation with 200 µg/mL and 2 µg/mL of EGCG was analyzed directly at the end of the treatment experiments by performing individual MTT assays for the Caco-2 located in the apical and the MDM located in the basolateral compartment.

4.1.5. Investigation of ECGC Effect on Caco-2 Monolayer

The potential effect of EGCG on the integrity of the intestinal barrier was investigated utilizing only the Caco-2 monolayer cultivated in the apical compartment of a Transwell® system without an inflammatory stimulus. The 200 µg/mL and 2 µg/mL EGCG were applied to the Transwell® plate in the basolateral compartment (simulating the blood side) or in the apical compartment (simulating the lumen side). TEER values were measured after 4 h, 24 h, and 48 h.

4.2. Analytical Methods

4.2.1. TEER Measurements

The TEER values of the Caco-2 cells in the Transwell® inserts were measured on a heating plate (W10, VWR, Germany) at 37 °C with the Epithelial Voltohmmeter EVOM2 (World Precision Instruments, Friedberg, Germany) and the STX2 Chopstick Electrode Set (World Precision Instruments, Friedberg, Germany). Before the measurements, the Chopstick Electrode set was cleaned in 70% 2-propanol (Th. Geyer, Renningen, Germany) for 10 min and dried at room temperature (RT).

4.2.2. ELISA Measurements

For performing ELISA to measure the cytokine release by the MDM in the co-culture system, the Human Uncoated ELISA Kits for the tested cytokines (TNF-α, IL-6, IL-8, and IL-10) from Thermo Fisher Scientific were used and carried out according to the provided protocol. In brief, the coating of 96-well plates (Corning, Glendale, AZ, USA) with the specific capture antibody followed by incubation at 4 °C overnight was performed in the first step. After washing the wells three times with wash buffer (1x DPBS (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) with 0.05% Tween-20 (Merck Millipore, Darmstadt, Germany)), the wells were blocked with 1x ELISA Spot Diluent and incubated at RT for one hour. The samples were prepared by centrifugation for five minutes at 1000× *g*. The following dilutions of the samples in MDM medium were transferred to the 96-well plates: 1:10 and 1:100 for the TNF-α ELISA; not diluted and 1:10 for the IL-6 ELISA; 1:100 and 1:1000 for the IL-8 ELISA; not diluted and 1:2 for the IL-10 ELISA. 100 µL of each sample was applied to the wells. The provided standard for each cytokine was reconstituted and transferred to the plates according to the protocol. The plates were stored at 4 °C overnight. Subsequently, the wells were washed five times with wash buffer, 100 µL detection antibody was added in each well, and the plates were incubated for one hour at RT. After that, the wells were washed again five times. The 100 µL/well Avidin–horseradish peroxidase (HRP) was added, and the plates were incubated for 30 min at RT. All wells were washed six times with wash buffer, and 100 µL/well 3, 3′, 5, 5-tetramethylbenzidine (TMB)-substrate was applied, followed by incubation for 15 min at RT protected from light. In the end, 100 µL/well 1 M ortho-phosphoric acid (stop solution) was added to stop the enzymatic reactions. The absorbance of the samples was measured at the wavelength 450 nm with the Plate Reader Synergy2 (BioTek Instruments GmbH, Bad Friedrichshall, Germany),

and background correction was performed by subtraction of the 570 nm reading. The concentration of each sample was calculated based on the respective calibration curve.

4.2.3. MTT Assay

To investigate the cell viability after the stimulation of the MDM and Caco-2 co-culture, MTT assays were performed at the end of the experiment. The cell viability was calculated individually for the MDM and the Caco-2 cells in the co-culture setup. The MTT assay was performed as described by Scherließ et al. with some adjustments due to the Transwell® system [50]. In brief: at the end of the co-culture experiment (see section Treatment with EGCG), the cells were washed twice with 500 µL (Caco-2) and 1000 µL (MDM) 1x HBSS (Gibco, Thermo Fisher Scientific, Waltham, MA, USA). The medium control wells were used as a positive control (100% viability), and 1% Triton-X-100 (AppliChem GmbH, Darmstadt, Germany) was used as negative control (0% viability). Subsequently, 0.5 mg/mL MTT reagent ((3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, Agros Organics, Thermo Fisher Scientific, Waltham, MA, USA) was added in a volume of 500 µL (apical) and 750 µL (basolateral). The cells were incubated for 4 h at 37 °C under permanent shaking at 35 rpm and exclusion of light. After removal of the MTT reagent, 500 µL apical and 750 µL basolateral DMSO were added to each well and incubated for 15 min at 37 °C under permanent shaking at 35 rpm and exclusion of light. The absorbance was measured at the wavelength 550 nm with the Plate Reader Synergy2 (BioTek Instruments GmbH, Bad Friedrichshall, Germany). The cell viability was calculated using the following formula:

$$Viability\ [\%] = \frac{absorbance_{test\ formulation} - absorbance_{Triton\ X-100}}{absorbance_{medium\ control} - absorbance_{Triton\ X-100}} * 100 \quad (1)$$

Formula (1) indicates the calculation of cell viability in %.

4.3. Statistical Analysis

The data are presented as mean ± standard deviation (SD) based on the performance of independent replicates in different numbers of repetitions (n). For the statistical analysis, the program OriginPro 2021 was used. When two groups were compared, a two sampled-t-test was performed; for the comparison of > two groups, one-way analysis of variance (ANOVA) and the Bonferroni test as a post hoc test were applied.

5. Conclusions

Following the investigation of the efficacy of EGCG in our human cell-based co-culture consisting of epithelial and immune cells, it is possible to conclude that EGCG is a potential complementary medicine for IBD patients. The treatment with EGCG at a concentration of 200 µg/mL showed a positive effect on the barrier integrity of the Caco-2 cells and the release of IL-6 and IL-8 by the MDM. Compared to APIs that are commonly applied in IBD, EGCG showed comparable barrier-stabilizing effects to the immunosuppressant 6-MP and the monoclonal antibody Infliximab. Furthermore, the attenuation of the IL-6 and IL-8 release was similar to the corticosteroid Prednisolone. The strongest effect on the TEER values was observed after 4 h of incubation. Hereafter, the effect decreased over time; however, the barrier integrity in the co-culture system was restored and intact according to the TEER, even after 48 h of incubation. The decreasing effect can be associated with the auto-oxidation and low stability of EGCG, which is reported as the main challenge for the transfer of in vitro data to the in vivo situation. Moreover, the biological effect and toxicity of the metabolites need to be critically investigated. Due to the human origin of the cells and the usage of primary donor-dependent immune cells, we could analyze the effect of EGCG addressing the human species; however, the processes of biotransformation of EGCG in vitro and in vivo should be addressed in further studies. In clinical trials, the efficacy of EGCG is often limited, and only low plasma levels have been achieved. To improve the stability and bioavailability of EGCG in the future, formulation strategies such as stabilizing by encapsulation in nanoparticles, conjugation to other compounds, or the

application of additional substances such as ascorbic acid as antioxidants are conceivable. Once the bottlenecks have been mitigated, the opportunities for EGCG exceed usage as a complementary medicine for IBD, other autoimmune diseases as well as a cancer treatment due to the numerous positive properties.

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5 Discussion

The development of an *in vitro* assay simulating IBD with the aim to reduce animal experimentation in preclinical phases of drug development was at the forefront of this thesis. Therefore, various acceptance criteria for the *in vitro* assay were defined. First, the co-culture provides an intact epithelial barrier integrity and a low release of IBD-related pro-inflammatory cytokines by macrophages in the healthy state. Second, related to the pathogenesis of IBD (see section 1.3 Pathophysiology of IBD), the stimulation with an inflammatory stimulus leads to a significant disturbance of the epithelial barrier and an increase of pro-inflammatory cytokines released by the macrophages. Third, the treatment of the induced inflammation with APIs commonly applied in IBD treatment and related to the severity level of IBD (Figure 4 'Treatment pyramid for IBD') is possible and reflects the *in vivo* effect that is defined in an AOP framework. Fourth, the *in vitro* assay can be applied for the evaluation of novel treatment options such as API combinations and anti-inflammatory natural products.

Addressing the acceptance criteria of a stable epithelial barrier integrity and a low release of pro-inflammatory cytokines in the healthy state, the selection of suitable cell lines was crucial. To mimic the epithelial barrier, the colon cancer-derived cell line Caco-2 was utilized in the apical compartment of the co-culture system. The Caco-2 cell line provides numerous advantages such as a high degree of enterocytic differentiation even under standard cultivation conditions [113] and the expression of numerous known transporter genes including the efflux transporter Permeability-glycoprotein (P-glycoprotein) [158]. Jarc *et al*. correlated the permeability coefficients of 21 drugs using a Caco-2 monolayer to data of human fractions absorbed, ranging from low to high absorption, resulting in a R^2 of 0.84, indicating a high IVIVC for the Caco-2 cell line [158]. In contrast to HT-29 cells, another cell line that is commonly used as *in vitro* model, Caco-2 cells show a higher degree of differentiation in normal culture conditions into a brush border microvilli phenotype, a higher enzymes activity, which is for some enzymes comparable to the *in vivo* small intestine, and the formation of epithelial domes indicating transepithelial ion transport [113,159,160]. Furthermore, the barrier integrity in healthy and inflamed state as well as the release of pro-inflammatory cytokines can be used to evaluate the intestinal barrier in IBD on a molecular level [108–111]. Although the Caco-2 line provides good suitability for mimicking the intestinal barrier, one of the main disadvantages of the cell line is the missing mucus production [120,161]. Furthermore, laboratory-to-laboratory (lab-to-lab) variability has been reported which might occur due to the fact that only small changes in culture conditions affect the phenotype and differentiation of the

Caco-2 cells [162–164]. The combination of Caco-2 and the mucus producing HT-29 cells used by different groups [121,142–144] might improve the simulation of the *in vivo* condition, however the ratio of both cell types is reported as crucial for a homogenous mucus production [120] which might further enhance lab-to-lab variability. The integration of primary cells obtained from GI-biopsies of healthy and IBD patients would be the closest to the human condition, but the limited accessibility and the genotype-related variances represent the most prominent challenges here [165]. Taking this into account, the Caco-2 cell line was selected based on the advantages and the aim to develop a simple but reproducible and predictive *in vitro* assay.

Furthermore, the integration of macrophages was necessary to simulate the immune system in IBD. In healthy state, the macrophages should release a low concentration of pro-inflammatory cytokines and should not disturb the Caco-2 barrier integrity. For the *in vitro* assay presented in this work, MDM isolated from human buffy coat were selected and applied in the basolateral compartment of the co-culture system. Focusing on the *in vivo* situation, the differentiation between tissue-resident macrophages located directly in the tissue ensuring homeostasis and blood circulating monocytes should be considered. Blood circulating macrophages can migrate into the tissue and differentiate into macrophages either in healthy conditions to renew the tissue-resident macrophages or in inflammatory conditions [166]. MDM derive from differentiation of blood monocytes that are generated in the bone marrow (BM) after birth [166]. During inflammatory conditions, a high number of BM-derived monocytes migrate into the respective tissue and differentiate into macrophages with a pro-inflammatory phenotype (M1 type) to support inflammatory reactions. Subsequently, the apoptosis of the MDM is induced during the reaction or the MDM renew tissue-resident macrophages including a change to the anti-inflammatory phenotype (M2 type) or become memory macrophages to memorise the inflammatory stimulus [166,167]. The GI-tract contains the highest number of functionally specialized macrophages located in the lamina propria in direct contact to the IECs [167,168]. These macrophages are mostly monocyte-derived and not of embryonic origin as a high rate of replenishment by blood monocytes is necessary due to the environmental changes in the gut [166]. Based on this, the usage of MDM for the *in vitro* IBD model might be the closest to the *in vivo* situation.

In comparison to other *in vitro* co-culture models simulating IBD using THP-1 macrophages (dTHP-1) described in literature [138,139,142], the MDM showed a higher standard deviation of the analysed cytokine release due to the donor to donor-variability which was observed in

literature before [150,169] and might be a disadvantage of MDM. However, despite the fact that THP-1 macrophages show comparable surface makers, cytokine release pattern and cell morphology to MDM [131,170] , the concentration of the stimuli and the time for differentiation and resting are crucial for the performance of the macrophages. A change of these factors can lead to different degrees of differentiation, regulation of genes, or an increased sensitivity towards the stimulation with LPS leading to variability in the cytokine release and to a decreased reproducibility [138,171–173]. Additionally, the malignant origin might influence the comparability with *in vivo* macrophages [169]. The main drawback of dTHP-1 usage in IBD *in vitro* models, is the potential negative effect on the epithelial barrier integrity. The cell ratio between dTHP-1 and epithelial cells is an important factor as several groups reported a barrier breakdown of the epithelial cells after the co-cultivation of 24 to 48 hours in the healthy state without the addition of a stimulus for an unknown reason [135–137]. The adjustment of the ratio led to a stable co-culture in healthy conditions, however a significant and lasting inflammatory state could only be achieved by pre-priming the epithelial cells and dTHP-1 with pro-inflammatory stimuli [138]. Based on the aforementioned similarity to the human *in vivo* situation and the reported barrier breakdown of the Caco-2 caused by dTHP-1 in a co-culture system, MDM were selected as immune component for the *in vitro* assay. The *in vitro* co-culture of Caco-2 and MDM presented in this thesis successfully provides stable TEER values indicating epithelial barrier integrity and low measurable levels of released pro-inflammatory cytokines by the MDM.

After the promising simulation of the healthy GI-tract on a cellular level, the induction of an inflammatory state related to the IBD pathophysiology (see section 1.3 Pathophysiology of IBD), characterized by the disturbance of the Caco-2 barrier and the increased release of pro-inflammatory cytokines by the MDM, was focused as the second acceptance criterium. In the IBD pathogenesis, a breakdown of the epithelial barrier and therefore an increase in the intestinal permeability is observed [37]. Furthermore, the infiltration of MDM into the tissue is significantly enhanced and in contrast to the healthy GI-tract, the macrophages maintain their pro-inflammatory phenotype characterized by the high release of pro-inflammatory cytokines. Although the mechanisms involved have not been fully understood yet, the pro-inflammatory milieu and the interactions with various Th cells and IECs including the secretion of numerous cytokines or inflammatory stimuli seems to promote the M1 type of the MDM [167]. In this thesis, the induction of the inflammatory state of the *in vitro* co-culture by the application of 200 ng/mL LPS led to a significant increase in the release of the analysed pro-inflammatory cytokines (IL-6, IL-8 and TNF-α) by the MDM and therefore to a decrease in TEER of the

Caco-2 cells indicating a disturbed barrier integrity. The analysis of the released concentration of the cytokines TNF-α, IL-6 and IL-8 by the MDM in the basolateral compartment showed that the stimulation with LPS leads to an enhanced release and simulates the pro-inflammatory state of the MDM in IBD similar to the aforementioned *in vivo* process. Nevertheless, the physiological process of macrophage migration into the apical compartment might not be simulated with the established co-culture due to the location of the MDM in the basolateral compartment. For the specific investigation of MDM migration in inflammatory conditions, Roh *et al.* developed a 3D bioengineered co-culture model consisting of primary organoids derived from large intestine biopsies as inner layer and of MDM as outer layer using a spongy biomaterial scaffold [151]. Despite the simulation of the healthy state, the established *in vitro* co-culture successfully simulates the inflammatory response of the MDM characterized by the increased release of pro-inflammatory cytokines and the disturbance of the epithelial barrier measured by the significant decrease of the TEER values of the Caco-2 cells.

The third acceptance criterium focused on the evaluation of the predictivity of the established *in vitro* co-culture. Concerning the previous results, the healthy and the inflammatory state of IBD could be simulated. However, the potential for an IVIVC remained unanswered. Therefore, the different readout parameters of the *in vitro* co-culture were evaluated by the application of one candidate from the class of 5-ASA derivates (5-ASA), corticosteroids (prednisolone), immunosuppressants (6-MP) and TNF-α antagonists (infliximab) respectively, that are related to the clinical treatment option connected to the disease severity. In the following, an AOP framework was developed that describes the causal relation of events *in vivo* after the exposure to LPS and the respective API. The AOP framework helps to evaluate if the *in vitro* assay meets important KE of IBD such as the decreased epithelial barrier integrity and an increased release of pro-inflammatory cytokines. In conclusion, the co-culture model proved its ability to display the efficacy of anti-inflammatory drugs on barrier integrity and the release of pro-inflammatory cytokines. The evaluation of the predictivity resulted in the highest IVIVC ($R^2 = 0.68$) for the TEER measurements of the epithelial barrier indicating that the barrier integrity is the most sensitive parameter of the established *in vitro* co-culture [52]. To further increase the strength of the evaluation of the IVIVC, the application of additional APIs and the comparison with human clinical data might be beneficial. Beside the application of TNF-α antagonists as biologicals, the α4β7-integrin antagonist vedolizumab and the IL-12/23 p40 subunit antagonist ustekinumab showed promising efficacies in CD and UC patients who experienced inadequate response, intolerance or unacceptable side effects from conventional therapy or from TNF-α

antibodies [174–177]. Nowadays, both drugs are applied as biologicals in the treatment of IBD [53,54] and are consequently suitable test candidates for the future predictivity evaluation of the *in vitro* co-culture. However, recently published long-term studies showed that 32.8% of patients treated with biologicals suffer from loss of response or intolerance and are in need of a second-line treatment with another available biological [178]. For instance, one third of CD patients that are treated with TNF-α antagonists had an annual risk to lose their response of 20.9% per patient-year [179]. Consequently, novel small molecules were recently under investigation in clinical trials while for some molecules the process is still ongoing to further improve the treatment of IBD patients [180]. One prominent example are JAK inhibitors like tofacitinib or upadacitinib that show promising results for induction and maintenance of remission in either UC or UC and CD patients in clinical trials [181–183]. JAK inhibitors intervene with the JAK-signal transducer and activator of transcription (STAT) signalling pathway by inhibiting the phosphorylation of both the cytokine receptors and STAT proteins by binding specific cytokines like IL-23 to their receptors. After the phosphorylation, STAT proteins are transferred to the nucleus and regulate gene transcription such as expression of pro-inflammatory cytokines [184]. Furthermore, novel small molecules such as anti-leukocyte trafficking agents or sphingosine-1-phosphate receptor modulators are currently surveyed for the treatment of IBD [180]. Moreover, by virtue of the persistent administration of drugs to manage the symptoms and the thereby caused adverse effects, new formulation strategies such as the preparation of nanoformulations with the corticosteroid budesonide or with 5-ASA derivates are investigated [148,185,186] and could be applied to the *in vitro* co-culture.

Depending on the patient, pharmacokinetics (PK) and pharmacodynamics (PD) can differ influenced by factors such as age, gender, albumin blood level or location and severity of the disease which may lead in the worst case to a non-response of the patient to the administered APIs. Due to that reason, the drug concentration and its related *in vivo* efficacy are difficult to predict [187]. Especially for TNF-α antagonists, the risk of ADA development is reported which diminishes the efficacy by elevating the clearance and by blocking the binding site for the TNF-α antagonist [187]. As a consequence, therapeutic drug monitoring (TDM) in regular intervals like the trough concentration (TC), meaning the lowest drug concentration before the next drug administration, are beneficial to adapt the dosage of the drug individually for the respective patient. The TC represents the drug concentration after the liberation, absorption, distribution, metabolism, and excretion (LADME) process. The determination of TC simplifies the maintaining of the therapeutic window to not exceed into a toxic concentration or to achieve only a insufficient level with low or no efficacy [187]. However, studies showed that notably

for infliximab a maximal efficacy concentration is existing and that the concentration-effect curve reaches a plateau where higher dosages do not lead to a further increase in efficacy [188,189]. As the concentration to effect-relationship is dependent on the PK and PD of the human body, the transfer of *in vitro* data related to a defined drug concentration to the bioavailable drug level and the required drug dosage *in vivo* remains as a great challenge [190,191]. Taking this into account, the here presented *in vitro* model is limited regarding the prediction of required drug dosage *in vivo* to obtain the observed effect *in vitro* and the prediction of bioavailability as it is missing PK processes. Nevertheless, the potential efficacy and cytotoxicity of APIs on the barrier integrity and pro-inflammatory cytokine release can be investigated including TEER identified as the most predictive readout. In this work, the investigation of the intravenous (IV) administration simulated by the application to the basolateral compartment representing the blood side was focused. Nonetheless, the change of application towards the apical side will enable the investigation of oral administration and drug absorption and transport over the epithelial barrier.

The fourth acceptance criterium was the investigation of the treatment effect of API combinations and an anti-inflammatory natural product. Combination therapy with different APIs enables the usage of additive or synergistic effects of drugs to enhance the effectiveness of the treatment [60]. The positive effect of API combination therapy was especially reported for the combination of the TNF-α antibody infliximab with immunosuppressants as not only a decreased ADA development was observed, but also the infliximab PK was improved [63,64]. The application of infliximab and the immunosuppressant 6-MP to the *in vitro* co-culture resulted in an increased stabilization of the barrier integrity compared to the single administration of infliximab. Focusing on the cytokine release by the MDM, the reduction of the LPS-induced TNF-α release was more significant after the coadministration of infliximab and 6-MP. Numerous trials proved the efficacy of the coadministration of infliximab with immunosuppressants such as azathioprine, the prodrug of 6-MP, for the treatment of CD and UC leading to corticosteroid-free clinical remission and to a significant improvement in mucosal healing [192–194]. After the coadministration of infliximab and 6-MP to the *in vitro* co-culture no significant change of the IL-8 level was observed which might be explained by potential drug interactions affecting the IL-8 release. Maini *et al.* showed in their studies addressing the treatment of rheumatoid arthritis with infliximab combined with the immunosuppressant methotrexate (MTX) a synergistic effect regarding the duration of response [195]. However, this observation was correlated to a defined concentration of 1 mg/kg infliximab and a weekly low-dose of MTX of 7.5 mg/week [195] indicating a dose-dependent

effect for the synergy of both APIs. Therefore, the adjustment of the administered concentration of the API combination to the *in vitro* co-culture might be beneficial to study the potential synergistic effect. Additionally, the approach DTT consisting of the combined administration of biologicals and/or small molecules to reach different targets simultaneously was recently performed in first clinical trials [60] and could be of interest to the further investigation of the established *in vitro* co-culture.

Although the combination of 5-ASA and immunosuppressants was discussed in literature, no significant change in disease activity or patient compliance compared to the monotherapy was observed in clinical trials. However, the risk of colon rectal cancer might be reduced by the coadministration [65]. The application of 5-ASA and 6-MP to the *in vitro* co-culture showed an increase in the barrier stabilization effect in comparison to the single administration of 5-ASA. Regarding the cytokine release, the significance of the TNF-α release reduction was increased, however this might be related to the improved standard deviation between the donor samples in comparison to the monotherapy. He *et al.* identified vorinostat, a histone deacetylase inhibitor, as potential drug that synergistically interacts with 5-ASA for the treatment of UC by the usage of an *in silico* screening. This finding was affirmed by the administration of the API combination to epithelial cells and to a DSS-induced colitis mouse model resulting in a lower cytotoxicity and alleviation of symptoms [196]. The coadministration of 5-ASA and vorinostat to achieve a potential synergistic treatment effect might be tested using the *in vitro* co-culture to investigate the effect on the epithelial barrier integrity and the release of pro-inflammatory cytokines.

The third API combination that was applied to the *in vitro* co-culture was the combination of the corticosteroid prednisolone and 6-MP. In comparison to the monotherapy with prednisolone, the administration of the API combination led to an increase in the barrier stabilization effect and a reduction of the TNF-α release by the inflamed MDM. However, the reduction of the IL-8 release was less significant. A population-based study from Sicilia *et al*. showed that 55% of the analysed immunosuppressed patients needed at least one course of corticosteroids to treat disease flare-ups. Although, the API combination was successful for 35% of the patients, the remaining patients (more likely CD patients) needed further administrations of corticosteroids, a change to biologicals or surgery in the following disease course [66]. Therefore, it remains unclear if the direct adjustment of the treatment to biologicals might be more beneficial.

Considering the treatment, not only novel drug substances are studied but T/CAM such as natural products or new formulation strategies of existing medication might also be of interest. In this thesis, the potential of the natural product and green tea extract EGCG was analysed. EGCG successfully restored the barrier integrity of the epithelial cells and significantly decreased the release of the pro-inflammatory cytokines IL-6 and IL-8 [197]. Focusing on IBD, further natural products like cinnamon extracts, marine organism products, for instance obtained from sponges, or algae and herbal extracts like myrrh, coffee charcoal, chamomile flower, curcumin and piperine showed anti-inflammatory effects in various models [139–142,198,199]. To evaluate the potential of natural compounds as complementary medicine, the combination with commonly applied APIs might be investigated utilizing the *in vitro* model.

Focusing on the next steps that are necessary after the development of the *in vitro* co-culture model to proceed towards the overall aim to reach regulatory application to achieve reduction of animal experiments, standardization and validation are important to improve robustness and within-laboratory and between-laboratory transferability and reproducibility. Describing the *in vitro* model according to the OECD 'Guidance Document for Describing Non-Guideline *In Vitro* Test Methods' including the definition of an AOP framework to investigate if the treatment with different APIs meet KE of IBD, which was performed in this thesis, is a promising first step to evaluate the predictivity and data quality on a first sight [52,154]. However, further standardizing and adjustments are necessary to meet regulatory criteria. Guidance documents such as the 'Guidance Document on Good Cell Culture and Tissue Culture Practice 2.0 (GCCP 2.0)' and the 'Guidance Document on Good In Vitro Method Practices (GIVIMP)' are supportive regarding the *in vitro* reproducibility and the quality of the obtained data [200,201].

Firstly, the understanding of the cell culture system and the factors that can affect it is crucial for the standardization. This includes the identification and confirmation of the genotype/phenotype [200,201] which is especially important for the Caco-2 cell line as described before, but also for the MDM as they consist of heterogenous phenotype that can be influenced by various microbial and environmental factors [202]. Furthermore, the confirmation of purity, for instance the absence of microbial contamination and the culture stability [200,201], especially for the Caco-2 cell line which can be affected over time in terms of enterocytic differentiation marker, TEER and proliferation [203,204], is important. The influence of the medium itself, medium additives and antibiotics as well as environmental factors such as temperature, pH, atmosphere and osmolarity must be carefully evaluated

[200,201]. In regard to the MDM, the stimulus to induce the differentiation of the isolated monocytes into MDM has an crucial influence on the differentiation into the M1 or M2 type [205,206], therefore the differentiation protocol should be standardized. Moreover, the *in vitro* model is inflamed by the addition of LPS obtained from *E. coli*, the main TLR4 activator, as TLR4 expression is strongly upregulated in IBD patients [207]. Stephens *et al.* showed in their studies that LPS from different gram-negative bacteria species that are significantly enriched in the microbiome of IBD patients, differently altered the NF-κB activation, the release of pro-inflammatory cytokines, the epithelial barrier TJ integrity, transport and TEER [109]. Although mostly *E. coli* lab strains are used in cell culture [109], the application of species-specific LPS is another factor that might influence lab-to-lab variability. Additionally, the investigation of medium additives and standardization of the cell culture protocols should consider serum-free alternatives such as synthetic formulations or human serum to replace fetal bovine serum (FBS) in the Caco-2 medium composition [208]. After the definition of detailed cell culture and experimental protocols for the *in vitro* co-culture, the development of standard operation procedures (SOPs) is critical to ensure transferability and reproducibility even with different experimenters [200,201].

Secondly, the GCCP includes the establishment of a quality management (QM) divided into quality assurance (QA) and quality control (QC). The QM serves to control the quality of the laboratory rooms, the laboratory cleaning program, the maintenance of the equipment and staff competency and the quality, suitability, correct handling and storage of the materials as well as data recording and documentation, monitoring and archiving. Moreover, for the aim of regulatory use, the description and evaluation of the data quality is essential. Therefore, GCCP acceptance criteria (GAC), including the parameter that should be assessed, the method of quantification and the acceptable range, need to be set [200,201]. For guidance, Krebs *et al.* published a test method questionnaire originally for toxicological test methods helping to define GAC, for instance GAC for the source cells, for assessing the system at the start of the experiments and at the end of the exposure to compounds [209]. This is specifically important for the MDM due to their primary origin and strong donor dependency. In addition, the QM should consist of a risk management system to protect individuals and the environment from potential hazards [200,201].

The validation and standardization of the *in vitro* model might improve the IVIVC of the *in vitro* readouts. In comparison to other models (see section 1.5.2 *In vitro* models), the implementation of mucus secreting cells like HT-29 cells or ECM simulating scaffolds, the

formation of organoids or the cultivation in a microphysiological microfluidic system could improve the physiological morphology of the cells and the simulation of the intestinal microenvironment [120,121,124,127,128]. However, the costs and the difficulty of reproducibility as well as the complexity of the handling is extending equally [101,125]. Although there are established IBD related models including DC [145–147,210], there is still an ongoing debate about the unique function of DC and the distinction between the role of DC and macrophages in inflammatory conditions [211–213]. The generation of tissue-derived organoids obtained from IBD patients for the investigation of new medication or the pathophysiology of IBD was recently reported [103–106]. As consequence, the application of patient tissue might be advantageous for the predictivity, however the characterization of such patient-derived organoids and the maintaining of the inflamed phenotype in culture conditions are important factors that need further optimization [103,105,106]. Furthermore, the accessibility of patient tissue is limited. In conclusion, the question if more complex IBD models are also more representative for the *in vivo* situation remains unanswered and requires further research.

6 Conclusion and future prospectives

Nowadays, the need for novel treatment options for IBD, affecting a persistent high number of people worldwide, is continuously increasing caused by the primary or secondary loss of response to the available treatment or the occurrence of unacceptable adverse effects. Simultaneously, the interest in *in vitro* models simulating the intestinal inflammatory processes is increasing in the fields of drug discovery and drug development. The focus of the presented work was the establishment of a cell-based co-culture simulating the epithelial barrier and the immune response in healthy conditions and the IBD pathophysiology in the inflamed state.

Until today, IBD-related animal models are established for the investigation of novel APIs and treatment options. However, animal experimentation is not only ethically questionable, but the physiology difference between species remains as challenge for the transfer of the data to the human conditions. Although various *ex vivo* and *in vitro* models for the simulation of the pathophysiology of IBD, such as human tissue combined with lab-on-chip technique, 3D cultures like organoids or the application of ECM scaffolds or 2D co-cultures were developed, the predictivity of the respective models remains undetermined. In this work, the established co-culture showed a stable epithelial barrier in healthy conditions and could be easily inflamed by the application of LPS resulting in a disturbance of the epithelial barrier and an increased release of pro-inflammatory cytokines. The evaluation with commonly applied APIs related to the severity levels, resulted in the highest IVIVC for the readout TEER and an efficacy ‘yes-no response’ concerning the release of pro-inflammatory cytokines. In addition, the anti-inflammatory effect of API combinations and of the natural product EGCG could be shown by the co-culture. However, further evaluation is necessary to investigate the full IVIVC of the model.

Hereby, the investigation of the potential efficacy of further clinically administered APIs such as small molecules or additional natural products might be conceivable. Nevertheless, the standardization and validation of *in vitro* models in general is essential to improve the reproducibility, robustness and within-laboratory and between-laboratory transferability and to achieve regulatory approval. Guidelines such as the OECD ‘Guidance Document of Describing Non-Guideline In Vitro Test Methods’ provide first approaches to start the evaluation of predictivity and the process of standardization. However, GCCP is mandatory to be able to meet regulatory criteria. As described in the ‘Guidance Document on Good Cell Culture Practice 2.0 (GCCP 2.0)’ and the ‘Guidance Document on Good In Vitro Method Practices (GIVIMP)’ the

evaluation of every influence from the environment, the cell genotype/phenotype, culture stability, medium, medium additives and the purity is crucial for the standardization. Furthermore, the definition of SOPs is essential to enable within-laboratory and between-laboratory transferability. Overall, GCCP requires the establishment of a QM to control the quality of the whole laboratory including equipment, cleaning, staff competency and the suitability, correct handling and storage of materials as well as data recording, documentation, data monitoring and archiving.

Taken everything into account, these comprehensive steps towards GCCP might often not be manageable for research groups due to the high costs and the time-consuming implementation of the respective guidelines. Various *in vitro* models for the simulation of IBD were established. Nevertheless, the potential and the predictivity of those models are often poorly evaluated hindering the proceeding development towards standardization and validation. Still, a paradigm shift can be noticed for the 3R principle becoming progressively more focused during research and development of novel medicines. Despite the anchoring in the European Directive 2010/63, the reduction, replacement, and refinement of animal experimentation was recently addressed by the FDA Modernization Act 2.0 in December 2022. These changes in regulatory and the increasing development of 3R networks might hopefully enable the interdisciplinary cooperation and combination of research and industry.

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Supplementary information

Investigation of the cytotoxicity of the API combinations (see section 3.2 Evaluation of API combinations)

The API combinations 5-aminosalicylic acid (5-ASA) + 6-mercaptopurin (6-MP); prednisolone + 6-MP and infliximab + 6-MP were applied in different concentrations to the Caco-2 cells and MDM, respectively. The concentrations that were tested combined the two APIs in a ratio of 1:1. For example the concentration 1 mg/mL contained 0.5 mg of API 1 and 0.5 mg of API 2. Figure S1 shows the calculated cell viability for each API combination for the Caco-2 and MDM.

For the Caco-2 cells, the API combinations 5-ASA + 6-MP and infliximab + 6-MP showed no cytotoxic effects in the concentration of ≥ 1 mg/mL. The combination of prednisolone + 6-MP led to full cell viability of Caco-2 in all tested concentrations (up to 5 mg/mL).

In regard to the MDM, the limited access to blood samples of the Blutspendezentrale Saar-Pfalz gGmbH due to lack of blood donations caused by the COVID-19 pandemic led to the performance of only one experiment for each API combination as the co-culture experiments were focused. Therefore, the MTT results should be considered as preliminary data. Nevertheless, the tested API combinations showed a comparable effect on the cell viability of the MDM as mentioned for the Caco-2 cells. The combination of infliximab + 6-MP even showed an increased cell viability compared to the Caco-2 cells for the two highest tested concentrations (3 mg/mL and 2 mg/mL).

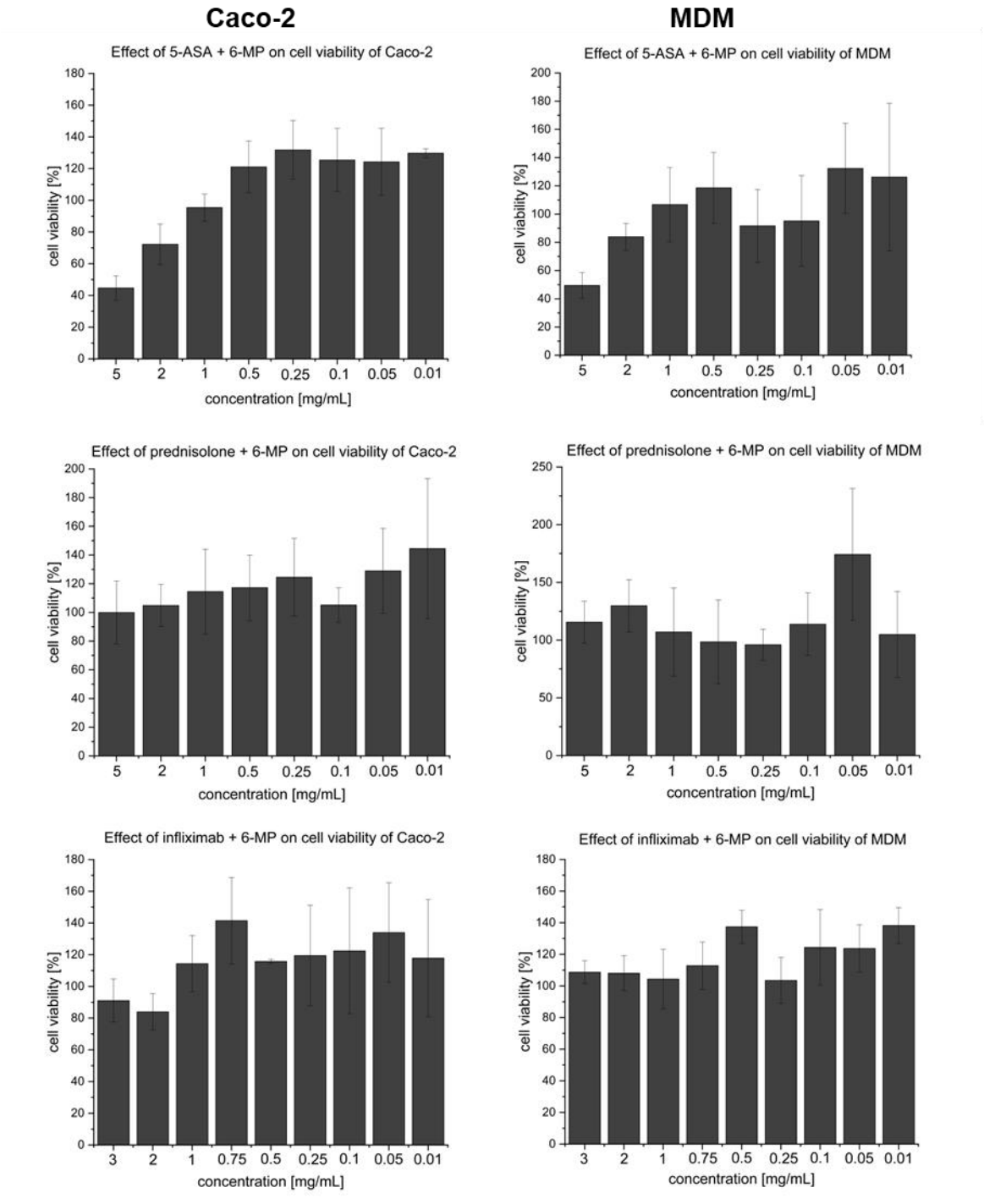

Figure S1. Cell viability of Caco-2 and MDM after the application of the three pairs of API combinations: 5-ASA + 6-MP; prednisolone + 6-MP and infliximab + 6-MP in different concentrations. No cytotoxic effects for concentrations ≥ 1 mg/mL were observed. Depending on the API combinations concentrations up to 5 mg/mL led to full cell viability of the cells. Results are represented as mean ± SD for in summary n=9 wells performed in three biological replicates for each concentration tested on Caco-2 and for in summary n=3 wells, performed in one preliminary experiment, for each concentration tested on MDM.

IV. Scientific Output

Publications

Schnur S, Hans F, Dehne A, Osti J, Schneemann MO, Schneider M, Hittinger M. **The Potential of Epigallocatechin-3-gallate (EGCG) as Complementary Medicine for the Treatment of Inflammatory Bowel Disease.** *Pharmaceuticals*. 2023 May 14;16(5):748. doi: 10.3390/ph16050748. PMID: 37242530

Schnur S, Wahl V, Metz JK, Gillmann J, Hans F, Rotermund K, Zäh RK, Brück DA, Schneider M, Hittinger M. **Inflammatory bowel disease addressed by Caco-2 and monocyte-derived macrophages: an opportunity for an in vitro drug screening assay.** *In Vitro Model.* 2022;1(4-5):365-383. doi: 10.1007/s44164-022-00035-8. Epub 2022 Nov 3. PMID: 37520160

Primavessy D, Metz JK, Schnur S, Schneider M, Lehr CM, Hittinger M. **Pulmonary in vitro instruments for the replacement of animal experiments.** *European Journal of Pharmaceutics and Biopharmaceutics*. 2021 Nov; 168:62-75. doi: 10.1016/j.ejpb.2021.08.005. Epub 2021 Aug 24. PMID: 34438019 Review.

Metz JK, Wiegand B, Schnur S, Knoth K, Schneider-Daum N, Groß H, Croston G, Reinheimer TM, Lehr CM, Hittinger M. **Modulating the Barrier Function of Human Alveolar Epithelial (hAELVi) Cell Monolayers as a Model of Inflammation.** *Alternatives to Laboratory Animals*. 2020;48(5-6):252-267. doi:10.1177/0261192920983015. Epub 2021 Jan 29. PMID: 33513307.

Metz JK, Scharnowske L, Hans F, Schnur S, Knoth K, Zimmer H, Limberger M, Groß H, Lehr CM, Hittinger M. **Safety assessment of excipients (SAFE) for orally inhaled drug products**. *ALTEX*. 2020;37(2):275-286. doi: 10.14573/altex.1910231. Epub 2020 Jan 29. PMID: 32052853.

Oral Presentations

In vitro efficacy testing for drugs targeting Inflammatory Bowel Disease;
Schnur S, Wahl V, Metz JK, Gillmann J, Hans F, Rotermund K, Zäh RK, Brück DA, Schneider M, Hittinger M. Globalisation of Pharmaceutics Education Network (GPEN) conference 2022, Minneapolis, Minnesota, USA

In vitro assay-based drug efficacy testing for the treatment of inflammatory bowel disease (IBD); Schnur S, Wahl V, Metz JK, Gillmann J, Hans F, Rotermund K, Zäh RK, Brück DA, Schneider M, Hittinger M. 23nd European Congress on Alternatives to Animal Testing (EUSAAT) 2022, Linz, Austria. Abstract published in ALTEX Volume 10, No. 2 (2022)

E-Bio Barriere - Entwicklung eines In Vitro-Darmmodells zur Simulation von chronisch entzündlichen Darmerkrankungen und Wirkstofftestung, participation at: ‚saarländischer Forschungspreis „Alternativen zu Tierversuchen 2022“, virtual event

Replacement & Reduction of animal experiments – Die Darmbarriere: Möglichkeiten und Herausforderungen, Invited presentation, lecture 'Replacement & Reduction of animal experiments' 2022, University of Applied Life Science Kaiserslautern, virtual event

Replacement & Reduction of animal experiments – Die biologische Barriere Darm
Invited presentation, lecture 'Replacement & Reduction of animal experiments' 2021, University of Applied Life Science Kaiserslautern, virtual event

When the co-culture does not do what it is supposed to do – a step "back" to ex vivo models, Schnur S, Zäh RK, Ruf C, Groß H, Lehr CM, Brück DA, Schneider M, Hittinger M. 22nd European Congress on Alternatives to Animal Testing (EUSAAT) 2019, Linz, Austria. Abstract published in ALTEX Volume 8, No. 1, (2019)

Poster Presentations

Correlation of readouts from an in vitro co-culture model mimicking inflammatory bowel disease to the clinical severity level, Schnur S, Vanessa W, Schneider M, Hittinger M. 13th International Conference on Biological Barriers 2021, virtual conference

Transepithelial Electrical Resistance as a fast read-out of in vitro and ex vivo models mimicking the GI-tract inflammation, Schnur S, Zäh RK, Hans F, Wahl V, Brück DA, Schneider M, Hittinger M. 11th World Congress on Alternatives and the Use of Animal in the Life Sciences (WC11) 2021, virtual congress

Entwicklung eines automatisierten Messverfahrens zur Erkennung (anti)-entzündlicher Reaktionen biologischer Barrieren, Schnur S, Zäh RK, Hans F, Brück DA, Schneider M, Hittinger M, E-Bio Barrier-project presentation European Regional Development Fund (ERDF) 2020, Saarland University, Saarbrücken, Germany

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