Defective epithelial barrier function in chronic inflammation of the intestinal mucosa

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To my wonderful family

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Abstract

The first part of this study investigated intestinal epithelial barrier in celiac disease (CeD) patients. Intestinal epithelial barrier is altered in CeD. However, the mechanism underlying disrupted barrier function in CeD is not clearly understood. Therefore, the aim of this study was to evaluate the effect of human monocytes (CD14+) isolated from peripheral blood mononuclear cells (PBMCs) from active and inactive CeD patients on the barrier function of intestinal epithelial cells. For this purpose, PBMCs were isolated from healthy controls, CeD patients on gluten-free diet and active CeD. Monocytes (CD14+) were sorted by MACS magnetic cell sorting. CacoBBe cells were co-cultured with PBMCs and CD14+ cells. Cells were treated with or without IL15/Tglia to verify the role of gliadin stimulation on barrier function. Moreover, CacoBBe cells were treated with IL15/Tglia alone to exclude possible toxic effects of gliadin on the epithelial barrier. Transepitelial electrical resistance (TER) was measured to evaluate the barrier integrity. Confocal microscopy after immunostaining was used to verify the localization of proteins with role in epithelial barrier function (Occludin and ZO-1). Monocytes were characterized by cytokines production and surface markers profile, through FACs analysis. Intestinal epithelial cells co-cultered with celiac monocytes presented a more pronounced decrease in TER in comparison with healthy controls. Also, Intestinal epithelial cells treated with IL15/Tglia alone, as observed in untreated cells, did not present decrease in TER. Decrease in occludin expression and an abnormal structure in ZO-1 were observed after co-culture of intestinal epithelial cells and celiac monocytes. Analysis of cytokine concentrations in monocyte supernatants revealed higher expression of proinflammatory cytokines, mainly interleukin-6 and MCP-1. However, surface marker expression did not reveal significant alterations in celiac monocytes. In conclusion, CeD peripheral monocytes reveal an intrinsically elevated proinflammatory cytokine pattern that is associated with the potential of peripheral monocytes to affect barrier function by altering TJ composition.

In the second part of the study, we investigated the impact of IL-22 in the barrier function integrity and cell polarity alterations in intestinal epithelia cells. Several cytokines have been related to directly affect the barrier function. One of these cytokines is IL-22, which might impact the integrity of the epithelial layer. IL-22 leads to the activation of various cellular signaling pathways including STAT-3, MAPK and PI3K/AKT. The effect of IL-22 on epithelial cells concerning cell polarity and barrier defect is not completely understood. Therefore, this study aimed to understand the mechanism underlying the development of dyspolar epithelia and barrier defect caused by IL-22. In order to answer this question, IECs were exposed to IL-22 at various concentrations. IECs implanted in Matrigel were grown to 3-dimensional cysts in the presence or absence of IL-22 and morphology and expression of polarity proteins were analyzed by confocal microscopy. To evaluate the barrier integrity and tight junction assembly,

measurements of transepithelial electrical resistance (TER) and calcium switch experiments were performed. TJ and cell polarity protein expression were assessed by western blotting and confocal microscopy. Cell motility was assessed through migration and invasion assays. Induction of epithelial-mesenchymal transition (EMT) was assessed by RT-qPCR analysis as well as western blotting. Activated signal transduction pathways were identified through Western blotting and inhibitors of STAT3 and MAPK/ERK were applied to uncover the signal transduction of barrier and polarity effects. We observed that IECs exhibited a barrier defect after IL-22 exposure in all tested concentrations. TJ protein distribution and expression were strongly impaired. Delayed recovery in the calcium-switch assay was observed suggesting a defect in TJ assembly. In our 3D-cyst model, multi-lumen and aberrant cysts as well as mislocalization of cell polarity proteins Par-3 and Dlg-1 was observed after IL-22 exposure. IL-22 induced cell motility with increased in cell migration and invasion as well as induction of EMT. Interestingly, inhibition of the MAPK pathway reverted IL-22 effects rescuing the TJal barrier defect, while blocking STAT3 led to apoptosis. In conclusion, we showed that IL-22 impairs intestinal epithelial cell barrier by inducing EMT, causing defects in epithelial cell polarity and increasing cell motility. Furthermore, we demonstrated that IL-22 modulates TJ protein expression and mediates tight junctional (TJal) barrier defects via ERK pathway.

Zusammenfassung

Der erste Teil dieser Studie untersuchte die Darmepithelbarriere bei Zöliakie (CeD) Patienten. Die Darmepithelbarriere ist bekanntermaßen bei Zöliakie defizient. Allerdings ist der zugrundliegende Mechanismus dieser gestörten Barrierefunktion noch nicht ausreichend erforscht. Ziel dieser Studie war es daher, zur Aufklärung dieses Barrieredefekts beizutragen. Genauer betrachtet wurde die Wirkung humaner CD14-positiver Monozyten, die aus dem peripheren Blut aktiver und inaktiver CeD-Patienten isoliert wurden, auf die epitheliale Barrierefunktion von Darmepithelzellen (IEC) untersucht. Zu diesem Zweck wurden periphere Blut-mononukleäre Zellen (PBMCs) gesunder Kontrollpersonen, CeD-Patienten mit einer glutenfreien Ernährung und CeD-Patienten mit aktiver Erkrankung, isoliert. CD14-positive Monozyten wurden mittels Magnetic-activated cell sorting (MASC) sortiert. Caco2BBe-Zellen wurden mit PBMCs oder mit CD14+ Zellen co-kultiviert. Die Zellen wurden zudem ±IL15/Tglia behandelt, um eine Gliadinabhängigkeit der epithelialen Barrierefunktion überprüfen zu können. Die Barrierefunktion wurde durch Vermessung des transepithelialen elektrischen Widerstands (TER) analysiert. Der Epithellayer wurde auf verschiedene Komponenten der Tight Junctions (TJs) immungefärbt und konfokalmikroskopisch hinsichtlich der Lokalisation von TJ-Proteinen (Occludin und ZO-1) untersucht. Darüber hinaus wurden Monozyten auf ihre Zytokinproduktion und die Expression von Oberflächenmarkern durchflusszytometrisch (FACS) vermessen. In den genannten Co-Kulturexperimenten ergab sich im Vergleich zur gesunden Kontrollgruppe ein erheblicher TER-Abfall der IEC-Layer bei Exposition mit CD14-positiven Monozyten, die von Zöliakie-Erkrankten isoliert wurden. Darmepithelzellen, die ausschließlich mit IL15/Tglia behandelt worden waren und unbehandelte Zellen zeigten keine Abnahme des TERs. Desweiteren wurden eine Abnahme der Occludin-Expression sowie eine abnormale ZO-1-Junktion nach Co-Kultivierung der Darmepithelzellen mit Zöliakie-Monozyten beobachtet. Die Bestimmung der Zytokinkonzentrationen in Monozyten-Überständen zeigte eine höhere Expression von pro-inflammatorischen Zytokinen, insbesondere Interleukin-6 und MCP-1. Die Expressionsanalyse der Oberflächenmarker ergab keine signifikanten Veränderungen bei Zöliakie-Monozyten im Vergleich zur Kontrollgruppe. Zusammengefasst ergab sich, dass periphere CeD-Monozyten eine pro-inflammatorisches Zytokin-Signatur aufweisen, die dazu beitragen kann, die epitheliale Barrierefunktion von IEC durch Veränderung der TJ-Proteinkomposition zu beeinflussen.

Im zweiten Teil der Studie untersuchten wir den Einfluss von Interleukin-22 (IL-22) auf die epitheliale Barrierefunktion und die epitheliale Polarität von IECs. IL-22 bindet an einen hauptsächlich auf IECs exprimierten heterodimeren Transmembranrezeptor. Die Bindung von IL-22 an den IL-22-Rezeptor führt zur Aktivierung intrazellulärer Signalkaskaden, insbesondere STAT-3, MAPK und PI3K/AKT. Um die IL-22-spezifische Rolle bei

Barrierefunktion und Zellpolarität zu klären, wurden IECs mit unterschiedlichen effektiven Konzentrationen und verschiedenen Expositionszeiten mit IL-22 exponiert. IECs wurden in Matrigel implantiert, wo sie zu 3-dimensionalen Zysten ±IL-22 differenzierten. Dann wurden die Zystenmorphologie/Lumenformation und Polaritätsprotein-Expression mittels konfokaler Mikroskopie untersucht. Transepithelial elektrischer Widerstand (TER) und Calciumswitch-Experimente wurden durchgeführt, um die Barrierefunktion bzw. die TJ-Assemblierung zu untersuchen. Zudem wurde die Expression der TJ- und Zellpolaritätsproteine mittels Western blotting und konfokaler Mikroskopie untersucht. Die Zellmotilität wurde mittel Migrations- und auch Invasionsassays untersucht. Hinweise für das Vorliegen einer Epithelial-zu-mesenchymalen Transition (EMT) wurden mittels RT-qPCR (RNA) und Western blotting (Protein) untersucht. Die Aktivität verschiedener Signaltransduktionswege wurde in An- und Abwesenheit verschiedener Inhibitoren der STAT3- und MAPK/ERK-Signalwege mittels Phosphoblotting bestimmt. Wir beobachteten, dass IL-22 bei IECs einen reproduzierbaren, Zelllinien-unabhängigen Barrieredefekt verursachte. TJ-Proteinexpression und -lokalisation waren deutlich verändert. Eine verspätete Erholung des TERs sprach im Calcium-switch-Versuch für das Vorhandensein eines IL-22-Effekts auf die TJ-Assemblierung. Bei unserem 3D-Zystenmodell zeigten sich Multilumen bzw. auch aberrante Zysten wie auch eine Fehllokalisation der Zellpolaritätsproteine Par-3 and Dlg-1 nach IL-22-Exposition. Die i.R. der o.g. Experimente nachweisbare, IL-22-induzierte, erhöht gemessene Zellmotilität und auch Zellinvasion brachten wir in Zusammenhang mit der Induktion EMT-typischer Transkriptionsfaktoren (Snail, Slug). Interessanterweise konnte man den Großteil der o.g. Effekte durch Inhibition der MAPK-Kaskade normalisieren. Dahingegen führte die Blockade des STAT3-Signalwegs zur IEC-Apoptose. Zusammengefasst konnten wir zeigen, dass IL-22 auf die intestinal-epitheliale Barrierefunktion einen vermindernden Effekt, was mutmaßlich auf die gleichzeitig stattfindende Induktion von EMT zurückgeht. Dies verursacht Defekte in der epithelialen Zellpolarität und erhöht die IEC-Motilität. Darüber hinaus haben wir gezeigt, dass IL-22 die TJ-Proteinexpression vermindert und TJ-assoziierte Barrieredefekte über den ERK-Signalweg vermittelt.

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Abbreviations	Meaning
ºC	Degree Celsius
ACeD	Active celiac disease
AJ	Adherens junction
APC	Antigen presenting cells
aPKC	Atypical protein kinase C
BSA	Bovine serum albumin
CD	Crohn's disease
CeD	Celiac disease
Da	Dalton
DAPI	4', 6'-diamidino-2-phenylindole
DC	Dendritic cells
DLG	Discs large
DMEM	Dulbecco's modified eagle's medium
DMSO	Dimethyl sulfoxide
dNTP	Deoxyribonucleotide triphosphate
DSS	Dextran sodium sulphate
DTT	Dithiothreitol
EATL	Enteropathy-associated T-cell lymphoma
ECM	Extracellular matrix
EMT	Epithelial-mesenchymal transition
ERK	Extracellular-signal-regulated kinase
FD4	4-kDa FITC-dextran
FITC	Fluorescein isothiocyanate
GALT	Gut-associated lymphoid tissu
GFD	Gluten-free diet
GM-CSF	Granulocyte macrophage colony stimulating factor
IBD	Inflammatory bowel diseases
IECs	Intestinal epithelial cells
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HLA	Human leukocyte antigen
JAM	Junctional adhesion molecule
JAK	Janus kinase
LGL	Lethal giant larvae
LSM	Confocal Laser Scanning Microscopy
MAPK	Mitogen-activated protein kinase
MMP	Metalloproteinases
IECs	Intestinal epithelial cells
IELs	Intraepithelial lymphocytes
IL-	Interleukin-
IL-22 BP	IL-22 binding protein
IL-22R	IL-22 Receptor
IL22RA2	IL-22 receptor-α2 gene
IFN-γ	Interferon-γ
IFNα2	Interferon-α2
ILC	Innate lymphoid cells
iNOS	Inducible nitric-oxide-synthase
ISC	Intestinal stem cells
MACS	Magnetic cell sorting
MCP-1	Monocyte chemotactic protein-1
NF-κB	Nuclear factor-κB
NK	Natural killer cells

PAR	Partitioning defective
PAGE	Polyacrylamide gel electrophoresis
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PGE_2	Lipid mediator prostaglandin E2
P/S	Penicillin–Streptomycin
RCD	Refractory celiac disease
RELMβ	Resistin-like molecule β
rpm	Rounds per minute
RPMI 1640	Medium Roswell Park Memorial Institute 1640
RT	Room temperature
SDS	Sodium dodecyl sulfate
STAT	Signal transducer and activator of transcription
TBS-T	Tris saline buffer and tween-20
TCR	T cell receptor
TER	Transepithelial electrical resistance
TEMED	Tetramethylethylenediamine
TGF- β	Transforming growth factor β
TG2	Tissue transglutaminase2
Th	T-helper
TNF	Tumor necrosis factor
TJ	Tight junction
TSLP	Thymic stromal lymphopoetin
UC	Ulcerative Colitis
wt	Wild type
ZO-1	Zonula occludens protein-1

1. Introduction

1.1 The intestinal mucosal barrier

The intestinal epithelium is organized as a single layer of cells that forms – by its surface – the largest barrier in our body against the external environment with seemingly contradictory functions. On the one hand, it acts as a permeable interface facilitating the absorption of electrolytes, water and nutrients. On the other hand, it constitutes a crucial barrier protecting against pathogens as well as other possible harmful substances from the external environment. For mechanistic studies, the selective permeability exerted by the epithelial barrier can be categorized into distinct pathways, a transcellular or transepithelial and a paracellular pathway[1]. Since in the transepithelial pathway, solutes are transported transcellular, i.e. through epithelial cells, it involves transporter proteins and pore proteins present in the cell membrane as well as endocytic processes. Complementary to this, within the paracellular pathway the passage of solutes occurs intercellular and is therefore regulated by junctional protein complexes that are localized to the apicolateral cell membrane and are known as tight junctions (TJ) and adherens junctions (AJ)[1,2].

When dissecting the barrier from the luminal side, the most luminal component of the intestinal mucosal barrier is mucus, followed by the intestinal epithelial cell layer and the lamina propria[1]. Furthermore, bone marrow-derived immune cells localized to the lamina propria and also intraepithelial. They have a central function in the homeostasis of the mucosal barrier as they interact closely with the compounds of the aforementioned defense layers, collect antigenic substances from the intestinal lumen and are crucial in the regulation of mucosal barrier function in conditions, that are found in all three layers, i.e. in the mucus, intraepithelial as well as in the lamina propria. Furthermore, components of the intestinal microbiota as well as anti-microbial peptides secreted by intestinal epithelial cells (IECs) significantly contribute to intestinal barrier function[1]. For this reason, the various components of this complex barrier are discussed in a sequential fashion.

1.1.1 Composition of the intestinal mucosal barrier

1.1.1.1 Mucus barrier

In both, small intestine and colon, the mucus layer is the first line of defense against luminal pathogens[3]. However, the composition of small intestinal mucus differs from colon mucus. In the colon, it consists of two layers, an inner layer attached to the epithelial cells, not allowing bacterial penetration and consequently free of bacteria; and a less dense and

unattached outer layer, habitat for commensal bacteria. In the small intestine the mucus is only single-layered and is permeable to macromolecules and bacteria-sized particles[4]. The difference in the small intestine and in the colon mucus layer was explained by distinct levels of proteases (as epithelial cell-anchored metalloprotease, meprin-β) that cleave Muc2 causing differences in density levels[5]. Besides, it is hypothesized that the difference in mucus composition is directly related to its function, especially the absorption of nutrients in the small intestine and barrier to bacterial components in the colon. One of the main functions of the mucus layer is to avoid adherence and subsequent invasion in the intestine by pathogens, as bacteria or fungi, thereby forming a protective layer covering the apical surface of the intestinal epithelium[3,6].

Mucus is produced and secreted by goblet cells and it is a complex viscoelastic adherent fluid composed to approx. 95% by water, which serves as a solvent and diffusion media for the other components, as electrolytes, lipids and proteins, including lysozyme, defensins, growth factors and soluble IgA[6]. The major protein component of mucus, however, are mucins, which are large proteins playing a pivotal role in the mucus generating process. Mucins are formed mainly by regions rich in serine and threonine and they are substrate to posttranslational modification resulting in highly glycosylated proteins. Their biochemical composition and structure enables O-glycosylation via serine and threonine residues contributing to the viscoelastic properties of the mucus[7]. The functional importance of the mucus layer has been neglected for a long time. However, Van der Sluis *et al* have uncovered the central function of intestinal mucus for barrier homeostasis, since mucus-deficient mice ($Muc2^{-/-}$) presented with weight loss and diarrhea, secondary to spontaneous development of colitis with a loss of physiological intestinal crypt architecture and absence of normal goblet cell morphology[8]. Interestingly, Velcich *et al* observed a higher incidence of adenomas in the small intestine of $Muc2^{-/-}$ mice, which consequently progressed to invasive adenocarcinoma and colorectal tumors[9]. Together, these studies point to a pivotal function of the mucus layer in preserving the structural integrity of the mucosal barrier.

1.1.1.2 Intestinal epithelial cell types and their function

The intestinal epithelial barrier is a single cell layer organized in several luminal projections (villous) and invaginations (crypts) covering the small intestine. It is composed of specialized cells kept together by cellular junctions, such as tight junctions (TJ) and adherens junctions (AJ). IECs are frequently renewed through cell division, maturation and cell migration[10]. The constant cell renewal in the small intestine occurs through active *Lgr5*$^{+}$ intestinal stem cells (ISC) at the basis of the crypts. After cell divisions, newly generated cells migrate from the crypts to the villous tips differentiating into various cell lineages presented in

the intestinal layer, such as enterocytes, goblet cells, Paneth cells, M cells and Tuft cells (Fig. 1.1)[11,12].

The most abundant cell type found in the intestinal epithelial barrier are the enterocytes, a highly polarized epithelial cell. Their central function is the absorption of electrolytes and nutrients. However, they also actively participate in the protection of the mucosal surface, where they secrete antimicrobial proteins to destroy harmful bacteria and pathogens[13]. In addition, it was hypothesized that they not only degrade but also present antigens directly to T-cells. Enterocytes present classical markers to antigen-presenting cells (APC) in their cell surface, such as CD14, CD35, CD43 and CD64, strongly suggesting that enterocytes may have a role as APCs inducing an immune response in the underlying intestinal lamina propria[13,14]. Goblet cells are secretory cells responsible to produce and secrete mucus in order to build the mucus barrier[10]. However, goblet cells are not only related to mucus production. These cells also produce and secrete a protein called resistin-like molecule β (RELMβ) that interacts with macrophages leading to the production of IL-12/23p40 after helminth infection. The goblet cell-macrophage crosstalk promotes adaptative immune response via T cells activation and Th1 response, which consequently leads to increased interferon(INF)-γ production and chronic intestinal inflammation[15]. Paneth cells play a crucial role in host defense against bacteria and microbiota regulation through production and secretion of α and β-defensins[16]. They are present at the base of the crypts alongside the stem cells and contain several secretory granules with antimicrobial proteins, and once they detect microbial signals, they release antimicrobial peptides into the intestinal lumen[17,18]. Reduced levels of antimicrobial peptides release by Paneth cells (α-defensins HD5 and HD6) are related to damage in mucosal defenses of the host and it might predispose to development of chronic intestinal inflammation as ileal Crohn's disease (CD)[19]. Additionally, Paneth cells are pivotal components of intestinal stem cells niche due to their expression of essential factors (EGF, TGF-α, Wnt3 and Notch signals) for intestinal stem cells maintenance. Therefore, genetic removal of Paneth cells is directly associated to loss of Lgr5 stem cells *in vivo*[20].

Another epithelial cell type, microfold cells (M cells), is the subset of IECs that covers the follicle-associated epithelium of the gastrointestinal (GI) tract. They are highly specialized cells in antigen sampling due to their high capacity for transcytosis and transepithelial transport of a variety of macromolecules and microorganisms from the lumen to lymphoid tissues (Peyer's patch) present in the lamina propria in order to initiate an immune response[21,22]. Rios *et al* revealed that antigen sampling of commensal bacteria mediated by M cells is an initial requested step for induction of antibodies (secretory IgA; SIgA) important to maintain homeostasis in mucosal surfaces as GI tract. They showed that mice lacking intestinal M cells presented delayed Peyer's patch maturation, resulting in decreased levels of SIgA[23]. Although Tuft cells were discovered decades ago, their function was elucidated only recently. Tuft cells

detect luminal helminth, such as *Tritrichomonas muris* via α-gutsducin, a GTP-binding protein[24]. Additionally, they produce and secrete interleukin (IL)-25 and thymic stromal lymphopoetin (TSLP) which induces Th2-immune response and consequently protection against helminth intestinal infection[25]. In accordance, Gerbe *et al* have demonstrated that mice without intestinal tuft cells presented compromised mucosal Th2 response and impaired worm expulsion[26]. More recently, Van Es *et al* demonstrated that Paneth cells depletion in mice, does not directly affect Lgr5 stem cells nurture, because tuft cells not only physically occupy Paneth cells position in nurture of stem cells but also can be an alternative source of essential signals, as Notch signals, for maintenance of Lgr5 stem cells[27].

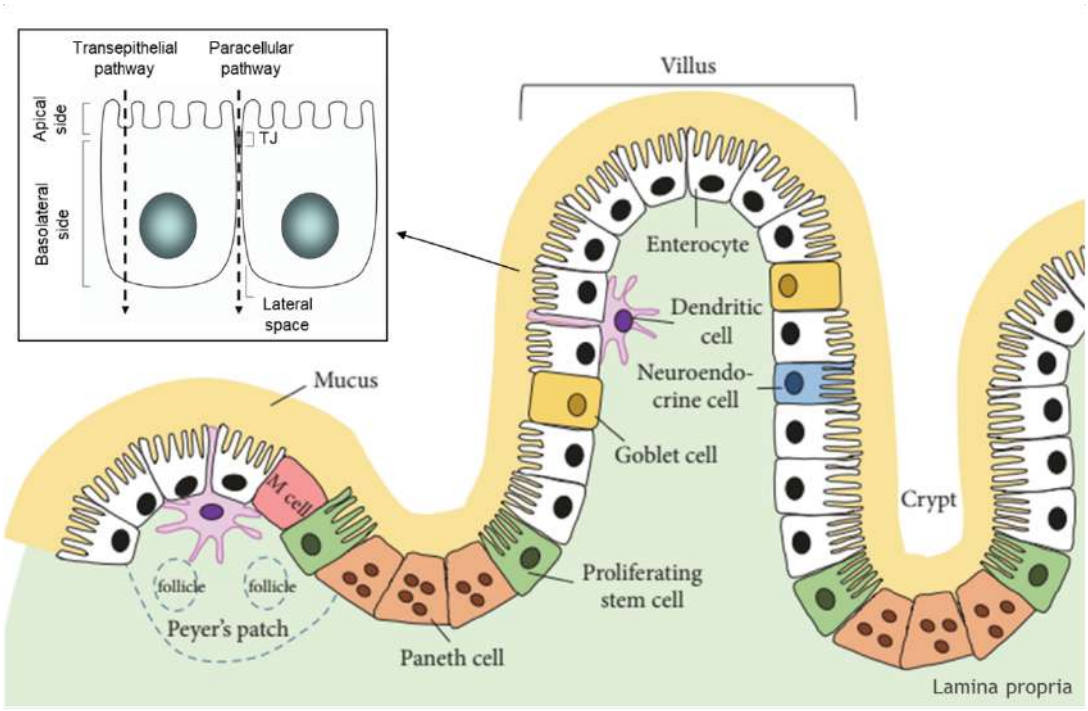

Figure 1.1: The small intestine mucosal barrier. The figure presents a simplified scheme showing the constitution of the small intestinal barrier: mucus and epithelial layer (Paneth, goblet, enterocytes, and stem cells), and underneath lamina propria where components of the immune system can be found (e.g. dendritic cells; Peyer's patch, well-organized portion of the immune system in the intestine). It is highlighted the two mainly permeability pathways: transepithelial and paracellular pathway, which is mediated by a complex of proteins known as tight junctions (TJ). Modified from Kong *et al* [28] and Sandek *et al* [29].

1.1.1.3 Apical junctional complex

IECs are connected through complexes of proteins localized in the apical-basal cell membrane. Their main function is to maintain the integrity and paracellular permeability of the epithelial barrier, cell polarity and tissue architecture. These complexes are known as adherens junctions (AJ) and tight junctions (TJ) (Fig. 1.2).

1.1.1.3.1 Adherens Junctions

AJ is composed of two complexes of proteins related to cell-cell adhesion: Nectin-afadin and cadherin-catenin. The extracellular region of these protein complexes mediates adhesion between neighboring cells and the intracellular region is involved in regulation of AJ dynamic, signaling pathways and interaction with components of cytoskeleton, such as actin. Nectins bind to the scaffolding protein afadin (AF-6, actin-binding protein) to form a structural adhesive complex directly linked to the cytoskeleton, thereby interacting with actin and the small GTPase RAP1[30]. In afadin-knockout mice, disorganized AJ and TJ in the ectoderm during embryogenesis were observed, leading to developmental defects[31]. Another study has indicated that nectin-afadin complexes exert a crucial role in AJ maturation, as it was observed that loss of afadin delays AJ formation[32].

The cadherin family is a superfamily of transmembrane glycoproteins involved in intercellular adhesion, which is in the extracellular interaction dependent on calcium (Ca^{+}). The N-terminal extracellular domain of E-cadherin binds to the identical cadherin domain of adjacent cells, while the C-terminal intracellular domain interacts with β-catenin and further components of the cytoskeleton, as actin and actin-binding proteins[33]. This complex is highly dynamic and is related to several growth and proliferation signaling pathways, as mitogen-activated protein kinase (MAPK) and PI3K signaling[33]. Alterations in these pathways contribute to tumor progression and metastasis, and can be associated to epithelial mesenchymal transition (EMT – more details in *1.2 Epithelial-mesenchymal transition* section).

1.1.1.3.2 Tight Junctions

TJs are found on the lateral cell membrane of IECs, apical to the AJs. TJ has a role in paracellular permeability acting as gate: where they create a permeable barrier, which selectively controls what goes through the interspace between the cells, as well as, fence: being crucial to restrict lateral diffusion of membrane proteins and lipids to either the apical or the basolateral compartment. Additionally, TJ complexes of proteins play an important role keeping the neighboring epithelial cells tightly together and maintenance of cell polarity[34,35]. TJ complex is mainly composed of transmembrane protein called occludin, claudins and junctional adhesion molecules (JAM)[30,35].

Studies have shown that occludin is involved in the regulation of paracellular permeability, and loss of occludin affects the localization of tricellulin (TJ protein localized at tricellular TJs, the specialized structures where three cells are connected[36]), which indicates a role in epithelial barrier function[37,38]. However, Saitou *et al* and Schulzke *et al* have shown that occludin-knockout mice present normal TJ strand formation[39,40]. Nonetheless, Van Itallie *et al*

demonstrated that overexpression of occludin is involved in signaling events related to barrier remodeling, with increase in TER measurements and paracellular flux of large molecules after exposure to proinflammatory cytokines (INF-γ and TNF-α)[41]. In accordance, Buschmann *et al* reported that occludin-knockdown monolayer presented increased TJ permeability to macromolecules, pointing to a role played by occludin in paracellular permeability[42]. Nonetheless, recently, Richter *et al* showed that occludin expression is not directly correlated to macromolecular flux, demonstrating that occludin alone may not be sufficient to stimulate epithelial leak pathway[43]. Another study demonstrated that occludin may also play a role on cell polarity. They reported that occludin knockdown led to disorganized and defective microtubule orientation. Moreover, it was observed that occludin is required for the localization of polarity proteins aPKC-Par3 and PATJ, which regulates the directional migration of epithelial cells[44]. Although, occludin was the first TJ component identified, its function has not been completely elucidated and more studies are required in order to clarify its impact on TJ formation and assembly.

Along with occludin, the family of claudin proteins are the major determinants of TJ structure and the so-called TJ strands, which is a structure of transmembrane proteins within the lipid bilayer. The organization of TJ strands creates a belt-like structure surrounding each cell to establish the epithelial barrier that control the molecules diffusion throughout the cellular sheet[35,45]. Furuse *et al* demonstrated that TJ-deficient fibroblast transfected with claudin-1 and -2 had their membranous strands similar to those usually found in epithelial cells. These results pointed to a pivotal role of claudins in TJ strands formation[46]. Moreover , the family of claudins might also be involved in the organization of the cytoskeleton, transport of vesicles and through signaling pathways that are directly associated with the scaffolding proteins ZO-1 and ZO-2[47]. These scaffolding proteins are pivotal for TJ polymerization and formation. Confocal microscopy revealed that ZO-1 and -2 knockout cells were well polarized with normal distribution of apical (syntaxin 3 and moesin) and basolateral markers (E-cadherin and erb2). However, these cells presented a complete lack of TJ formation with diffuse localization along the cytoplasm and basolateral membrane of TJ proteins as claudin-3, occludin and JAM-A showing that TJ formation was affected in ZO-1 and -2 knockout cells[48]. Moreover, the proteins within the claudin family regulate the selectivity of TJs with regard to ions. Interestingly, the claudin family of proteins includes not only members that play a role in barrier function to solutes (claudin-1, -4, -5 or -8), but also others members that form channels such as claudin-2 and claudin-17. These channels allow the selective passage of charged ions (Na^+, K^+: claudin-2, -15; Cl^-: claudin-17) and water (claudin-2) and thus act as a high capacity route for these solutes[49,50]. Therefore, changes in claudin expression and localization are correlated to disturbance of homeostasis and contribute to development of several diseases including inflammatory bowel diseases (IBD), such as CD, as well as various types of cance[51–53]. In

samples of CD patients, levels of claudin-2 were upregulated whereas occludin, claudin-5, and -8 (sealing TJ proteins) levels were downregulated and their localization was altered leading to barrier dysfunction[51]. Prasad *et al* also observed upregulation of claudin-2 along the inflamed tissue in samples of active ulcerative colitis (UC) by immunochemistry in comparison with normal colon, which presented no or very reduced levels of claudin-2. Claudin-3 and -4 were reduced and redistributed in active UC[54]. Corroborating these data, through western blotting and immunochemistry analysis, another study showed that levels of claudin -4 and -7 were reduced, while claudin-2 was upregulated[55]. These data indicate that alterations in claudins levels may be related to impaired epithelial barrier in pathological conditions.

Another family of proteins that composes the TJ structure is the JAM family. The JAM family is composed of three members: JAM-A, JAM-B and JAM-C. However, only JAM-A is directly involved in the maintenance of TJ structure[56]. Itoh *et al* have shown that JAM-A overexpression in mouse fibroblasts lacking TJs was not capable to induce the formation of TJ strand-like structures, suggesting that JAM-A alone was not directly associated to TJ formation[57]. Nonetheless, further studies have shown that intestinal mucosal explant of JAM-A-deficient-mice revealed a functionally disturbed intestinal epithelial barrier, specifically an increased permeability to 4 kDa-FITC-dextran as well as a decreased transepithelial electrical resistance (TER)[58]. In addition, both CD and UC tissue samples as well as dextran sodium sulfate (DSS)-induced colitis samples from mice, presented reduction in epithelial expression of JAM-A. Furthermore, in *in vivo* experiments, JAM-A-knockout mice showed a strong DSS-colitis-susceptibility, increased intestinal permeability and higher production of proinflammatory cytokines in comparison with the wild-type mice. In *in vitro* experiments, JAM-A knockdown resulted in increased paracellular permeability[59]. These studies provide evidence for JAM-A having a role in the regulation of paracellular permeability. JAM-A can also be associated with mitotic spindle orientation during epithelial morphogenesis. In an elegant study, Tuncay *et al* demonstrated that JAM-A regulates the formation of cortical actin cytoskeleton via transient activation of Cdc42 and PI3K. In conclusion, absence of JAM-A expression caused misaligning of the mitotic spindle with mislocalization of dynactin, and impairment of epithelial morphogenesis in three-dimensional culture [60]. Additionally, studies by Ebnet *et al* suggest a potential role played by JAM-A in cell polarity through directly interaction with essential polarity proteins – PAR-3/aPKC – in cell-cell contact of epithelial cells via specific domains, as PDZ. In accordance JAMs proteins can directly interact with PDZ-domain-proteins, as AF-6 and possibly ZO-1, confirming the possibility of function on polarity in epithelial cells[61,62].

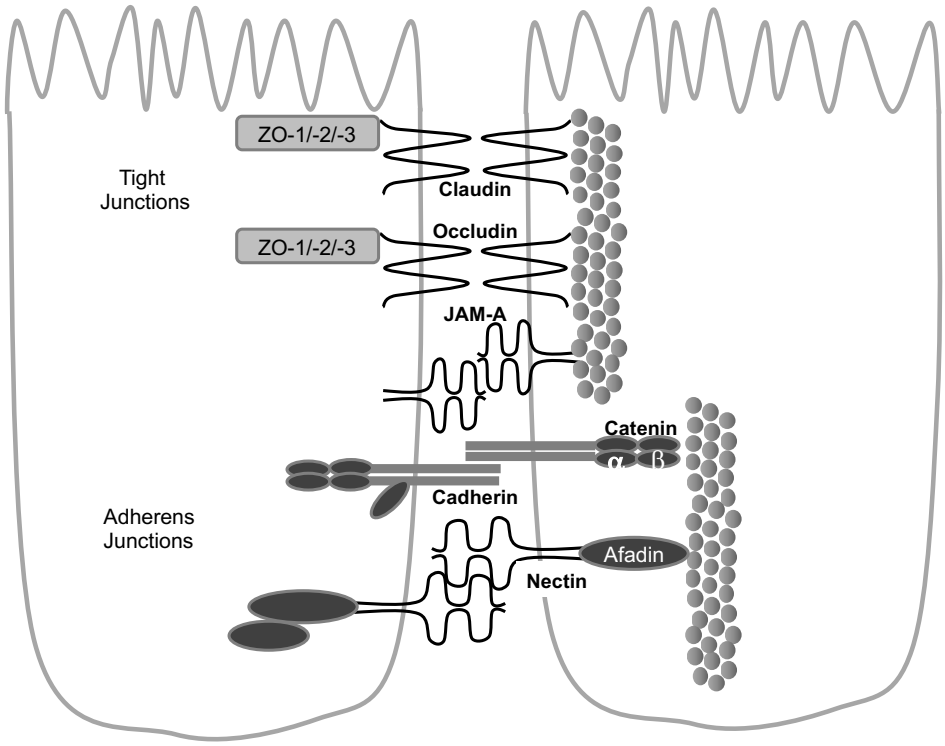

Figure 1.2: Apical complex junction. Simplified figure with the main proteins of tight and adherens junction complexes. Adapted from Schumann & Kühnel[63]

1.1.1.4 Intraepithelial lymphocytes

Mucosal T-cells also comprise a heterogeneous cell population, some of which are located beneath or between epithelial cell layer and which are known as intraepithelial lymphocytes (IELs), mostly $CD3^+$ $CD8^+$ T-cells. Due to their localization, intestinal IELs can directly interact with enterocytes through expression of the integrin CD103 (integrin α_E), C-type lectins and the activation marker CD69, as well CD244 (NK cell inhibitor receptor 2B4). IELs can contribute to tissue homeostasis and pathogenesis of diseases[64–66] . Different from other T-cell populations, IELs do not circulate through blood and they are rather tissue-resident. However, IELs present a very dynamic behavior. Edelblum *et al* have reported that IELs actively migrate into the lamina propria and within the epithelial layer to make contacts with IECs. They also have shown that occludin is a pivotal TJ protein to IELs migration, which provides comprehension into the regulation of molecular mechanisms responsible to IELs/IECs interactions [67]. Kuhn *et al* have shown that IELs interact with commensal microorganisms in the gut to secrete cytokines, as IL-6, promoting alterations on epithelial barrier function. In IL-6 knockout mice, it was noted that paracellular permeability was found to be increased, the mucus layer was significantly thinned and barrier function was defective as expression of claudin-1 which is a TJ-sealing claudin was reduced[68].

Interestingly, all of these features were reversible once levels of IEL-derived IL-6 were rescued, showing their importance in the maintenance of epithelial barrier[68]. It also illustrates

that IELs need to be tightly regulated. In the contrary case, they can contribute to the induction of inflammatory diseases by deterioration of barrier function. In line with this, an increased number of $CD3^+$ $CD8^+$ IELs is defined as a hallmark for another common chronic inflammatory disease of the small intestine, celiac disease (CeD; more details in *1.5 Celiac disease – a model disease for barrier dysfunction*), where it is found in such a reproducible manner, that it is used in daily clinic for diagnosis[69,70]. In active and refractory celiac disease (RCD), it has been shown that intraepithelial CD8αβ cytotoxic T lymphocytes are activated by higher levels of IL-15, produced by monocytes, leading to destruction of IECs and consequently a dysfunctional intestinal barrier[71].

1.1.1.5 Lamina propria cells: their effect on intestinal barrier function and inflammation

The lamina propria is the histological designation of the layer directly basal of the epithelial sheet and is separated from the epithelial cells only by the basal lamina. Cells from the lamina propria have to fulfill a complex array of tasks as they play a pivotal role in the defense against intraluminal pathogens, toxins, and other harmful substances. At the same time these cells are important contributors to epithelial barrier function and as such determine the homeostasis of the IECs by directly interacting with them or regulating their function through production and secretion of cytokines[72]. With regard to the various cell types included in the intestinal lamina propria I will focus on those cells that have been shown to exert effects directly on the intestinal barrier.

1.1.1.5.1 Mononuclear phagocytes

Mononuclear phagocytes, as macrophages and dendritic cells (DC), are noticeable, since these cells play an important function in phagocytosis of pathogens and/or antigens. These cells act as APC shaping an immune response through activation of T helper cells and later, regulatory T cells (Tregs)[73]. Remarkably, DCs collect luminal antigens with their dendrites and pass through the epithelial/TJ layer without significantly altering overall barrier function. These DCs express at their dendrites high levels of TJ proteins, as claudin-1, -7 and ZO-2 and thus – by building TJ-like complexes at their protrusions with which they are able to interact with IECs – seal the epithelial layer although traversing it at the same time[74]. Another aspect is that these cells are top producer of pro and anti-inflammatory cytokines that have a major impact on barrier function and small intestine homeostasis, such as IL-1, IL-6, tumor necrosis factor (TNF)-α and IL-10[75,76].

Furthermore, macrophages and DC can affect IECs in a different manner in an inflammatory subset. For example, IECs co-cultured with macrophages derived from patients suffering from IBD, presented alterations in TJ proteins, as upregulation of claudin-2 and

disruption in ZO-1 architecture with ZO-1 localized in the basal side of epithelial cells, which led to barrier integrity disruption. The deleterious effect on the barrier was due to production of proinflammatory cytokines, such as IL-1β, IL-18 and TNF-α[77]. Additionally, mononuclear phagocytes produce the eicosanoid PGE_2 (lipid mediator prostaglandin E2) that upon binding to its G protein-coupled receptor on the basal membrane of IECs triggers production of cAMP thereby increasing intracellular Ca^{2+} which contributes to stabilizing intestinal barrier function[78]. In addition, PGE_2 regulates immune response mediated by neutrophils contributing to maintenance of intestinal epithelial barrier integrity[79,80]. In accordance, Miyoshi *et al* demonstrated that PGE_2 via its receptor Pterg4 drove morphological and transcriptional differentiation in wound-associated cells leading to wound healing and renewal of intestinal epithelium mediated by nuclear translocation of β-catenin[81]. Denning *et al* have demonstrated that intestinal $CD11b^+$ DC and macrophages present in the lamina propria interacted with each other directly affecting the delicate balance between immune response and tolerance. Specifically, lamina propria macrophages induced the differentiation of $Foxp3^+$ regulatory T cells by an IL-10-dependent mechanism, resulting in the inhibition of a proinflammatory immune response. In addition, they observed that $CD11b^+$ DCs might have opposing effects by inducing the differentiation of T cells that produce proinflammatory IL-17. These results highlight the complexity of interactions within the monocytic cell population of the lamina propria[82].

1.1.1.5.2 Intestinal T cells

Historically, two subsets of T lymphocytes were described by the cell surface markers CD4 and CD8. On the one hand, $CD8^+$ cytotoxic T cells play an important role in adaptative immunity against tumors, virus, bacteria and parasites infection[83–86]. On the other hand, $CD4^+$ T cells, also known as T helper cells, regulate the immune reaction by secreting various cytokines once they are specifically activated via their T-cell receptor. Further, this subset is divided into polarized (Th1) or Th2 lymphocytes that produce pro- and anti-inflammatory cytokines which directly affect intestinal barrier function, such as IL-4, IL-5 and INF-γ[87].

Firstly, lymphocytes develop in primary lymphoid organs, i.e. thymus and bone marrow. These lymphocytes migrate from the primary organs to peripheral (or secondary) lymphoid organs, where they can react with foreign antigen and become active[88]. Naïve T cells can migrate to gut-associated lymphoid tissue (GALTs) via blood circulation. There, naïve $CD4^+$T and $CD8\alpha\beta^+$ T cells become active after antigen presentation by APCs and acquire the capacity to migrate to intestinal tissues secondary to the expression of molecules, such as integrin α4β7, activation marker CD44, the chemokine receptor CCR9, adhesion molecule LFA-1, and very late antigen-4 (VLA-4, also known as α4β1) [88,89]. Chemokines as CCL25 recruit CCR9-expressing T cells to enter in the intestinal mucosa where they can interact,

mainly by cytokine production and secretion, with IECs[90,91]. Intestinal $CD4^+$ T cells present in the lamina propria in UC patients produce high levels of IL-13 that can be cytotoxic for IECs leading to defects in barrier function. On the other hand, it became clear that the specific role of a T-cell depends on its affiliation to a distinct T-cell subset. For γδ-T cells Sun *et al* and Tsuchiya *et al* demonstrated that these T cell subsets can be mucosa-protective, as mice deficient in T-cell receptor (TCR) δ-chain developed severe DSS-induced colitis. These mice were rescued once IL-17-producing γδ T cells were substituted. Moreover, they showed that γδ T cells preserved the epithelial surface integrity suggesting that γδ T cells play a protective role in ulcerative colitis[92,93]. In both, patients with Crohn's disease and ulcerative colitis, deficiencies in Treg function and elevated Th1 and Th17-associated cytokines are strongly related to development of disease activity[94–96]. As such, it has been reported that both, regulatory B cells (Bregs) and Tregs, are induced by IL-33 and that the Th17 cell response was suppressed in chronic DSS colitis suggesting that IL-33-dependent Breg and Treg function can play a protective role in the homeostasis of the intestinal mucosal immune system[97]. The development of Th17 cells correlates with the progression of colitis, and neutralization of their produced cytokines IL17A and IL17F was hypothesized to become a strategy in the treatment of IBD[95]. However, within their randomized, double-blind placebo-controlled trial, Hueber *et al* reported that blockage of IL17A was ineffective for treating Crohn's disease. Contrary to the hypothesis, CD patients presented even an increase in inflammatory activity when compared to the placebo group[98].

1.1.1.5.3 Innate lymphoid cells

Innate lymphoid cells (ILCs) are a heterogeneous population of lymphocytes. They are important players mediating immune responses against pathogens and additionally contribute to the maintenance of intestinal tissue homeostasis. ILCs are divided into three subgroups based on their cytokine production: ILC1, ILC2 and ILC3[99,100]. ILC1 cells produce and secrete IFN-γ and TNFα in response to IL-12 and IL-15. They work closely together with Th1 cells against viruses, bacteria or parasites[101,102]. ILC2 cells secrete Th2 cell-associated cytokines, such as IL-4, IL-5, IL-9 and IL-13, required for the immune responses against helminths and other extracellular parasites[99,103,104]. The third group of ILCs, ILC3, can be found at mucosal surfaces, such as bronchial system and lung. Furthermore, these cells have been implicated to play a role in intestinal homeostasis inducing T cell tolerance and also conveying protection against infection by extracellular bacteria and fungi. This is crucially mediated by IL-22 secretion[105,106]. However, ILC3 can play a non-beneficial role in the GI tract. Buonocore et al have demonstrated that ILC3 cells have been associated with *Helicobacter hepatitis*-induced colitis increasing the production of IL-17 and IFN-λ[107]. In addition, ILC3 were shown to induce

cell death of IECs and consecutive barrier dysfunction secondary to overexpression of IL-22[108]. Altogether, these studies show the complexity of the crosstalk between lamina propria cells, barrier function in intestinal inflammation. Therefore, further studies are necessary to completely elucidate the role of these cells on intestinal homeostasis and disease progression.

1.1.1.6 Intestinal microbiota and its effect on intestinal inflammation

The human intestinal microbiota is a complex and dynamic system of microorganisms composed of certain viruses, fungi, helminthic parasites as well bacteria classified as commensal bacteria, the predominant member of this complex[109]. The intestinal microbiota exerts an important role in digestion and absorption as well synthesis of vitamins and protection against overgrowth of harmful bacteria in the gut promoting further development of the immune system of the host. Together with immune cells, the intestinal microbiota interacts with IECs and influences the structure of intestinal barrier as well mucus layer, determining intestinal permeability and mucus production. Lack of regulation and altered composition of intestinal microbiota are tightly associated with intestinal barrier dysfunction in the small intestine and together they may predispose the host to develop IBD, as CD[110,111].

Pull *et al* have shown that commensal bacteria-depleted mice were more susceptible to develop mucosal injury when exposed to DSS, a well-established model of experimental colitis, than control mice[112]. *Faecalibacterium prausnitzii* is a commensal bacterium with anti-inflammatory properties with increased levels of anti-inflammatory cytokines, such as IL-10, and decreased levels of proinflammatory cytokines, such as INF-γ and IL-12. Sokol *et al* have found an association with decreased of *F. prausnitzii* and higher risk of ileal Crohn's disease recurrence[113].. Similarly, *Lactobacillus casei* reduced the secretion of the proinflammatory cytokines TNF-α, IFN-γ, IL-2, IL-6 and IL-8 in surgical mucosal explants *in* an *ex vivo* experimental setting, implying a protective role for this bacterium in IBD. It even had the potential to downregulate proinflammatory effects caused by preincubation with *Escherichia coli* in the CD mucosal explants[114]. Regarding fungal composition present in segments of the intestine (ileum, cecum and colon), Qiu *et al* have revealed that the localization of fungal colonization is severely altered in intestinal inflammation. Using a an acute DSS colitis mouse model, they showed that *Penicillium, Wickerhamomyces, Alternaria*, and *Candida* populations were increased in numbers, but *Cryptococcus, Phialemonium, Wallemia* and a *Saccharomycetales* genus were decreased in the guts of DSS-treated mice. Even more interestingly, fungi-depleted mice suffered from aggravated DSS colitis associated with gain of *Hallella, Barnesiella, Bacteroides, Alistipes*, and *Lactobacillus* and loss of butyrate-producing *clostridium XIVa*, and *Anaerostipes*, suggesting that certain intestinal fungi populations might play a pivotal role in preventing inflammation triggered by pro-inflammatory bacteria in a setting prone to develop IBD.[115]. In addition, the microbiota can play a role on the regulation of

intestinal barrier function[116,117]. Laval *et al* have shown that *Lactobacillus rhamnosus*, a well-known probiotic, was able to increase the levels of occludin and E-cadherin, partially restoring intestinal barrier[118]. Another study has analyzed soluble factors produced and secreted by *Bifidobacteria infantis* and its effects on IECs. In T84 cells, *Bifidobacteria infantis*-conditioned medium had an effect on intestinal barrier function increasing TER and levels of ZO-1 and occludin, mediated by ERK (extracellular-signal-regulated kinase) inhibition. In IL-10-deficient mice, administration of oral *Bifidobacteria infantis*-conditioned medium reduced the inflammation and secretion of IFN-γ[119]. Taken together, these studies demonstrate that microbiota have a pivotal impact on intestinal epithelial physiology and function.

1.2 Epithelial-mesenchymal transition

EMT is a reversible biological process where polarized epithelial cells undergo molecular changes which enable them to assume a mesenchymal phenotype increasing invasion and migration capacity[120]. This process is crucial during embryonic development mainly in the gastrulation phase, in neural crest cell migration and in organ formation, but also in processes occurring in finally differentiated organisms as wound healing or in pathophysiology in carcinogenesis and metastasis [121–123].

EMT is executed in progressive stages. Firstly, cell-cell contacts of epithelial cells are lost which leads to redistribution of cytoskeletal proteins and disruption of apical junctional complexes. This stage is characterized by downregulation of TJ proteins, such as claudin-1 and -4, JAM-A, occludin and ZO-1, as well as, integrins related to cell polarity and responsible to mediate cell-cell junctions and cell/basement membrane connections[124]. During this stage, cytokeratins are replaced by vimentin and increased levels of F-actin is induced. Also, AJ re-organization occurs with decreasing the levels of E-cadherin, which can be considered as a hallmark of EMT, and a partial replacement by N-cadherin (regarded as a mesenchymal marker). Subsequently, dynamic cytoskeletal changes are observed, as formation of actin stress fibers that bind to focal adhesion complexes in order to initiate cell migration[122]. During this process, epithelial cells obtain the ability to migrate along the extracellular matrix (ECM) forming protrusions of actin-rich membranes – lamellipodia – and spike-like extensions – filopodia. These protrusions interact with the ECM via several proteins, such as small Rho GTPases, myosin kinases, and α5β1 integrin, all of which are required for initiating the migration process. After that, cells produce and secrete matrix-metalloproteinases (MMPs) and ECM components as collagen type I and fibronectin in order to decompose basement membrane components and remodel ECM to facilitate migration and invasion (Fig. 1.3)[121].

EMT induction is orchestrated by changing the expression profile of the target cell, which is accomplished by induction of so-called EMT-transcription factors, including ZEB,

Snail, Slug and Twist. Expression of EMT-transcription factors reduces the expression of epithelial markers, as E-cadherin or cytokeratins, and concomitantly induces the expression of mesenchymal markers, such as vimentin, fibronectin and β1 and β3 integrins[121,125,126]. Several extracellular ligands, including tyrosine kinase receptors (epidermal growth factor, fibroblast growth factor, insulin-like growth factor), integrins, Wnt, nuclear factor (NF)-κB and transforming growth factor β (TGF-β) pathways may trigger EMT[125,127,128]. The transcription factors Snail and Slug induce the formation of a complex that binds to the promoter region of genes of various members of the TGF-β family, as TGF-β3, thereby increasing their transcription, subsequently initiating EMT[129]. Moreover, Ikenouchi *et al* demonstrated that there is a strong relation between Snail and repression of TJ protein levels. In this study, it was noticed that Snail overexpression in cultured mouse epithelial cells led to EMT induction and decrease in protein and mRNA expression levels of TJ as claudins (-3, -4, -7) and occludin[130].

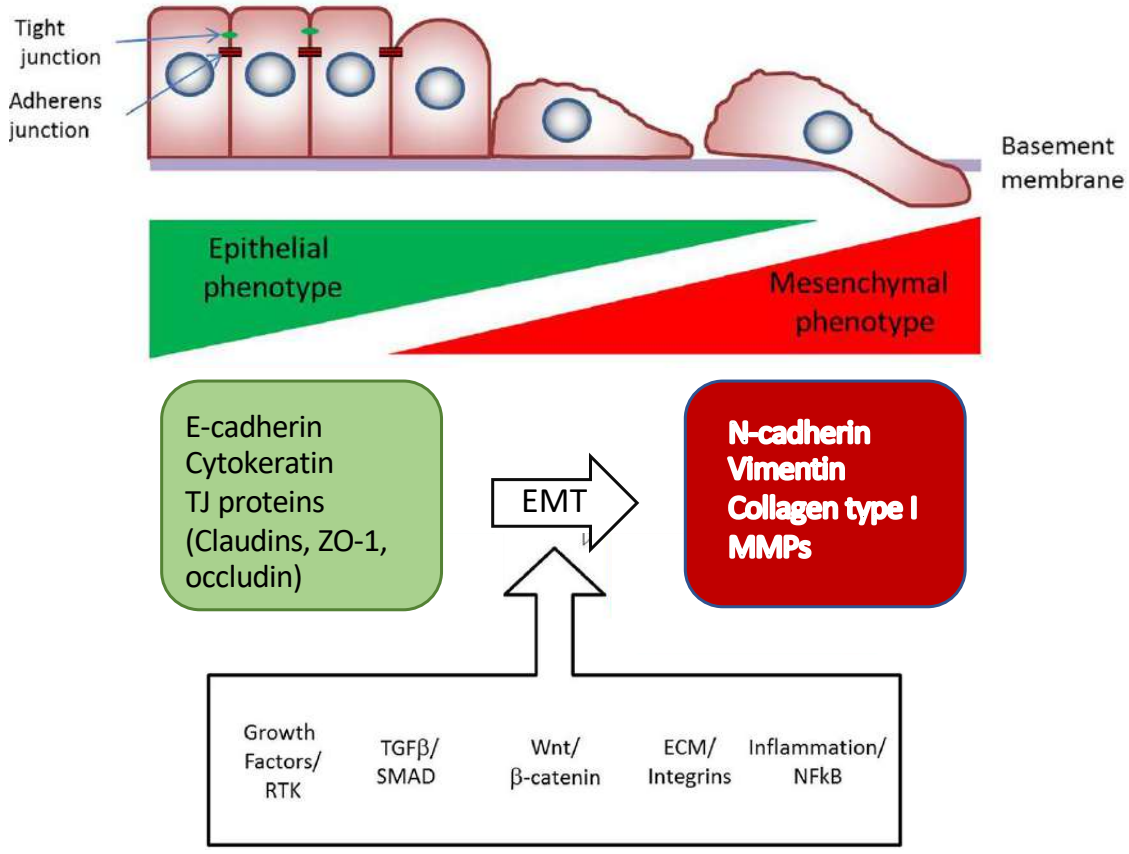

Figure 1.3: The features of epithelial-mesenchymal transition. EMT is triggered by several pathways, such including activation of tyrosine kinase receptors (epidermal growth factor, fibroblast growth factor, insulin-like growth factor), integrins, Wnt, nuclear factor (NF)-κB and transforming growth factor β (TGF-β) pathways. The cells lose expression of decisive genes that define epithelial properties (green box) and gain expression of markers for mesenchymal differentiation (red box). These newly gained qualities facilitate including cell migration and the invasion of cells through the basement membrane. Adapted from Bartis *et al*[131].

1.3 Cell polarity

Polarized epithelial cells form a continuous layer at interfaces that ascertains compartmentalization. In the case of the intestinal mucosa, this interface confines the luminal, microbiota- and nutrient-containing compartment from the mostly sterile subepithelial compartment. At the level of the epithelial sheet, a complex composition of epithelial cell membranes, apical junctional complexes and intraepithelial immune cells (mostly IELs) determines the barrier function of this structure[132,133]. The resulting barrier function is crucially regulated by luminal stimuli from microbiota and the mucus as well as basolateral stimuli, coming from the diverse set of lamina propria immune cells. To ascertain this crucial function, epithelia are strongly polarized cells, as their various structures at the cell apex differs widely in composition and function from equivalent structures of the cell base[134]. To build up a safe barrier, one key structure within the epithelial layer is the TJs (described in detail in *1.1.1.3.2 Tight Junctions*). As common enterocytes evolve from intestinal epithelial stem cells in the deep crypt and move upward to the surface epithelia (or in the case of the small intestine to the villous tips) these pivotal structures need to be assembled in a tightly regulated process that involves various polarity complex proteins[135,136]. Interestingly, the set of proteins involved reveals striking parallels to proteins defining directed cell movement in migrating cells as phagocytes that in these cells orchestrate the highly dynamic cell protrusions at leading edges as lamellipodia or filopodia, that are required to perform this fluidic type of cell movement[133,137].

The polarization process is strongly regulated and, at least, three evolutionary conserved complexes are crucial in the execution of the polarization process: The scribble (SCRIB) complex, crumbs (CRB) complex and partitioning defective (PAR) complex[138]. *SCRIB complex:* The maintenance of the basolateral side is essentially due to SCRIB complex, which is composed of Scribble, Discs large (DLG) and Lethal giant larvae (LGL) that are located at the basolateral side of the epithelium. *PAR complex:* The PAR complex is composed of PAR3, PAR6 and atypical protein kinase C (aPKC) and interacts with proteins that form an early junctional complex that later on will evolve in TJs and AJs. *CRB complex:* Together with the PAR complex, the CRB complex is associated with apical junction formation. This complex is constituted by transmembrane protein, localized mainly in the apical membrane compartment, CRB and proteins associated to cytosolic proteins, such as Lin seven 1 (PALS1) and Pals1-associated tight junction protein (PATJ)[135,139,140]. Michel *et al* demonstrated that PATJ is associated with TJ proteins and plays an important role in TJ assembly and organization in IECs[141]. In addition, Crumbs3, together with aPKC, are important to regulate the early lumen formation and alterations in their function lead to no-lumen or multilumen 3D cysts[142].

Disturbances in these complexes result in barrier dysfunction, changes in cell morphology, cell survival and development of pathological disorders as Crohn's disease and

cancer[143]. Homozygous knockout mice of many polarity genes are lethal, pointing to an important function played by cell polarity proteins in the embryonic development. Murdoch *et al* have demonstrates that mutations in Scrib, alone or in combination with *Celsr1* and *Vangl2*, caused lethal defect on the neural tube formation, called craniorachischisis, where the brain and spinal cord remain open, and subsequently there is a congenital malformation of the central nervous system[144,145]. Additionally, Dauber *et* al demonstrated that alterations in Scrib led to human birth defects including coloboma, microcephaly, as well as cardiac and renal defects[146]. Mehalow *et al* have shown that CRB1 is essential for photoreceptor morphogenesis in the retina of mammalian. Therefore, mutations in CRB1 gene lead to several forms of retinal disorders in humans, and it can be related to retinitis pigmentosa and Leber congenital amaurosis[147]. Mutations on DLG genes, as *DLG5* and *DLG3* contribute to IBD development as well as severe X-linked mental retardation[148,149]. Genetic studies suggest that deregulation of polarity protein levels may also be considered in cancer development[150,151]. Studies using *Drosophila* support the idea and provide evidence that a group of membrane-associated proteins act in order to regulate epithelial cell structure and proliferation, and subsequently, cell polarity[152–154]. Recently, McCaffrey *et al* reported that loss of Par3 is associated with increased levels of metastasis and tumorigenesis in breast cancer[155]. In accordance, Zen *et al* noticed that expression levels of *PARD3* was not detected and might be a novel mechanism of action driving the cancer progression in human esophageal squamous cell carcinoma[156]. In contrast, other studies have shown that genes encoding PKCζ, Par3, Dlg and Scribble can be overexpressed in tumors, suggesting that they might have a tumorigenic role[157–159].

In the intestine, studies have demonstrated that enteropathogenic *Escherichia coli* may destabilize apico-basal polarity via redistribution of polarity proteins (Crb3 and Pals1) from the CRB complex in IECs and colonocytes of infected mice[160], changes the PAR complex recruiting active aPKC[161], disrupting TJ assembly. In CeD patients, polarity proteins Par3 and PP-1 and TJ protein were altered in their levels, suggesting that cell polarity is directly associated to barrier function[162]. Ivanov *et al* investigated the role of Scribble in TJ regulation in intestinal epithelium after a pro-inflammatory stimulus. Scribble localized in the TJ structure and the SiRNA-knockdown inhibited TJ reassembly and suppressed the formation of epithelial barrier. Furthermore, it was described that Scribble immunoprecipitated with ZO-1, indicating interaction between these proteins and pointing to a role played by Scribble in TJ assembly[163]. Moreover, loss of polarity proteins, as Dlg1, promoted invasive behavior in epithelial cells after exposure to proinflammatory cytokines, TNF-α and IL-6[164]. Taken together, these studies pointed out the importance of polarity process for cell morphology, cell survival, barrier function and that disturbances lead to various pathologies.

1.4 IL-22 as a model cytokine in intestinal mucosa inflammation

IL-22 belongs to the IL-10 superfamily of cytokines. Initially, IL-22 was described as a cytokine produced mainly by T helper (Th) cells (Th1, Th17 and Th22). IL-22 can be produced by a wide variety of cells from the lymphoid lineage, including αβ T cells, γδ T cells, natural killer T cells, and innate lymphoid cells[100,165]. Nonetheless, recent studies have shown that myeloid cells, as macrophages[166], neutrophils[167] and dendritic cells[168], as well nonhematopoietic cells as fibroblasts[169] are also capable of producing and secreting IL-22.

A variety of molecules and cytokines, such as IL-23, IL-7, IL-25 and IL-22 binding protein (IL-22BP), regulate the expression of IL-22[165,170,171]. IL-22BP is encoded by the IL-22 receptor-α2 gene (IL22RA2) and acts as a direct antagonist of IL-22 preventing the binding of IL-22 and its receptor (IL-22R1). It is a soluble IL-22 receptor lacking a transmembrane domain and specifically binds exclusively to IL-22 and not to any other IL-10 family member [172,173]. Huber *et al* generated both, IL22BP and IL-22 knockout-mice and made use of the AOM/DSS model (AOM, azoxymethane; DSS, dextran sodium sulphate), an established model for colitis-associated colon cancer, to investigate the role of IL-22 and IL-22BP in inflammation-associated carcinogenesis[174]. Interestingly, the results pointed to the necessity to delicately balance the concentrations of IL-22 and its counterpart IL-22-BP, as mice lacking IL-22BP expression revealed epithelial protection by wound healing effects resulting in less severe inflammation, but were prone to develop colon cancers in a significantly accelerated fashion. These results suggest that IL-22 has a role not only in inflammation but also in carcinogenesis, and emphasize the importance to critically regulate the IL-22-IL-22BP axis[174].

The IL-22 receptor (IL-22R) is a heterodimeric type II cytokine receptor formed by the two subunits IL22R1 and IL10R. The receptor complex is mainly expressed on non-hematopoietic cells, as epithelial cells[175]. Therefore, the central target of IL-22 are epithelial cells present in a diversity of tissues including liver, pancreas, lung and the GI tract. Once IL-22 binds to its receptor, downstream signaling pathway are activated, including the JNK, p38, STAT3 and MAPK signaling pathways (more details in *1.4.1 Signaling pathways activated by IL-22* section). As briefly pointed out above, IL-22 has overlapping roles in regulating cell proliferation and survival, wound healing, tissue protection and regeneration, host defense and inflammation (overview in Fig. 1.4)[165,170,176]. Studies have shown evidence pointing to IL-22-mediated tissue protection and regeneration, and also host defense in intestinal epithelial tissues. Zheng *et al* have shown that IL-22 knockout mice infected with *Citrobacter rodentium* developed increased intestinal epithelial damage, bacterial burden and mortality. Furthermore, IL-22 directly induced RegIIIβ and RegIIIγ, member of the Reg family of antimicrobial proteins[177]. Besides, Aujla *et al* have shown similar results in lung tissues, where IL-22 together with IL-17A played an important role in bacteria control and mucosal host defense against

Klebsiella pneumoniae, regulating production of CXC chemokines and granulocyte colony-stimulating factor[178]. In addition, Liang *et al* have shown that IL-22 in combination with IL-17A and IL-17F synergistically induced several antimicrobial peptides, such as β-defensin2, S100A9, S100A7 and S100A8[179]. Altogether, these results demonstrate the functional importance of IL-22 in host protective immunity against extracellular bacteria.

As pointed out before, IL-22 induces protective effects on the intestinal epithelia. which is supported by data from intestinal epithelial cell culture, organoid models and murine models of intestinal inflammation. With regard to the latter model systems, IL-22 enhanced intestinal mucus production in a mouse model of Th2-mediated colitis, thereby contributing to a reduction in local inflammation[180]. Epithelial regeneration was the focus in the work of Lindemans *et al*, showing that IL-22 affected directly intestinal stem cells (ISCs) leading to increased levels of cell proliferation and ISC expansion. Specifically, IL-22 treatment contributed to the cure of murine graft versus host disease which occurred after allogenic bone marrow transplantation, by reducing intestinal damage and mortality, increasing recovery of ISCs and consequently, inducing intestinal epithelial regeneration[181]. Conversely, using a model of murine ileal organoids, a recent study revealed that exposition to higher concentrations of IL-22 might induce the opposite effect, downregulating ISC self-renewal and expansion[182]. Interestingly, another study suggests a role of IL-22 in intestinal barrier function. It has been shown that IL-22 alters TJ assembly of proteins by upregulation claudin-2 expression leading to reduction of TER and increased intestinal permeability[183]. In accordance, Tsai *et al* also showed IL-22-mediated upregulation of claudin-2, which led to diarrhea and pathogen clearance[184]. In addition, high levels of IL-22 could be associated to some inflammatory diseases. Recent studies have indicated that patients suffering from IBD, mainly active CD, have higher levels of IL-22 in inflamed colonic lesions compared to noninflamed tissue[185–187]. Further, IL-22 can also lead to proinflammatory features inducing and working tightly together with other proinflammatory cytokines, such as IL-1, IL-6, IL-8, IL-17 and TNF-α [138,187,188]. Taken together, these studies point to IL-22 having complex effects on epithelial survival growth and differentiation, but also suggest that resulting clinical phenotypes are an integral of IL-22 and its direct counterparts, implying a role for local concentration-dependent hotspots. IL-22 have a dual role; on the one hand IL-22 may be beneficial for regeneration of inflamed tissue, on the other hand, a chronic upregulation of IL-22 expression may lead to production of proinflammatory cytokines and chemokines which might induce inflammation.

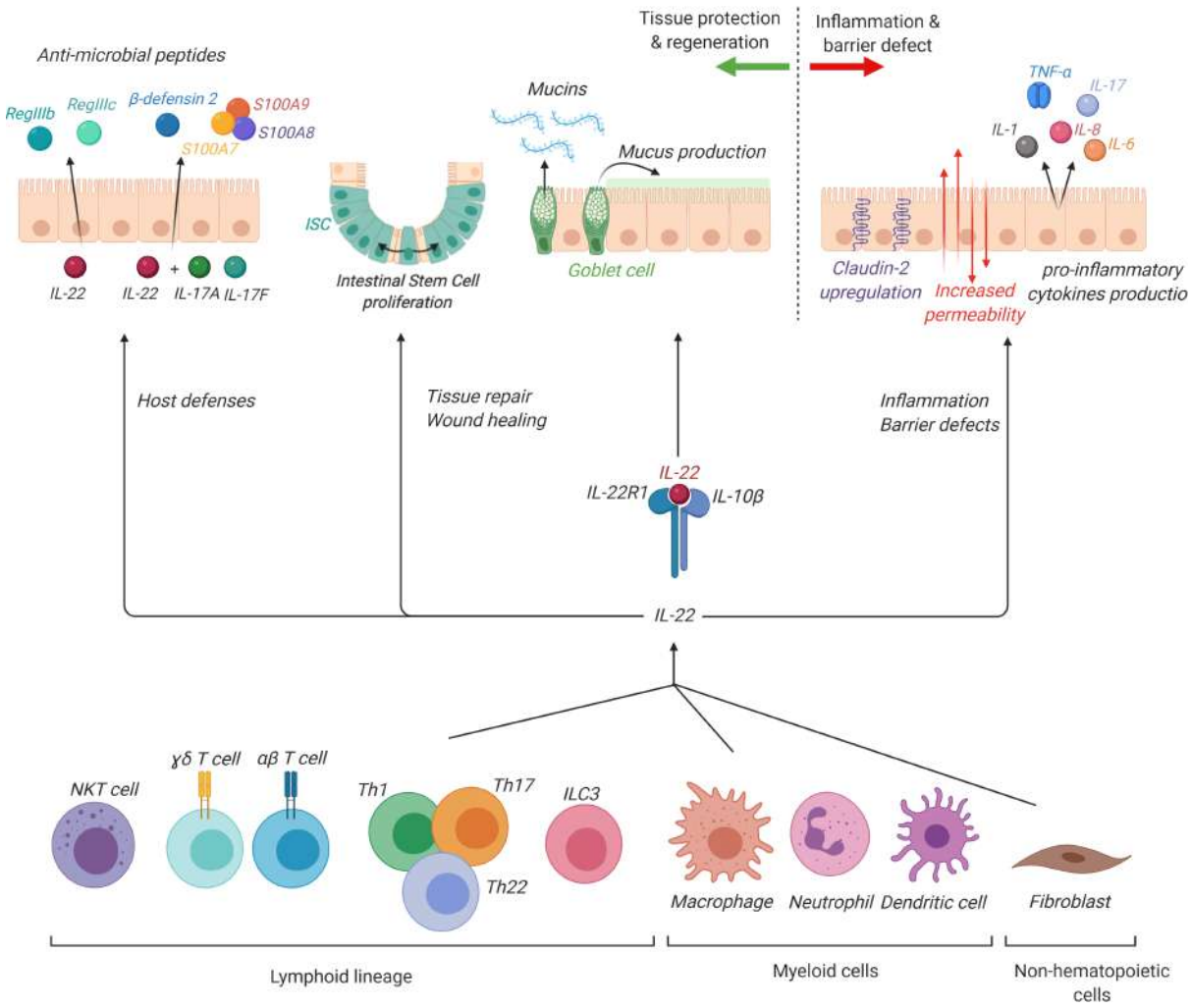

Figure 1.4: Diverse actions of IL-22 on the intestinal mucosa. Simplified scheme demonstrating the principal cellular sources of IL-22 and its functions, including host defense by regulation of antimicrobial peptide secretion, tissue repair and wound healing with increased levels of cell proliferation and tissue protection by increased the levels of mucins secreted by goblet cells; and inflammation. ILC: innate lymphoid cells; NKT cells: Natural killer T cells; Th cells: T helper cells**.** Created with BioRender.com

1.4.1 Signaling pathways activated by IL-22

The binding of IL-22 to the IL-22R complex triggers activation of a number of intracellular signaling pathways, such as STAT and MAPK signaling transduction (Fig. 1.5). As other IL-10 family members, IL-22 induces activation of the Jak-STAT pathway through phosphorylation and activation of Janus kinase 1 (Jak1) and Tyk2[165,170,171]. Consequently, this leads to STAT3 phosphorylation mainly on the tyrosine-705 residue. However, phosphorylation of serine-727 has also been described [189]. Additionally, IL-22 can activate STAT1 and STAT5[189]. In the intestinal epithelium, IL-22-mediated STAT3 activation has been associated with mucosal wound healing in epithelial cells. Pickert *et al* have demonstrated that mice with impaired STAT3 activity presented susceptibility to colitis and defects in epithelial

regeneration, suggesting a regulation of intestinal homeostasis exerted by IL-22-mediated STAT3 activation[190], Sovran *et al* have shown that in Muc2 knockout mice, mucus barrier is compromised, leading to an increased exposure to microorganisms which in turn, triggered an upregulation of the IL-22/STAT3 pathway[191]. However, other studies have shown an opposite role after activation of the IL-22/STAT3 axis. In one study, the effect of IL-22 on tumor growth and metastasis *in vivo* was investigated using a colon cancer cell line in a model of subcutaneous cell transplantation. It was observed in an *in vitro* setting that tumor growth and expression of anti-apoptotic proteins were enhanced due to IL-22-mediated STAT3 activation[192]. Other studies pointed to a role exerted by the IL-22/STAT3 pathway in intestinal inflammation status increasing levels of inducible nitric-oxide-synthase (iNOS), a mediator of colonic inflammation and cancer development and upregulation of IL-10 in colon epithelial cells[193,194].

Another important signaling transduction induced by IL-22 is the MAPK pathway. Akil *et al* have demonstrated that IL-22 induced the activation of the MAPK signaling pathway leading to cell survival in a glioblastoma cell model which suggests a role of this pathway in tumorigenesis[195]. In another study using a gastric cancer model, IL-22 stimulated cancer cell invasion via phosphorylation of ERK1/2 (an important member of the MAPK cascade) as well as STAT3[196]. Additionally, in rat hepatoma cell line, it was observed that IL-22 induced activation of JNK, p38 and ERK1/2, members of the MAPK cascade[189]. In the intestinal context, using a colon cancer cell line as a model, one study investigated the effect of IL-20, IL-22 and IL-24 on epithelial proliferation and renewal. Only IL-22 expression was capable to induce cell proliferation via activation of ERK1/2 phosphorylation[197]. Similarly, another study has shown that IL-22-induced MAPK activation is able to induce proliferation of IECs through phosphorylation of MAPK cascade members, including JNK and ERK1/2[186]. Altogether, these studies suggest a crucial role for IL-22/STAT3 and IL-22/MAPK activation in promoting tumor growth, apoptosis inhibition and inflammation. Therefore, these pathways are an interesting target for therapeutic intervention in treatment of inflammatory diseases, including IBD.

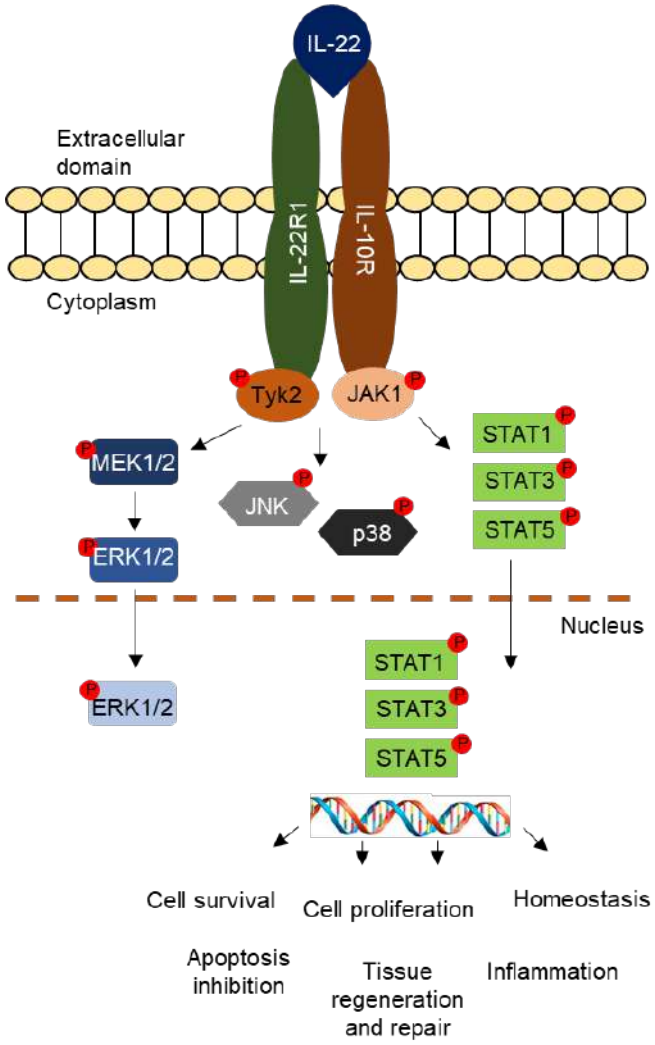

Figure 1.5: Signaling pathways active by IL-22. IL-22 binds to its receptor complex, a heterodimer composed of the two subunits IL22R1 and IL10R, which is mainly expressed in epithelial cells. Binding of IL-22 to IL-22R leads to activation of various signaling pathways, including MAPK (MEK1/2 and ERK1/2), JNK, p38 and STAT1, STAT3 and STAT5. Activation of these pathways is responsible for a variety of cellular functions, comprising cell survival, proliferation, apoptosis inhibition, tissue regeneration and repair, homeostasis and inflammation.

1.5 Celiac disease – a model disease for understanding barrier dysfunction

Celiac disease (CeD) is a T cell-mediated autoimmune disease affecting approximately 1% of the population in Western countries. CeD has a polygenetic background that is partially explained by the human leukocyte antigen (HLA)-DQ2 or DQ8 haplotype[198]. CeD is characterized by chronic, T-cell -dependent inflammation in the small intestine triggered by the ingestion of gluten, a protein mixture that is a major constituent of grains including wheat, barley and rye. The T-cell immune reaction guided towards gluten leads to a defective small intestinal mucosa with villous atrophy and crypt hyperplasia, thereby resulting in malabsorption of nutrients. However, studies have shown that, not only T-cells, but also monocytes and

macrophages, may contribute to the pathogenesis of celiac disease by affecting the barrier function of IECs. These cells recognize gluten peptides, release proinflammatory cytokines, which leads to adaptive responses with activation of lymphocytes, and subsequently, defects in the barrier of IECs[199]. Also, Innate immunity has an important role in barrier dysfunction especially through IL-15 secretion. Gliadin peptides (components of gluten) induce IL-15 expression by monocytes, consequently, it mediates innate immunity activating T cells which leads to enterocytes apoptosis[200]. Yokoyama et al have demonstrated that transgenic mice overexpressing human IL-15 showed villous atrophy as well severe duodenal-jejunal inflammation with high levels of NK-like $CD8^+$ lymphocytes. Using these mice, they observed that blockage of IL-15 levels restored intestinal homeostasis, suggesting that uncontrolled IL-15 expression may be associated to CeD development and maintenance[201].

The Fig. 1.6 shows an overview on CeD immune pathology. Gluten, especially the alcohol soluble prolamin fraction, called gliadin peptides in wheat, cross the intestinal epithelial barrier via paracellular and transcellular pathways to reach the lamina propria, where they are deamidated by the enzyme tissue transglutaminase2 (TG2). This process increases dramatically their affinity to the binding groove of the MHC-II antigen-presenting protein HLA-DQ2 or HLA-DQ8 Deamidated gliadin peptides that are taken up by professional APCs are thus very effectively presented to gliadin-specific CD4-positive T-cells. This is emphasized by the fact that nearly 100% of CeD patients express either HLA-DQ2.2, HLA-DQ2.5 or HLA-DQ8 haplotypes. The gliadin specificity of the $CD4^+$ T cells is determined by the expression of a T-cell receptor, that optimally binds the gliadin-MHC-II complex. Once activated, the CD4+ T cells secrete cytokines, stimulate $CD8^+$ T and NK (natural killer) cells, which contribute to enterocytes apoptosis, matrix metalloprotease activation in the small intestinal mucosa and B cell activation, thereby causing the hallmarks of CeD histopathology. At the same time, gluten- and TG2-specific B-cells differentiate into plasma cells and produce antibodies against deamidated gliadin peptides (DGPs) and TG2, which are used as specific markers of CeD[202]. Accurate diagnosis of CeD includes a positive celiac serology (anti-TG2-IgA antibodies) and a duodenal histology compatible with CeD, i.e. evidence for villous atrophy and crypt hyperplasia [203]. Determining the HLA status is in most cases facultative, since it has a low positive predictive value as approx. 30% of the western population are either HLA-DQ2- or HLA-DQ8-positive[204,205].

Currently, the only successful treatment for CeD is a strict gluten-free diet (GFD). This elimination diet prevents the intestinal mucosa from being exposed to gliadin, the antigen triggering the immune response and therefore leads to mucosal healing[206,207]. Although most of the CeD patients are successfully treated with a GFD, a significant percentage of patients, called refractory celiac disease (RCD) patients, does not respond to the treatment and may require additional therapeutic intervention. Despite strict adherence to a GFD for more than 12

months, these patients still present a persistence or a recurrence of malabsorption of nutrients and – in line with this – villous atrophy in the small intestinal histology. RCD patients are classified according their IEL population as RCD type 1, characterized by a normal IEL phenotype, with no evidence for T-cell receptor (TCR) clonality, or RCD type 2, characterized by an abnormal IEL phenotype. RCD type 2 patients show various evidence for abnormal IEL populations with loss of normal surface markers, including CD3 and CD8 but with expression of cytoplasmatic CD3ε and detection of clonal TCR rearrangements. While RCD type 1 patients improve after treatment with a combination of nutritional support, and immunosuppressive therapies, RCD type 2 patients have a significantly poorer prognosis, since they are threatened to develop an enteropathy-associated T-cell lymphoma (EATL), which in most cases is fatal. Thus RCD type 2 patients have a significantly reduced 5-year survival rate (approx. 60%) and are treated with cytostatic treatments (cladribine) or targeted approaches (anti-IL15, anti-CD52) to prevent transformation from RCD type 2 into an EATL[208].

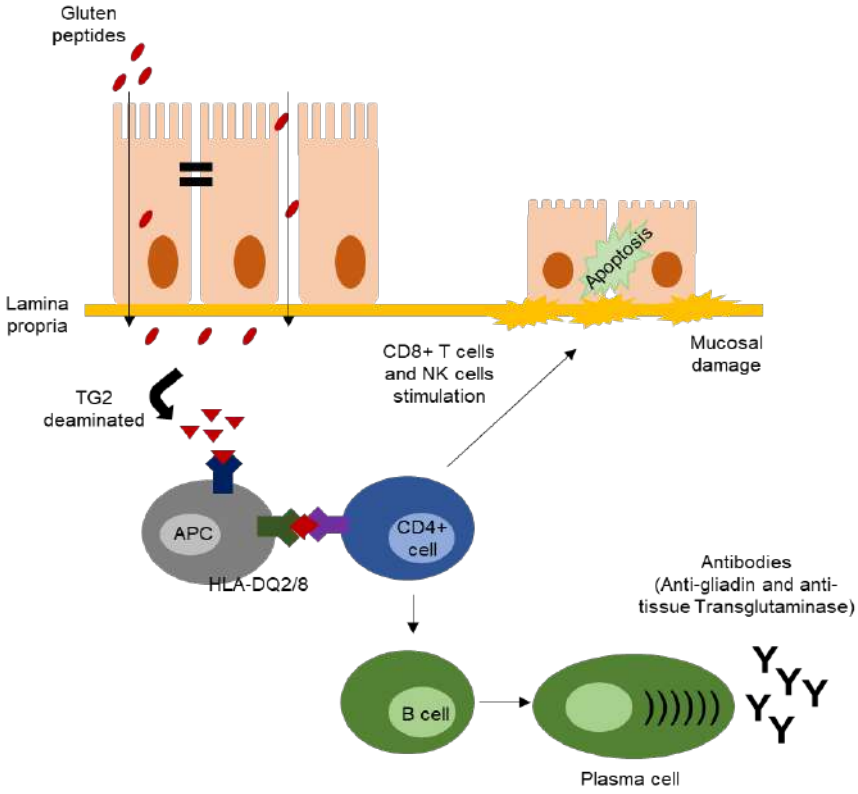

Figure 1.6: Celiac disease pathophysiology. Simplified scheme demonstrating the pathophysiology of celiac disease. Gluten peptides trigger the inflammatory reaction in CeD. Once ingested, gluten peptides cross the intestinal epithelial barrier via paracellular and transcellular pathways to the lamina propria, where they are deamidated by the enzyme TG2 (tissue transglutaminase 2). Subsequently, gliadin-specific CD4+ T cells are activated, leading to secretion of pro-inflammatory cytokines (IFN-γ, TNF-α, IL-21) contributing to activation of $CD8^+$ T cells and NK cells (causing enterocytes apoptosis and mucosal damage), differentiation of B cells into plasma cells and – secondary to that – production of antibodies. APC: antigen presenting cell; HLA: human leukocyte antigen; NK cells: natural killer cells.

1.5.1 Intestinal barrier function in celiac disease

Back in the 1970's, studies have reported first evidence for a dysfunctional mucosal barrier in CeD by analyzing the transfer of sugars from the intestinal lumen to the urine. These studies uncovered that CeD patients with villous atrophy had an increased permeability ratio to disaccharides and a decreased permeability to monosaccharides as evaluated by determining lactulose/mannitol, lactulose/L-rhamnose or cellobiose/mannitol ratios, proving alterations in intestinal barrier function in CeD patients for the first time[209–211].

The observed changes in permeability to sugars were interpreted to be caused by an increased paracellular permeability secondary to altered TJs. In accordance, Schulzke et al revealed a decreased number of TJ strands and an increased number of discontinued TJ strands in children with active CeD was noted. In children responding clinically to a GFD, the TJ network had recovered, but revealed a significantly smaller number of TJ strands when compared to healthy controls[212]. Further studies revealed that the epithelial resistance of small intestinal mucosa in active CeD patients – measured by one-path impedance spectroscopy – was reduced by approximately 50%. In addition, patients responding to GFD presented a significant recovery, although epithelial resistance was partially recovered compared to control individuals[213]. In line with the data previously described, paracellular permeability in active CeD is strongly related to changes in TJ proteins, as claudins (-2, -3, -4, -5 and -7) and ZO-1, and these alterations might be regulated by the cell polarity defining proteins Par-3 and PP-1, that also had been found changed[162]. Regarding RCD patients, our group published a study showing a reduction in epithelial resistance similar to active CeD patients, in comparison with control subjects. In addition, RCD patients presented alterations in TJ proteins levels with increased levels of claudin-2, a pore-forming claudin, and decreased levels of claudin-5, a barrier-forming claudin[214]. Altogether, these results point to alterations that may impact TJ assembly during active disease that are mostly reversed by GFD.

In an elegant study, the authors performed analyses of ZO-1 immunoprecipitation from biopsies specimens derived from active, GFD-treated CeD patients and controls[215]. Although they did not find any differences in ZO-1 and occludin total protein levels in ZO-1-immunoprecipitates, they found almost a complete decrease in anti-tyrosine-phosphorylated ZO-1 and anti-occludin levels in active CeD patients, in accordance with data found on immunofluorescence analysis by confocal microscopy. In AJ, the expression of β-catenin or E-cadherin were similar among the groups, however, β-catenin was found to be largely phosphorylated in small intestinal samples from active CeD patients. Using confocal LSM, the authors showed that β-catenin and E-cadherin stainings were intensively localized at the lateral membrane of the IECs in normal mucosa and treated CeD presented a similar pattern. Nonetheless, in active CeD, levels of β-catenin and E-cadherin staining and they were found

at the cell surface and in the cytoplasm as well. Therefore, alterations in epithelial resistance and TJ ultrastructure observed in the studies previously mentioned are associated with molecular changes in both TJ and AJ protein in CeD patients, mainly active CeD patients in comparison to control subjects. More studies have shown alterations of TJ structure and assembly, which may be responsible for the increase permeability observed in CeD. Szakál *et al* found changes in TJ content with increased levels of claudin-2 and -3 in proximal and distal duodenum biopsies of children with CeD compared with control tissue[216]. More recently, our group have performed further investigations on claudin protein levels and localization in duodenal biopsy samples from CeD patients[162]. We demonstrated decreased protein levels of occludin and increased levels of pore-forming claudins, as claudin-2 and -15; and decreased levels of barrier-forming claudins, as claudin-3, -5 and -7. In addition, claudin-2 was localized mainly to TJs of crypts of CeD patients Interestingly, claudin-5 and -15 were found in intracellular vesicles in CeD, which suggests that these TJ proteins were taken out of their normal function as sealing and pore-forming TJ-proteins in CeD. As observed by Ciaccocioppo *et al*[215], in our study, ZO-1 were reduced in protein levels and localization to the TJ and was rather spread along the whole lateral membrane as a sign for dyspolarization.

As mentioned above, immune cells may have a role in barrier regulation. In this context, monocytes/macrophages are strongly associated with the barrier function regulation, by secreting cytokines and/or interacting directly with IECs. Monocytes adhere to vascular endothelium and migrate through the tissue to reach the inflamed area and alter TJ structure and consequently, affecting the barrier function[217,218]. Cinova *et al* have shown that monocytes derived from CeD patients after gluten peptide exposure, secreted higher levels of proinflammatory cytokines, such as TNF-α and IL-8[199]. As mentioned before, IL-15 plays a pivotal role in CeD pathogenesis. Using peripheral blood mononuclear cells derived from healthy individuals, Harris *et al* have shown that IL-15-stimulated have a higher capacity to produce and secrete proinflammatory cytokines related to barrier dysfunction, including IL-6, IL-23, TNF-α and IL1β potentially contributing to CeD barrier dysfunction[219].

1.6 Aims

As detailed in the introduction section, several immunopathological conditions are thought to alter TJ assembly in intestinal epithelial cells, thereby causing barrier dysfunction. These conditions include exposure to pro-inflammatory cytokines and the interaction with specific immune cell subsets. In the present study, we intended to investigate alterations in TJ assembly under these conditions.

Thus, the aim of the study can be summarized by further specifying:

1. Understanding the impact of celiac monocytes on epithelial barrier function, specifically with regard to the integrity and composition of TJs by using a co-culture model of intestinal epithelial cells and monocytes isolated from CeD patients.

2. Understanding the impact of the TH17 cytokine interleukin-22 on intestinal epithelial barrier function and intestinal epithelial cell polarity using a cell culture-based model system.

2. Materials and methods

2.1 Materials

2.1.1 Devices

Table 2.1 Devices

Device	Version	Supplier
Centrifuge	PerfectSpin 24R Refrigerated Microcentrifuge	PEQLAB Biotechnology GmbH, Germany
	Universal 320R	Hettich, Wehingen, Germany
CO_2 incubator	Model CB-60 170 260	Binder GmbH, Tuttlingen, Germany
Electric pipetting device	Pipetboy acu	INTEGRA Biosciences, Zizers, Switzerland
Freezing Container	CoolCell® LX	Corning Inc., NY, USA
Heating block	AccuBlock™ Digital Dry Bath	Labnet International, Inc. NJ, USA
Inverted microscope	Olympus CK2	Olympus Optical Co. (Europa) GmbH, Hamburg, Germany
Lab Water System	Arium® pro UV Ultrapure Water System	Sartorius, Göttingen, Germany
Laminar Flow Workbench	SAFE 2020	Thermo Electron Corporation, Waltham, MA, USA
	LaminAir HB2472	Heraeus Instruments, Hanau, Germany
	HERA safe	Thermo Electron Corporation, Waltham, MA, USA
Laser-Scanning Microscope	LSM 780	Carl Zeiss Microscopy GmbH, Jena, Germany
Magnetic stirring	Ikamag® REO/RCT	Merck, Darmstadt, Germany
Micropipette	10, 100, 200 and 1000 µL	Eppendorf, Hamburg, Germany
Microplate reader	Tecan Infinite M200 PRO (Absorbance 96 well plates)	Tecan Trading AG, Switzerland
	Tecan Sunrise (Absorbance 96 well plates)	Tecan Trading AG, Switzerland
Mini centrifuge	Sprout	Thermo Fisher Scientific Inc., Waltham, MA, USA
Objective 63x Immersion oil	Plan-Apochromat 63x/1.4 Oil DIC M27	Carl Zeiss Microscopy GmbH, Jena, DE
Osmometer	Osmomat 3000	Gonotec®, Berlin, Germany
pH meter	HI 9017 microprocessor	Hanna Instruments, Kehl, Germany
Power supply	Blotting device 200/2.0	Bio-Rad Laboratories GmbH, Munich, Germany

Resistance measuring device	--	Institut für Klinische Physiologie, CBF, Charité Berlin, Germany
Scale	--	Musahl Waagenservice GmbH, Berlin, Germany
Shaker	Rocking platform VWR	VWR International GmbH, Vienna, Austria
	Rocking platform WT12	Biometra, Göttingen, Germany
	Rocking platform WT17	Biometra, Göttingen Germany
Vortex device	LSE™ Vortex mixer	Corning Inc., NY, USA
Water bath	GFL 1083	Burgwedel Biotech GmbH, Rheinland-Pfalz, Germany
	B4E5	Medingen, Dresden, Germany
	SW20C (Shaking)	JULABO GmbH, Seelbach, Germany

2.1.2 Consumable supplies

Table 0.2. Consumables supplies

Consumable supplies	Supplier
25 cm^2-tissue culture flask	Corning Inc., NY, USA
75 cm^2-tissue culture flask	Corning Inc., NY, USA
15 mL PPN tube	Corning Inc., NY, USA
50 mL PPN tube	Greiner Bio-One GmbH, Frickenhausen, Germany
6-well-tissue culture plate (10 cm^2 per well)	Corning Inc., NY, USA
12-well-tissue culture plate (4 cm^2 per well)	Corning Inc., NY, USA
24-well-tissue culture plate (2 cm^2 per well)	Corning Inc., NY, USA
96-well microplates for BCA assays	Corning Inc., NY, USA
Biosphere filter tips 10, 200, 1000 µL	SARSTEDT AG & CO. KG, Nümbrecht, Germany many
Cell culture dish (35x10 mm)	SPL, Life Sciences
Stirring rod 120 mm	SARSTEDT AG & CO. KG, Nümbrecht, Germany
CryoPure Tubes 1.6 mL	SARSTEDT AG & CO. KG, Nümbrecht, Germany
Gel-Blotting-papers, Whatman® 3MM	Carl Roth, Karlsruhe, Germany
Microscope slides	Menzel/Glaser, Braunschweig, Germany
Microtiter plate 96 wells (Round bottom)	SARSTEDT AG & CO. KG, Nümbrecht, Germany
Serological Pipet (5, 10 and 25 mL)	Corning Incorporated, NY, USA

Polyscreen (R) PVDF transfer membrane	PerkinElmer, Boston, MA, USA
SafeSeal tube 1.5 mL	SARSTEDT AG & CO. KG, Nümbrecht, Germany
Safe-Lock tubes 0.5 mL	Eppendorf AG, Hamburg, Germany
Surgical disposable scalpels	B Braun™, Thermo Fisher Scientific
Syringe 20 mL	BD Discardit™ II, Spain
Tissue culture dish (100x20 mm)	Corning Incorporated, NY, USA
Transwell filters (Millicell-HA, 0,6 cm²)	MilliporeSigma, Darmstadt, Germany

2.1.3 Chemicals and kits

Table 2.3 Chemicals and kits

Chemicals	Supplier
1,4 Dithiothreitol (DTT)	Sigma-Aldrich Chemie GmbH, Steinheim, Germany
4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)	Sigma-Aldrich Chemie GmbH, Schnelldorf, Germany
4', 6-diamidino-2-phenylindole (DAPI)	Sigma-Aldrich Chemie GmbH, München, Germany
4-kDa dextran (Lot: 181176 and 200190)	Serva, Heidelberg, Germany
4-kDa FITC-dextran (FD4)	TdB Consultancy, Uppsala, Sweden
β-Mercaptoethanol	Clontech, Heidelberg, Germany
Ammonium persulfate (APS)	Sigma-Aldrich, Schnelldorf, Germany
AquaResist	VWR International GmbH, Vienna, Austria
BCA-Protein Assay (Reagents A and B)	Pierce, Rockford, Illinois, USA
Bovine serum albumin (BSA)	Biomol GmbH, Hamburg, Germany
Calcium chloride, dihydrate	Carl Roth GmbH, Karlsruhe, Germany
Carbogen	Linde AG, München, DE
cOmplete mini, EDTA free, Protein Inhibitor Cocktail	Roche, Basel, Switzerland
DMSO (cell culture quality)	Carl Roth GmbH, Karlsruhe, Germany
Dulbecco's PBS with Mg^{2+}/Ca^{2+}	Gibco, Waltham, Massachusetts, U.S.
Dulbecco's PBS without Mg^{2+}/Ca^{2+}	Gibco, Waltham, Massachusetts, U.S.
Emersion oil for microscopy	VWR International GmbH, Darmstadt, Germany
Ethanol 100%	Fisher scientific, UK/Acros organics, Belgium
	T.J. Baker, Poland
Ethanol 80%	Chemsolute, TH Geyer, Renningen, Germany
Fetal Bovine Serum (FBS)	Gibco, ThermoFisher Scientific, Berlin, Germany
Glucose	Carl Roth GmbH, Karlsruhe, Germany
Glycine	Carl Roth GmbH, Karlsruhe, Germany
Hydrochloric acid (32%)	Merck, Darmstadt, Germany
Methanol	Merck, Berlin, Germany

Milk powder (Blotting grade blocker non-fat dry milk)	Carl Roth, Karlsruhe, Germany
N,N,N',N'-Tetramethylethylenediamine (TEMED)	ThermoFisher Scientific, Waltham, MA, USA
PageRuler Plus Prestained Protein Ladder	ThermoFisher Scientific, Waltham, MA, USA
Paraformaldehyde 16%	Electron microscopy sciences, Hatfield, PA, USA
Penicillin/Streptomycin (P/S)	Corning, Manassas, VA, USA
Polyacrylamide mix (30%)	Carl Roth GmbH, Karlsruhe, Germany
Potassium chloride	Carl Roth GmbH, Karlsruhe, Germany
ProTaqs® MountFluor	Biocyc, Luckenwalde, Germany
Puromycin (Hydrochloride)	Cayman Chemical Company, Michigan, USA
Sodium azide	Carl Roth GmbH, Karlsruhe, Germany
Sodium chloride	Carl Roth GmbH, Karlsruhe, Germany
Sodium dihydrogen phosphate, monohydrate	Carl Roth GmbH, Karlsruhe, Germany
Sodium Dodecyl Sulfate (SDS)	Carl Roth GmbH, Karlsruhe, Germany
Sodium hydrogen carbonate	Carl Roth GmbH, Karlsruhe, Germany
Sodium hydroxide	Fisher scientific, UK/Acros organics, Belgium
Sodium phosphate dibasic, dodecahydrate	Carl Roth GmbH, Karlsruhe, Germany
SuperSignal™ West Pico PLUS Luminol/Enhancer solution	ThermoFisher Scientific, Waltham, MA, USA
SuperSignal™ West Pico PLUS Stable peroxide solution	ThermoFisher Scientific, Waltham, MA, USA
Tris-Base	Carl Roth GmbH, Karlsruhe, Germany
Tris-Hydrochloride	Carl Roth GmbH, Karlsruhe, Germany
Triton X-100	Roche, Basel, Switzerland
Trypsin/EDTA	Biochrom, Berlin, Germany
Tween-20	Fisher scientific, UK/Acros organics, Belgium
Water for molecular biology	MilliporeSigma, Darmstadt, Germany
Water for cell culture	MilliporeSigma, Darmstadt, Germany
Water (HPLC Gradient Grade)	J.T. Baker, Gliwice, Poland

2.1.4 Buffers and solutions

Table 2.4 Buffers and solutions

Buffers and solutions	Composition
Immunofluorescence labeling	
Blocking buffer	PBS (pH 7.4) 5% goat serum 1% BSA 0.05% Tween 20 0.01% Triton X-100
Washing buffer	PBS (pH 7.4) 1% BSA
Protein purification	
Cell lysis extraction buffer	10 mM Tris-HCl pH 7.5 150 mM NaCl 0.5% Triton X-100 0.1% SDS protease inhibitor (1 tablet per 10 mL)

2.2 Methods

2.2.1 Information on enrolled patients

The study was approved by the Ethics Committees of the Charité – Universitätsmedizin Berlin, Germany (protocol number EA4/116/18, accepted on Jan 22nd, 2019). Heparinized whole blood samples were collected from healthy individuals and CeD patients. Inactive (GFD) patients received a GFD for >1 year. All patients declared their informed consent (signed consent form). Healthy controls were individuals without a history of enteropathy and without clinical signals of CeD or other autoimmune diseases (Table 2.5). Further characteristics of CeD patients is described in Table 2.6.

Table 2.5: CeD patients enrolled in the study

Number of subjects		17
Female/Male		14/3
Age at enrolment, median (range)		46 (23-83)
Age at CeD diagnosis, median (range)		32 (6-73)
Marsh Grade at enrolment, n (%)		
	0	5 (29)
	1	2 (12)
	2	0 (0)
	3a	1 (6)
	3b	3 (18)
	3c	0 (0)
	not available	6 (35)
tTG at enrolment, n (%)		
Positive		6 (35)
negative		2 (12)
not available		9 (53)
HLA DQ status, n (%)		
DQ2+		11 (65)
DQ8+		0 (0)
not available		6 (35)
GFD status, n (%)		
Active CeD		6 (35)
- New CeD diagnosis		4
- CeD, non-compliant to GFD		2
CeD on GFD		11 (65)

CeD, celiac disease; tTG, transglutaminase antibodies; GFD, gluten-free diet.

Table 2.6: Characteristics of CeD patients enrolled in the study

Patient ID	Sex	Age	Age at diagnosis	Group	GFD	HLA-DQ status	Data at CeD diagnosis		Data at enrolment*	
							tTG#	Marsh	tTG#	Marsh
1	Female	33	32	CeD on GFD	yes	n.a.	41.9 U/mL	3a	15.5 U/mL	n.a.
2	Female	47	45	active CeD	no (non-compliant)	DQ2+	190 U/mL	3b	n.a.	n.a.
3	Female	24	24	active CeD	no (new diagnosis)	DQ2+	84 U/mL	1	-	-
4	Male	33	29	CeD on GFD	yes	n.a.	n.a.	n.a.	n.a.	n.a.
5	Female	28	28	active CeD	no (new diagnosis)	DQ2+	>200 U/mL	3b	-	-
6	Female	26	26	active CeD	no (new diagnosis)	DQ2+	35 U/mL	3b	-	-
7	Female	46	32	CeD on GFD	yes	DQ2+	n.a.	n.a.	1.8 U/mL	n.a.
8	Female	46	42	CeD on GFD	yes	DQ2+	>200 U/mL	3b	2.2 U/mL	0
9	Female	60	51	CeD on GFD	yes	DQ2+	positive	3a	1.6 U/mL	0
10	Female	57	50	CeD on GFD	yes	n.a.	positive	3b	2.9 U/mL	0
11	Female	65	6	CeD on GFD	yes	n.a.	n.a.	3	3.9 U/mL	n.a
12	Female	40	31	CeD on GFD	yes	n.a.	positive	3b	2.2 U/mL	0
13	Female	59	41	CeD on GFD	yes	DQ2+	32 U/mL	3c	2.7 U/mL	1
14	Male	74	n.a.	CeD on GFD	yes	DQ2+	n.a.	n.a.	3.3 U/mL	0
15	Female	55	n.a.	CeD on GFD	yes	n.a.	n.a.	n.a.	1.3 U/mL	n.a.
16	Female	23	23	active CeD	no (new diagnosis)	DQ2+	38 U/mL (IgG)	3a	-	-
17	Male	83	73	active CeD	no (non-compliant)	DQ2+	positive	3	23 U/mL	3b

GFD, gluten-free diet; CD, celiac disease; tTG, transglutaminase antibodies (IgA unless otherwise specified); n.a., data not available
*in case of new diagnosis, data at enrolment coincide with data at diagnosis
normal values for tTG: <10 U/ml, in some cases by values >10 U/ml only “positive” was reported in the clinical records

2.2.2 Isolation of peripheral blood mononuclear cells and magnetic CD14$^+$ cell sorting

Peripheral blood mononuclear cells (PBMCs) were isolated from peripheral blood collected from healthy donors or celiac disease patients. PBMCs isolation was performed by Biocoll (Merk Millipore) with density gradient centrifugation. Subsequently, PBMCs were sorted using CD14 MACS MicroBeads (Miltenyi Biotech, Bergisch Gladbach, Germany) according to manufacturer's instruction. Preparations containing >90% CD14$^+$ cells were used, as determined by flow cytometry. CD14$^+$ cells were plated in 24-well dishes with RPMI-1640 as media (Gibco), supplemented with 10% of fetal bovine serum (Gibco) and 1% of penicillin and streptomycin (Corning). CD14$^+$ cells were exposed to human granulocyte macrophage colony stimulating factor (GM-CSF; 10 ng/mL) for 24 h before they were transferred to the co-culture. Cell culture supernatants were collected for further cytokine analysis.

2.2.3 Cell culture

The human colon epithelial cell lines, CacoBBe, Caco-2 and HT29/B6 were maintained in Dulbecco's Modified Eagle's Medium (DMEM) + GlutaMAX (Gibco), with 10μM HEPES-buffer and 1M non-essential amino acids (Merck Millipore), Minimum Essential Medium Eagle's (MEM – Gibco) and RPMI-1640 supplemented with 10% fetal bovine serum (FBS, Gibco), 1% penicillin and streptomycin (Corning), respectively. Cells were kept at 37° C in a 5% CO_2 environment. Culture medium was changed three times per week. Cells were treated with IL-13 (Prepotech, Hamburg, Germany; 10 ng/ml) and IL-22 (Biolegend; 10 ng/ml) at different times and concentrations. In some experiments, IEC filters were basolaterally exposed to other pro-inflammatory cytokines including TNF-α (1000 U/ml); IFN-γ (100 U/ml); TGF-β1 (20 ng/ml). These cytokines were from Peprotech (Hamburg, Germany).

2.2.4 Co-culture with monocytes

Intestinal epithelial cells (CacoBBe) were plated on permeable transwell polycarbonate filter supports (0.4 μm; 0.6 cm^2, Merck Millipore) and kept at 37 °C in a 5% CO_2 environment. Culture medium was changed three times per week. On days 10 to 16 after plating, filters were transferred to 24-well dishes containing CD14$^+$ cells (5x10^5 cells per well). In addition, IL-15/Tglia (10 mg/ml) was added (gift by W. Dieterich; LPS-free) to filters with IECs.

2.2.5 Transepithelial electrical resistance

IECs were seeded on PCF filters (0.4 µm; 0.6 cm2, Merck Millipore) and grown to confluence for 7, 10 and 12-14 days in culture at 37 °C in a 5% CO2 environment, respectively. IL-22 (Biolegend; 10 ng/ml) was added to the apical and basolateral compartment of the transwell filters for the indicated times (24 and 48 h) and transepithelial resistance (TER) was measured using chopstick electrodes.

2.2.6 Immunostaining of IECs

Epithelial cell layers were washed 3x with PBS, then fixed with PFA 4% pH 7,5 and kept in 4 °C with PBS for maximally seven days prior to immunostaining. Epithelial cell layers were stained using the following primary antibodies: ZO-1 (1:100; BD Biosciences, NJ, USA). For immunstaining with secondary antibodies Alexa Fluor 488 goat anti-mouse or rabbit IgG, and Alexa Fluor 594 goat anti-mouse or rabbit IgG were selected (1:500; Thermo Fisher Scientific, MA, USA). To determine occludin expression and cellular distribution, an occludin mouse monoclonal antibody (OC-3F10) was used as an Alexa Fluor® 594 Conjugate (Thermofischer). Nuclei were stained using DAPI (4′,6-Diamidin-2-phenylindol, conc. 1:2000). Immunofluorescence staining was analyzed by confocal laser scanning microscopy (LSM 780, Carl Zeiss, Jena).

2.2.7 Caco-2 3D cysts

Caco-2 cells (1 x 10^4 cells) were seeded in freshly prepared Matrigel (150 µl, Corning), which was kept a 4°C to ensure the fluidic character of the Matrigel while implanting the cells in the Matrigel. Lab-tek slides (ThermoFischer) were then kept for 30 minutes in 37 °C 5% CO_2. Subsequently, 500 µl of media (Minimum Essential Medium Eagle's - MEM – Gibco, supplemented with 10% of fetal bovine serum – Gibco) was added and cells were left to grow for 3 to 5 days in 37 °C 5% CO_2, Cells were fixed with 4% paraformaldehyde pH 7,5 for 30 minutes then the Lab-teks were kept in 4 °C with PBS for a maximum of 7 days and immunofluorescence was performed.

2.2.7.1 Immunostaining of 3D Caco-2 cysts

For immunostaining, cells were washed with PBS+ and fixed using PFA (4%, pH 7.5) for 30 min at RT. Extensive PBS+ washes were followed by permeabilization/blocking using PBL-solution (0.7% fish skin gelatin and 0.025% saponin, in PBS+; 2 h, RT), followed by PBS-washes and quenching using 75 mM NH4Cl and 20 mM glycine in PBS+ (10 min, RT). After one wash using PBL, cells were incubated overnight with PFA-fixed 3D-cysts at 4°C with E-cadherin antibody (1:100; Alexa Fluor647-conjugate, BD Biosciences, San Jose, CA, USA) and DY-594-phalloidin (1:100; Dyomics, Jena, Germany) to stain actin. Nuclei were stained using DAPI (4′,6-Diamidin-2-phenylindol, 1:2000) for 1.5 h at room temperature. Microscopy was performed using a confocal laser scanning microscopy (LSM 780, Carl Zeiss, Jena).

2.2.8 Treatment with inhibitors of signaling pathways

To inhibit STAT3 phosphorylation, various inhibitors were used. Stattic and STAT3 Inhibitor IV (S31-201) are cell-permeable molecules that inhibit by selective binding of the STAT3-SH2 domain impairing STAT3 activation, dimerization and nuclear translocation [220–222]. Furthermore, a cell-permeable peptide analogue, which is also a selective blocker of STAT3 activation, was used[223]. As an indirect inhibitor, WP1066 was used, blocking STAT3 phosphorylation by binding to JAK2, a kinase upstream of STAT3 [224,225]. To inhibit the MAPK signalling, the inhibitor U0196 was used. It acts as a selective inhibitor of MEK1 and MEK2 preventing activation of MAP kinases p42 and p44 (ERK1/2) [226]. Specifically, after seven days in culture, HT29/B6 cells growing on transwell filters were exposed to the inhibitors mentioned above for two hours (Table 2.7). Subsequently, IL-22 (10 ng/ml) was added for one hour and then cells were lysed, or were kept for a maximum of 72 hours to determine TER (48 and 72 h) and prepare cell lysate in order to perform Western blotting.

Table 2.7: Inhibitors characteristics

STAT3 Inhibitors	Manufacturer	Concentration (µM)	Catalogue number
Stattic	Calbiochem (San Diego, USA)	0.1; 3; 10	573099
WP1066	Calbiochem (San Diego, USA)	10; 50	573097
STAT3 Inhibitor VI (S3I-201)	Calbiochem (San Diego, USA)	100	573192
Cell permeable – STAT3 Inhibitor Peptide	Calbiochem (San Diego, USA)	100	573096
MAPK/ERK Inhibitor	**Manufacturer**	**Concentration (µM)**	**Catalogue number**
U0126	Biogems International (Westlake Village, USA)	10	1095821

2.2.9 Real-time quantitative PCR

2.2.9.1 RNA extraction

Total RNA was extracted using the *mir*Vana™ mRNA Isolation Kit (Thermo Fisher) according to manufacturer's instructions. Briefly, IECs (HT29/B6 cells) after IL-22 (10 and 100 ng/ml) exposure for 5 and 30 hours were scraped with Lysis Binding buffer, and the RNA Homogenate solution was added at a 1/10 of the Lysis buffer volume. Samples were vortexed and kept on ice for 10 minutes. A volume equal to the Lysis buffer of Phenol Chloroform was added, then samples were thoroughly vortexed and centrifuged at 10000 x g for 5 minutes at room temperature. The aqueous phase was collected in a new tube and 1.25 volume of 100% ethanol was added. Samples were transferred to filter cartridges and washed once with washing buffer 1 and twice with washing buffer 2/3. The RNA was then eluted in nuclease-free water at 95 °C and stored at -80 °C.

2.2.9.2 RNA quantification and cDNA synthesis

To quantify the extracted RNA, RNA concentration was determined using the NanoDrop 1000 (Thermo Fisher) was. 800 ng to 1 µg of total RNA was used to synthetize cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer's instructions. According to Table 2.8, reactions were prepared and the reverse transcription reaction was performed through one cycle at 25 °C for 10 minutes, one cycle at

37 °C for 120 minutes and one cycle at 85 °C for 5 minutes. Samples were stored at -80 °C until qPCR reaction was performed.

Table 2.8: Reverse transcription mixes

RT Master Mix	Volume per reaction:
10x RT Buffer Mix	2 µl
10x Random Primers	0.8 µl
25x dNTP Mix (100mM)	2 µl
MultiScribe™ Reverse Transcriptase (50 U/µl)	1 µl
Nuclease-free H_2O	4,2 µl
RNA sample	**Volume per reaction:**
RNA sample	up to 10 µl
Nuclease-free H_2O	Q.S.* to 10 µl
Total per reaction	20 µl

*Q.S. = Quantity sufficient

2.2.9.3 qPCR reaction

Real time-qPCR reactions were performed using 1 µL of cDNA template, 1 µL of the desired probe, 10 µL of RT-qPCR Master Mix (Applied Biosystems) and nuclease-free water to a final volume of 20 µL. Comparative CT reactions were performed in triplicates using the StepOnePlus™ instrument (Applied Biosystems). Calculations for gene expression changes were performed using the $2^{-\Delta\Delta CT}$ method. Human probes (Applied Biosystems) used were *SNAI1* (Hs00195591_m1), *SNAI2* (Hs00161904_m1), *MMP -2* (Hs01548727_m1)*, -7* (Hs01042796_m1) *and -9* (Hs00957562_m1)*. ACTB* (Hs01060665_g1) was used as control of the reaction amplification.

2.2.10 Flow cytometry assessment – surface markers and cytokine expression analysis

CD14+ cells were washed twice with PBS, and the surface markers described in the Table 2.9, were checked. Dead cells were excluded by DAPI staining. Samples were assessed by flow cytometry using a FACSCanto II and the FACS Diva software (version 6; BD Biosciences). Supernatants of the cultures after 24 h of culture with GM-CSF (10 ng/ml) were tested for cytokine expression (IL-1, IFN-α2, IFN-λ, TNF-α, MCP-1, IL-6, IL-8, IL-19, IL-12p70,

IL-17A, IL-18, IL-23 and IL-33) using the LEGENDplex Multi-Analyte Flow Assay kit–Human Inflammation Panel (13-plex) (Biolegend) according to the manufacturer's protocol. FACS data were analyzed using FlowJo (v10.6.1) and LEGENDplex v8.0 software (BioLegend, San Diego, CA, USA).

Table 2.9: Antibodies for flow cytometry

Antigen	Channel	Clone number	Dilution	Manufacturer	Catalogue number
CD80	FITC	2D10.4	1:40	eBioscience (San Diego, USA)	11-0809-42
CD16	PE	3G8	1:40	Biolegend (San Diego, USA)	302056
HLA-DR	PerCP-Cy5.5	LN3e	1:100	eBioscience (San Diego, USA)	45-9956-42
CD163	PE-Cy7	GHI/61	1:20	Biolegend (San Diego, USA)	333606
CD14	APC	MφP9	1:40	BD Biosciences (Franklin Lakes, USA)	340436
CD11b	APC-Cy7	ICRF44	1:100	Biolegend (San Diego, USA)	301342

2.2.11 Western Blotting

For protein quantification, epithelial cells were washed twice with ice-cold PBS^+. Protein extraction was done using ice-cold lysis buffer (150 mM NaCl, 10 mM Tris buffer pH of 7.5, 0.5% Triton X-100, and 1% SDS). A volume of 10 ml lysis buffer was supplemented with one Complete Protease Inhibitor Cocktail tablet; Roche AG, Basel, Switzerland). Cells were scraped from the filters, incubated for 60 min on ice, and vortexed every 10 min. The supernatant was collected after centrifugation (30 min, 15,000× g at 4 °C). To determine the protein content, Pierce BCA assay (Thermo Scientific, Waltham, MA, USA) was performed according to the product instructions using a Tecan plate reader (Tecan GmbH, Maennedorf, Switzerland) at an absorbance of 562 nm. Protein samples (20 µg) were mixed with 5xLaemmli buffer and loaded on SDS polyacrylamide gel (premade). After electrophoretic separation, proteins were transferred to a PVDF membrane (Thermo Scientific, Waltham, MA, USA) using the Trans-Blot system (Bio-Rad Laboratories, Inc., Hercules, CA, USA) at 25 V for 7-10 min and membranes were blocked for 2 h at RT with 1% PVP-40 (Polyvinylpyrrolidone; Sigma Aldrich, St. Louis, MO, USA) in TBST/0.05% Tween-20 buffer. Primary antibodies (Table 2.10)

were incubated overnight at 4 °C. Peroxidase-conjugated secondary antibodies were incubated for 2 h at RT. For protein detection, SuperSignal West Pico PLUS Stable Peroxide Solution (Thermo Scientific, Waltham, MA, USA) was used and signals were detected with Fusion FX7 imaging system (Vilber Lourmat Deutschland GmbH, Eberhardzell, Germany).

Table 2.10: Primary antibodies for Western Blotting

Antigen	Dilution	Host animal	Manufacturer	Catalogue number
AKT	1:1000	Rabbit	Cell Signaling Technology (Frankfurt am Main, Germany)	9272S
pAKT (Thr308)	1:1000	Rabbit	Cell Signaling Technology (Frankfurt am Main, Germany)	9275S
ERK	1:1000	Rabbit	Cell Signaling Technology (Frankfurt am Main, Germany)	4695S
pERK	1:1000	Rabbit	Cell Signaling Technology (Frankfurt am Main, Germany)	91015S
STAT3	1:1000	Rabbit	Cell Signaling Technology (Frankfurt am Main, Germany)	30835S
pSTAT3 (Tyr705)	1:1000	Rabbit	Cell Signaling Technology (Frankfurt am Main, Germany)	9145S
Claudin 1	1:1000	Rabbit	Invitrogen (Carlsbad, USA)	51-9000
Claudin 2	1:1000	Mouse	Invitrogen (Carlsbad, USA)	516100
Claudin 4	1:1000	Mouse	Invitrogen (Carlsbad, USA)	329400
E-cadherin	1:1000	Mouse	Cell Signaling Technology (Frankfurt am Main, Germany)	14472S
JAM-A	1:1000	Rabbit	Thermo Fisher Scientific (Massachusetts, USA)	36-1700
β-actin	1:1000	Mouse	Sigma Aldrich (St. Louis, USA)	A5441
MMP-7	1:500	Rabbit	Abcam (Cambridge, UK)	EPR17888-71

The peroxidase-conjugated secondary antibodies used were goat anti-rabbit IgG or goat anti-mouse IgG (Jackson ImmunoResearch, Ely, UK) in 1:10000 dilution.

2.2.12 Statistical analysis

Statistical analysis was performed using the GraphPad Prism software (GraphPad Software, La Jolla, CA). For descriptive statistics, means and standard error of the mean values (SEM) were calculated. For performing inferential statistics, the non-parametric Mann–Whitney U test was applied. $p < 0.05$ was considered significant.

3. Results

3.1 Part one: Monocytes isolated from patients with celiac disease - effect on intestinal barrier function

3.1.1 Monocytes isolated from patients with celiac disease disrupt intestinal barrier function

As outlined in the *Introduction*, it has previously been described that barrier integrity is altered in CeD patients. To investigate whether monocytes isolated from the peripheral blood of patients with celiac disease contribute to a dysfunctional intestinal barrier and alterations in barrier integrity, CacoBBe cells, an intestinal epithelial cell (IEC) line were co-cultured with CeD monocytes. To determine epithelial barrier function, TER was measured as described in the *Material & Methods* section.

Firstly, peripheral blood mononuclear cells (PBMCs) were isolated from peripheral blood derived from healthy individuals and celiac disease patients with active (ACeD) or inactive (GFD: gluten-free diet) celiac disease. PBMCs were sorted for CD14 expression and seeded onto 24-well plates. In parallel, IECs were seeded on transwell filters until confluence was reached and a stable barrier function had established. Transwells with IECs seeded on the filter membrane were then transferred to 12-wells containing CeD monocytes (Fig 3.1). It is relevant to emphasize that no direct contact of IECs and $CD14^+$ monocytes can occur in this model as the two cell types are separated from each other. Thus, the interaction presumably exclusively takes place through soluble factors in the media that are capable of passing the filter membrane.

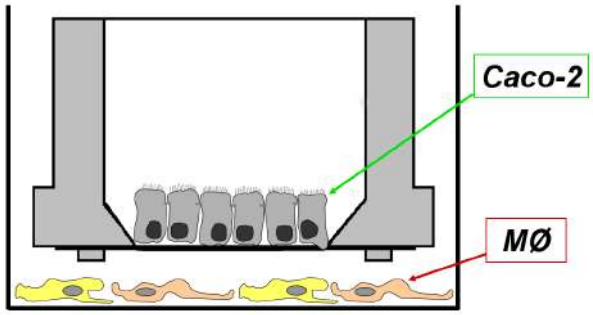

Figure 3.1: Setup of the IEC-human CeD monocyte co-culture model. A transwells with IECs seeded on the filter membrane was transferred to a well within a 12-well-dish containing previously seeded CeD monocytes.

After 48 h of co-culture, the presence of monocytes derived from patients with CeD, and independent of their disease status, resulted in a significant reduction in TER when compared to IECs exposed to monocytes derived from healthy controls (Fig. 3.2A). Similar effects on TER were observed, when IECs were co-cultured with unsorted PBMCs (Fig. 3.2B). Thus, these data provide evidence that CeD-derived monocytes are capable of destabilizing IEC barrier function.

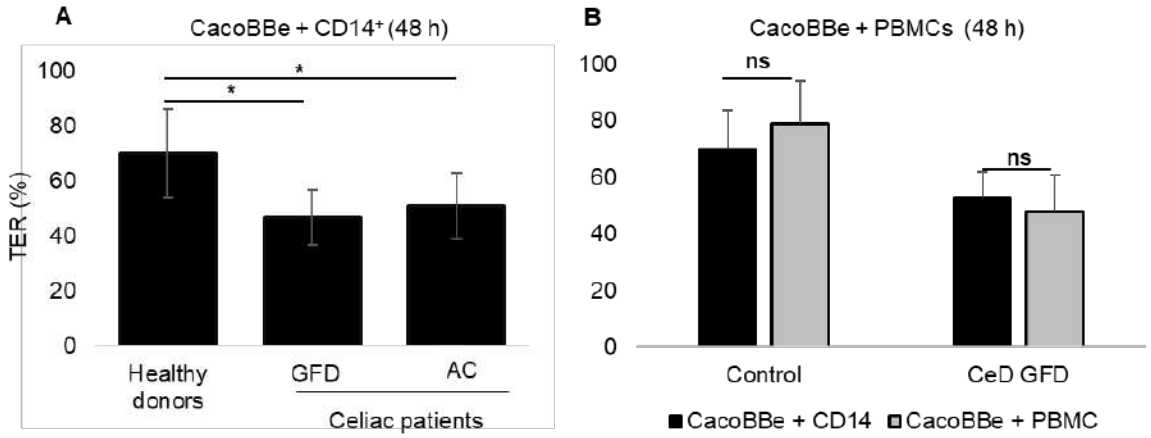

Figure 3.2: Effects on epithelial integrity after co-culture with mononuclear cells. (**A**) Peripheral blood mononuclear cells were isolated and CD14+ cells purified as described in the *Methods* section. Intestinal epithelial cells were co-cultured with CD14+ monocytes from healthy donors or celiac disease patients on gluten-free diet (GFD) or active disease (ACeD). The transepithelial resistance (TER) was measured after 48 h of co-culture and expressed as % of TER prior to addition of monocytes. Mean TERs of the analysis of n=36 (healthy donors), n=15 (GFD) and n=20 (AC) individual filters are shown. Monocytes used for these experiments were isolated from n=8 (healthy donors), n=4 (GFD) and n=5 (ACeD). (**B**) Co-culture of IECs with total PBMCs from healthy donors or CeD patients on GFD (CeD GFD) was performed. TER was measured after 48 h of co-culture. The mean of n=8 (healthy donors), n=8 (GFD) individual filters measurements is shown. Monocytes used for these experiments were isolated from n=2 (healthy donors) and n=2 (GFD). Mann-Whitney U *$p<0.05$, comparison between co-cultures with monocytes from healthy donors and CeD patients; ns: non-significant.

Gliadin is the alcohol-soluble protein component of the protein mixture gluten, capable of inducing CeD immune pathology. It can initiate CeD inflammation mainly mediated by T-cells leading to mucosal damage. The next experiments aimed at exploring whether gliadin exerts a direct effect on IECs or on monocytes. Thus, IECs were exposed to IL-15/Tglia in the presence or absence of $CD14^+$ monocytes isolated from either healthy controls or patients with celiac disease. The presence of IL-15/Tglia alone did not affect the TER. The combined addition of IL-15/Tglia and $CD14^+$ monocytes did not differ from the effects described for the exclusive presence of $CD14^+$ monocytes from healthy controls or monocytes (Fig. 3.2). These results indicate that the effect on epithelial barrier integrity observed after co-culture of IECs with $CD14^+$ monocytes is gliadin-independent and appears to be predominantly mediated by the celiac monocytes.

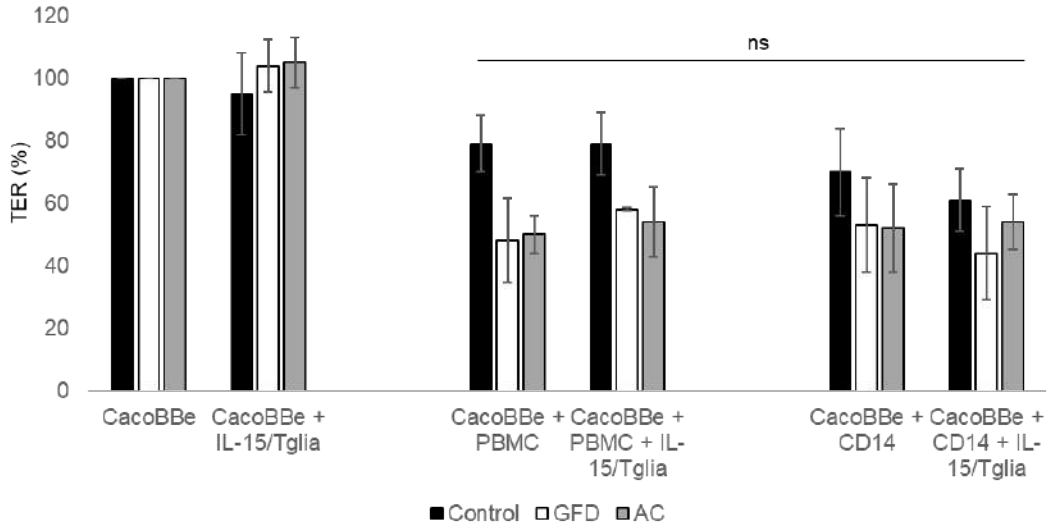

Figure 3.3: Comparison of the effects on epithelial integrity after addition of IL-15/Tglia on CacoBBe cells with or without monocytes or PBMCs exposure. Intestinal epithelial cells were co-cultured with CD14+ monocytes or total PBMCs isolated from healthy donors or celiac disease patients on glten-free diet (GFD) or with active disease (AC). Subsequently, the TER was measured after 48 h of co-culture and expressed as % of TER prior to addition of monocytes. The mean of n=36 (healthy donors), n=15 (GFD) and n=20 (AC) individual filters measurements is shown. Monocytes used for these experiments were isolated from n=8 (healthy donors), n=4 (GFD) and n=5 (active CeD). Mann-Whitney U; non-significant.

3.1.2 $CD14^+$ monocytes from celiac disease patients induce alterations in tight junction expression of intestinal epithelial cells

In our previous experiments, TER was significantly reduced in IECs co-cultured with $CD14^+$ monocytes isolated from CeD patients. To explore whether alteration in the expression of TJ proteins are responsible for this functional barrier defect, the expression of TJ proteins was evaluated. The expression and localization of ZO-1 as well as occludin in the IECs (CacoBBe cells) after 48 h of co-culture with $CD14^+$ monocytes isolated from healthy controls of patients with celiac disease was analyzed.

As shown in Fig. 3.4A, the expression of occludin was found to be reduced in the presence of $CD14^+$ monocytes from celiac disease patients but not in the presence of $CD14^+$ monocytes from healthy controls. In addition, in the presence of $CD14^+$ monocytes from celiac disease patients, ZO-1 presented an abnormal structure, which was not observed after co-culture with $CD14^+$ monocytes from healthy controls. In addition, the presence of $CD14^+$ monocytes from CeD patients resulted in a patchy pattern where the expression was

significantly reduced. In addition, XZ-projections revealed irregular distribution of ZO-1 as well as an uneven structure of the apical membrane after co-culture of IECs with CD14+ monocytes from CeD patients (Fig. 3.4B).

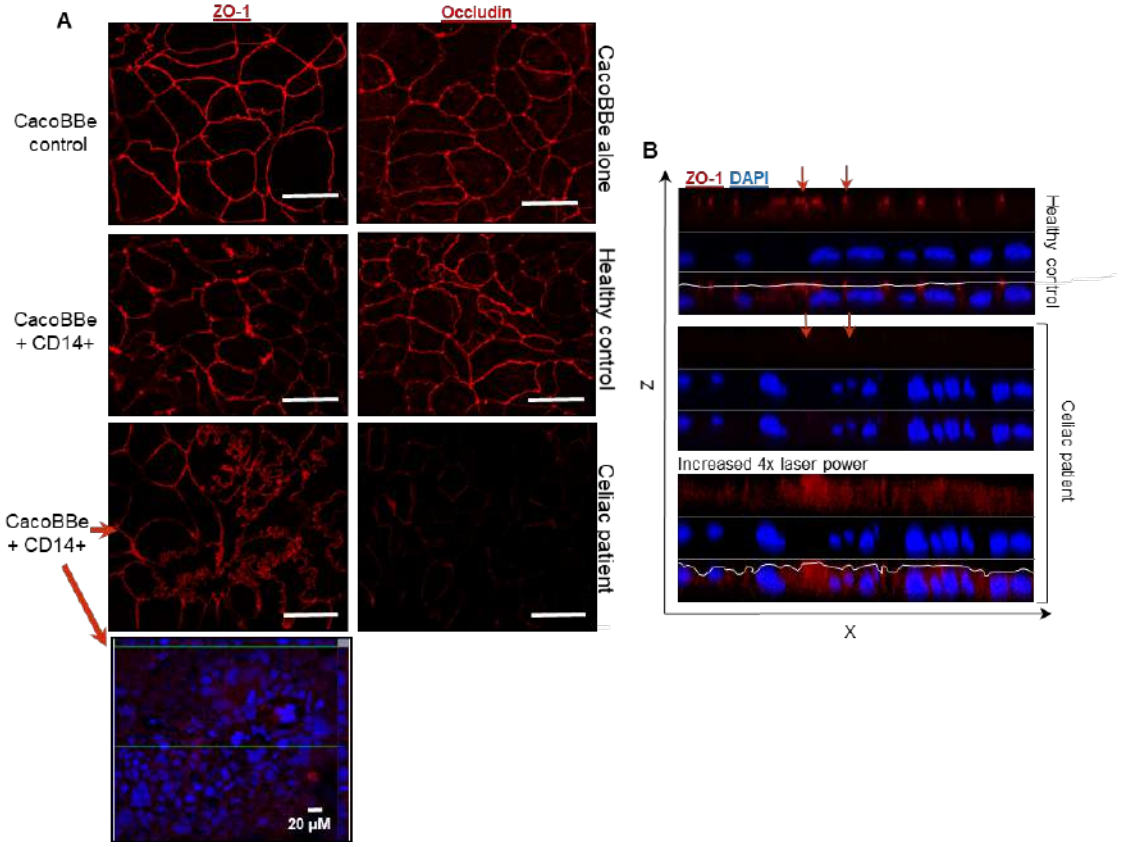

Figure 3.4: Effects on TJ assembly after co-culture with monocytes derived from celiac disease patients. (A) Intracellular localization of TJ proteins with a key role in epithelial barrier function (occludin and ZO-1) were investigated using confocal microscopy after immunostaining. Representative images from n=5 (healthy donors), n=3 (CeD on GFD) and n=3 (ACeD patients). Scale bar: 50 µM. (B) Collapsed Z-stack projections revealing two findings: Firstly, ZO-1 levels were found to be reduced in IECs exposed to CeD monocytes. Moreover, the apical cell surface of IECs appears to be irregular in shape. The putative apical cell surface is indirectly imaged by immunostaining of ZO-1 (apical surface is denoted by a white line in the merged images), thereby uncovering the irregularly shaped apical cell surface of IECs that had been exposed to CeD monocytes for 48 h. Representative images are shown (n=3).

Subsequently, to confirm alterations in TJ assembly, protein levels of occludin and the TJ-sealing protein, claudin-5, which has previously been related to the barrier defect in CeD, were analyzed. As shown in Fig. 3.5, the expression of occludin and claudin-5 were reduced

after co-culture with CD14$^+$ monocytes from celiac disease patients when compared to IECs co-cultured with CD14$^+$ monocytes from healthy controls. Therefore, these data provide evidence that CD14$^+$ monocytes derived from CeD patients induce a reduction in protein expression of occludin and claudin-5 as well as an altered structure and expression of ZO-1. Altogether, these data showed an effect of CD14$^+$ celiac monocytes on TJ protein composition, which was not observed after co-culture with heathy monocytes. In addition, it indicates the structural changes within the IEC layer for the decreased TER described above.

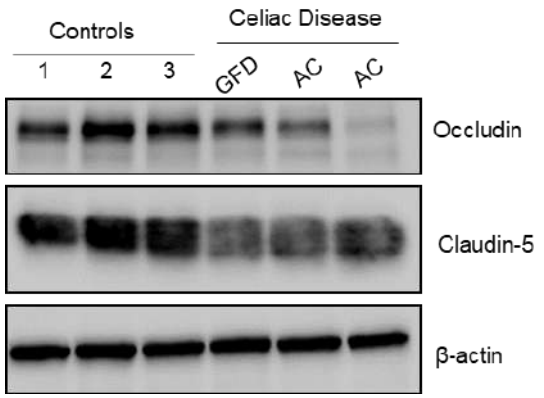

Figure 3.5: Effects on TJ assembly after co-culture with monocytes derived from celiac disease patients. Occludin and Claudin-5 protein levels of intestinal epithelial cells were evaluated after co-culture with monocytes by Western blotting.

3.1.3 Characterization of CD14$^+$ monocytes from celiac disease patients

As we had observed that celiac monocytes have a role in barrier defect in IECs, the next step was to characterize isolated CD14$^+$ monocytes from healthy controls and CeD patients to reveal potential differences with regard to surface markers and cytokine expression. Flow cytometer analysis was performed after cell sorting for either freshly isolated CD14$^+$ cells or after 24 h of culture in the presence of GM-CSF (10 ng/ml). The gating strategy underlying the flow cytometric analysis is outlined in Fig. 3.6. The population of interest was subsequently analyzed for expression of classical inflammatory markers, specifically CD11b, CD80, HLA-DR, CD163 and CD16.

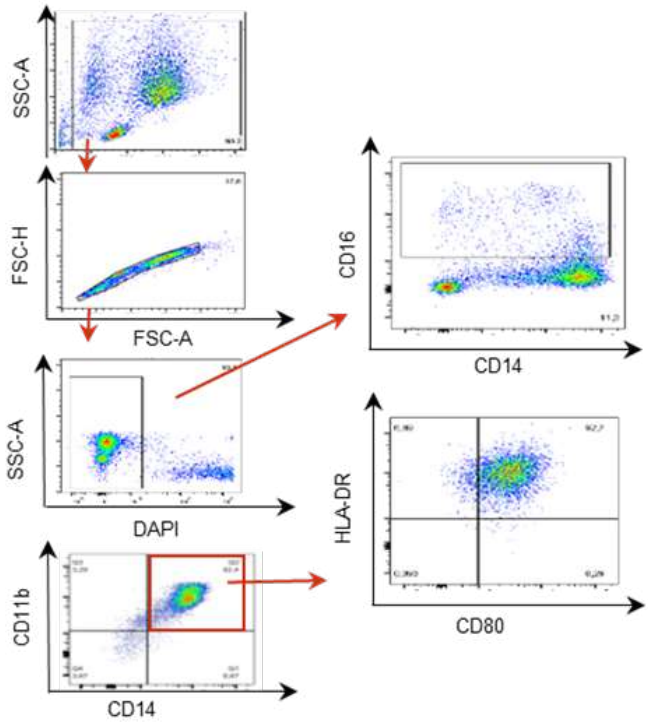

Figure 3.6: Gating strategy applied to analyze the monocyte population. Peripheral blood mononuclear cells were isolated and sorted for CD14+ as described in the *Methods* section. The stepwise gating approach is highlighted by revealing various steps of the flow cytometric analytic process. The sequence can be anticipated by following the red arrows. Representative plots from a healthy control are shown.

When comparing freshly isolated $CD14^+$ monocytes with monocytes after 24 h of culture in the presence of GM-CSF (10 ng/ml), no differences in the expression of any of the analyzed surface markers was detected (Fig. 3.7A-F). We also analyzed for cells being double positive for CD80 and HLA-DR, which are both markers of inflammation. However, this analysis proved not to be significantly different between the groups.

Another interesting marker, we analyzed, was CD16, which is a marker for intermediate/non-classical monocytes involved in different autoimmune diseases, including Crohn's disease but also in atherosclerosis. Interestingly, CD16 expression revealed a slight shift toward higher frequencies in celiac monocytes after 24 h exposure to GM-CSF. Nevertheless, it did not reach significance levels (Fig. 3.7C).

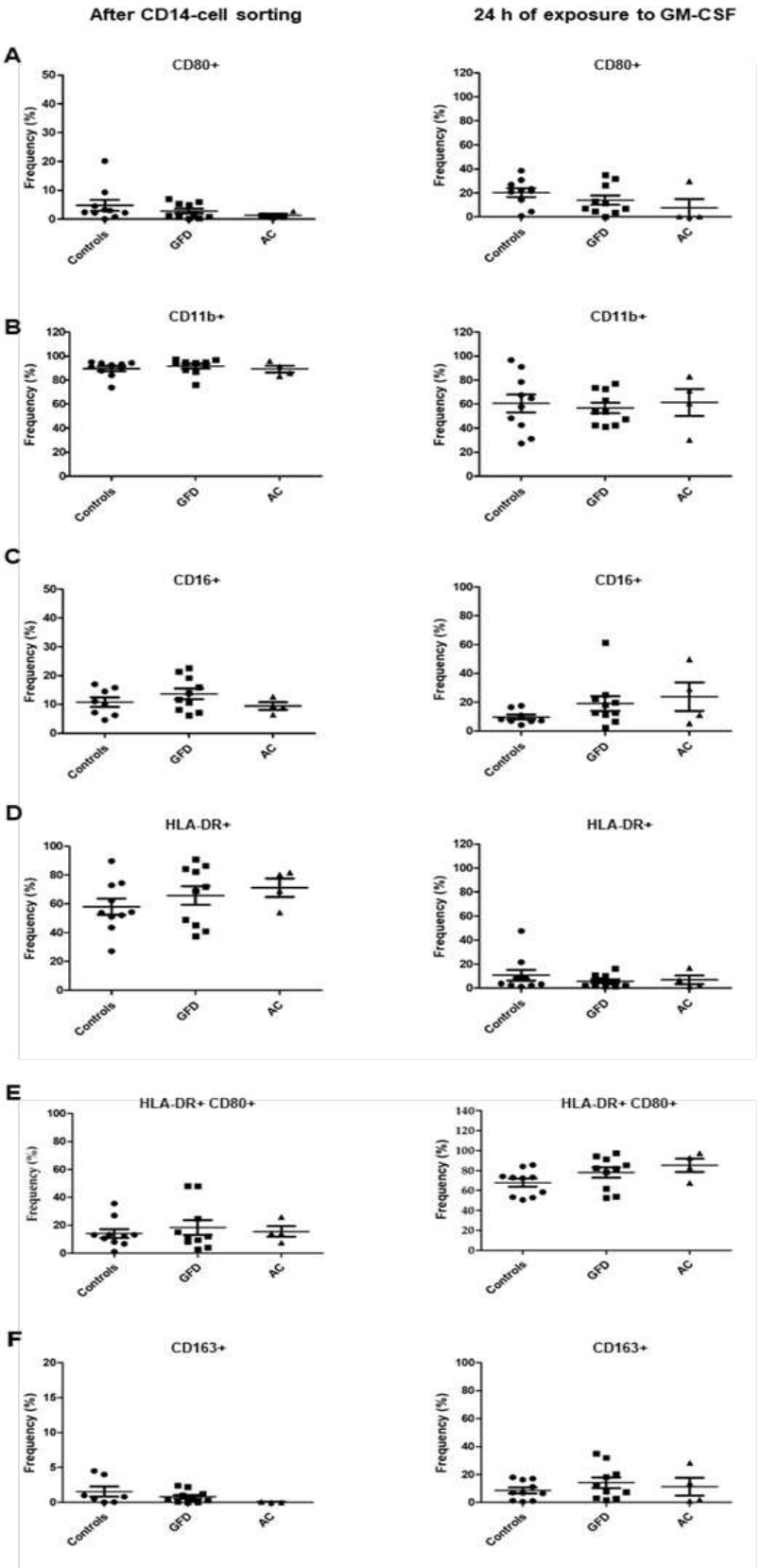
After CD14-cell sorting
24 h of exposure to GM-CSF
A
CD80+
B
CD11b+
C
CD16+
D
HLA-DR+
E
HLA-DR+ CD80+
F
CD163+
Frequency (%)
Controls
GFD
AC

Figure 3.7: Expression of surface markers on peripheral CD14+ monocytes from celiac disease patients and healthy controls. Peripheral blood mononuclear cells were isolated and sorted for CD14 as described in the *Methods* section. Subsequently, monocytes were cultured in the presence of GM-CSF (10 ng/ml) for 24 h and evaluated by flow cytometry. Each dot represents the expression of a surface marker in a single patient. Mean values ± SEM are shown. Mann Whitney test.

Subsequently, we investigated the concentration of pro-inflammatory cytokines in the supernatants of $CD14^+$ monocytes derived from ACeD patients, CeD patients on GFD and healthy individuals. As shown in Fig. 3.8A, IFN-α2, IFN-λ, IL-19, IL-12p70, IL-17A, IL-18, IL-23, IL-33 did not reach the detection level of the assay neither in control nor in the CeD group. Although not statistically significant, a tendency towards a higher concentration of IL-1β, TNF-α, IL-8 and IL-10 in the CeD group in comparison to healthy controls was found. More interestingly, IL-6 and monocyte chemotactic protein-1 (MCP1) were significantly increased in the supernatants of $CD14^+$ monocytes from CeD on GFD patients when compared to $CD14^+$ monocytes from healthy controls (Fig. 3.8E-F). These data indicate that $CD14^+$ monocytes isolated from CeD patients carry a more pro-inflammatory phenotype. Since there was no differene between ACeD and CeD on GFD patients, we concluded that this pro-inflammatory phenotype is, however, independent of disease activity.

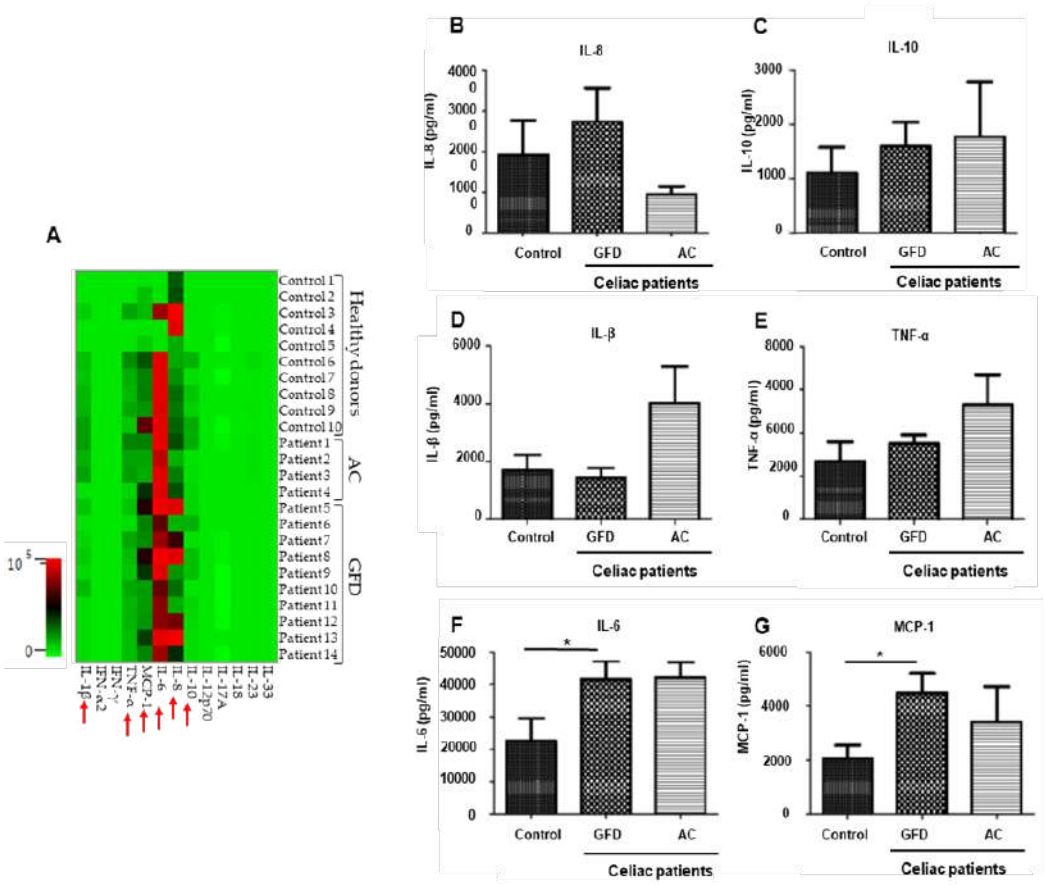

Figure 3.8: Increased levels of pro-inflammatory cytokines in the supernatant of CD14+ monocytes from CeD patients. Peripheral blood mononuclear cells (PBMCs) were isolated from 10 controls, 10 patients with CeD on a gluten-free diet (GFD) and 4 patients with ACeD. CD14+ monocytes were sorted using CD14 MACS MicroBeads (Miltenyi Biotech Bergisch-Gladbach, Germany) as described in the *Methods* section. Subsequently, cells were cultured for 24 h in the presence of GM-CSF (10 ng/ml). The supernatant was collected after 24 h and a LEGENDplex assay to determine cytokine concentrations was performed as described in the *Methods* section. (**A**) Data are illustrated as a heat map revealing color-coded concentrations of cytokines (green: low concentration; red: high concentration). (**B–G**) Results for individual cytokine measurements are shown: Interleukin- (IL-)8, IL-10, IL-1, TNF-α, IL-6, and MCP-1. Statistics: Mann Whitney test with *p<0.05.

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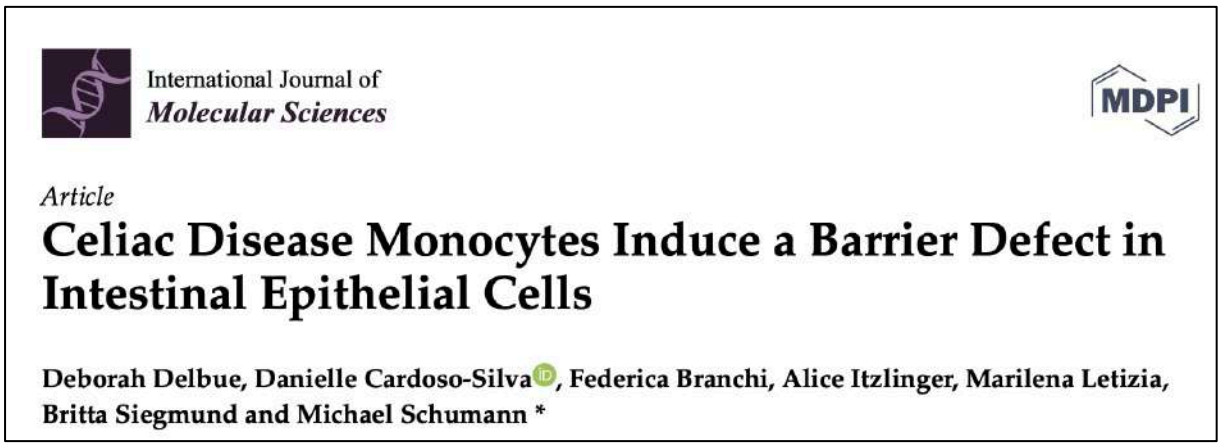


Some of the experiments (specifically the Sandwich assay, Calcium Switch assay and Cell motility assays) included in this part of results were not performed by myself. However, they were included in the thesis because me and my supervisor believed them to be essential assays for understanding the whole study. I was responsible to carry on with the experiments and analysis, and finalize the project.

3.2 Part Two: IL-22 as a model cytokine and its effects on cell polarity and barrier function in intestinal epithelial cells

3.2.1 Paracellular intestinal epithelial barrier is impaired after IL-22 exposure

To investigate the role of IL-22 on barrier integrity, IECs seeded on transwell filters were exposed to IL-22 (apical and basolateral compartment). A stable epithelial barrier was established in HT-29/B6 cells on day 7 and in T84 cells on day 14. Subsequently, apical and basolateral cell surfaces were exposed to IL-22. TER was monitored throughout the experiment (Fig. 3.9). Exposure to IL-22 induced a significant decrease in TER in a dose-dependent (Fig. 3.9A) and time dependent manner (Fig. 3.9A-B) with reductions in TER as much as 60% of control level at 10 ng/ml and 100 ng/ml of IL-22 (72 h exposure). Furthermore, IECs were exposed to other proinflammatory cytokines. Interestingly, the reduction in TER was comparable to the result found on IECs exposed to IL-22 (Fig. 3.9C). Interestingly, we could visualize the IL-22-induced barrier leak allowing the passage of macromolecules like TMR-dextran3000 as shown by the sandwich assay (Fig. 3.9D), indicating impairment on barrier function.

In order to evaluate the impact of IL-22 exposure on TJ assembly, IECs were challenged in a calcium switch assay. Calcium is the crucial cation that interlinks the intercellular homophilic E-cadherin interaction thereby conveying cell-cell adhesion of IECs in adherens junctions (ACs). Therefore, IEC filters mounted to Ussing chambers were seeded on filters to establish an epithelial barrier in the absence or presence of IL-22 and were then deprived of calcium for 30 min, which triggered consecutive loss of cell-cell adhesion of AJs and as a consequence of that also of TJs. Substitution of Ca^{2+} in the buffer triggered reassembly of TJs. The course of TER was monitored in 10 s-intervals throughout the experiment and measured every 60 minutes for 6 hours. The reestablishment of calcium allowed for the formation of protein complexes of cell-cell adhesion and therefore, triggered a steady increase in TER. Fig. 3.9E illustrates that IECs exposed to IL-22 reveal a delay in the TER rescue effect, and within the experimental procedure did not reach the final TER levels of

the control group of filters. This result shows proof that IL-22 exposure of IECs delays TJ assembly. Altogether, these results show that IL-22 impairs the paracellular barrier function of IECs and promotes an increased permeability of small ions (measured by TER) and macromolecules (as measured by the sandwich assay).

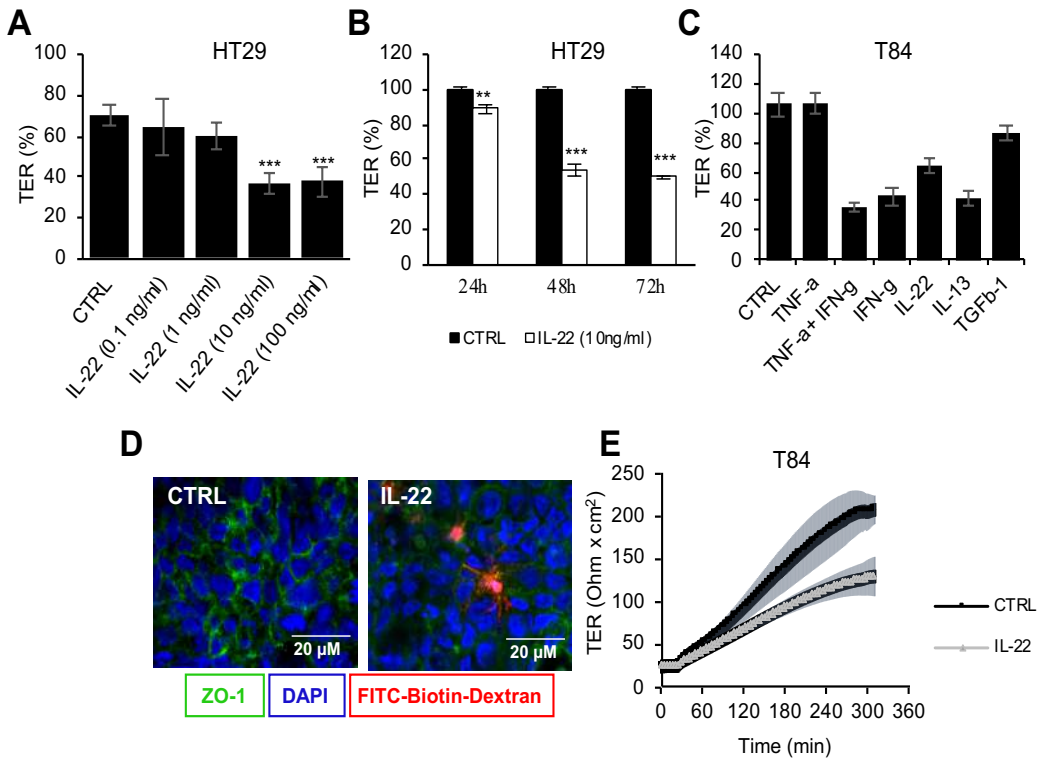


Figure 3.9: Barrier integrity is affected by IL-22. (A) Transepithelial resistance (TER) was determined in HT-29/B6 cells grown on transwell filters. Cells were exposed to IL-22 at different concentrations (0.1, 1, 10 and 100 ng/ml). TERs after 72 h of IL-22 exposure are shown. n= 25 (B) TER time course in HT29/B6 cells exposed to IL-22 (10 ng/ml); n=32. (C) Comparative analysis of TER in T84 cells after a 48 hour-exposure to various cytokines (TNFα: 1000 U/ml, IFN-γ: 100 U/ml, IL-22: 10 ng/ml; IL-13: 10 ng/ml; TGF-b1: 20 ng/ml); n=8. (D) Sandwich assay (it was not performed by myself) revealing transepithelial passage of macromolecules, specifically TexasRed-dextran3000 (red fluorescence) in control and IL-22-treated Caco-2 cells. E-cadherin, green; nuclei, blue; n=3. (E) Calcium switch experiment (it was not performed by myself): T84 cells growing on transwell filters were exposed to IL-22 (10 ng/ml, 48 h) and mounted to Ussing chambers, where TER was monitored in 10 s-intervals throughout the experiment. Transepithelial resistance was measured every 60 minutes for 6 hours; n=3. Mann Whitney U test **p<0,01; ***p<0,001.

3.2.2 IL-22 exposure causes formation of atypical cysts formation and defective epithelial polarity

After describing the impact of IL-22 exposure on paracellular barrier integrity, the next step was to investigate a potential role of IL-22 in altering epithelial polarity. Thus, a well-established model for epithelial cell polarity, the 3-dimensional (3D) Caco2 cyst model was selected. Formation of cysts after exposure to IL-22 was compared to untreated cysts. Specifically, the development of the central cyst lumen was analyzed as this is known to reflect the integrity of the polarization process. Cells were immunostained and analyzed by confocal laser scanning microscopy. Untreated cysts presented a single polarized epithelial layer, as observed by the actin staining, and a central and single lumen. Interestingly, IL-22 exposure affected cyst formation with the occurrence of multilumen cysts and a completely disorganized cyst structure (Fig. 3.10A-B).

Furthermore, we immunostained key cell polarity proteins, including Par-3, that has been described to orchestrate the assembly of apical junctions in epithelial cells and Dlg-1, that is physiologically localized to the basolateral cell compartment and excludes certain apical proteins from the basolateral cell domains. In general, these expected protein localizations were confirmed in established cysts (i.e. five days after seeding). Par-3 was localized to the most apical part of the lateral cell membrane in control cysts and Dlg1 was restricted to the basolateral membrane. In contrast to that, in IL-22-treated cysts, Par-3 was dislocated as it was found diffusely along the entire lateral membrane, similar to what we had previously found when immunostaining ZO-1. Furthermore, membranous Dlg-1 staining was reduced compared to controls and was shifted to an intracellular compartment (Fig. 3.10C, white arrows). Taken together, these results suggest that IL-22 impairs intestinal epithelial polarity and can significantly change organization of polarity proteins within 3D cysts.

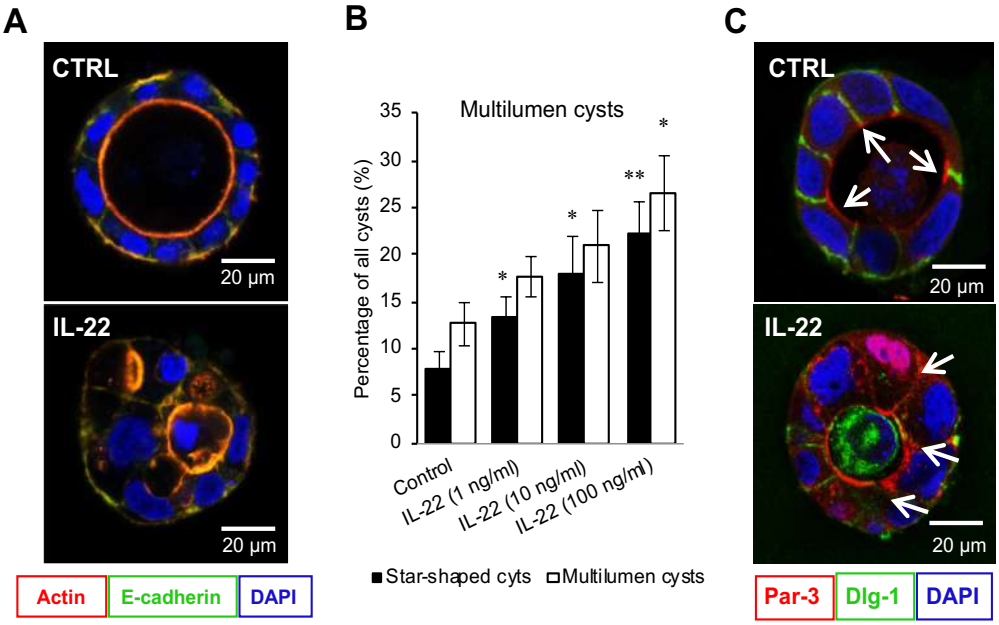


Figure 3.10: IL-22 exposure altered 3D cysts formation and polarization. (A and C) Caco-2 cells were seeded in Matrigel and grown for 5-7 days to form 3D cysts. Cysts were treated with IL-22 (10 ng/ml) starting at the day after seeding. Subsequently, they were fixed and immunostained. (A) Blue, nuclei; red, actin; green: E-cadherin. (C) Blue: nuclei; red: Par-3; green: Dlg-1. n=4 independent experiments. (B) Quantification of the 3D-cyst experiments (it was not performed by myself): Caco-2 cysts growing in Matrigel were analyzed by confocal LSM. Multilumen, cysts were microscopically quantified; n=6.

3.2.3 IL-22 exposure increases cell motility and induces epithelial-mesenchymal transition on IECs

As we had observed IEC polarity defects after IL-22 exposure, we next investigated, whether IL-22 might also impact in cell motility increasing migratory and invasive properties of IECs. Thus, we carried out a wound healing assay by performing uniform scratches into a single Caco-2 layer that stably expressed actin-GFP and monitored live by confocal LSM. Exposure to IL-22 (10 ng/ml) resulted in a statistically significant increased IEC migration (Fig.3.11A-B). Similarly, IL-22 had the capacity to induce invasion of cells in a combined Matrigel/filter-based assay. After IL-22 exposure, the number of colonies invading the basal compartment was approx. 3-fold higher as compared to the control situation (Fig. 3.11C). Taken together, these results show that IL-22 increases cell motility and cell invasion.

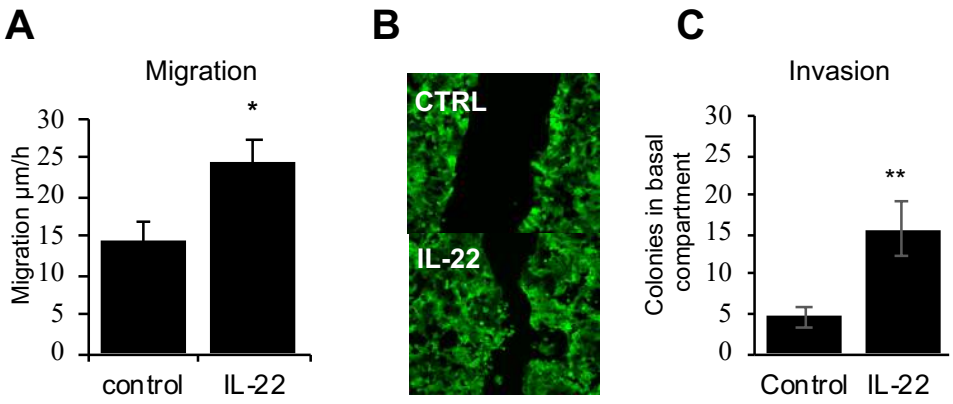


Figure 3.11: IL-22 induces migration and invasion on intestinal epithelial cells. (A) A defined scratch (diameter 100 µm) was introduced to filter-grown Caco-2 cells and they kept with medium with 1% of fetal bovine serum (Gibco) to avoid cell proliferation. Cells were exposed to IL-22 (10 ng/ml) and migration was evaluated by measuring the distance at 24 and 48 hours after scratching through fluorescence microscopy. (B) IECs were seeded on transwell inserts coated with Matrigel and then exposure to IL-22. Subsequently, IECs that passed through the membrane formed colonies in the basal compartment, were fixed and stained for DAPI. Subsequently, these colonies were counted through fluorescence microscopy. Mann-Whitney test; * *p<0,05; ** p<0,01.* These experiments were not performed by myself.

The data previously observed suggest that EMT might be triggered by exposure of cells to IL-22. In short, EMT is a process, in which epithelial cells partially lose properties defining cell polarity as well as cell-cell adhesion and on the hand gain migratory and invasive properties. To follow-up on this thought, further experiments were designed. Since expression of a distinct set of proteins is regulated within EMT, we firstly quantified E-cadherin and matrix metalloprotease-7 (MMP-7) by western blotting in an experimental course of exposing IECs to IL-22. While E-cadherin levels declined significantly after 72 h of IL-22 exposure, MMP-7 expression peaked 24 h after IL-22 addition (Fig. 3.12A-B), as observed by the densitometry analysis (Fig.3.12C-D).

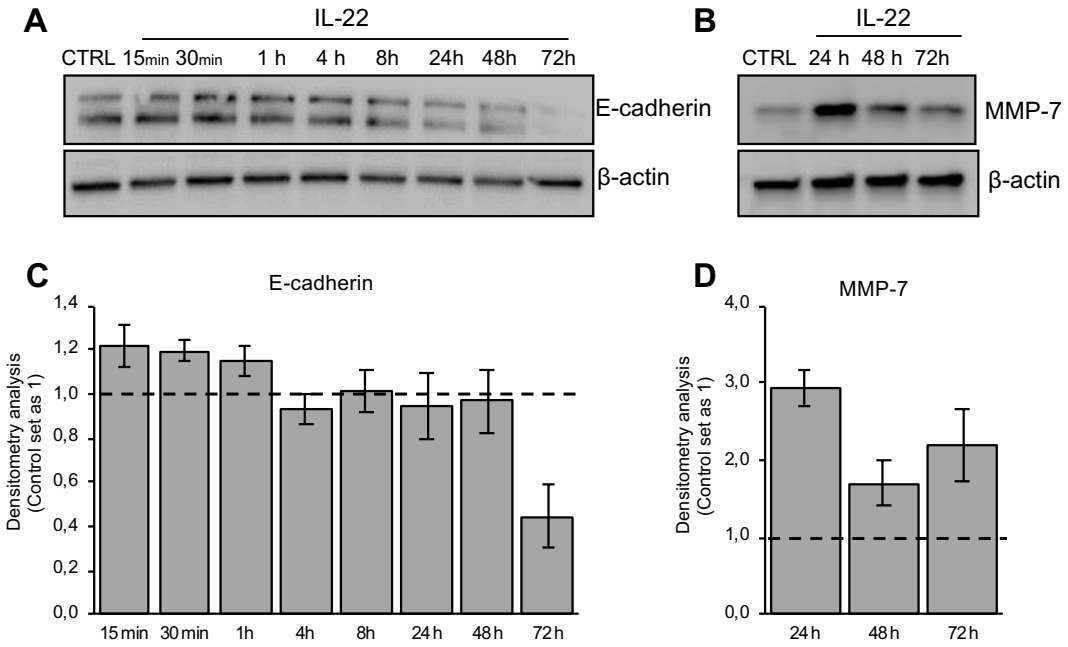


Figure 3.12: IL-22 induces epithelia-mesenchymal transition on intestinal epithelial cells. IECs were exposed to IL-22 (10 ng/ml) for different lengths in time (15 and 30 minutes, 1, 4, 8, 24, 48 and 72 hours for western blotting analysis). (A-B) Cells were lysed and protein levels of E-cadherin and MMP-7 were investigated through western blotting. Representative blot of 3 and 2 independent experiment, respectively (C-D) Densitometric analysis of E-cadherin and MMP-7 protein levels (n=3 and n=2, respectively). The dashed line represents the expression level of the control condition. β-actin was used as an internal control for normalization to protein content.

To support our hypothesis that EMT is induced by IL-22, *SNAI1* (Snail), *SNAI2* (Slug) and MMP-7 messenger RNA (mRNA) was assessed by RT-qPCR, after 3 and 24 h of IL-22 exposure in two different concentrations, 10 and 100 ng/ml. In accordance to the previous data, IL-22 increased *SNAI1* and *SNAI2* gene expression, mainly after 24 h of IL-22 exposure, in comparison with untreated IECs. Surprisingly, no pronounced difference in mRNA levels for Snail and Slug of cells treated with 10 or 100 ng/ml of IL-22 was found. The concentration of 100 ng/ml of IL-22 induced higher levels of MMP-7 mRNA than 10 ng/ml of IL-22 in 3 h of exposure. However, after 24 h of IL-22 exposure, there is no pronounced difference and the levels of mRNA are similar between these concentrations (Fig. 3.13A-C). Our data indicate that IL-22 is a strong inductor of EMT in IECs, which might contribute to migratory as well as invasive properties of IL-22-treated IECs.

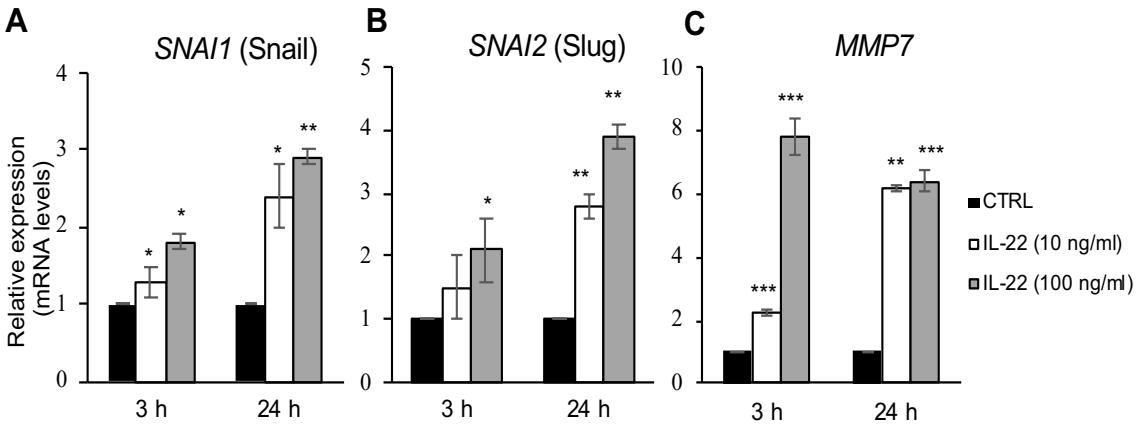


Figure 3.13: IL-22 induces increased in Snail, Slug and MMP-7 mRNA levels. IECs were exposed to IL-22 (10 ng/ml) for 3 and 24 hours for Real Time-PCR. IECs were exposed to IL-22 for 3 and 24 hours and then qPCR analysis for the transcription factors Snail (SNAI1, A), Slug (SNAI2, B) and MMP-7 (C) was performed. Expression changes were calculated using the 2-ΔΔCT method. Mann Whitney U test *$p<0,05$; **$p<0,01$: ***$p<0,001$ n=3 independent experiments.

3.2.4 IL-22 affects tight junction protein levels

The next step was to investigate the impact of IL-22 on expression and subcellular localization of TJ proteins. In a first step, we monitored the expression of claudins in the course of IL-22-exposure by western blotting. After exposing the cells to IL-22, the protein level of claudin-1, a barrier-forming claudin, decreased with a pronounced effect after 48 and 72 h of IL-22 exposure, whereas protein levels of the pore-forming claudin-2 and claudin-4, which are TJ-proteins that had previously been linked to EMT, started to increase after 8 h of IL-22 addition (Fig. 3.14A-D).

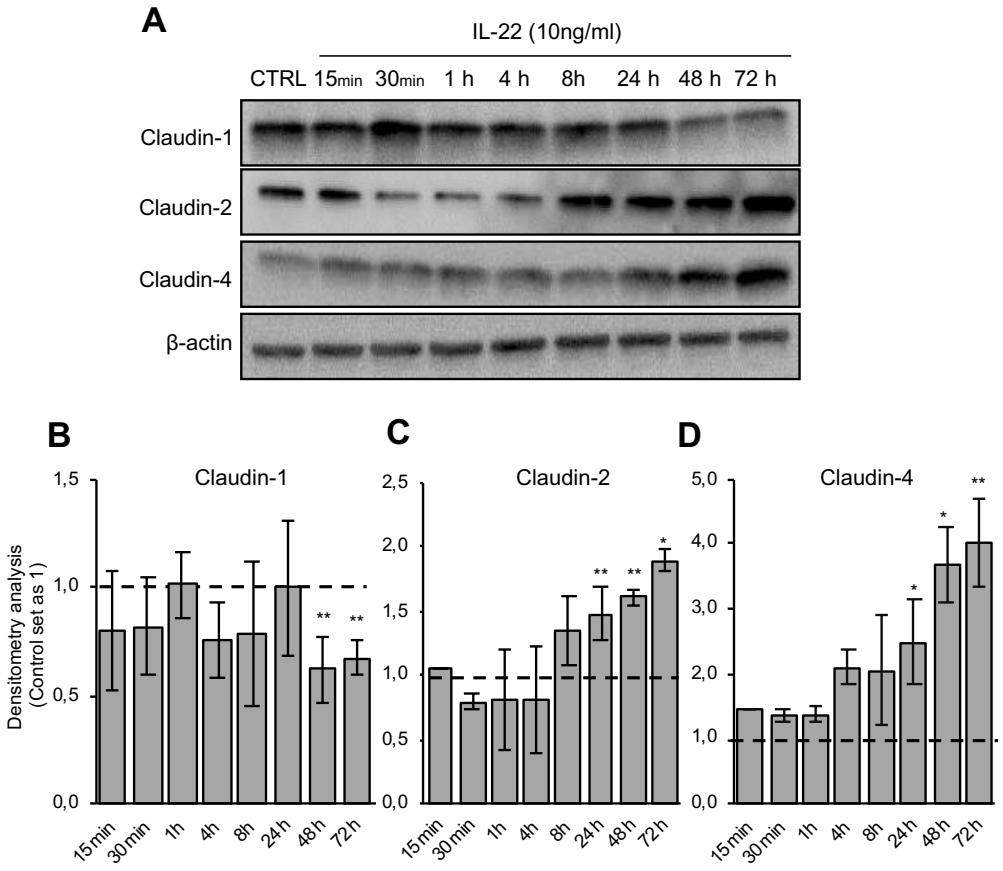


Figure 3.14: IL-22 alters protein expression levels of claudins. IECs were exposed to IL-22 (10 ng/ml) for different lengths in time (15 and 30 minutes, 1, 4, 8, 24, 48 and 72 hours). (A) Subsequently, cells were lysed and protein levels of TJ-proteins were investigated by western blotting. A representative blot of 3 independent experiments is shown. (C-D) Densitometric analysis of protein levels for claudin-1, -2 and -4 (n=3). The dashed line represents the expression of the control condition. β-actin was used as an internal control for normalization of protein content. Mann Whitney U test *$p<0,05$; **$p<0,01$: ***$p<0,001$; n=3 independent experiments.

We also determined the localization of TJ-proteins using confocal laser scanning microscopy. Localization of occludin was shifted to an intracellular compartment and to the lateral membrane in the 2D transwell and the 3D cyst model, respectively (Fig. 3.15, white arrows). Corroborating our previous data, these results suggest that in fact IL-22 has an effect on TJ, which contributes to a defect on barrier function observed after IL-22 exposure in IECs.

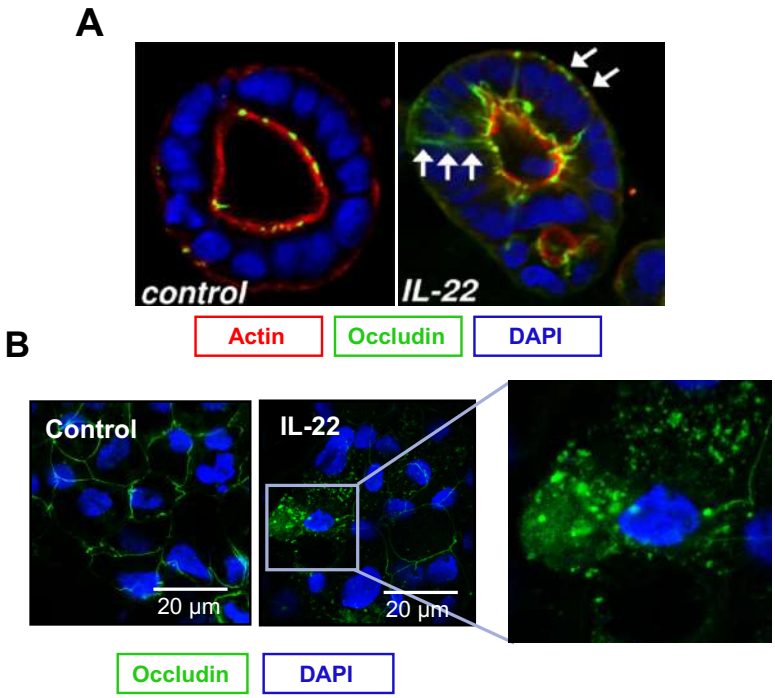


Figure 3.15: IL-22 alters occludin localization. Confocal microscopy after immunostaining 3D and 2D cultures of IECs (A) Caco-2 cells were seeded in Matrigel and grown for 5-7 days to form 3D cysts. Cysts were treated with IL-22 (10 ng/ml) starting at the day after seeding. Subsequently, they were fixed and immunostained. (B) HT-29/B6 cells grown on transwell filters were fixed after 7 days on culture and then immunostained. Dislocation of occludin by IL-22 treatment is highlighted by white arrows. Green, occludin; red, actin; blue, nuclei; n=3 independent experiments.

3.2.5 IL-22 induces phosphorylation of STAT3 and ERK

According to our previous experiments, IL-22 affects barrier integrity and cell polarity leading to multilumen cysts formation, as well as increases cell motility inducing EMT in IECs. As shown in various previous studies, the STAT3 pathway as well as MAPK/ERK pathways are activated upon binding of IL-22 to its receptor. We exposed IECs (HT29/B6 cells) to IL-22 in different lengths of time (15 and 30 minutes, 1, 4, 8, 24, 48 and 72 hours) to evaluate the kinetics of activation of these signaling pathways. As shown on Fig. 3.16A-B, after 15 minutes, IL-22 induced phosphorylation of STAT3.This was maintained until 48h when the levels of phosphorylation decreased reaching similar levels as observed in the control. ERK was phosphorylated after exposing cells to IL-22 for ≥4 h (Fig. 3.16A; C). Interestingly, we did not

detect any phosphorylation of AKT in our model system (Fig. 3.16A; D). Our data confirm that STAT3 and MAPK/ERK signaling pathways are activated after IL-22 exposure.

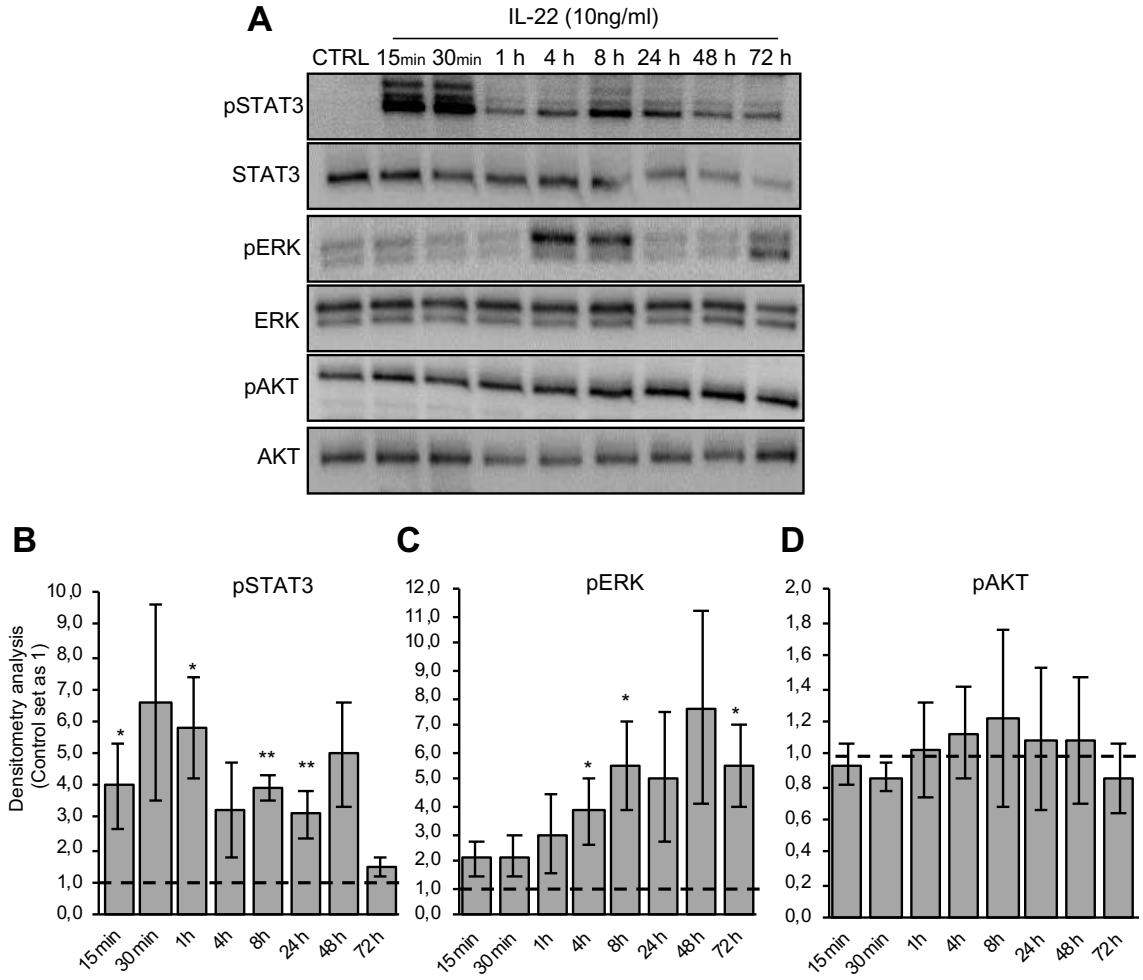


Figure 3.16: IL-22 signaling activated STAT-3 and MAPK/ERK signaling pathways. IECs were exposed to IL-22 (10 ng/ml) for different time points (15 and 30 minutes, 1, 4, 8, 24, 48 and 72 hours). Subsequently, cells were lysed and protein levels of members of signaling pathways possibly activated by IL-22 were investigated through western blotting. (B-D) Densitometric analysis of protein levels of claudin -1, -2 and -4 (n=3). The dashed line represents the expression of the control condition. β-actin was used as an internal control for normalization to protein content. Mann Whitney U test; *$p<0,05$; **$p<0,01$: n=3 independent experiments.

3.2.6 STAT3 inhibitors are not able to abrogate STAT3 phosphorylation after IL-22 exposure

In our previous results, we have shown that at least two signaling pathways may be crucial for the mechanism of action of IL-22 in IECs. Since activation of STAT3 signaling had previously been reported to be pivotal for epithelial protection, we next determined the effect of various strategies to inhibit STAT3 signaling. As a control, we exposed IECs (HT29/B6) to IL-13, which also induces phosphorylation of STAT3 at 30 min and 48 h, and exposed IECs to IL-22 for 48 h. IL-13 induced phosphorylation of STAT3 after 30 min of exposure. Levels of phosphorylation decreased again starting at 48 h of IL-13 exposure. Furthermore, the STAT3 phosphorylation inhibitor Stattic blocked STAT3 phosphorylation levels in the two concentrations used in the experiment. However, when IECs were exposed to IL-22, the same effects of Stattic was not observed. Instead, STAT3 continued to be strongly activated by IL-22 in the presence of the inhibitor Stattic, which obviously was not able to inhibit STAT3 phosphorylation (Fig.3.17A).

Moreover, we evaluated three additional STAT3 inhibitors with different mechanisms of STAT3 phosphorylation inhibition. IECs were exposed to WP1066, which inhibits JAK2 phosphorylation, which is a protein kinase upstream of STAT3. Thus, when applying WP1066, STAT3 phosphorylation becomes consecutively downregulated. Another inhibitor used was SI3-201 (STAT3 Inhibitor VI). It is a cell-permeable compound that binds the STAT3-SH2 domain and prevents STAT3 phosphorylation/activation, DNA binding and STAT3-dependent transcription. With a similar mode of action as SI3-201, the STAT3 Inhibitor Peptide was used, which is an analogue of the STAT3-SH2 domain-binding phosphopeptide and therefore acts as a highly selective inhibitor of STAT3 activation. Once we treated the cells with IL-22, STAT3 is phosphorylated and none of the inhibitors was able to significantly reduce STAT3 activation. Thus, no alteration of TER levels was observed (Fig. 3.17B-C)**.** Altogether, our data show that IL-22 strongly induces activation of the STAT3 signaling pathway. Furthermore, STAT3 inhibitors were not capable of inhibiting STAT3 activation after IECs were exposed to IL-22.

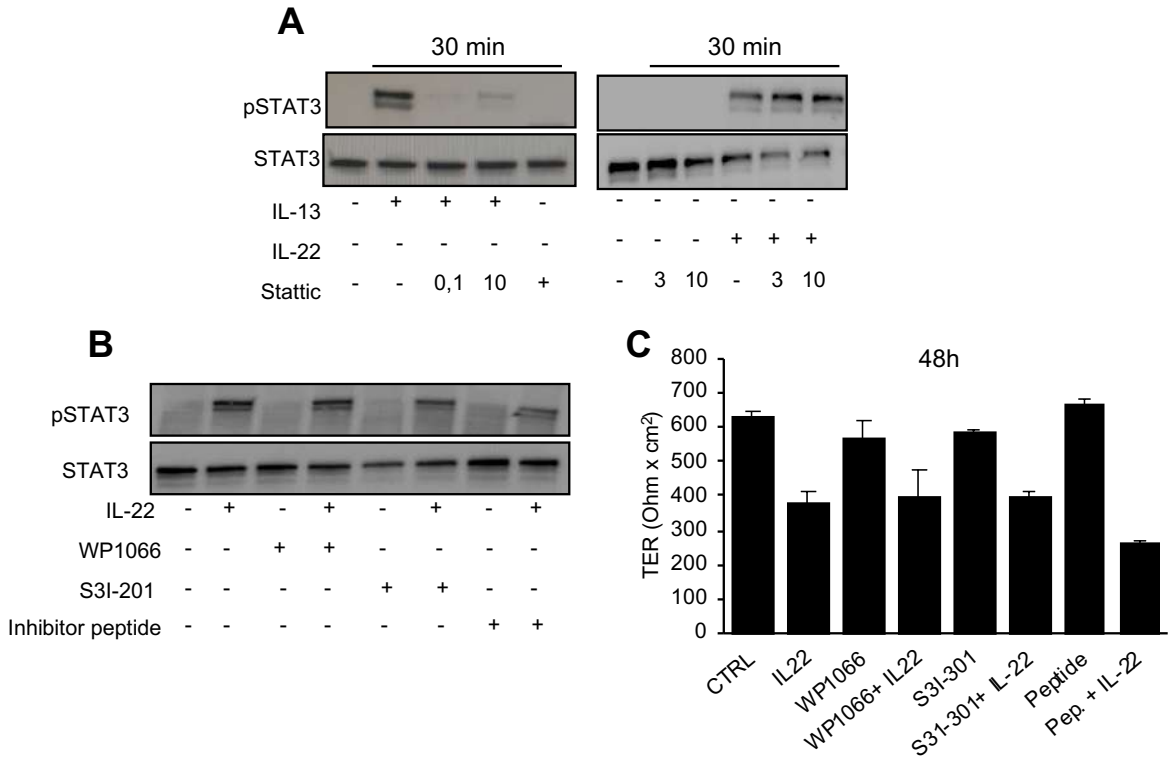


Figure 3.17: STAT3 inhibitors are not able to inhibit STAT3 phosphorylation after IL-22 exposure. HT-29/B6 cells were treated with (A) IL-22 (10 ng/ml) or IL-13 (10 ng/ml) and the (B) STAT3 inhibitors Stattic, WP1066, S3I-201 and the inhibitor peptide as described in the Methods section. (C) TERs of transwell filters treated with STAT3 inhibitors with or without exposing additionally to IL-22 (10 ng/ml) were determined after 48 hours. n=16 filters.

Additionally, IECs were exposed to WP1066 at a higher concentration (50 µM) in order to evaluate whether STAT3 activation could be blocked at this high concentration. Surprisingly, the inhibitor was able to block STAT3 activation. However, we noted that STAT3 total protein levels were also reduced and that TERs of treated IECs that were only exposed to the inhibitor were dropping (Fig. 3.18), suggesting a toxic effect of the inhibitor on the IECs at this concentration. In line with this, levels of cleaved-caspase-3, a marker for apoptosis, increased (Fig. 3.18A-B). In summary, these results indicated that STAT3 is crucial for intestinal cell survival. As a result of this, blockage of STAT3 leads to cell death.

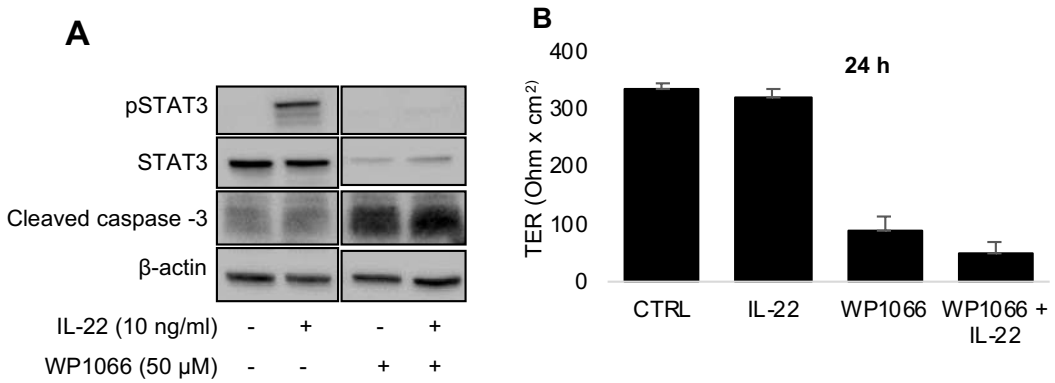


Figure 3.18: STAT3 signaling pathway is crucial for intestinal cell survival. IECs were exposure to WP1066 with or without IL-22. (A) IECs were lysate and western blotting was performed to evaluated protein levels of STAT3 total and phospho-STAT3 (pSTAT3), and cleaved caspase -3. (B) TER was measured after 24 h of IL-22 exposure on filters treated with WP1066 (50 μM) with or without IL-22 (10 ng/ml) exposure.

3.2.7 MAPK signal transduction is pivotal to IL-22 effects on intestinal epithelial cells

The following step was to evaluate effects of blockage of MAPK/ERK signaling pathway in IECs and its effect on IL-22 mechanism of action. To address this question, the well-known inhibitor of MEK, U0126, was used as described on *Materials & Methods* section. We observed total blockage of ERK phosphorylation levels after exposing IECs to the inhibitor (HT29/B6), showing that contrary to what was observed previously with STAT3 inhibition after IL-22 exposure, IL-22-dependent activation of MAPK/ERK signaling was blocked after U0126 exposure (Fig. 3.19A). In line with the findings on MAPK/ERK phosphorylation, the IL-22-induced reduction on TER was reversed (Fig.3.19B). This result suggests that the MAPK/ERK cascade conveys the intracellular signals that are decisive for paracellular barrier function. This is also confirmed by the intracellular localization of occludin in the confocal LSM study, which equivalently revealed a reversal of the occludin dislocation when MAPK was inhibited (Fig. 3.19C).

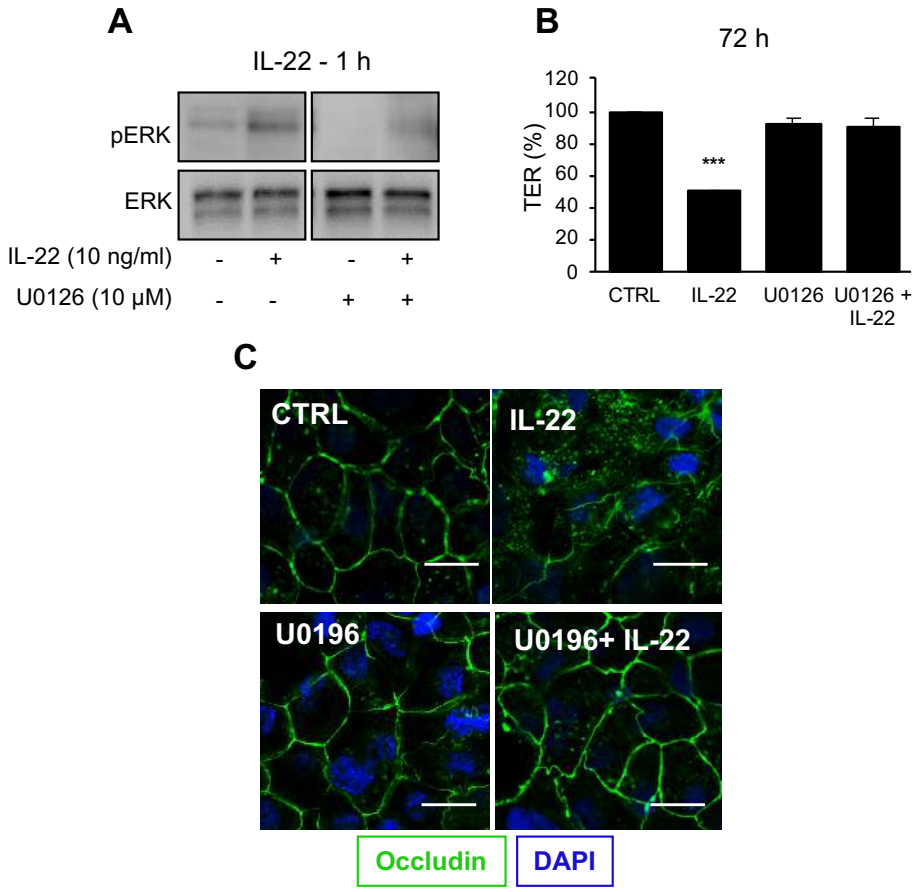


Figure 3.19: Inhibition of ERK phosphorylation reverses IL-22 effects on barrier integrity in IECs. IECs were exposed to U0126 (10 µM) for 1 h to evaluate ERK inhibition and 72 h to measure TER levels, in the presence or absence of IL-22 (10 ng/ml). (A) IECs were lysed and western blotting was performed to determine protein levels of ERK total and phospho-levels of ERK (pERK). A representative blot is presented. (B) TERs of treated cells with U0126 with or without IL-22 (10 ng/ml) was measured after 72 h. Mann Whitney U test ****p<0,001.* (C) IECs were fixed with 4% PFA, immunofluorescence for occludin was performed and images were obtained using confocal laser scanning microscopy. Blue: nuclei; green: occludin. Representative images. n=3 independent experiments.

As previously noticed, IL-22 exposure induced changes in claudin protein levels, and decreased E-cadherin levels as well as increased MMP-7 protein levels. Interestingly, once MAPK/ERK signaling was blocked by treating cells with U0196, the reduction in E-cadherin and claudin-1 protein levels as well as the increases in claudin-2, -4 and MMP7 returned to similar levels compared to the levels found in untreated cells (Fig. 3.20 3A-F). Altogether, our results indicate that MAPK/ERK signaling is central in mediating IL-22-dependent barrier and EMT signaling in IECs.

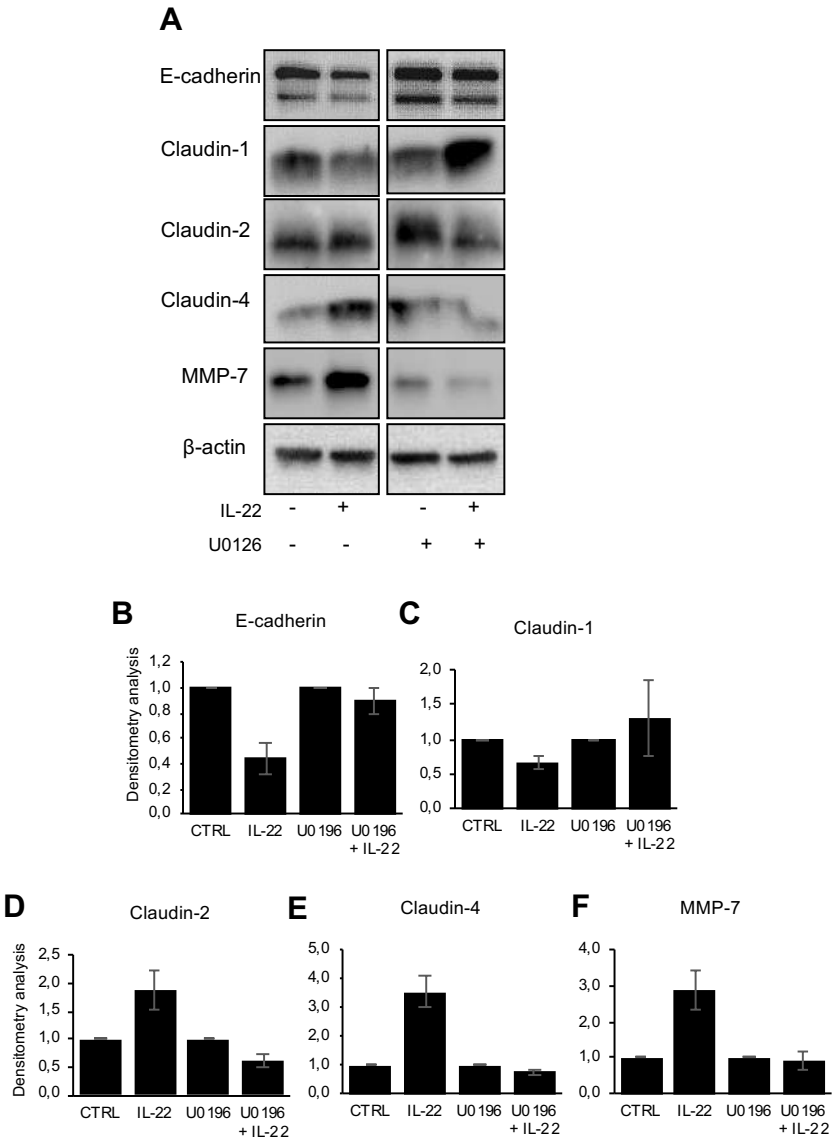
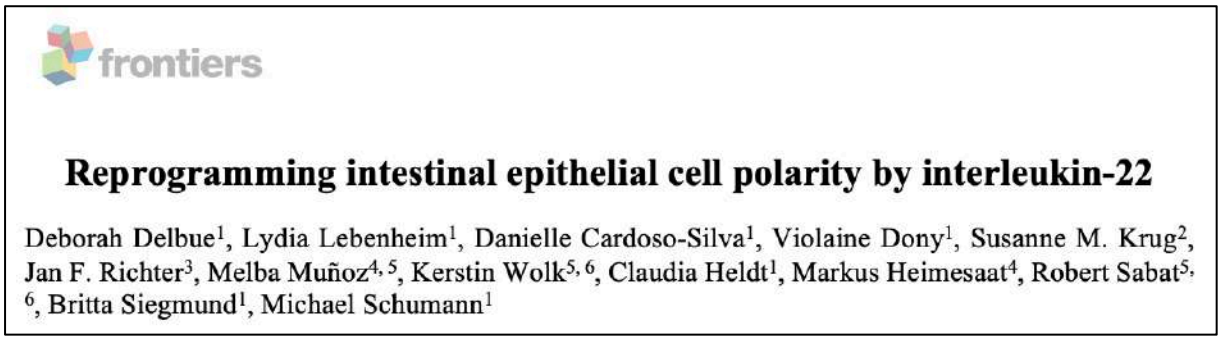


Figure 3.20: Inhibition of ERK phosphorylation reverses IL-22 effects on TJ-protein levels and E-cadherin. IECs were exposed to U0126 (10 µM) for 72 h with or without IL-22 treatment (10 ng/ml). (A) Subsequently, they were lysed and western blotting was performed to evaluate protein levels of E-cadherin, claudin-1, -2, -4, MMP-7. (D-F) Densitometric analysis of protein levels for claudin-1, -2 and -4 (n=3) are shown. β-actin was used as an internal control for normalization of the protein content. *n=2* independent experiments.

Some of the experiments (specifically the Sandwich assay, Calcium Switch assay and Cell motility assays) were not performed by myself. However, they were included in the thesis because me and my supervisor believed them to be essential assays for understanding the whole study. I was responsible to carry on with the experiments and analysis, and finalize the project. In addition, I wrote the manuscript. This study was already submitted to the journal "Frontiers in Medicine – Gastroenterology" and was recently accepted for publication.



frontiers

Reprogramming intestinal epithelial cell polarity by interleukin-22

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4 Discussion

4.1. Monocytes in celiac disease

4.1.1 Alterations in barrier integrity caused by CeD monocytes

CeD is a T cell-mediated autoimmune disease that affects the small intestine of genetically predisposed individuals and is triggered by gluten ingestion. Activation and impact of T cells on CeD is a well-stablished model in order to understand the pathology and progression of the disease [198]. However, it is unclear how the barrier defect in CeD is triggered. Our hypothesis was induced by an early published study that showed that in intestinal inflammation, blood monocytes infiltrate in the mucosa, differentiate into macrophages and further secrete pro-inflammatory cytokines rather than tissue-resident macrophages [227]. Since monocytes are classified as mediators of the mucosal barrier defect, we have investigated in the present study whether monocytes as members of the innate immune system might have an impact on intestinal barrier function. More specifically, we asked, if monocytes from CeD patients have an impact distinct of monocytes from healthy individuals and thereby asked, if celiac monocytes carry a barrier-deleterious signature.

As detailed in the *Introduction,* previous studies have demonstrated that barrier integrity is altered in CeD patients [162,212,213]. Here, we reported that not only T cells, but also, monocytes derived from CeD patients have effects on barrier integrity of IECs. Our first result showed the impact on barrier integrity of IECs co-cultured with monocytes derived from CeD patients on GFD or active CeD. After isolation of PBMCs and cell sorting for $CD14^+$ cells, we observed that co-culture with CeD monocytes affected barrier integrity of IECs with decreased TER levels, regardless of the disease activity, i.e. GFD patients had a similar impact on barrier function compared to ACeD patients. This is appears to be somewhat astonishing as GFD is known to be the only successful treatment in CeD and – when stringently carried out – leads to clinical normalization [206,207]. In addition, we performed the co-culture of IECs with unsorted PBMCs and a similar result in TER levels was found. Once gliadin is a component of gluten and it can initiate uncontrolled inflammatory response, the next experiment explored whether gliadin had a direct effect on IECs and/or monocytes. We observed that IL-15/Tglia alone or IL-15/Tglia and CeD monocytes did not affect the TER levels. These experiments suggest that the effect on barrier integrity of IECs is mainly caused by co-culture with CeD monocytes themselves.

The next step was to evaluate the expression and localization of TJ proteins, once we observed decrease in TER levels, which suggest barrier dysfunction and alterations on barrier integrity. It is known that TJ are key proteins responsible for the maintenance and proper function of the intestinal epithelial barrier [34,35]. Studies from our group and others have shown that TJ assembly is disrupted in IBD and CeD with increased paracellular permeability, decreased in TJ strands formation and alterations on TJ proteins levels (more details in *1.5.1 Intestinal barrier function in celiac disease*). In our next experiment, we demonstrated that CeD-derived monocytes play a role in regulation of barrier function in IECs, as observed by Lissner *et al* in monocytes derived from IBD patients [77]. In accordance, we observed alterations in expression of crucial TJ protein, such as ZO-1, occludin and a TJ-sealing claudin, claudin-5. These data show that monocytes from active CeD or GFD patients change the structure of the TJ, leading to dysfunction of the intestinal barrier. In line with our findings, other studies have shown that alterations on TJ proteins occurred in CeD with reduction of occludin, claudin-3, -5, and -7, and altered phosphorylation of ZO-1 [162,215]. Although cell death was not analyzed in our study, it is important to mention that apoptosis might also contribute to paracellular barrier dysfunction in epithelial sheets. This needs specifically to be considered as monocytes might induce apoptosis of IECs, either vy secretion of pro-inflammatory cytokines (e.g. TNF-α) or by direct cell-cell contact.[162,228]. In addition, a dysfunctional barrier by disruption of apical junctions might contribute to the paracellular passage of gliadin peptides (gliadin as a protein component of gluten and accepted CeD trigger) through the intestinal epithelium, which leads to activation of immune responses, causing CeD development in genetically predisposed individuals. This activation of immune response due to intestinal permeability is crucial for CeD pathogenesis [199,229,230]. Taken together, these findings suggest that monocytes could also exert a function in the development of CeD by altering the barrier function and TJ composition of IECs, contributing to the passage of gliadin peptides, which enhance the immune response increasing the inflammation status.

4.1.2 CeD monocytes: Surface markers and cytokine production

As observed, co-culture of intestinal epithelial cells and CeD monocytes caused impairment of barrier function with alterations of TJ proteins regarding their protein levels and their subcellular localization. Therefore, we characterized the monocytes isolated from CeD by surface marker analysis and cytokine expression finding a more proinflammatory type of celiac

monocyte population with IL-6 and MCP-1 production and a tendency towards increased expression of TNF-α and IL-1β. A similar proinflammatory cytokine signature had also been observed in intestinal monocytes and macrophages in IBD in other studies [231,232]. Interestingly, Manavalan *et al* have demonstrated that significantly higher levels of proinflammatory cytokines, as IFN-γ, IL-1β, TNF-α, and IL-8 occur in the serum of CeD patients [233]. Interestingly, O'Keeffe *et al* have reported that increased levels of proinflammatory cytokines, as TNF-α and MIF (migration inhibition factor) were even found in GFD patients, who revealed a histologically normal (i.e. completely healed) duodenal mucosa, [234]. Altogether, these findings imply that monocytes from CeD patients carry a more proinflammatory phenotype, producing cytokines that have a deleterious function on the intestinal barrier. However, we did not inhibit this presumably cytokine-related effect on the barrier by applying anti-TNF or IL1 receptor antagonist Anakinra as Lissner et al. did in their study. However, when extrapolating these data, one comes to the conclusion that it is suggestive that pro-inflammatory cytokines are the cause for the barrier-depressing effect of celiac monocytes. Nevertheless, our cytokine expression analysis of IL-1β and TNF-α, which were previously shown by Lissner et al. to be crucial for the IEC barrier defect in the M1- and M0-polarized macrophage model, only revealed a non-significant tendency towards higher levels of these cytokines [77].

Interestingly, Cinova *et al* reported that CeD monocytes presented a more proinflammatory activation expressing higher levels of M1 macrophages surface markers, as CD80, CD86 and CD40, and activation of NF-κB signaling pathway [199]. In accordance to these data, our experiments showed that proinflammatory cytokine secretion coincides with higher levels of proinflammatory surface markers, as CD80 and HLA-DR, which also suggests a more proinflammatory phenotype of the monocytes derived from CeD patients. The expression of CD14 and CD16 can be used in order to characterize human monocytes. The majority of monocytes in the circulating blood carry $CD14^{+}CD16^{-}$ and they are defined as classical monocytes. However, a small subset of $CD14^{+}CD16^{+}$ monocytes, called non-classical/intermediate monocytes, was also identified as a population of approx. 15% of total monocytes circulating in the peripheral blood of individuals [235]. Interestingly, the non-classical/intermediate monocytes carry inflammatory features, since they produce more proinflammatory cytokines. In addition, these non-classical monocytes are observed in inflammatory autoimmune diseases, as multiple sclerosis, lupus erythematodes and neuromyelitis optica [236,237]. Accordingly, we observed that CeD patients revealed higher

percentages of $CD14^{+}CD16^{+}$ monocytes, a finding that also can be interpreted as a accentuation of the proinflammatory phenotype observed in these cells.

In summary, our findings revealed celiac monocytes to have an impact on epthelial barrier function with a specific effect on TJ protein composition. Furthermore, we also observed a tendency to higher frequencies of CD16-positive monocytes, presumably reflecting a pro-inflammatory status of celiac monocytes. In line with this, celiac monocytes secreted higher levels of the proinflammatory cytokines IL-6 and MCP-1, allowing for the conclusion that celiacs – even when successfully on a GFD – reveal a significantly more pro-inflammatory subset of peripheral monocytes.

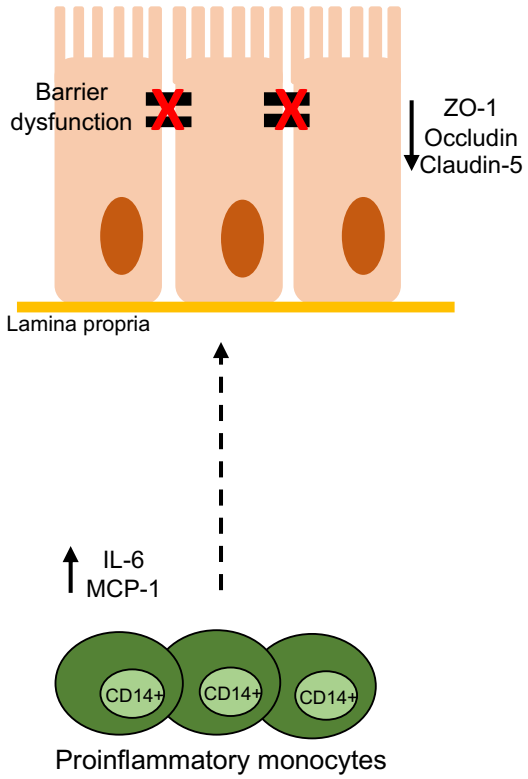

Figure 4.1: Simplified scheme with the main results. CeD-derived monocytes carry a stronger proinflammatory phenotype. As such, proinflammatory cytokines, including IL-6 and MCP-1, are increased. This result might explain the barrier dysfunction observed after co-culture of IECs and CeD monocytes.

4.2 IL-22 as a model cytokine

4.2.1 IL-22: Barrier integrity

IL-22 is a cytokine that with regard to its role in intestinal inflammation was shown to induce inflammation in some and reduce inflammation status in other *in vivo* models of intestinal inflammation[165,180,184]. On the one hand, studies have shown that during infection and inflammation, IL-22 induces tissue repair and maintains integrity of the epithelial layer[165]. On the other hand, other studies indicated a pro-inflammatory role of IL-22 on intestinal epithelial cells, where IL-22 induced mucosal barrier defects and also, together with other proinflammatory cytokines, as IL-17, IL-18 and TNF-α, increased inflammation status[138,187,188]. These on the first view conflicting results suggested that IL-22 action might depend on the inflammatory context, the at the relevant location effective IL-22 concentration and the target structure/tissue.

In this research context, the present study aimed to functionally analyze the intestinal epithelial barrier when exposed to IL-22 and, furthermore, to characterize epithelial cell polarity altered of IL-22-exposed IECs as we hypothesized, that polarity processes might be fundamental in orchestrating barrier function [238,239]. As IL-22 was identified as a crucial mediator in inflammation for intestinal tissue repair and regeneration [180,240], it came to our surprise, that IL-22 rather de-stabilized epithelial barrier function in various IEC models of intestinal barrier function. This appears on the first view to be in contrast to the report by Pickert *et al*, who showed that a barrier-protective role by an increase in wound healing in murine models of intestinal inflammation. However, in our study IL-22 destabilized intestinal barrier function, inducing defects on barrier integrity with a profound reduction of IEC-TER that corresponds to a relevant increase in transepithelial small ion flux which was shown to be secondary to an increase in paracellular permeability (as revealed by 2-path impedance spectroscopy by PD Dr. Susanne Krug, see our accepted manuscript Frontiers in Med – Gastroenterology - 2021) [190,191]. These initially surprising finding were validated in three different intestinal epithelial cell lines, thereby showing dose- and time-dependence of the IL-22 effects. Importantly, the barrier function defect is not limited to small ion flux as shown by the sandwich assay that allows for visualization of leaks allowing macromolecular flux. These data suggested a role for IL-22 in disrupting TJs. Our findings on the other hand were in accordance with a study that showed an IL-22- and claudin-2-dependent mechanism in

triggering a leak-flux diarrhea in the murine *Citrobacter rodentium* as well as a study on epithelial barrier defects for small solutes in the CaCo-2BBE model [183,184]. Further results of our study included an IL-22-induced delay of the reassembly of TJs. Nevertheless, our cautious reasoning at this point in time was, that IL-22 might rather trigger a junctional release of cells (which would be measurable as a functional barrier defect) in order to enable IECs to migrate into wounds. This explanation might cover both aspects of the findings mentioned above.

4.2.2 IL-22: Cell polarity and EMT induction

Further, we noticed, by confocal microscopy, that IL-22 exposure dramatically rearranged the structure of Caco-2 cysts with the occurrence of multilumen cysts, connected with a significantly altered localization of essential cell polarity proteins for apical complex formation, such as Par3 and Dlg1[137]. In line with this, TER monitoring of IL-22-exposed IECs after calcium switch provided functional evidence for a defective assembly of TJs. In accordance to several published studies, we confirmed that IL-22 exposure of IECs increased cell motility and also invasion of IECs into surrounding structures [186,241,242].

Our *in vitro* data thus showed that IL-22 changed TJ structure and severely altered barrier function as well as cell polarity with increased cell motility with EMT induction. Specific transcription factor including Snail and Slug mediate EMT induction as elucidated in the introduction chapter, thereby inducing expressional changes including a decrease in epithelial markers as E-cadherin [120]. In the present study, we showed that IL-22 is a potent inductor of EMT. In that regard, it displays some similarities to IL13, a cytokine that has been previously described as an EMT inductor. In their study, Scharl *et* al reported that TGF-β together with IL-13 led to EMT-like phenotype in IECs [243]. Accordingly, we reported that IL-22 increased expression of Snail and Slug (transcription factors related to EMT induction), and decreased expression of E-cadherin (epithelial marker). These results point to a reprogramming of IECs to allow migration but at the risk of increasing the chance for epithelial invasion to occur. This EMT-process includes the reorganization of TJs leading to a release in junctional tightness and to contributes to IEC migration into mucosal wounds, suggesting that epithelial polarity is altered [244]. As found in the present study, Ji *et al* showed IL-22 exposure stimulated increased EMT-like features in gastric cancer cells as accessed through migration and invasion assay. This effect occurred via regulating the AKT/MMP-9 signaling axis [245]. It differs from our findings,

where although we noticed increased cell motility and EMT induction, we could not observe induction of AKT phospho-levels nor MMP-9 activity, instead we observed activation of MAPK signaling pathway via ERK phosphorylation and increased protein levels of MMP-7.

4.2.3 IL-22: Active signaling pathway

IL-22 binds to its receptor complex (IL10Rb and IL22Ra1) leading to activation of downstream signaling pathways via phosphorylation of Tyk2 and JAK1, such as MAPK, JNK/p38 and STAT3 that might play a role in the IL-22 mechanism of action [170,171]. In our experiments, we did not observe activation of AKT, which suggests that in the models used in our study, this pathway was not activated by IL-22. Brand *et al* and Pickert *et al* have demonstrated an important function of IL-22 regarding wound healing and intestinal healing after inflammation [186,190]. In contrast, STAT3 pathway did not play a role on TJal and polarity reprogramming in our model. In fact, we showed that IL-22 exposure strongly induced STAT3 phosphorylation, which could only be related to survival signaling, as previously described [246,247]. Interestingly, we found activation of MAPK signaling pathway via ERK phosphorylation to be important in IL-22-dependent signaling to barrier function and epithelial polarity. In line, inhibiting this pathway reversed almost completely the effects of IL-22 on IECs regarding cell polarity, EMT induction and barrier function. Altogether, our data point to a crucial role for ERK signaling in the IL-22-dependent programming of IECs.

We reported that IL-22 exposure has a profound effect on barrier function in IECs, leading to impairment in barrier integrity and TJ formation and expression, and effects on cell polarity with formation of aberrant 3D-cysts as well changes in the localization of cell polarity proteins. In addition, we showed that IL-22 induced cell motility and cell invasion, which may be strongly associated to EMT induction in IECs. In our cell model, we found that STA3 is crucial for cell survival and blockage of this signaling pathway leads to cell death. We also described a signaling pathway that seems to be crucial for IL-22 mechanism of action, MAPK/ERK, and once this pathway is blocked, the effects of IL-22 regarding barrier integrity and TJ expression levels are impaired. Taken together, our results imply that IL-22 affects IECs through MAPK/ERK signaling pathway rather than STAT3 pathway.

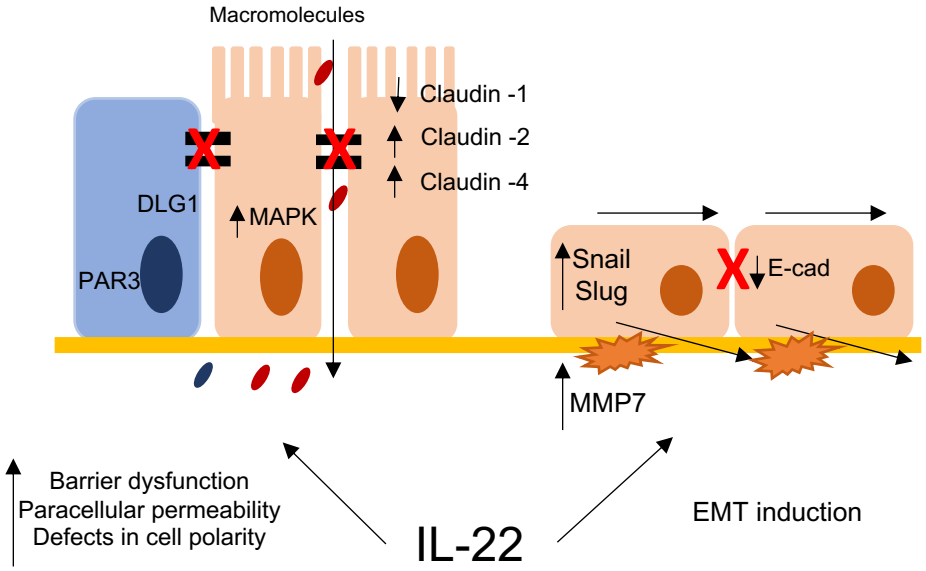

Figure 4.2: Simplified scheme highlighting the major effects caused by IL-22 on IECs. IL-22 exposure caused increased barrier dysfunction and increased paracellular permeability with alterations on claudin levels. At the same time, IL-22 induced defects on cell polarity with mislocation of pivotal polarity proteins, as PAR3 and DLG1. In addition, IL-22 exposure induced EMT by increasing the expression of the transcription factors Snail and Slug. In line with IL-22 being a strong inductor of EMT, E-cadherin protein levels were decreased while MMP7 protein levels were increased.

5 Conclusions

The main goal of the present study was to understand alterations in TJ assembly that might cause inflammation, and consequently, inflammatory diseases in the intestine. We can summarize our findings in two conclusions:

1 In the first part of the thesis, we have shown that $CD14^+$ monocytes isolated from peripheral blood of celiac disease patients induced an intestinal barrier defect mediated by a decrease of occludin and claudin-5 expression as well as altered ZO-1. Furthermore, we also observed a tendency to higher levels of frequency of CD16-positive monocytes, which was related to the enhanced proinflammatory status of these cells. In line, monocytes isolated from celiac disease patients produced more proinflammatory cytokines, specifically IL-6 and MCP-1. Taken together, our results showed that celiac monocytes carry a more proinflammatory phenotype, which was the presumed cause for the depressing effects on IEC barrier function.

2 In the second part of the thesis data were presented that reveal an epithelial reprogramming by the TH17-cytokine IL-22. This includes IEC barrier function as well as cell polarity of IECs. Data were provided that revealed altered TJ composition and assembly. Also, we demonstrated IL-22 effects on cell polarity with formation of Multilumen, aberrant 3-dimensional IEC cysts. In addition, we showed that IL-22 induces EMT in IECs, with decreased protein levels of E-cadherin and increased levels of *SNAI1* (Snail) and *SNAI2* (Slug) gene expression. The induction of EMT by IL-22 presumably explains the increased cell motility and increased cell invasion. In our cell model, we found that STA3 is crucial for cell survival and blockage of this signaling pathway leads to cell death. We also described the MAPK/ERK signaling pathway to be crucial for barrier, polarity and EMT as once this pathway is blocked, IL-22-dependent effects are reversed. Taken together, our results show that IL-22 has an effect on reprogramming intestinal epithelial cells through MAPK/ERK signaling pathway.

6. References

1. Groschwitz KR, Hogan SP. Intestinal Barrier Function: Molecular Regulation and Disease Pathogenesis. *J Allergy Clin Immunol*. 2009;124(1):3-22. doi:10.1016/j.jaci.2009.05.038

2. Hu YJ, Wang YD, Tan FQ, Yang WX. Regulation of paracellular permeability: Factors and mechanisms. *Mol Biol Rep*. 2013;40(11):6123-6142. doi:10.1007/s11033-013-2724-y

3. Pelaseyed T, Bergström JH, Gustafsson JK, et al. The mucus and mucins of the goblet cells and enterocytes provide the first defense line of the gastrointestinal tract and interact with the immune system. *Immunol Rev*. 2014;260(1):8-20. doi:10.1111/imr.12182

4. Johansson MEV, Sjövall H, Hansson GC. The gastrointestinal mucus system in health and disease. *Nat Rev Gastroenterol Hepatol*. 2013;10(6):352-361. doi:10.1038/nrgastro.2013.35

5. Schütte A, Ermund A, Becker-Pauly C, et al. Microbial-induced meprin β cleavage in MUC2 mucin and a functional CFTR channel are required to release anchored small intestinal mucus. *Proc Natl Acad Sci U S A*. 2014;111(34):12396-12401. doi:10.1073/pnas.1407597111

6. France MM, Turner JR. The mucosal barrier at a glance. *J Cell Sci*. 2017;130(2):307-314. doi:10.1242/jcs.193482

7. Dhanisha SS, Guruvayoorappan C, Drishya S, Abeesh P. Mucins: Structural diversity, biosynthesis, its role in pathogenesis and as possible therapeutic targets. *Crit Rev Oncol Hematol*. 2018;122:98-122. doi:10.1016/j.critrevonc.2017.12.006

8. Van der Sluis M, De Koning BAE, De Bruijn ACJM, et al. Muc2-Deficient Mice Spontaneously Develop Colitis, Indicating That MUC2 Is Critical for Colonic Protection. *Gastroenterology*. 2006;131(1):117-129. doi:10.1053/j.gastro.2006.04.020

9. Velcich A, Yang WC, Heyer J, et al. Colorectal cancer in mice genetically deficient in the mucin Muc2. *Science (80-)*. 2002;295(5560):1726-1729. doi:10.1126/science.1069094

10. Peterson LW, Artis D. Intestinal epithelial cells: Regulators of barrier function and immune homeostasis. *Nat Rev Immunol*. 2014;14(3):141-153. doi:10.1038/nri3608

11. Okumura R, Takeda K. Roles of intestinal epithelial cells in the maintenance of gut homeostasis. *Exp Mol Med*. 2017;49(5):e338-e338. doi:10.1038/emm.2017.20

12. Martini E, Krug SM, Siegmund B, Neurath MF, Becker C. Mend Your Fences: The Epithelial Barrier and its Relationship With Mucosal Immunity in Inflammatory Bowel Disease. *CMGH*. 2017;4(1):33-46. doi:10.1016/j.jcmgh.2017.03.007

13. Miron N, Cristea V. Enterocytes: active cells in tolerance to food and microbial antigens in the gut. *Clin Exp Immunol*. 2012;167(3):405-412. doi:10.1111/j.1365-2249.2011.04523.x

14. Martín-Villa JM, Ferre-López S, López-Suárez JC, Corell A, Pérez-Blas M, Arnaiz-Villena A. Cell surface phenotype and ultramicroscopic analysis of purified human enterocytes: A possible antigen-presenting cell in the intestine. *Tissue Antigens*. 1997;50(6):586-592. doi:10.1111/j.1399-0039.1997.tb02916.x

15. Nair MG, Guild KJ, Du Y, et al. Goblet Cell-Derived Resistin-Like Molecule β Augments CD4 + T Cell Production of IFN-γ and Infection-Induced Intestinal Inflammation . *J Immunol*. 2008;181(7):4709-4715. doi:10.4049/jimmunol.181.7.4709

16. Gassler N. Paneth cells in intestinal physiology and pathophysiology. *World J Gastrointest Pathophysiol*. 2017;8(4):150-160. doi:10.4291/wjgp.v8.i4.150

17. Armbruster NS, Stange EF, Wehkamp J. In the Wnt of Paneth Cells: Immune-Epithelial Crosstalk in Small Intestinal Crohn's Disease. *Front Immunol*. 2017;8(SEP):1204. doi:10.3389/fimmu.2017.01204

18. Bevins CL, Salzman NH. Paneth cells, antimicrobial peptides and maintenance of intestinal homeostasis. *Nat Rev Microbiol*. 2011;9(5):356-368. doi:10.1038/nrmicro2546

19. Wehkamp J, Salzman NH, Porter E, et al. Reduced Paneth cell α-defensins in ileal Crohn's disease. *Proc Natl Acad Sci U S A*. 2005;102(50):18129-18134. doi:10.1073/pnas.0505256102

20. Sato T, Van Es JH, Snippert HJ, et al. Paneth cells constitute the niche for Lgr5 stem cells in intestinal crypts. *Nature*. 2011;469(7330):415-418. doi:10.1038/nature09637

21. Kucharzik T, Lugering N, Rautenberg K, et al. Role of M Cells in Intestinal Barrier Function. *Ann N Y Acad Sci*. 2006;915(1):171-183. doi:10.1111/j.1749-6632.2000.tb05240.x

22. Ohno H. Intestinal M cells. *J Biochem*. 2015;159(2):151-160. doi:10.1093/jb/mvv121

23. Rios D, Wood MB, Li J, Chassaing B, Gewirtz AT, Williams IR. Antigen sampling by intestinal M cells is the principal pathway initiating mucosal IgA production to commensal enteric bacteria. *Mucosal Immunol*. 2016;9(4):907-916. doi:10.1038/mi.2015.121

24. Howitt MR, Lavoie S, Michaud M, et al. Tuft cells, taste-chemosensory cells, orchestrate parasite type 2 immunity in the gut. *Science (80-)*. 2016;351(6279):1329-1333. doi:10.1126/science.aaf1648

25. O'Leary CE, Schneider C, Locksley RM. Tuft Cells—Systemically Dispersed Sensory Epithelia Integrating Immune and Neural Circuitry. *Annu Rev Immunol*. 2019;37(1):47-72. doi:10.1146/annurev-immunol-042718-041505

26. Gerbe F, Sidot E, Smyth DJ, et al. Intestinal epithelial tuft cells initiate type 2 mucosal immunity to helminth parasites. *Nature*. 2016;529(7585):226-230. doi:10.1038/nature16527

27. Van Es JH, Wiebrands K, López-Iglesias C, et al. Enteroendocrine and tuft cells support Lgr5 stem cells on Paneth cell depletion. *Proc Natl Acad Sci U S A*. 2019;116(52):26599-26605. doi:10.1073/pnas.1801888117

28. Kong S, Zhang YH, Zhang W. Regulation of intestinal epithelial cells properties and functions by amino acids. *Biomed Res Int*. 2018;2018(Figure 1). doi:10.1155/2018/2819154

29. Sandek A, Anker S, Haehling S. The Gut and Intestinal Bacteria in Chronic Heart Failure. *Curr Drug Metab*. 2009;10(1):22-28. doi:10.2174/138920009787048374

30. Campbell HK, Maiers JL, DeMali KA. Interplay between tight junctions & adherens junctions. *Exp Cell Res*. 2017;358(1):39-44. doi:10.1016/j.yexcr.2017.03.061

31. Ikeda W, Nakanishi H, Miyoshi J, et al. Afadin: A key molecule essential for structural organization of cell- cell junctions of polarized epithelia during embryogenesis. *J Cell Biol*. 1999;146(5):1117-1131. doi:10.1083/jcb.146.5.1117

32. Sato T, Fujita N, Yamada A, et al. Regulation of the assembly and adhesion activity of E-cadherin by nectin and afadin for the formation of adherens junctions in Madin-Darby canine kidney cells. *J Biol Chem*. 2006;281(8):5288-5299. doi:10.1074/jbc.M510070200

33. Tian X, Liu Z, Niu B, et al. E-Cadherin/β-Catenin Complex and the Epithelial Barrier. *BioMed Biotechnol*. 2011;2011:1-6. doi:10.1016/j.biocel.2008.09.027

34. Anderson JM, Van Itallie CM. Physiology and function of the tight junction. *Cold Spring Harb Perspect Biol*. 2009;1(2):2584-2585. doi:10.1101/cshperspect.a002584

35. Günzel D, Fromm M. Claudins and other tight junction proteins. *Compr Physiol*. 2012;2(3):1819-1852. doi:10.1002/cphy.c110045

36. Mariano C, Sasaki H, Brites D, Brito MA. A look at tricellulin and its role in tight junction formation and maintenance. *Eur J Cell Biol*. 2011;90(10):787-796. doi:10.1016/j.ejcb.2011.06.005

37. Ikenouchi J, Sasaki H, Tsukita S, Furuse M, Tsukita S. Loss of occludin affects tricellular localization of tricellulin. *Mol Biol Cell*. 2008;19(11):4687-4693. doi:10.1091/mbc.E08-05-0530

38. Balda MS, Flores-Maldonado C, Cereijido M, Matter K. Multiple domains of occludin are involved in the regulation of paracellular permeability. *J Cell Biochem*. 2000;78(1):85-96. doi:10.1002/(SICI)1097-4644(20000701)78:1<85::AID-JCB8>3.0.CO;2-F

39. Saitou M, Furuse M, Sasaki H, et al. Complex phenotype of mice lacking occludin, a component of tight junction strands. *Mol Biol Cell*. 2000;11(12):4131-4142. doi:10.1091/mbc.11.12.4131

40. Schulzke JD, Gitter AH, Mankertz J, et al. Epithelial transport and barrier function in occludin-deficient mice. *Biochim Biophys Acta - Biomembr*. 2005;1669(1):34-42. doi:10.1016/j.bbamem.2005.01.008

41. Van Itallie CM, Fanning AS, Holmes J, Anderson JM. Occludin is required for cytokine-induced regulation of tight junction barriers. *J Cell Sci*. 2010;123(16):2844-2852. doi:10.1242/jcs.065581

42. Buschmann MM, Shen L, Rajapakse H, et al. Occludin OCEL-domain interactions are required for maintenance and regulation of the tight junction barrier to macromolecular flux. *Mol Biol Cell*. 2013;24(19):3056-3068. doi:10.1091/mbc.E12-09-0688

43. Richter JF, Hildner M, Schmauder R, Turner JR, Schumann M, Reiche J. Occludin knockdown is not sufficient to induce transepithelial macromolecule passage. *Tissue Barriers*. 2019;7(2). doi:10.1080/21688370.2019.1608759

44. Du D, Xu F, Yu L, et al. The Tight Junction Protein, Occludin, Regulates the Directional Migration of Epithelial Cells. *Dev Cell*. 2010;18(1):52-63. doi:10.1016/j.devcel.2009.12.008

45. Günzel D, Yu ASL. *Claudins and the Modulation of Tight Junction Permeability*. Vol 93.; 2013. doi:10.1152/physrev.00019.2012

46. Furuse M, Sasaki H, Fujimoto K, Tsukita S. A single gene product, claudin-1 or -2, reconstitutes tight junction strands and recruits occludin in fibroblasts. *J Cell Biol*. 1998;143(2):391-401. doi:10.1083/jcb.143.2.391

47. Van Itallie CM, Tietgens AJ, Anderson JM. Visualizing the dynamic coupling of claudin strands to the actin cytoskeleton through ZO-1. *Mol Biol Cell*. 2017;28(4):524-534. doi:10.1091/mbc.E16-10-0698

48. Umeda K, Ikenouchi J, Katahira-Tayama S, et al. ZO-1 and ZO-2 Independently Determine Where Claudins Are Polymerized in Tight-Junction Strand Formation. *Cell*. 2006;126(4):741-754. doi:10.1016/j.cell.2006.06.043

49. Krug SM, Günzel D, Conrad MP, et al. Claudin-17 forms tight junction channels with distinct anion selectivity. *Cell Mol Life Sci*. 2012;69(16):2765-2778. doi:10.1007/s00018-012-0949-x

50. Rosenthal R, Milatz S, Krug SM, et al. Claudin-2, a component of the tight junction, forms a paracellular water channel. *J Cell Sci*. 2010;123(11):1913-1921. doi:10.1242/jcs.060665

51. Zeissig S, Bürgel N, Günzel D, et al. Changes in expression and distribution of claudin 2, 5 and 8 lead to discontinuous tight junctions and barrier dysfunction in active Crohn's disease. *Gut*. 2007;56(1):61-72. doi:10.1136/gut.2006.094375

52. Kominsky SL, Argani P, Korz D, et al. Loss of the tight junction protein claudin-7 correlates with histological grade in both ductal carcinoma in situ and invasive ductal carcinoma of the breast. *Oncogene*. 2003;22(13):2021-2033. doi:10.1038/sj.onc.1206199

53. Zhu Y, Brännström M, Janson PO, Sundfeldt K. Differences in expression patterns of the tight junction proteins, claudin 1, 3, 4 and 5, in human ovarian surface epithelium as compared to epithelia in inclusion cysts and epithelial ovarian tumours. *Int J Cancer*. 2006;118(8):1884-1891. doi:10.1002/ijc.21506

54. Prasad S, Mingrino R, Kaukinen K, et al. Inflammatory processes have differential effects on claudins 2, 3 and 4 in colonic epithelial cells. *Lab Investig*. 2005;85:1139-1162. doi:10.1038/labinvest.3700316

55. Oshima T, Miwa H, Joh T. Changes in the expression of claudins in active ulcerative colitis. In: *Journal of Gastroenterology and Hepatology (Australia)*. Vol 23. Blackwell Publishing; 2008:S146-50. doi:10.1111/j.1440-1746.2008.05405.x

56. Ebnet K, Suzuki A, Ohno S, Vestweber D. Junctional adhesion molecules (JAMs): More molecules with dual functions? *J Cell Sci*. 2004;117(1):19-29. doi:10.1242/jcs.00930

57. Itoh M, Sasaki H, Furuse M, Ozaki H, Kita T, Tsukita S. Junctional adhesion molecule (JAM) binds to PAR-3: A possible mechanism for the recruitment of PAR-3 to tight junctions. *J Cell Biol*. 2001;154(3):491-497. doi:10.1083/jcb.200103047

58. Laukoetter MG, Nava P, Lee WY, et al. JAM-A regulates permeability and inflammation in the intestine in vivo. *J Exp Med*. 2007;204(13):3067-3076. doi:10.1084/jem.20071416

59. Vetrano S, Rescigno M, Rosaria Cera M, et al. Unique Role of Junctional Adhesion Molecule-A in Maintaining Mucosal Homeostasis in Inflammatory Bowel Disease. *Gastroenterology*. 2008;135(1):173-184. doi:10.1053/j.gastro.2008.04.002

60. Tuncay H, Brinkmann BF, Steinbacher T, et al. JAM-A regulates cortical dynein localization through Cdc42 to control planar spindle orientation during mitosis. *Nat Commun*. 2015;6(1):1-12. doi:10.1038/ncomms9128

61. Ebnet K, Schulz CU, Meyer Zu Brickwedde MK, Pendl GG, Vestweber D. Junctional adhesion molecule interacts with the PDZ domain-containing proteins AF-6 and ZO-1. *J Biol Chem*. 2000;275(36):27979-27988. doi:10.1074/jbc.M002363200

62. Ebnet K, Suzuki A, Horikoshi Y, et al. The cell polarity protein ASIP/PAR-3 directly associates with junctional adhesion molecule (JAM). *EMBO J*. 2001;20(14):3738-3748. doi:10.1093/emboj/20.14.3738

63. Schumann M, Kühnel A. Pathophysiological role of TNF in inflammatory bowel disease: TNF and its impact on barrier function. In: *Anti-Tumor Necrosis Factor Therapy in Inflammatory Bowel Disease*. S. Karger AG; 2015:35-48. doi:10.1159/000381386

64. Saurer L, Mueller C. T cell-mediated immunoregulation in the gastrointestinal tract. *Allergy*. 2009;64(4):505-519. doi:10.1111/j.1398-9995.2009.01965.x

65. Inagaki-Ohara K, Dewi FN, Hisaeda H, et al. Intestinal intraepithelial lymphocytes sustain the epithelial barrier function against Eimeria vermiformis infection. *Infect Immun*. 2006;74(9):5292-5301. doi:10.1128/IAI.02024-05

66. Konjar Š, Ferreira C, Blankenhaus B, Veldhoen M. Intestinal barrier interactions with specialized CD8 T cells. *Front Immunol*. 2017;8(OCT). doi:10.3389/fimmu.2017.01281

67. Edelblum KL, Shen L, Weber CR, et al. Dynamic migration of γδ intraepithelial lymphocytes requires occludin. *Proc Natl Acad Sci U S A*. 2012;109(18):7097-7102. doi:10.1073/pnas.1112519109

68. Kuhn KA, Schulz HM, Regner EH, et al. Bacteroidales recruit IL-6-producing intraepithelial lymphocytes in the colon to promote barrier integrity. *Mucosal Immunol*. 2018;11(2):357-368. doi:10.1038/mi.2017.55

69. Ferguson A, Murray D. Quantitation of intraepithelial lymphocytes in human jejunum. *Gut*. 1971;12(12):988-994. doi:10.1136/gut.12.12.988

70. Mayassi T, Jabri B. Human intraepithelial lymphocytes. *Mucosal Immunol*. 2018;11(5):1281-1289. doi:10.1038/s41385-018-0016-5

71. Jabri B, Abadie V. IL-15 functions as a danger signal to regulate tissue-resident T cells and tissue destruction. *Nat Rev Immunol*. 2015;15(12):771-783. doi:10.1038/nri3919

72. Moens E, Veldhoen M. Epithelial barrier biology: good fences make good neighbours. *Immunology*. 2012;135(1):1-8. doi:10.1111/j.1365-2567.2011.03506.x

73. Varol C, Zigmond E, Jung S. Securing the immune tightrope: Mononuclear phagocytes in the intestinal lamina propria. *Nat Rev Immunol*. 2010;10(6):415-426. doi:10.1038/nri2778

74. Sung S-SJ, Fu SM, Rose CE, Gaskin F, Ju S-T, Beaty SR. A Major Lung CD103 (α E)-β 7 Integrin-Positive Epithelial Dendritic Cell Population Expressing Langerin and Tight Junction Proteins . *J Immunol*. 2006;176(4):2161-2172. doi:10.4049/jimmunol.176.4.2161

75. Morhardt TL, Hayashi A, Ochi T, et al. IL-10 produced by macrophages regulates epithelial integrity in the small intestine. *Sci Rep*. 2019;9(1):1-10. doi:10.1038/s41598-018-38125-x

76. Wang S, Ye Q, Zeng X, Qiao S. Functions of macrophages in the maintenance of intestinal homeostasis. *J Immunol Res*. 2019;2019. doi:10.1155/2019/1512969

77. Lissner D, Schumann M, Batra A, et al. Monocyte and M1 macrophage-induced barrier defect contributes to chronic intestinal inflammation in IBD. *Inflamm Bowel Dis*. 2015;21(6):1297-1305. doi:10.1097/MIB.0000000000000384

78. Blikslager AT, Roberts MC, Rhoads JM, Argenzio RA. Prostaglandins I2 and E2 have a synergistic role in rescuing epithelial barrier function in porcine ileum. *J Clin Invest*. 1997;100(8):1928-1933. doi:10.1172/JCI119723

79. Grainger JR, Wohlfert EA, Fuss IJ, et al. Inflammatory monocytes regulate pathologic responses to commensals during acute gastrointestinal infection. *Nat Med*. 2013;19(6):713-721. doi:10.1038/nm.3189

80. Grainger JR, Konkel JE, Zangerle-Murray T, Shaw TN. Macrophages in gastrointestinal homeostasis and inflammation. *Pflugers Arch Eur J Physiol*. 2017;469(3-4):527-539. doi:10.1007/s00424-017-1958-2

81. Miyoshi H, VanDussen KL, Malvin NP, et al. Prostaglandin E2 promotes intestinal repair through an adaptive cellular response of the epithelium. *EMBO J*. 2017;36(1):5-24. doi:10.15252/embj.201694660

82. Denning TL, Wang YC, Patel SR, Williams IR, Pulendran B. Lamina propria macrophages and dendritic cells differentially induce regulatory and interleukin 17-producing T cell responses. *Nat Immunol*. 2007;8(10):1086-1094. doi:10.1038/ni1511

83. Sahin U, Derhovanessian E, Miller M, et al. Personalized RNA mutanome vaccines mobilize poly-specific therapeutic immunity against cancer. *Nature*. 2017;547(7662):222-226. doi:10.1038/nature23003

84. Pamer EG. Immune responses to Listeria monocytogenes. *Nat Rev Immunol*. 2004;4(10):812-823. doi:10.1038/nri1461

85. Masopust D. Developing an HIV cytotoxic T-lymphocyte vaccine: issues of CD8 T-cell quantity, quality and location. *J Intern Med*. 2009;265(1):125-137. doi:10.1111/j.1365-2796.2008.02054.x

86. Epstein JE, Tewari K, Lyke KE, et al. Live attenuated malaria vaccine designed to protect through hepatic CD8+ T cell immunity. *Science (80-)*. 2011;334(6055):475-480. doi:10.1126/science.1211548

87. Tanaka H, Demeure CE, Rubio M, Delespesse G, Sarfati M. Human monocyte-derived dendritic cells induce naive T cell differentiation into T helper cell type 2 (Th2) or Th1/Th2 effectors: Role of stimulator/responder ratio. *J Exp Med*. 2000;192(3):405-411. doi:10.1084/jem.192.3.405

88. Malissen B, Grégoire C, Malissen M, Roncagalli R. Integrative biology of T cell activation. *Nat Immunol*. 2014;15(9):790-797. doi:10.1038/ni.2959

89. Agace WW. T-cell recruitment to the intestinal mucosa. *Trends Immunol*. 2008;29(11):514-522. doi:10.1016/j.it.2008.08.003

90. Ma H, Tao W, Zhu S. T lymphocytes in the intestinal mucosa: defense and tolerance. *Cell Mol Immunol*. 2019;16(3):216-224. doi:10.1038/s41423-019-0208-2

91. Turner JR. Intestinal mucosal barrier function in health and disease. *Nat Rev Immunol*. 2009;9(11):799-809. doi:10.1038/nri2653

92. Tsuchiya T, Fukuda S, Hamada H, et al. Role of γδT Cells in the Inflammatory Response of Experimental Colitis Mice. *J Immunol*. 2003;171(10):5507-5513. doi:10.4049/jimmunol.171.10.5507

93. Sun X, Cai Y, Fleming C, et al. Innate γδT17 cells play a protective role in DSS-induced colitis via recruitment of Gr-1+CD11b+ myeloid suppressor cells. *Oncoimmunology*. 2017;6(5). doi:10.1080/2162402X.2017.1313369

94. Leppkes M, Becker C, Ivanov II, et al. RORγ-Expressing Th17 Cells Induce Murine Chronic Intestinal Inflammation via Redundant Effects of IL-17A and IL-17F. *Gastroenterology*. 2009;136(1):257-267. doi:10.1053/j.gastro.2008.10.018

95. Schmidt EGW, Larsen HL, Kristensen NN, et al. TH17 cell induction and effects of IL-17A and IL-17F blockade in experimental colitis. *Inflamm Bowel Dis*. 2013;19(8):1567-1576. doi:10.1097/MIB.0b013e318286fa1c

96. Powrie F, Leach MW, Mauze S, Menon S, Barcomb Caddle L, Coffman RL. Inhibition of Thl responses prevents inflammatory bowel disease in scid mice reconstituted with CD45RBhi CD4+ T cells. *Immunity*. 1994;1(7):553-562. doi:10.1016/1074-7613(94)90045-0

97. Zhu J-F, Xu Y, Zhao J, et al. IL-33 Protects Mice against DSS-Induced Chronic Colitis by Increasing Both Regulatory B Cell and Regulatory T Cell Responses as Well as Decreasing Th17 Cell Response. *J Immunol Res*. 2018;2018:1-12. doi:10.1155/2018/1827901

98. Hueber W, Sands BE, Lewitzky S, et al. Secukinumab, a human anti-IL-17A monoclonal antibody, for moderate to severe Crohn's disease: Unexpected results of a randomised, double-blindplacebo- controlled trial. *Gut*. 2012;61(12):1693-1700. doi:10.1136/gutjnl-2011-301668

99. Artis D, Spits H. The biology of innate lymphoid cells. *Nature*. 2015;517(7534):293-301. doi:10.1038/nature14189

100. Diefenbach A, Gnafakis S, Shomrat O. Innate Lymphoid Cell-Epithelial Cell Modules Sustain Intestinal Homeostasis. *Immunity*. 2020;52(3):452-463. doi:10.1016/j.immuni.2020.02.016

101. Bernink JH, Peters CP, Munneke M, et al. Human type 1 innate lymphoid cells accumulate in inflamed mucosal tissues. *Nat Immunol*. 2013;14(3):221-229. doi:10.1038/ni.2534

102. Fuchs A, Vermi W, Lee JS, et al. Intraepithelial type 1 innate lymphoid cells are a unique subset of il-12- and il-15-responsive ifn-γ-producing cells. *Immunity*. 2013;38(4):769-781. doi:10.1016/j.immuni.2013.02.010

103. Moro K, Yamada T, Tanabe M, et al. Innate production of TH 2 cytokines by adipose tissue-associated c-Kit+ Sca-1+ lymphoid cells. *Nature*. 2010;463(7280):540-544. doi:10.1038/nature08636

104. Neill DR, Wong SH, Bellosi A, et al. Nuocytes represent a new innate effector leukocyte that mediates type-2 immunity. *Nature*. 2010;464(7293):1367-1370. doi:10.1038/nature08900

105. Tumanov A V., Koroleva EP, Guo X, et al. Lymphotoxin controls the IL-22 protection pathway in gut innate lymphoid cells during mucosal pathogen challenge. *Cell Host Microbe*. 2011;10(1):44-53. doi:10.1016/j.chom.2011.06.002

106. Guo X, Qiu J, Tu T, et al. Induction of innate lymphoid cell-derived interleukin-22 by the transcription factor STAT3 mediates protection against intestinal infection. *Immunity*. 2014;40(1):25-39. doi:10.1016/j.immuni.2013.10.021

107. Buonocore S, Ahern PP, Uhlig HH, et al. Innate lymphoid cells drive interleukin-23-dependent innate intestinal pathology. *Nature*. 2010;464(7293):1371-1375. doi:10.1038/nature08949

108. Eken A, Singh AK, Treuting PM, Oukka M. IL-23R+ innate lymphoid cells induce colitis via interleukin-22-dependent mechanism. *Mucosal Immunol*. 2014;7(1):143-154. doi:10.1038/mi.2013.33

109. Thursby E, Juge N. Introduction to the human gut microbiota. *Biochem J*. 2017;474(11):1823-1836. doi:10.1042/BCJ20160510

110. Ostaff MJ, Stange EF, Wehkamp J. Antimicrobial peptides and gut microbiota in homeostasis and pathology. *EMBO Mol Med*. 2013;5(10):1465-1483. doi:10.1002/emmm.201201773

111. Ince MN, Blazar BR, Edmond MB, Tricot G, Wannemuehler MJ. Understanding Luminal Microorganisms and Their Potential Effectiveness in Treating Intestinal Inflammation. *Inflamm Bowel Dis*. 2016;22(1):194-201. doi:10.1097/MIB.0000000000000599

112. Pull SL, Doherty JM, Mills JC, Gordon JI, Stappenbeck TS. Activated macrophages are an adaptive element of the colonic epithelial progenitor niche necessary for regenerative responses to injury. *Proc Natl Acad Sci U S A*. 2005;102(1):99-104. doi:10.1073/pnas.0405979102

113. Sokol H, Pigneur B, Watterlot L, et al. Faecalibacterium prausnitzii is an anti-inflammatory commensal bacterium identified by gut microbiota analysis of Crohn disease patients. *Proc Natl Acad Sci U S A*. 2008;105(43):16731-16736. doi:10.1073/pnas.0804812105

114. Llopis M, Antolin M, Carol M, et al. Lactobacillus casei downregulates commensals' inflammatory signals in Crohn's disease mucosa. *Inflamm Bowel Dis*. 2009;15(2):275-283. doi:10.1002/ibd.20736

115. Qiu X, Zhang F, Yang X, et al. Changes in the composition of intestinal fungi and their role in mice with dextran sulfate sodium-induced colitis. *Sci Rep*. 2015;5(1):1-12. doi:10.1038/srep10416

116. Dieterich W, Schink M, Zopf Y. Microbiota in the Gastrointestinal Tract. *Med Sci*. 2018;6(4):116. doi:10.3390/medsci6040116

117. Alam A, Neish A. Role of gut microbiota in intestinal wound healing and barrier function. *Tissue Barriers*. 2018;6(3). doi:10.1080/21688370.2018.1539595

118. Laval L, Martin R, Natividad JN, et al. Lactobacillus rhamnosus CNCM I-3690 and the commensal bacterium faecalibacterium prausnitzii A2-165 exhibit similar protective effects to induced barrier hyper-permeability in mice. *Gut Microbes*. 2015;6(1):1-9. doi:10.4161/19490976.2014.990784

119. Ewaschuk JB, Diaz H, Meddings L, et al. Secreted bioactive factors from Bifidobacterium infantis enhance epithelial cell barrier function. *Am J Physiol - Gastrointest Liver Physiol*. 2008;295(5):1025-1034. doi:10.1152/ajpgi.90227.2008

120. Kalluri R, Weinberg RA. The basics of epithelial-mesenchymal transition. *J Clin Invest*. 2009;119(6):1420-1428. doi:10.1172/JCI39104

121. Gilles C, Newgreen DF, Sato H, Thompson EW. Matrix Metalloproteases and Epithelial-to-Mesenchymal Transition: Implications for Carcinoma Metastasis. Published online 2013. Accessed February 14, 2021. https://www.ncbi.nlm.nih.gov/books/NBK6387/

122. Daniele V, Simeone P, Frank J, et al. Translating epithelial mesenchymal transition markers into the clinic: Novel insights from proteomics. *EuPA Open Proteomics*. 2016;10:31-41. doi:10.1016/j.euprot.2016.01.003

123. Kim YS, Yi BR, Kim NH, Choi KC. Role of the epithelial-mesenchymal transition and its effects on embryonic stem cells. *Exp Mol Med*. 2014;46(8):e108-e108. doi:10.1038/emm.2014.44

124. Dongre A, Weinberg RA. New insights into the mechanisms of epithelial–mesenchymal transition and implications for cancer. *Nat Rev Mol Cell Biol*. 2019;20(2):69-84. doi:10.1038/s41580-018-0080-4

125. Gehren AS, Rocha MR, de Souza WF, Morgado-Díaz JA. Alterations of the apical junctional complex and actin cytoskeleton and their role in colorectal cancer progression. *Tissue Barriers*. 2015;3(3):1-12. doi:10.1080/21688370.2015.1017688

126. Peinado H, Portillo F, Cano A. Transcriptional regulation of cadherins during development and carcinogenesis. *Int J Dev Biol*. 2004;48(5-6):365-375. doi:10.1387/ijdb.041794hp

127. Nelson WJ. Regulation of cell-cell adhesion by the cadherin-catenin complex. *Biochem Soc Trans*. 2008;36(2):149-155. doi:10.1042/BST0360149

128. Baum B, Georgiou M. Dynamics of adherens junctions in epithelial establishment, maintenance, and remodeling. *J Cell Biol*. 2011;192(6):907-917. doi:10.1083/jcb.201009141

129. Medici D, Hay ED, Olsen BR. Snail and slug promote epithelial-mesenchymal transition through β-catenin-T-cell factor-4-dependent expression of transforming growth factor-β3. *Mol Biol Cell*. 2008;19(11):4875-4887. doi:10.1091/mbc.E08-05-0506

130. Ikenouchi J, Matsuda M, Furuse M, Tsukita S. Regulation of tight junctions during the epithelium-mesenchyme transition: Direct repression of the gene expression of claudins/occludin by Snail. *J Cell Sci*. 2003;116(10):1959-1967. doi:10.1242/jcs.00389

131. Bartis D, Mise N, Mahida RY, Eickelberg O, Thickett DR. Epithelial-mesenchymal transition in lung development and disease: Does it exist and is it important? *Thorax*. 2014;69(8):760-765. doi:10.1136/thoraxjnl-2013-204608

132. Knust E. Regulation of epithelial cell shape and polarity by cell-cell adhesion (Review). *Mol Membr Biol*. 2002;19(2):113-120. doi:10.1080/09687680210137219

133. Piroli ME, Blanchette JO, Jabbarzadeh E. Polarity as a physiological modulator of cell function. *Front Biosci - Landmark*. 2019;24(3):451-462. doi:10.2741/4728

134. Schneeberger K, Roth S, Nieuwenhuis EES, Middendorp S. Intestinal epithelial cell polarity defects in disease: Lessons from microvillus inclusion disease. *DMM Dis Model Mech*. 2018;11(2). doi:10.1242/dmm.031088

135. Klunder LJ, Faber KN, Dijkstra G, Van Ijzendoorn SCD. Mechanisms of cell polarity – Controlled epithelial homeostasis and immunity in the intestine. *Cold Spring Harb Perspect Biol*. 2017;9(7):a027888. doi:10.1101/cshperspect.a027888

136. Lee JL, Streuli CH. Integrins and epithelial cell polarity. *J Cell Sci*. 2014;127(15):3217-3225. doi:10.1242/jcs.146142

137. Campanale JP, Sun TY, Montell DJ. Development and dynamics of cell polarity at a glance. *J Cell Sci*. 2017;130(7):1201-1207. doi:10.1242/jcs.188599

138. Fabre T, Molina MF, Soucy G, et al. Type 3 cytokines IL-17A and IL-22 drive TGF-–dependent liver fibrosis. *Sci Immunol*. 2018;3(28). doi:10.1126/sciimmunol.aar7754

139. Orlando K, Guo W. Membrane organization and dynamics in cell polarity. *Cold Spring Harb Perspect Biol*. 2009;1(5):a001321. doi:10.1101/cshperspect.a001321

140. Assémat E, Bazellières E, Pallesi-Pocachard E, Le Bivic A, Massey-Harroche D. Polarity complex proteins. *Biochim Biophys Acta - Biomembr*. 2008;1778(3):614-630. doi:10.1016/j.bbamem.2007.08.029

141. Michel D, Arsanto JP, Massey-Harroche D, Béclin C, Wijnholds J, Le Bivic A. PATJ connects and stabilizes apical and lateral components of tight junctions in human intestinal cells. *J Cell Sci*. 2005;118(17):4049-4057. doi:10.1242/jcs.02528

142. Schlüter MA, Pfarr CS, Pieczynski J, et al. Trafficking of Crumbs3 during cytokinesis is crucial for lumen formation. *Mol Biol Cell*. 2009;20(22):4652-4663. doi:10.1091/mbc.E09-02-0137

143. Liu J, Li J, Ren Y, Liu P. DLG5 in cell polarity maintenance and cancer development. *Int J Biol Sci*. 2014;10(5):543-549. doi:10.7150/ijbs.8888

144. Murdoch JN, Henderson DJ, Doudney K, et al. Disruption of scribble (Scrb1) causes severe neural tube defects in the circletail mouse. *Hum Mol Genet*. 2003;12(2):87-98. doi:10.1093/hmg/ddg014

145. Murdoch JN, Damrau C, Paudyal A, et al. Genetic interactions between planar cell polarity genes cause diverse neural tube defects in mice. *DMM Dis Model Mech*. 2014;7(10):1153-1163. doi:10.1242/dmm.016758

146. Dauber A, Golzio C, Guenot C, et al. Scrib and puf60 are primary drivers of the multisystemic phenotypes of the 8q24.3 copy-Number variant. *Am J Hum Genet*. 2013;93(5):798-811. doi:10.1016/j.ajhg.2013.09.010

147. Mehalow AK, Kameya S, Smith RS, et al. CRB1 is essential for external limiting membrane integrity and photoreceptor morphogenesis in the mammalian retina. *Hum Mol Genet*. 2003;12(17):2179-2189. doi:10.1093/hmg/ddg232

148. Stoll M, Corneliussen B, Costello CM, et al. Genetic variation in DLG5 is associated with inflammatory bowel disease. *Nat Genet*. 2004;36(5):476-480. doi:10.1038/ng1345

149. Tarpey P, Parnau J, Blow M, et al. Mutations in the DLG3 gene cause nonsyndromic X-linked mental retardation. *Am J Hum Genet*. 2004;75(2):318-324. doi:10.1086/422703

150. Rothenberg SM, Mohapatra G, Rivera MN, et al. A genome-wide screen for microdeletions reveals disruption of polarity complex genes in diverse human cancers. *Cancer Res*. 2010;70(6):2158-2164. doi:10.1158/0008-5472.CAN-09-3458

151. Halaoui R, McCaffrey L. Rewiring cell polarity signaling in cancer. *Oncogene*. 2015;34(8):939-950. doi:10.1038/onc.2014.59

152. Bilder D, Schober M, Perrimon N. Integrated activity of PDZ protein complexes regulates epithelial polarity. *Nat Cell Biol*. 2003;5(1):53-58. doi:10.1038/ncb897

153. Bilder D, Li M, Perrimon N. Cooperative regulation of cell polarity and growth by Drosophila tumor suppressors. *Science (80-)*. 2000;289(5476):113-116. doi:10.1126/science.289.5476.113

154. Gateff E. Malignant neoplasms of genetic origin in Drosophila melanogaster. *Science (80-)*. 1978;200(4349):1448-1459. doi:10.1126/science.96525

155. McCaffrey LM, Montalbano JA, Mihai C, Macara IG. Loss of the Par3 Polarity Protein Promotes Breast Tumorigenesis and Metastasis. *Cancer Cell*. 2012;22(5):601-614. doi:10.1016/j.ccr.2012.10.003

156. Zen K, Yasui K, Gen Y, et al. Defective expression of polarity protein PAR-3 gene (PARD3) in esophageal squamous cell carcinoma. *Oncogene*. 2009;28(32):2910-2918. doi:10.1038/onc.2009.148

157. Dugay F, Le Goff X, Rioux-Leclerq N, et al. Overexpression of the polarity protein PAR-3 in clear cell renal cell carcinoma is associated with poor prognosis. *Int J Cancer*. 2014;134(9):2051-2060. doi:10.1002/ijc.28548

158. Anastas JN, Biechele TL, Robitaille M, et al. A protein complex of SCRIB, NOS1AP and VANGL1 regulates cell polarity and migration, and is associated with breast cancer progression. *Oncogene*. 2012;31(32):3696-3708. doi:10.1038/onc.2011.528

159. Yao S, Bee A, Brewer D, et al. Prkc-ζ expression promotes the aggressive phenotype of human prostate cancer cells and is a novel target for therapeutic intervention. *Genes and Cancer*. 2010;1(5):444-464. doi:10.1177/1947601910376079

160. Tapia R, Kralicek SE, Hecht GA. EPEC effector EspF promotes Crumbs3 endocytosis and disrupts epithelial cell polarity. *Cell Microbiol*. 2017;19(11):e12757. doi:10.1111/cmi.12757

161. Tapia R, Kralicek SE, Hecht GA. Enteropathogenic Escherichia coli (EPEC) recruitment of PAR polarity protein atypical PKCζ to pedestals and cell–cell contacts precedes disruption of tight junctions in intestinal epithelial cells. *Int J Mol Sci*. 2020;21(2). doi:10.3390/ijms21020527

162. Schumann M, Günzel D, Buergel N, et al. Cell polarity-determining proteins Par-3 and PP-1 are involved in epithelial tight junction defects in coeliac disease. *Gut*. 2012;61(2):220-228. doi:10.1136/gutjnl-2011-300123

163. Ivanov AI, Young C, Beste K Den, et al. Tumor suppressor scribble regulates assembly of tight junctions in the intestinal epithelium. *Am J Pathol*. 2010;176(1):134-145. doi:10.2353/ajpath.2010.090220

164. Chatterjee S, Seifried L, Feigin ME, et al. Dysregulation of cell polarity proteins synergize with oncogenes or the microenvironment to induce invasive behavior in epithelial cells. *PLoS One*. 2012;7(4). doi:10.1371/journal.pone.0034343

165. Dudakov JA, Hanash AM, Van Den Brink MRM. Interleukin-22: Immunobiology and Pathology. Published online 2015. doi:10.1146/annurev-immunol-032414-112123

166. Liu Y, Verma VK, Malhi H, et al. Lipopolysaccharide downregulates macrophage-derived IL-22 to modulate alcohol-induced hepatocyte cell death. *Am J Physiol Cell Physiol*. 2017;313:305-313. doi:10.1152/ajpcell.00005.2017.-Interleu

167. Zindl CL, Lai JF, Lee YK, et al. IL-22-producing neutrophils contribute to antimicrobial defense and restitution of colonic epithelial integrity during colitis. *Proc Natl Acad Sci U S A*. 2013;110(31):12768-12773. doi:10.1073/pnas.1300318110

168. Mann ER, Bernardo D, Ng SC, et al. Human Gut Dendritic Cells Drive Aberrant Gut-specific T-cell Responses in Ulcerative Colitis, Characterized by Increased IL-4 Production and Loss of IL-22 and IFNγ. *Inflamm Bowel Dis*. 2014;20(12):2299-2307. doi:10.1097/MIB.0000000000000223

169. Ikeuchi H, Kuroiwa T, Hiramatsu N, et al. Expression of interleukin-22 in rheumatoid arthritis: Potential role as a proinflammatory cytokine. *Arthritis Rheum*. 2005;52(4):1037-1046. doi:10.1002/art.20965

170. Parks OB, Pociask DA, Hodzic Z, Kolls JK, Good M. Interleukin-22 signaling in the regulation of intestinal health and disease. *Front Cell Dev Biol*. 2016;3(JAN):85. doi:10.3389/fcell.2015.00085

171. Lanfranca MP, Lin Y, Fang J, Zou W, Frankel T. Biological and Pathological Activities of Interleukin-22. *J Mol Med*. 2016;94(5):523-543. doi:10.1007/s00109-016-1391-6

172. Mühl H, Bachmann M. IL-18/IL-18BP and IL-22/IL-22BP: Two interrelated couples with therapeutic potential. *Cell Signal*. 2019;63. doi:10.1016/j.cellsig.2019.109388

173. Dumoutier L, Lejeune D, Colau D, Renauld J-C. Cloning and Characterization of IL-22 Binding Protein, a Natural Antagonist of IL-10-Related T Cell-Derived Inducible Factor/IL-22. *J Immunol*. 2001;166(12):7090-7095. doi:10.4049/jimmunol.166.12.7090

174. Huber S, Gagliani N, Zenewicz LA, et al. IL-22BP is regulated by the inflammasome and modulates tumorigenesis in the intestine. *Nature*. 2012;491(7423):259-263. doi:10.1038/nature11535

175. Wolk K, Kunz S, Witte E, Friedrich M, Asadullah K, Sabat R. *IL-22 Increases the Innate Immunity of Tissues*. Vol 21.; 2004.

176. Sonnenberg GF, Fouser LA, Artis D. Border patrol: Regulation of immunity, inflammation and tissue homeostasis at barrier surfaces by IL-22. *Nat Immunol*. 2011;12(5):383-390. doi:10.1038/ni.2025

177. Zheng Y, Valdez PA, Danilenko DM, et al. Interleukin-22 mediates early host defense against attaching and effacing bacterial pathogens. *Nat Med*. 2008;14(3):282-289. doi:10.1038/nm1720

178. Aujla SJ, Chan YR, Zheng M, et al. IL-22 mediates mucosal host defense against Gram-negative bacterial pneumonia. *Nat Med*. 2008;14(3):275-281. doi:10.1038/nm1710

179. Liang SC, Tan XY, Luxenberg DP, et al. Interleukin (IL)-22 and IL-17 are coexpressed by Th17 cells and cooperatively enhance expression of antimicrobial peptides. *J Exp Med*. 2006;203(10):2271-2279. doi:10.1084/jem.20061308

180. Sugimoto K, Ogawa A, Mizoguchi E, et al. IL-22 ameliorates intestinal inflammation in a mouse model of ulcerative colitis. *J Clin Invest*. 2008;118. doi:10.1172/JCI33194

181. Lindemans CA, Calafiore M, Mertelsmann AM, et al. Interleukin-22 Promotes Intestinal Stem Cell-Mediated Epithelial Regeneration. *Nature*. 2015;528(7583):560-564. doi:10.1038/nature16460

182. Zwarycz B, Gracz AD, Rivera KR, et al. IL22 Inhibits Epithelial Stem Cell Expansion in an Ileal Organoid Model. *Cell Mol Gastroenterol Hepatol*. 2019;7:1-17. doi:10.1016/j.jcmgh.2018.06.008

183. Wang Y, Mumm JB, Herbst R, Kolbeck R, Wang Y. IL-22 Increases Permeability of Intestinal Epithelial Tight Junctions by Enhancing Claudin-2 Expression. *J Immunol*. 2017;199(9):3316-3325. doi:10.4049/jimmunol.1700152

184. Tsai PY, Zhang B, He WQ, et al. IL-22 Upregulates Epithelial Claudin-2 to Drive Diarrhea and Enteric Pathogen Clearance. *Cell Host Microbe*. 2017;21(6):671-681.e4. doi:10.1016/j.chom.2017.05.009

185. Li L-J, Gong C, Zhao M-H, Feng B-S. Role of interleukin-22 in inflammatory bowel diseases. *World J Gastroenterol*. 2014;20(48):18177-18188. doi:10.3748/wjg.v20.i48.18177

186. Brand S, Beigel F, Olszak T, et al. IL-22 is increased in active Crohn's disease and promotes proinflammatory gene expression and intestinal epithelial cell migration. *Am J Physiol Gastrointest Liver Physiol*. 2006;290:827-838. doi:10.1152/ajpgi.00513.2005.-IL-22

187. Andoh A, Zhang Z, Inatomi O, et al. Interleukin-22, a Member of the IL-10 Subfamily, Induces Inflammatory Responses in Colonic Subepithelial Myofibroblasts. Published online 2005. doi:10.1053/j.gastro.2005.06.071

188. Mühl H. Pro-inflammatory signaling by IL-10 and IL-22: Bad habit stirred up by interferons? *Front Immunol*. 2013;4(FRB):18. doi:10.3389/fimmu.2013.00018

189. Lejeune D, Dumoutier L, Constantinescu S, Kruijer W, Jacob Schuringa J, Renauld J-C. Interleukin-22 (IL-22) Activates the JAK/STAT, ERK, JNK, and p38 MAP Kinase Pathways in a Rat Hepatoma Cell Line. *J Biol Chem*. 2002;277(13):33676-33682. doi:10.1074/jbc.M204204200

190. Pickert G, Neufert C, Leppkes M, et al. STAT3 links IL-22 signaling in intestinal epithelial cells to mucosal wound healing. *J Exp Med*. 2009;206(7):1465-1472. doi:10.1084/jem.20082683

191. Sovran B, Loonen LMP, Lu P, et al. IL-22-STAT3 pathway plays a key role in the maintenance of ileal homeostasis in mice lacking secreted mucus barrier. *Inflamm Bowel Dis*. 2015;21(3):531-542. doi:10.1097/MIB.0000000000000319

192. Jiang R, Wang H, Deng L, et al. IL-22 is related to development of human colon cancer by activation of STAT3. *BMC Cancer*. 2013;13(59):1-11. doi:10.1186/1471-2407-13-59

193. Ziesché E, Bachmann M, Kleinert H, Pfeilschifter J, Mühl H. The Interleukin-22/STAT3 Pathway Potentiates Expression of Inducible Nitric-oxide Synthase in Human Colon Carcinoma Cells. *J Biol Chem*. 2007;282(22):16006-16015. doi:10.1074/jbc.M611040200

194. Nagalakshmi ML, Rascle A, Zurawski S, Menon S, de Waal Malefyt R. Interleukin-22 activates STAT3 and induces IL-10 by colon epithelial cells. *Int Immunopharmacol*. 2004;4(5):679-691. doi:10.1016/j.intimp.2004.01.008

195. Akil H, Abbaci A, Lalloué F, et al. IL22/IL-22R Pathway Induces Cell Survival in Human Glioblastoma Cells. Ryffel B, ed. *PLoS One*. 2015;10(3):e0119872. doi:10.1371/journal.pone.0119872

196. Fukui H, Zhang X, Sun C, et al. IL-22 produced by cancer-associated fibroblasts promotes gastric cancer cell invasion via STAT3 and ERK signaling. *Br J Cancer*. 2014;111(4):763-771. doi:10.1038/bjc.2014.336

197. Moniruzzaman M, Wang R, Jeet V, McGuckin MA, Hasnain SZ. Interleukin (IL)-22 from IL-20 subfamily of cytokines induces colonic epithelial cell proliferation predominantly through ERK1/2 pathway. *Int J Mol Sci*. 2019;20(14):1-17. doi:10.3390/ijms20143468

198. Mustalahti K, Catassi C, Reunanen A, et al. The prevalence of celiac disease in Europe: Results of a centralized, international mass screening project. *Ann Med*. 2010;42(8):587-595. doi:10.3109/07853890.2010.505931

199. Cinova J, Palová-Jelínková L, Smythies LE, et al. Gliadin peptides activate blood monocytes from patients with celiac disease. *J Clin Immunol*. 2007;27(2):201-209. doi:10.1007/s10875-006-9061-z

200. Maiuri L, Ciacci C, Ricciardelli I, et al. Association between innate response to gliadin and activation of pathogenic T cells in coeliac disease. *Lancet*. 2003;362(9377):30-37. doi:10.1016/S0140-6736(03)13803-2

201. Yokoyama S, Watanabe N, Sato N, et al. Antibody-mediated blockade of IL-15 reverses the autoimmune intestinal damage in transgenic mice that overexpress IL-15 in enterocytes. *Proc Natl Acad Sci U S A*. 2009;106(37):15849-15854. doi:10.1073/pnas.0908834106

202. Gujral N, Freeman HJ, Thomson ABR. Celiac disease: Prevalence, diagnosis, pathogenesis and treatment. *World J Gastroenterol*. 2012;18(42):6036-6059. doi:10.3748/wjg.v18.i42.6036

203. Stamnaes J, Sollid LM. Celiac disease: Autoimmunity in response to food antigen. *Semin Immunol*. 2015;27(5):343-352. doi:10.1016/j.smim.2015.11.001

204. Tye-Din JA, Cameron DJS, Daveson AJ, et al. Appropriate clinical use of human leukocyte antigen typing for coeliac disease: An Australasian perspective. *Intern Med J*. 2015;45(4):441-450. doi:10.1111/imj.12716

205. Van Heel DA, Franke L, Hunt KA, et al. A genome-wide association study for celiac disease identifies risk variants in the region harboring IL2 and IL21. *Nat Genet*. 2007;39(7):827-829. doi:10.1038/ng2058

206. Itzlinger A, Branchi F, Elli L, Schumann M. Gluten-free diet in celiac disease—forever and for all? *Nutrients*. 2018;10(11):1-14. doi:10.3390/nu10111796

207. Cardoso-Silva D, Delbue D, Itzlinger A, et al. Intestinal barrier function in gluten-related disorders. *Nutrients*. 2019;11(10):1-19. doi:10.3390/nu11102325

208. Rubio-Tapia A, Murray JA. Classification and management of refractory coeliac disease. *Gut*. 2010;59(4):547-557. doi:10.1136/gut.2009.195131

209. Menzies IS, Pounder R, Heyer S, et al. Abnormal Intestinal Permeability To Sugars in Villous Atrophy. *Lancet*. 1979;314(8152):1107-1109. doi:10.1016/S0140-6736(79)92507-8

210. Pearson ADJ, Eastham EJ, Laker MF, Craft AW, Nelson R. Intestinal permeability in children with Crohn's disease and coeliac disease. *Br Med J*. 1982;285(285):20-21. doi:10.1136/bmj.285.6334.20

211. Cobden I, Dickinson RJ, Rothwell J, Axon ATR. Intestinal permeability assessed by excretion ratios of two molecules: Results in coeliac disease. *Br Med J*. 1978;2(6144):1060. doi:10.1136/bmj.2.6144.1060

212. Schulzke JD, Bentzel CJ, Schulzke I, Riecken EO, Fromm M. Epithelial tight junction structure in the jejunum of children with acute and treated celiac sprue. *Pediatr Res*. 1998;43(4 I):435-441. doi:10.1203/00006450-199804000-00001

213. Schulzke JD, Schulzke I, Fromm M, Riecken EO. Epithelial barrier and ion transport in coeliac sprue: Electrical measurements on intestinal aspiration biopsy specimens. *Gut*. 1995;37(6):777-782. doi:10.1136/gut.37.6.777

214. Schumann M, Kamel S, Pahlitzsch ML, et al. Defective tight junctions in refractory celiac disease. *Ann N Y Acad Sci*. 2012;1258(1):43-51. doi:10.1111/j.1749-6632.2012.06565.x

215. Ciccocioppo R, Finamore A, Ara C, Di Sabatino A, Mengheri E, Corazza GR. Altered expression, localization, and phosphorylation of epithelial junctional proteins in celiac disease. *Am J Clin Pathol*. 2006;125(4):502-511. doi:10.1309/DTYRA91G8R0KTM8M

216. Nagy Szakál D, Győrffy H, Arató A, et al. Mucosal expression of claudins 2, 3 and 4 in proximal and distal part of duodenum in children with coeliac disease. *Virchows Arch*. 2010;456(3):245-250. doi:10.1007/s00428-009-0879-7

217. Pollard JW. Trophic macrophages in development and disease. *Nat Rev Immunol*. 2009;9(4):259-270. doi:10.1038/nri2528

218. Weber B, Saurer L, Mueller C. Intestinal macrophages: Differentiation and involvement in intestinal immunopathologies. *Semin Immunopathol*. 2009;31(2):171-184. doi:10.1007/s00281-009-0156-5

219. Harris KM, Fasano A, Mann DL. Monocytes differentiated with IL-15 support Th17 and Th1 responses to wheat gliadin: Implications for celiac disease. *Clin Immunol*. 2010;135(3):430-439. doi:10.1016/j.clim.2010.01.003

220. McMurray JS. A New Small-Molecule Stat3 Inhibitor. *Chem Biol*. 2006;13(11):1123-1124. doi:10.1016/j.chembiol.2006.11.001

221. Schust J, Sperl B, Hollis A, Mayer TU, Berg T. Stattic: A Small-Molecule Inhibitor of STAT3 Activation and Dimerization. *Chem Biol*. 2006;13(11):1235-1242. doi:10.1016/j.chembiol.2006.09.018

222. Siddiquee K, Zhang S, Guida WC, et al. Selective chemical probe inhibitor of Stat3, identified through structure-based virtual screening, induces antitumor activity. *Proc Natl Acad Sci U S A*. 2007;104(18):7391-7396. doi:10.1073/pnas.0609757104

223. Turkson J, Ryan D, Kim JS, et al. Phosphotyrosyl Peptides Block Stat3-mediated DNA Binding Activity, Gene Regulation, and Cell Transformation. *J Biol Chem*. 2001;276(48):45443-45455. doi:10.1074/jbc.M107527200

224. Iwamaru A, Szymanski S, Iwado E, et al. A novel inhibitor of the STAT3 pathway induces apoptosis in malignant glioma cells both in vitro and in vivo. *Oncogene*. 2007;26(17):2435-2444. doi:10.1038/sj.onc.1210031

225. Ferrajoli A, Faderl S, Van Q, et al. WP1066 disrupts janus kinase-2 and induces caspase-dependent apoptosis in acute myelogenous leukemia cells. *Cancer Res*. 2007;67(23):11291-11299. doi:10.1158/0008-5472.CAN-07-0593

226. Duncia J V., Santella JB, Higley CA, et al. MEK inhibitors: The chemistry and biological activity of U0126, its analogs, and cyclization products. *Bioorganic Med Chem Lett*. 1998;8(20):2839-2844. doi:10.1016/S0960-894X(98)00522-8

227. Grimm M, Pullman W, Bennett G, Sullivan P, Pavli P, Doe W. Direct evidence of monocyte recruitment to inflammatory bowel disease mucosa. *J Gastroenterol Hepatol*. 1995;10(4):387-395. doi:10.1111/j.1440-1746.1995.tb01589.x

228. Bojarski C, Gitter AH, Bendfeldt K, et al. Permeability of human HT-29/B6 colonic epithelium as a function of apoptosis. *J Physiol*. 2001;535(2):541-552. doi:10.1111/j.1469-7793.2001.00541.x

229. Lammers KM, Lu R, Brownley J, et al. Gliadin Induces an Increase in Intestinal Permeability and Zonulin Release by Binding to the Chemokine Receptor CXCR3. *Gastroenterology*. 2008;135(1):194. doi:10.1053/j.gastro.2008.03.023

230. Barilli A, Rotoli BM, Visigalli R, et al. Gliadin-mediated production of polyamines by RAW264.7 macrophages modulates intestinal epithelial permeability in vitro. *Biochim Biophys Acta - Mol Basis Dis*. 2015;1852(9):1779-1786. doi:10.1016/j.bbadis.2015.06.001

231. Kamada N, Hisamatsu T, Okamoto S, et al. Unique CD14+ intestinal macrophages contribute to the pathogenesis of Crohn disease via IL-23/IFN-γ axis. *J Clin Invest*. 2008;118(6):2269-2280. doi:10.1172/JCI34610

232. Lampinen M, Waddell A, Ahrens R, Carlson M, Hogan SP. CD14 + CD33 + myeloid cell-CCL11-eosinophil signature in ulcerative colitis . *J Leukoc Biol*. 2013;94(5):1061-1070. doi:10.1189/jlb.1212640

233. Manavalan JS, Hernandez L, Shah JG, et al. Serum cytokine elevations in celiac disease: Association with disease presentation. *Hum Immunol*. 2010;71(1):50-57. doi:10.1016/j.humimm.2009.09.351

234. O'Keeffe J, Lynch S, Whelan A, et al. Flow cytometric measurement of intracellular migration inhibition factor and tumour necrosis factor alpha in the mucosa of patients with coeliac disease. *Clin Exp Immunol*. 2001;125(3):376-382. doi:10.1046/j.1365-2249.2001.01594.x

235. Kapellos TS, Bonaguro L, Gemünd I, et al. Human monocyte subsets and phenotypes in major chronic inflammatory diseases. *Front Immunol*. 2019;10(AUG):2035. doi:10.3389/fimmu.2019.02035

236. Kong BS, Kim Y, Kim GY, et al. Increased frequency of IL-6-producing non-classical monocytes in neuromyelitis optica spectrum disorder. *J Neuroinflammation*. 2017;14(1):191. doi:10.1186/s12974-017-0961-z

237. Mukherjee R, Kanti Barman P, Kumar Thatoi P, Tripathy R, Kumar Das B, Ravindran B. Non-Classical monocytes display inflammatory features: Validation in Sepsis and Systemic Lupus Erythematous. *Sci Rep*. 2015;5(1):1-14. doi:10.1038/srep13886

238. Lamouille S, Xu J, Derynck R. Molecular mechanisms of epithelial-mesenchymal transition. *Nat Rev Mol Cell Biol*. 2014;15(3):178-196. doi:10.1038/nrm3758

239. Bryant DM, Mostov KE. From cells to organs: Building polarized tissue. *Nat Rev Mol Cell Biol*. 2008;9(11):887-901. doi:10.1038/nrm2523

240. Mizoguchi A. Healing of intestinal inflammation by IL-22. *Inflamm Bowel Dis*. 2012;18(9):1777-1784. doi:10.1002/ibd.22929

241. Rui J, Chunming Z, Binbin G, Na S, Shengxi W, Wei S. IL-22 promotes the progression of breast cancer through regulating HOXB-AS5. *Oncotarget*. 2017;8(61):103601-103612. doi:10.18632/oncotarget.22063

242. Komine-Aizawa S, Aizawa S, Takano C, Hayakawa S. Interleukin-22 promotes the migration and invasion of oral squamous cell carcinoma cells. *Immunol Med*. 2020;43(3):121-129. doi:10.1080/25785826.2020.1775060

243. Scharl M, Frei S, Pesch T, et al. Interleukin-13 and transforming growth factor β synergise in the pathogenesis of human intestinal fistulae. *Gut*. 2013;62(1):63-72. doi:10.1136/gutjnl-2011-300498

244. Thiery JP, Sleeman JP. Complex networks orchestrate epithelial-mesenchymal transitions. *Nat Rev Mol Cell Biol*. 2006;7(2):131-142. doi:10.1038/nrm1835

245. Ji Y, Yang X, Li J, et al. IL-22 promotes the migration and invasion of gastric cancer cells via IL-22R1/AKT/MMP-9 signaling. *Int J Clin Exp Pathol*. 2014;7(7):3694-3703. Accessed December 2, 2020. www.ijcep.com/

246. Kanda N, Seno H, Konda Y, et al. STAT3 is constitutively activated and supports cell survival in association with survivin expression in gastric cancer cells. *Oncogene*. 2004;23(28):4921-4929. doi:10.1038/sj.onc.1207606

247. Lin L, Fuchs J, Li C, Olson V, Bekaii-Saab T, Lin J. STAT3 signaling pathway is necessary for cell survival and tumorsphere forming capacity in ALDH +/CD133 + stem cell-like human colon cancer cells. *Biochem Biophys Res Commun*. 2011;416(3-4):246-251. doi:10.1016/j.bbrc.2011.10.112

7. Appendices

7.1 List of publications

Accepted publications:

1. Delbue D., L. Lebenheim, D. Cardoso-Silva, V. Dony, S. M. Krug, J. Richter, M. Munoz, K. Wolk, C.Heldt, M. Heimesaat, R. Sabat, B. Siegmund, M. Schumann. Reprogramming intestinal epithelial cell polarity by interleukin-22. Frontiers in Medicine – Gastroenterology. 2021 (accepted),

2. Delbue, D., Cardoso-Silva, D.; Branchi, F.; Itzlinger, A.; Letizia, M.; Siegmund, B.; Schumann, M. Celiac Disease Monocytes Induce a Barrier Defect in Intestinal Epithelial Cells. Int. J. Mol. Sci. 2019, 20, 5597.

3. Cardoso-Silva, D*., Delbue, D*.; Itzlinger, A.; Moerkens, R.; Withoff, S.; Branchi, F.; Schumann, M. Intestinal Barrier Function in Gluten-Related Disorders. Nutrients 2019, 11, 2325. (*shared first authorship)

In progress:

1. Cardoso-Silva D., Sehn M., Manna S., Weiner J., Delbue D., Weixler B., Gröne J., Siegmund B., Elezkurtaj S., Hummel M., Schumann M. “Osteopontin in colitis-associated carcinoma” (in progress)

7.2 Presentations at scientific conferences

1. Lecture at Sanofi – Aug 24, 2020. Deborah Delbue. IL22 and epithelial barrier; role in IBD. Impact of monocytes from celiac patients on epithelial barrier (Oral presentation). Frankfurt, Germany.

2. Programm der Arbeitsgruppe auf der DGVS 2019 in Wiesbaden. Transport- und Barrierefunktionen in Darm und Leber: Methoden zur Untersuchung von Struktur und Funktion. Oct 04, 2019. Wiesbaden, Germany. Deborah Delbue da Silva, LPP and C1orf106 in intestinal barrier function (Oral presentation).

3. Deborah Delbue, Alice Itzlinger, Bosse Jessen, Hella Pfeiffert, Federica Branchi, Donata Lissner, Walburga Dieterich, Britta Siegmund, Michael Schumann. Effect of monocytes derived from celiac disease patients on the intestinal barrier function. ICDS – Set 05-07, 2019. Paris, France.

4. Deborah Delbue, Lydia Lebenheim, Claudia Heldt, Britta Siegmund, Michael Schumann. IL-22 affects barrier function and cell polarity by MAPK/PI3 kinase signal transduction. ECCO – Mar 06-09, 2019. Copenhagen, Denmark.

5. Deborah Delbue, Alice Itzlinger, Bosse Jessen, Hella Pfeiffert, Claudia Heldt, Federica Branchi, Donata Lissner, Walburga Dieterich, Michael Schumann. Disruption of epithelial barrier function by celiac peripheral blood mononuclear cells. ECCO – Mar 06-09, 2019. Copenhagen, Denmark.

6. Deborah Delbue, Alice Itzlinger, Bosse Jessen, Hella Pfeiffert, Claudia Heldt, Federica Branchi, Donata Lissner, Walburga Dieterich, Michael Schumann. Peripheral monocytes impair the barrier function of intestinal epithelial cells: new insights on the pathogenesis of celiac disease. UEG – Oct 20-24, 2018. Vienna, Austria.

Declaration of Authorship

I hereby certify that this thesis first submitted has been composed by me and is based on my own work, unless specified otherwise. No other person's work has been used without acknowledgement in this thesis. All references and literal extracts have been cited, and all sources of information, including graphs and data sets, have been specifically acknowledged.

March 2021, Berlin, Germany

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Deborah Delbue da Silva