Aus dem Institut für Veterinär-Physiologie
des Fachbereichs Veterinärmedizin
der Freien Universität Berlin

Effects of the pro-inflammatory cytokine TNF on functional and molecular barrier properties of porcine intestinal epithelial models

Inaugural Dissertation
zur Erlangung des Grades einer
Doktorin der Veterinärmedizin
an der
Freien Universität Berlin

vorgelegt von
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Berlin 2023
Journal-Nr.: 4382

Gedruckt mit Genehmigung
des Fachbereichs Veterinärmedizin
an der Freien Universität Berlin

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Deskriptoren (nach CAB-Thesaurus): pigs, animal models, intestinal diseases, intestinal mucosa, cytokines, tumor necrosis factor, antiinflammatory properties, inflammation, epithelium, lymph nodes, peyer patches, tight junctions (MeSH), claudins (MeSH), receptors, tumor necrosis factor, typ I (MeSH)

Tag der Promotion: 02.03.2023

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List of abbreviations

CD	Crohn's disease
ECS	Extracellular segment
FAE	Follicle-associated epithelium
GALT	Gut-associated lymphatic tissue
IBD	Inflammatory bowel disease
IFNγ	Interferon gamma
I_{SC}	Short-circuit current
JAM	Junctional adhesion molecule
ML-7	1-(5-iodonaphthalene-1-sulfonyl)-1H-hexahydro-1,4-diazepine hydrochloride
MLC	Myosin II light chain
MLCK	Myosin light chain kinase
NF-κB	Nuclear factor kappa B
PP	Peyer's patch
PWD	Post-weaning diarrhea
sTNF	Soluble tumor necrosis factor
TER	Transepithelial resistance
TJ	Tight junction
tmTNF	Transmembrane-bound tumor necrosis factor
TNF	Tumor necrosis factor
TNFR	Tumor necrosis factor receptor
UC	Ulcerative colitis
VE	Villus epithelium
ZO-1	Zonula occludens-1

Chapter 1: Introduction

Because of its participation in physiological and pathological processes, the pro-inflammatory cytokine tumor necrosis factor (TNF) and the associated molecular signaling mechanisms are currently the subjects of intense research. Following the release of TNF mainly by macrophages, it binds the specific TNF receptors TNFR-1 or -2 and exerts its numerous functions by inducing complex signaling cascades (Loetscher *et al.,* 1990). As a pro-inflammatory cytokine, its primary tasks are to activate other immune cells and to initiate immune responses (Lee *et al.,* 2013). However, additional functions of TNF are known, as it not only can induce cell necrosis and apoptosis, but is also capable of stimulating tissue regeneration (Baud and Karin, 2001). An exaggerated expression of TNF is often associated with inflammation and is involved in the pathogenesis of various diseases (Jang *et al.,* 2021). During inflammatory disorders in the intestine, TNF is known to be one of the major cytokines that perturb the epithelial barrier function (Pagnini and Cominelli, 2021).

As the gastrointestinal tract is in constant contact with external factors, an efficient epithelial barrier is essential to ensure the controlled uptake of nutritional ingredients and to protect against antigens and bacterial toxins from the gut lumen (Vancamelbeke and Vermeire, 2017). Transport through the epithelial barrier can occur *via* two different pathways: the transcellular pathway across the apical and the basolateral membranes and the paracellular pathway between two epithelial cells (Powell, 1981). By forming a belt-like attachment at the apical part of adjacent cells, tight junctions (TJs) are one of the main components that control and regulate the paracellular pathway (Schneeberger and Lynch, 1992). The TJ strands are built up of multiple integral membrane proteins, the main component being constituted by the claudin gene family, which was first discovered in 1998 (Furuse *et al*., 1998a). Now, 27 members of the claudin family are known (Mineta *et al.*, 2011). In addition to their barrier-forming function, some claudins have been described to mediate the paracellular passage of cations, anions, and water (for review, see Markov *et al.,* 2015).

Hence, an altered composition of claudins is often associated with irregular intestinal permeability and correlates with pathophysiological disorders of the intestine (Zeissig *et al.,* 2007; Amasheh *et al.,* 2009a). In cases of intestinal inflammatory diseases, TNF is considered to play an essential role by altering the claudin composition within TJ strands (Breese *et al.,* 1994; Clayburgh *et al*., 2004). However, the exact pathomechanisms of inflammatory disorders of the intestine have not been completely analyzed and remain the subject of major research projects.

Animal models are frequently used during translational research, although the application of some models is questionable with regard to the absence of comparability (Ribitsch *et al.,*

2020). The gastrointestinal tract of the pig shares close similarities to that of humans, particularly concerning digestion and nutrition (Deglaire and Moughan, 2012). This makes porcine models a useful tool for analyzing gastrointestinal (patho-)physiology (Deglaire and Moughan, 2012). However, data are lacking about the detailed effect of TNF on the porcine intestinal epithelial barrier, especially with regard to TJ proteins.

Thus, in the work presented in this thesis, two different models have been used to analyze the effect of the pro-inflammatory cytokine TNF on the porcine intestinal epithelial barrier function *in vitro* and *ex vivo*:

1. The non-transformed porcine intestinal epithelial cell line IPEC-J2

The physiological properties of the improved cell line IPEC-J2, cultured under species-specific conditions, strongly resemble those of porcine jejunal epithelium, and this cell line has been characterized as a suitable *in vitro* model for the analysis of porcine intestinal barrier function (Zakrzewski *et al.,* 2013). Therefore, a functional and molecular analysis of the direct TNF effect on the paracellular pathway of IPEC-J2 cells was investigated.

2. Comparison of porcine Peyer's patches and neighboring villus epithelium

The Ussing chamber technique can be used for a direct comparison of porcine Peyer's patches (PP) and villus epithelium (VE) under inflammatory conditions induced by TNF. PPs are a major part of the gut-associated lymphatic tissue (GALT) and represent an important component of intestinal immunity (Jung *et al.,* 2010). PP and VE tissue samples *ex vivo* present useful models for the analysis of changes in barrier properties during intestinal inflammation because of the good comparability of their characteristics with intestinal properties *in vivo.*

Chapter 2: Literature review

2.1. The pro-inflammatory cytokine TNF

Tumor necrosis factor (TNF; also referred to as TNFα, TNFSF2) is a cytokine that is mainly released by macrophages, although it can also be produced by other cell types such as monocytes, T lymphocytes, or natural killer cells (Steeland *et al.,* 2018). It is expressed as a 26 kDa precursor transmembrane-bound structure (tmTNF) that can be converted into the 17 kDa soluble TNF form (sTNF) by the action of the TNF alpha converting enzyme (Luettig *et al.,* 1989; Black *et al.,* 1997). Moreover, lipopolysaccharides and other bacterial toxins are often associated with an increased release of sTNF (Wajant *et al.,* 2003). Both forms of TNF can exert their functions by binding to their specific receptors TNFR-1 or -2 (Loetscher *et al.,* 1990; see **chapter 2.1.1**). A number of proteins that are structurally related to TNF have been identified, and 19 ligands and 29 receptors of the TNF superfamily are now known that play a crucial role in immunological processes (Aggarwal *et al.,* 2012).

Originally, TNF was discovered as being toxic and to induce necrosis in malignant tumor cells, lending its name to these functions (Carswell *et al*., 1975). However, clinical trials as an anti-cancer treatment failed because of the induction of severe side effects such as fever, headache, and hypotension after systemic therapy with TNF (Schwartz *et al.,* 1989). Since then, numerous actions of the pleiotropic cytokine on physiological processes have been investigated, such as apoptosis and necrosis or cell proliferation and differentiation (Baud and Karin, 2001).

With such a wide-ranging spectrum of effects, TNF is unsurprisingly involved in the pathogenesis of numerous diseases. It plays a pivotal role in pathologic states such as inflammation, infection, or tumor development and is one of the major cytokines in the pathogenesis of inflammatory bowel diseases (IBDs), such as Crohn's disease (CD) or ulcerative colitis (UC; Reinecker *et al.,* 1993). After its release, TNF acts as a pro-inflammatory cytokine by activating immune cells and mediating the release of other cytokines, leading to an up-regulation of inflammatory states (Lee *et al.,* 2013). Another potential action of TNF in IBDs is its ability to disrupt the intestinal epithelial barrier (Amasheh *et al.,* 2010; Marchiando *et al.,* 2011), allowing toxins and exogenous substances to penetrate the usually mostly impermeable intestinal epithelium (Lu *et al.,* 2013). The integrity of the gastrointestinal epithelium is constituted by the presence of cell-cell junctions that seal the paracellular space (for detailed information, see **chapter 2.2**) and that are influenced by the action of TNF (Ma *et al.,* 2004; Wang *et al.,* 2006). Moreover, disturbance of barrier function by TNF is also associated with the increased activation of the myosin light chain kinase (MLCK; Cunningham and Turner, 2012). An overexpression of MLCK leads to phosphorylation of the myosin II light

chain (MLC) resulting in a contraction of the perijunctional actomyosin ring and a rearrangement of cell-cell junctions (Clayburgh *et al.,* 2004). The molecular mechanism behind TNF-induced MLCK expression is the activation of the protein transcription factor nuclear factor kappa B (NF-κB) by TNF (Ye *et al.,* 2006). These substantial changes in epithelial barrier function, which are related to increased mucosal permeability and antigen-uptake, often result in diarrhea in IBDs (Mankertz and Schulzke, 2007).

However, TNF is not only a crucial factor for IBDs. Other diseases, such as rheumatoid arthritis, psoriasis, and noninfectious uveitis, are associated with an increased serum concentration of TNF (Jang *et al.,* 2021). The most recent studies have revealed that the cytokine is also a critical factor in the pathogenesis of viral infections such as COVID-19, as it might be involved in cytokine shock and cell death during SARS-CoV-2 infection (Karki *et al.,* 2021; Frisoni *et al.,* 2022).

The relevance of TNF in the development of various inflammatory diseases is of great interest for therapeutical approaches. Barrier dysfunction in CD, for example, might be improved by TNF-neutralizing antibodies (Suenaert *et al.,* 2002). Currently, four different TNF-inhibitors are approved for treating intestinal inflammatory diseases such as CD or UC (Peyrin-Biroulet *et al.,* 2021). However, some patients either do not respond to the medical approach with anti-TNF drugs or develop intolerances to the therapy (Peyrin-Biroulet *et al.,* 2021). Therefore, the medical treatment of these diseases needs to be improved, with more attention being paid to the specific TNF receptors and the detailed mechanism of barrier disturbance.

2.1.1. TNF receptors

The functions of TNF can be exerted by its binding to specific cell surface receptors, namely TNFR-1 and -2 (Loetscher *et al.,* 1990). From the initiation of inflammation to the suppression of immune cells, TNF exerts a wide variety of biological functions, and so do its receptors. An understanding of the signaling pathways of the two specific receptors might lead to the better analysis of the role of TNF in the pathogenesis of various diseases and its pleiotropic actions.

TNFR-1 (also referred to as TNFRSF1A, CD120a, p55) is a 55 kDa receptor that is constitutively found on most nucleated cell types (Speeckaert *et al.,* 2012), and can be activated by both tmTNF and sTNF (Grell *et al.,* 1995). Signaling *via* TNFR-1 plays a crucial role in the induction of apoptosis, as the TNFR-1-associated death domain is activated after the binding of TNF to this receptor (Hsu *et al.,* 1995). However, the type I receptor is not only responsible for the induction of programmed cell death. By activating the NF-κB signaling pathway, TNF signaling *via* TNFR-1 plays an important role in the initiation and promotion of inflammation and can also induce anti-apoptotic pathways (Hayden and Ghosh, 2014).

The 75 kDa type II receptor TNFR-2 (also referred to as TNFRSF1B, CD120b, p75) is mainly expressed by immune cells, such as lymphocytes or macrophages (Gough and Myles, 2020).

In contrast to TNFR-1, TNFR-2 lacks the intracellular death domain and is preferably activated by tmTNF (Grell *et al*., 1995) mainly leading to cell survival and proliferation (Rauert *et al.*, 2010; Fischer *et al.*, 2011).

Because of the numerous biological effects of TNF and the differential signaling of its two receptors, the specific targeting of TNF receptors is of great interest for medical approaches.

2.2. Epithelial cell junctions

Epithelial cells cover all surfaces of our body and form the outermost layer of our skin, building a protective barrier against physical, chemical, and microbial influences (Jensen and Proksch, 2009). As not only our skin, but also our internal organs are exposed to the external environment, reliable protection is needed to maintain health. Therefore, all body cavities and hollow organs, such as the digestive tract, the respiratory tract, and the urogenital tract are lined with epithelium (Bragulla and Homberger, 2009). Moreover, epithelial cells can be arranged in single or multiple layers and can appear in squamous, cuboidal, or columnar arrangements depending on their function (Bragulla and Homberger, 2009). The intestinal mucosa, for example, is covered by simple columnar epithelium and maximizes its surface area by folds, villi, and microvilli enabling effective digestion and absorption (Helander and Fändriks, 2014). Furthermore, the epithelial cells are tightly linked, as they also act as a barrier to prevent the uncontrolled passage of luminal contents to the mucosa (Laukoetter *et al.,* 2008).

This selectively impermeable barrier is structured by various paracellular junctions that seal the extracellular space between the lateral membrane and that connect various cytoskeletal structures of neighboring cells (Alberts *et al.,* 2002). This results in an integrated and continuous cell-layer across the tissue (Garcia *et al.,* 2018). The various cellular junctions can be distinguished in terms of their structure, localization, and function as described below. A schematic overview of the various cell-cell junctions is given in **Figure 1**.

Tight junctions (TJs) are located at the most apical part of the cell and are crucial for polarization of epithelia, as they create a barrier between the apical and the basal side of each cell (Gumbiner and Louvard, 1985). As the name suggests, TJs form a tight connection between adjacent cells and build strands that surround each cell in a belt-like manner (Goodenough and Revel, 1970). Moreover, the major regulation of paracellular solute movement is mainly limited by TJs, as they can act as barrier builders and channel formers (Amasheh *et al.,* 2009b). Detailed information about TJs is given in **chapter 2.2.1** below.

Adherens junctions (AJs) are localized adjacent and basal to the TJs and form cell-cell adhesions that are characterized by an intercellular space of ~20 nm (Farquhar and Palade, 1963). The cell adhesion of AJs is provided by membrane-spanning cadherins that accumulate at the AJs and that can interact with cadherins from neighboring cells (Sandquist and Bement, 2010). The cytoplasmic domain of cadherins, in turn, is associated with catenins that can bind actin filaments, and thus, AJs have an important function in, for example, cellular movement or epithelial reorganization after the loss of cell-cell contact (Meng and Takeichi, 2009; Sandquist and Bement, 2010).

Desmosomes represent the third and the most basally located intercellular junction that Farquhar and Palade defined in 1963. Unlike the belt-like structure of TJs and AJs, desmosomes form discontinuous button-like structures (Farquhar and Palade, 1963). However, they share the function of intercellular adhesion and are structurally related to AJs (Stokes, 2007). In contrast to AJs, desmosomes are linked to the cytoskeleton *via* desmoglein and desmocollin, the two desmosomal cadherin subtypes (Chitaev and Trovanovsky, 1997; Delva *et al.,* 2009). Autoantibodies against desmosomal cadherins, as is the case in autoimmune diseases such as pemphigus foliaceus or pemphigus vulgaris, lead to the loss of cell-cell adhesion in the skin and result in acantholysis (Waschke, 2008).

Gap junctions act as communicating junctions unlike the above-mentioned tightening and anchoring junctions (Goodenough and Paul, 2009). A few years after the junctional complex was described, an intercellular gap of 2 nm between adjoining cells was visualized by using colloidal lanthanum as a tracer (Revel and Karnovsky, 1967). In contrast to the aforementioned intercellular junctions, gap junctions connect the cytoplasm of neighboring cells by channels that allow the transport of ions, nutrients, or metabolites (Villanelo *et al.*, 2017). Gap junction channels are formed by a variety of connexin isoforms (Yeager and Harris, 2007).

Hemidesmosomes are specialized junctions found along the basal membrane of epithelial cells and connect the epithelium with the underlying tissue (Staehelin, 1974). The transmembrane structures of hemidesmosomes connecting the intermediate filaments to the extracellular matrix involve integrin-based mechanisms (Green and Jones, 1996). After the loss of cell-substrate adhesion in injury, hemidesmosome proteins play an essential role in epithelial wound healing (Gipson *et al.,* 1993; Underwood *et al.,* 2009).

Tricellular junctions have been identified as sealing the space between three cells (Higashi and Miller, 2017). The structure and protein composition of tricellular TJs differ from those of bicellular TJs, as tricellulin (Ikenouchi *et al.,* 2005) and angulin (Higashi *et al.,* 2013) represent the main components. A low expression of tricellulin in the central tube of tricellular TJs results in an increased permeability of macromolecules, explaining a major function in paracellular permeability (Krug *et al*., 2009).

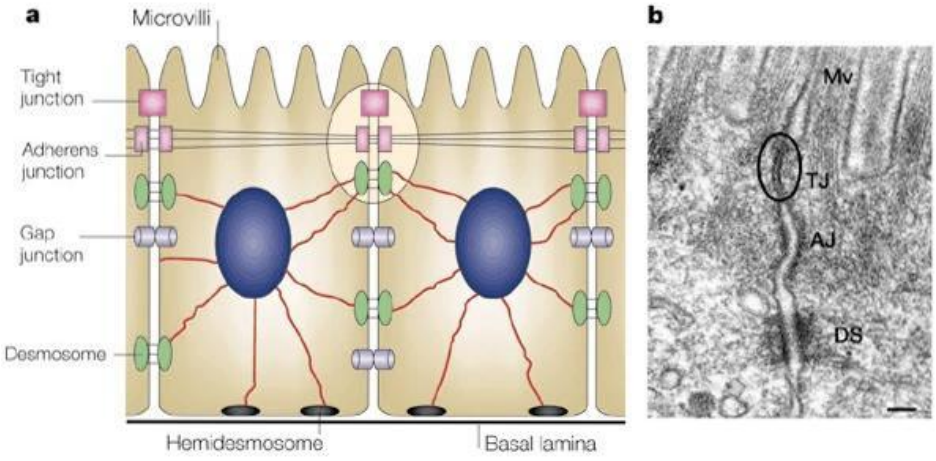

Figure 1. Intercellular junctions in intestinal epithelial cells. (A) Tight junctions (TJs) constitute the most apical junctions between adjacent cells and form a closely sealed intercellular space. Adherens junctions (AJs) and desmosomes represent the structures underlying the TJs and are connected to cytoskeletal structures. The junctional complex, consisting of TJ, AJ, and desmosome, is encircled. Gap junctions form pores that connect the cytoplasm of neighboring cells. By connecting epithelial cells with the underlying tissue, hemidesmosomes are located at the basal membrane and, just like desmosomes, are linked to the cytoskeleton. **(B)** Visualization of intercellular junctions in an electron micrograph. At the TJ, the intercellular space seems obliterated, whereas a gap between adjacent cells can be seen at the AJ and desmosome. Mv = Microvilli, DS = Desmosome (Tsukita *et al.,* 2001).

However, epithelial cells and their intercellular junctions are not only responsible for barrier properties and the regulation of paracellular solute transport. The complex communication and information exchange between adjacent cells by intercellular junctions also represents a critical function for the maintenance of tissue homeostasis such as cell proliferation, migration, and apoptosis (Garcia *et al.,* 2018). For example, inflammatory diseases or cancers of the intestine often accompany abnormalities in expression, composition, and function of cell-cell junctions (Gassler *et al.,* 2001; Luissint *et al.,* 2016; Bhat *et al.,* 2019). The majority of studies regarding the epithelial homeostasis in the intestine have focused on the effects on TJs and AJs, also referred to as the apical junctional complex. The main focus of the work described in this thesis, however, is the analysis of TJ proteins under inflammatory states in the porcine intestinal epithelial barrier.

2.2.1. The tight junction

The invention of electronic microscopy enabled a detailed observation and characterization of cell-cell junctions, based on their various localization and appearance. As outlined in **chapter 2.2**, this resulted in the classification of three structures, namely TJ, AJ, and the desmosome, involved in intercellular sealing (Farquhar and Palade, 1963). In the following years, an in-depth analysis of the junctions between neighboring cells, especially of the most apical located TJs, was implemented. At first, the TJ was thought to create an impermeable barrier between adjacent cells, but subsequent studies revealed that TJs not only seal the paracellular area, but are also permeable to ions and solutes (Frömter and Diamond, 1972). Based on this

finding, epithelial junctions were then differentiated into “tight” or “leaky” ones (Claude and Goodenough, 1973).
Freeze-fracture electron microscopy, a method depicting the hydrophobic inner faces of membranes, revealed the complex architecture of TJs: At the sites of membrane contact of adjacent cells, TJs form a belt-like structure with branching and anastomosing strands (Chalcroft and Bullivant, 1970; Staehelin, 1973). The number of junctional strands appearing to correlate with the tightness of epithelia (Claude and Goodenough, 1973).
Even now, a half-century after the first description of junctional strands, extensive research continues into the structure and function of TJs. A general overview of the various proteins that constitute the molecular components of TJs is given in the following section.

Zonula occludens-1 (ZO-1), a 225 kDa polypeptide that lacks transmembrane domains, was the first discovered protein to be associated with TJs (Stevenson *et al.,* 1986). Several more proteins belonging to the ZO protein family (ZO-2, ZO-3) have subsequently been described. Multifarious functions are exerted by ZO proteins, such as the connection of transmembranous junctional proteins to the cytoskeletal F-actin filaments (Hervé *et al.,* 2014), or the regulation of cell proliferation (Bauer *et al.,* 2010). However, additional transmembrane proteins that accomplish both barrier- and pore-forming functions were clearly needed.
The 65 kDa transmembrane protein **occludin** was the first integral part of the TJ to be identified (Furuse *et al.,* 1993), followed by the discovery of **claudin-1** and **-2** a few years later (Furuse *et al.,* 1998a). The claudin protein family has now grown to include 27 members (Mineta *et al.*, 2011) having manifold functions (see **chapter 2.2.1.1**).
Further proteins that are associated with the TJ have recently been described: **marvelD3**, which, together with **occludin** and **tricellulin**, belongs to the family of TJ-associated MARVEL proteins (Raleigh *et al.,* 2010), **junctional adhesion molecules (JAMs)**, and **angulin** (for review, see Piontek *et al.,* 2020).

The transmembrane TJ proteins either occur as tetra-spanning (having four transmembrane domains) or single-spanning (with only one transmembrane domain) proteins. Whereas the tetra-spanning proteins (claudin, occludin, marvelD3, and tricellulin) mostly affect barrier function, the single-spanning proteins (angulin, JAM) exert more adhesive or regulatory properties (Piontek *et al.,* 2020).
Two main functions are fulfilled by the barrier that is built by TJs: the fence and the gate functions (Mandel *et al.,* 1993). The fence function is thought to support apicobasal cell polarity by preventing the intermixing of apical and basolateral proteins, lipids, and macromolecules, and is linked to the intramembranous strands (Zihni *et al.,* 2016). However, although TJs play an important role in the establishment of cell polarity, cells that lack TJs are nevertheless able

to polarize (Ikenouchi *et al*., 2012). The gate function regulates the passive paracellular transport of molecules through two different pathways. Large solutes and macromolecules can pass *via* the leak pathway, which is restricted to a size limit of up to 60 Å (Van Itallie *et al.,* 2008; Buschmann *et al.,* 2013). The second pathway, referred to as the pore pathway, is thought to be related to TJ-associated claudins that form a pore that is selective to ions and small uncharged molecules (Amasheh *et al.,* 2002; Yu *et al.,* 2009).

In addition to the barrier building properties, TJs fulfill further functions. They can act as a bidirectional signaling platform: together with AJs and focal adhesions, TJs are part of adhesion complexes that can crosstalk with each other to modify their assembly and function (Zihni *et al.,* 2016). Furthermore, depending on information from the cell interior, TJs can adapt their function by altering the composition of claudins and, *vice versa*, can transmit signals to the cell for the regulation of cell differentiation, proliferation, and migration (Balda and Matter, 2000; Steed *et al.,* 2014). Because of their numerous functions, the disturbance in TJ proteins is often associated with the pathogenesis of many diseases.

2.2.1.1. Claudins, the key components of tight junction strands

Claudins constitute one of the most important components of TJ strands (Furuse *et al.,* 2002). TJs that lack claudins are unable to form the typical belt-like structure that is visualized by freeze-fracture electron microscopy, and thus, major attention has been paid to claudins as the main architects of TJ structure (Otani *et al.,* 2019). Even cells such as fibroblasts that naturally lack TJs can form strand-like networks after being transfected with claudin cDNA (Furuse *et al.,* 1998b).

Claudins have a complex structure, which is shown in **Figure 2**. The molecular weight of these integral membrane proteins varies between 21 to 34 kDa (Günzel and Yu, 2013). They contain four transmembrane domains, a cytoplasmic N- and C-terminus, and two extracellular segments (ECS; also referred to as ECL; Krause *et al,* 2008). The first segment (ECS1) consists of ca. 50 amino acids and is described as the pore-lining segment, which is substantial for the permeability of small ions (Colegio *et al.,* 2003). The smaller ECS2 with ca. 25 amino acids plays an essential role in trans-interactions, i.e., interactions with proteins from the membrane of adjacent cells (Piontek *et al.,* 2008). All claudins share the same basic structure but vary in their extracellular regions. The composition of amino acids in the ECSs is the main factor for the claudin-specific differences in the permeability of ions with different charges and sizes (Krause *et al.,* 2008).

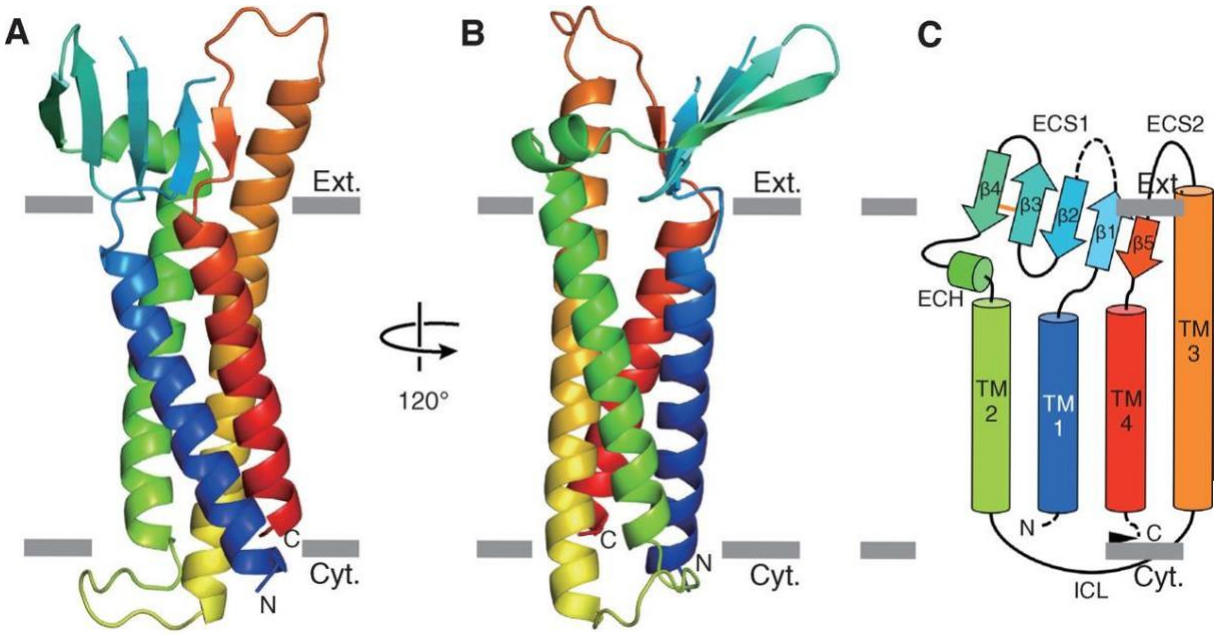

Figure 2. Structural claudin protein model. (A, B) Claudin structure as in the example of monomeric mClaudin15 within the membrane. The gray bars represent the inner (Cyt.) and outer (Ext.) leaflets of the lipid bilayer. **(C)** The complex structure of claudins consists of four transmembrane domains (TM 1-4). All claudins include two extracellular segments (ECS1, -2), and a N- and C-terminus both of which are located at the cytoplasmic compartment. The extracellular helix (ECH) at the end of β4 is connected to TM 2. The orange line between β3 and β4 represents a disulfide bond. ICL represents the cytoplasmic loop between TM 2 and TM 3 (Suzuki *et al.,* 2014).

Hence, as each claudin has different permeability characteristics, epithelia can either be tight or leaky depending on the interplay of the various claudins at the TJ (Krause *et al.,* 2008). This depends on the numerous functions that epithelia fulfill in the various organs: Whereas the epithelium of the urinary bladder is mainly impermeable, the epithelium lining the small intestine is much more permeable to small ions and solutes (Alberts *et al.,* 2002).

Questions concerning the mechanism of leaky epithelia were answered by the finding that the overexpression of claudin-2 leads to a decreased transepithelial resistance (TER) and an increased permeability to small cations (Amasheh *et al.,* 2002) and water (Rosenthal *et al.,* 2010). Based on this, claudins can be classified with regard to their sealing or pore-forming properties. To date, several more claudins that form paracellular channels to cations (claudin-2, -10b, -15, -21), anions (claudin-10a, -17), or water (claudin-2, -15) are known (Günzel, 2017). Particularly in epithelia in which a strong exchange of ions and water is achieved, e.g., in the proximal tube of the nephron, an increased paracellular flux through pore-forming claudins is highly energy efficient (Pei *et al.,* 2016). Some claudins even show a selectivity for specific ions: For example, claudin-8 is associated with the absorption of Na^+ in the colon (Amasheh *et al.,* 2009c), whereas claudin-16 and -19 are required for the renal re-absorption of Mg^{2+} (Hou and Goodenough, 2010).

With regard to their diverse functions, many diseases are associated with a disturbance of the physiological claudin composition (Amasheh *et al.,* 2011). Inflammatory states that are related

to TNF show disturbed claudin composition, such as a decrease of claudin-1 (Poritz *et al.,* 2004) and claudin-3 (Haines *et al.,* 2016) or an up-regulation of the channel-building claudin-2 (Weber *et al.,* 2008). In the intestine, a down-regulation and reorganization of claudins can lead to an impaired barrier function and, hence, an exaggerated permeability to toxins and bacteria (Lu *et al.,* 2013). On the other hand, a disturbed barrier function can result in an increased flux of Na^+ and Cl^-, which activates secretory mechanisms and can lead to diarrhea (Krug *et al.,* 2014). Because of the major role of claudins in the pathophysiology of numerous diseases, new studies concerning the function of claudins as a therapeutic target are currently attracting major attention, which will be discussed in **chapter 6** of this thesis.

2.3. Porcine models for the analysis of intestinal epithelial barrier function

Animal models are essential components in translational research and are frequently used to study the pathophysiology of various diseases and to develop treatment strategies (Da Costa *et al.,* 2022). However, the application of some animal models for scientific purposes is highly controversial, as the translation of findings from rodents to clinical applications for humans is questionable (Ribitsch *et al.,* 2020). Compared with the gastrointestinal tract of other non-primates, that of the pig shares many similarities with that of humans with regard to anatomy, physiology, and nutritional requirements (Deglaire and Moughan, 2012). Therefore, the pig represents a good model not only for the research of gastrointestinal physiology, but also for the deeper analysis of the pathophysiology of various inflammatory gastrointestinal disorders in humans, such as IBD (Wang *et al.,* 2013). Moreover, an analysis of porcine gastrointestinal health is also useful for research into porcine gastrointestinal diseases: Diarrheal diseases in pigs, such as post-weaning diarrhea (PWD), are one of the most frequently occurring diseases in pig farming and are often associated with a high mortality rate and immense economic losses (Fairbrother *et al.,* 2005). Therefore, a detailed understanding of porcine barrier function during healthy and inflammatory conditions is essential for investigating new therapeutic approaches in both human and veterinary medicine.

Nevertheless, the use of pigs as an *in vivo* model has several drawbacks concerning ethical considerations and high costs (Ribitsch *et al.,* 2020). The establishment of *in vitro* and *ex vivo* models that optimally resemble gastrointestinal physiology *in vivo* is thus of great interest (Joshi *et al.,* 2022).

In gastrointestinal models, a well-established barrier function is mandatory to provide a solid basis before proceeding with further experiments focusing on epithelial barrier function (Srinivasan *et al.,* 2015). Numerous qualitative and quantitative methods are available to analyze the barrier integrity of *in vitro* and *ex vivo* models:

Qualitative analysis of barrier function

Qualitative analyses of the barrier integrity of tissue samples can be carried out by immunostaining and confocal laser scanning immunofluorescence microscopy of specific TJ proteins (Srinivasan *et al.,* 2015). Furthermore, the exact localization of TJ proteins can be observed by these methods. Information can thus be gained as to whether the proteins are functionally located to the apicolateral membrane of neighboring cells or whether they are relocated or internalized (Cornelius *et al.,* 2022).

Quantitative techniques to measure the barrier integrity

i) Transepithelial resistance measurements

The transepithelial resistance (TER), recorded in ohms, represents a quantitative method for the measurement of epithelial barrier function. This is reflected by the transepithelial movement of ions and solutes across the epithelial barrier by using two electrodes in the apical and basolateral compartment, respectively (Tang and Goodenough, 2003). A TER measurement setup and the according measurement circuit is depicted in **Figure 3**. The TER of the respective tissue can be calculated by subtracting the resistance values of the blank resistance:

$$R_{TISSUE} = R_{TOTAL} - R_{BLANK}$$

After recording, the resistance value is multiplied by the surface area (M_{AREA}), resulting in TER values in units of Ω x cm^2 (Bednarek, 2022).

$$TER_{REPORTED} = R_{TISSUE} \times M_{AREA}$$

Information regarding the tightness of epithelia can be obtained based on the calculated TER values. Tight epithelia have high TER values, whereas epithelia with TER values below 1000 Ω x cm^2, for example, are considered to be leaky (Chen *et al.,* 2015).

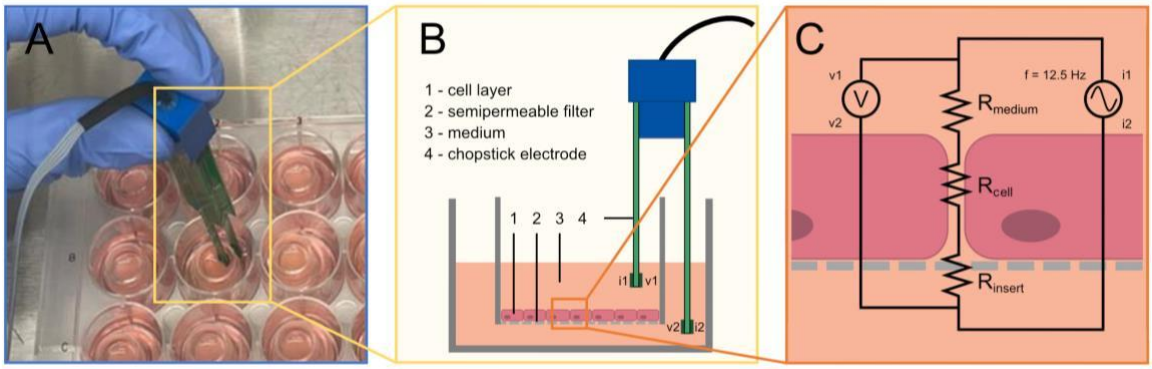

Figure 3. TER measurement with chopstick electrodes. (A) Photograph of a cell culture setup with semipermeable Milicell® cell culture inserts. TER is measured using a chopstick electrode and an Epithelial Volt-Ohm Meter (EVOM). **(B)** Schematic drawing of the electrode arrangement for TER measurements. The cell monolayer is grown on the semipermeable filter of the cell culture insert positioned in a multi-well plate, both compartments being filled with medium. For the electrical measurement of barrier integrity, two electrodes are placed in the setup, one in the apical and the other in the basolateral compartment on either side of the cell filter. **(C)** A simplified TER measurement circuit. Based on Ohm's law, the ohmic resistance is calculated as the ratio of voltage and current. To avoid any direct current that might damage the cells and electrodes, a constant alternating current signal with a square wave at a frequency of 12.5 Hz is applied through i1 and i2 electrodes. The resulting voltage is measured *via* v1 and v2 electrodes to calculate the total electrical resistance. The total electrical resistance consists of the resistance of the cell culture medium (R_{medium}), of the cell layer (R_{cell}), and of the cell culture insert (R_{insert}). Figure modified according to Raut *et al.,* 2021.

ii) Permeability tracer assays

Another method for the quantitative analysis of the TJ barrier function is the measurement of the paracellular permeability between the apical and basolateral compartments of the epithelium. This can be investigated by flux measurements with tracer molecules of various molecular weights (Srinivasan *et al.,* 2015). Commonly used tracer molecules are mannitol, polyethylene glycol, or polysaccharides, such as dextran or inulin (Bednarek, 2022). The variable size of tracer compounds provides information concerning the pore size of TJ proteins (Srinivasan *et al.,* 2015). The calculation of tracer flux has been described in detail by Zakrzewski (2014).

2.3.1. *In vitro* models

In vitro models allow the examination of microorganisms or cells outside of their natural environment and are often used as a first step in biomedical research (Joshi *et al.,* 2022). One essential advantage of *in vitro* cell culture models is that the behavior of the cells can be studied under specific conditions in a controlled context without environmental influences. By using cell lines derived from the gastrointestinal epithelium, intestinal epithelial barrier function can be precisely analyzed during their interactions with a variety of drugs or food compounds (Costa and Ahluwalia, 2019).

Commonly used epithelial cell culture models, such as Caco-2 (Hidalgo *et al.*, 1989), HT29 (Zweibaum *et al.,* 1985) or T84 (Murakami and Masui, 1980), share a common feature: they are all immortalized cell lines derived from tumor tissue. However, a few limitations arise when

employing tumor cell lines, as their comparability with *in vivo* conditions is questionable by virtue of their malignant origin (Rahman *et al.,* 2021). Therefore, the use of non-transformed cell lines is of great interest, as they lack malignant characteristics (Geraghty *et al*., 2014).
In 1989, Helen Berschneider established the intestinal porcine enterocyte cell line (IPEC-J2), which stems from the jejunal epithelia of an unsuckled neonatal piglet (Berschneider, 1989). This non-transformed permanent cell line shows morphological and functional parallels to porcine intestinal enterocytes *in vivo* and has previously been characterized as a good model for studies of porcine microbial pathogenesis (Schierack *et al.,* 2006). The cells form polarized monolayers when cultured on filters of 0.4 μm pore size and build stable TER values within 1 – 2 weeks after being seeded (Vergauwen*,* 2015). Whereas the culturing with conventional fetal bovine serum leads to atypical high resistance values and low transport rates, cultivation under species-matching conditions by using porcine serum results in significantly lower TER values that are more comparable to those *in vivo* (Zakrzewski *et al.,* 2013). Moreover, this optimized culturing protocol for IPEC-J2 changes the morphology of the cells, their TJ ultrastructure being more similar to that of jejunal enterocytes (Zakrzewski *et al.,* 2013). Because of their suitable comparability to *in vivo* situations, IPEC-J2 cells can be used to reduce expensive feeding trials on piglets (Spitzer *et al.,* 2016).

2.3.2. *Ex vivo* models

Ex vivo models involve the use of tissue samples or organs extracted from an organism and cultured outside of its usual environment (Shi *et al.,* 2019). All epithelial cell types present in native tissue can be considered by use of this technique. Various *ex vivo* models are currently available for experimental purposes, such as the Ussing chamber technique, the everted gut sac model, or the microfluidic gut-on-chip technology (Joshi *et al.,* 2022). The main focus of the *ex vivo* models in this thesis will be the Ussing chamber technique.
In order to gain a better understanding of the transport mechanisms within intestinal tissues, the Danish zoologist Hans Ussing invented the chamber that bears his name in the 1950s (Ussing and Zerahn, 1951). This chamber consists of two halves separated by the epithelial sample, as shown in **Figure 4**. Therefore, both the apical and the basolateral compartment can be examined directly. By measuring the TER and the short-circuit current (I_{SC}), information can be obtained with regard to the barrier integrity and ionic currents across the epithelium (Thomson *et al.,* 2019). Such data can be employed to characterize the epithelial barrier properties across the longitudinal axis of the gastrointestinal tract in terms of specific local characteristics (Markov *et al.,* 2010). Moreover, studies concerning the paracellular transport of various tracer molecules from one compartment to another can provide helpful information about epithelial barrier function (Thomson *et al.,* 2019). Such studies can be executed in

combination with incubation experiments in which the addition of food compounds and their impact on epithelial barrier function can elucidate the situation *in vivo* (Grilli *et al.,* 2015; Radloff *et al.,* 2017a). Therefore, with regard to the closer similarity of the cell composition to the intestinal epithelium *in vivo* and the manifold options for electrophysiological measurements, the *ex vivo* Ussing chamber technique provides a good model for the analysis of intestinal physiology and barrier integrity under a variety of conditions (Ghiselli *et al.,* 2021).

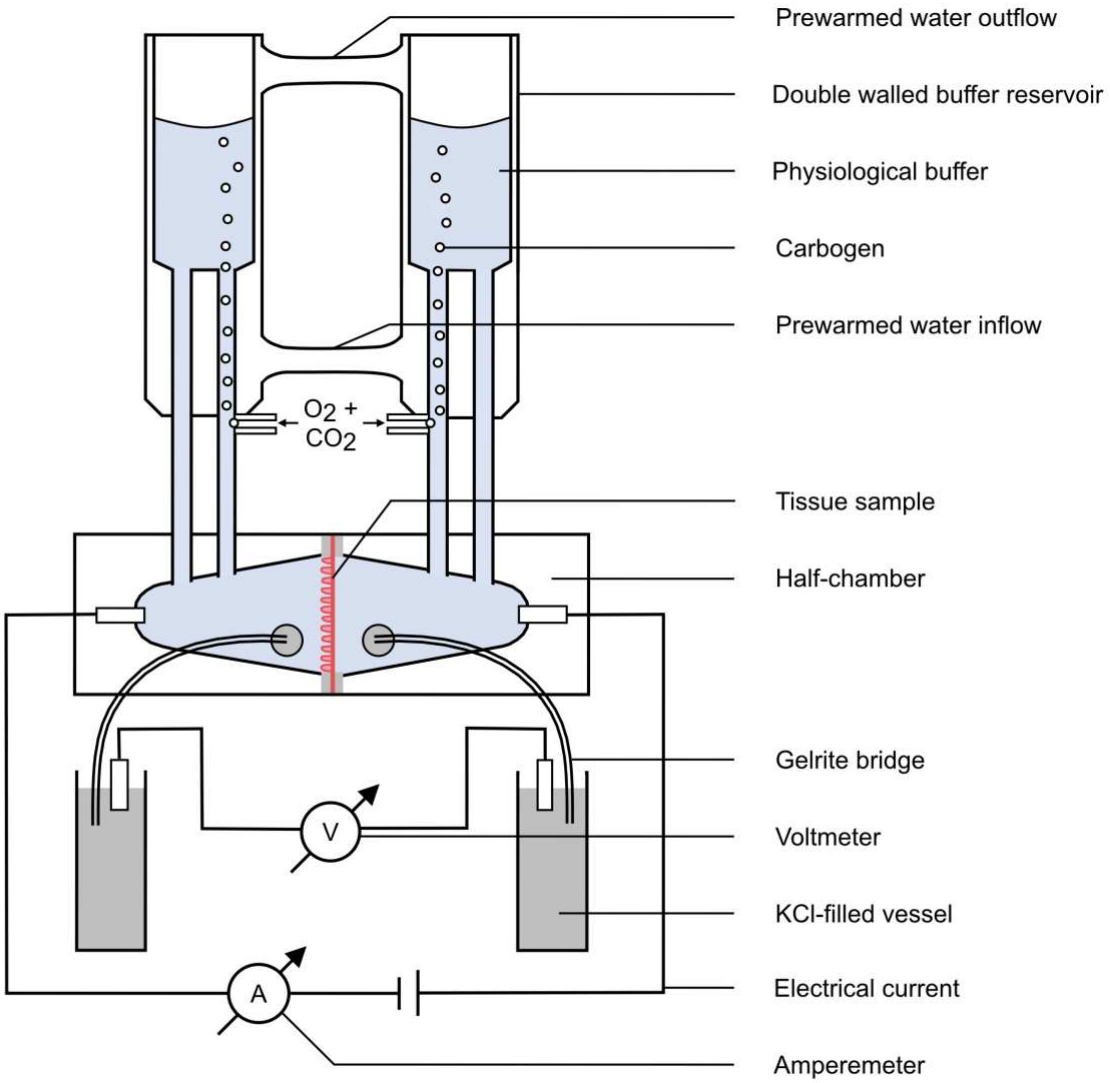

Figure 4. Schematic drawing of a conventional Ussing chamber setup. The double walled glass tube is connected to a prewarmed water bath supply and filled with experimental buffer. For continuous mixing, the buffer is constantly gassed with carbogen (95 % O_2 and 5 % CO_2). Tissue samples are mounted between the two halves of the Ussing chamber. The Voltmeter measures the arising potential difference between the two electrodes that are connected to the half-chambers *via* gelrite bridges. An electrical current is required to compensate the potential difference to zero. This current represents the sum of all ionic currents across the tissue sample and is called short-circuit current (I_{SC}). The resistance of the respective tissue sample can be calculated subsequently (Mießler *et al.,* 2018).

2.3.2.1. Peyer's patches

As the epithelium of the intestine constantly comes into contact with exogen substances during ingestion, an efficient epithelial barrier and a good working intestinal immune system is necessary. As a preventative measure against infections, the gut-associated lymphatic tissue (GALT) contains a majority of endogenous immune cells (Jung *et al.,* 2010). Porcine Peyer's patches (PPs), which consist of aggregated lymphoid follicles and are a part of the GALT, are mainly located in sections of the small intestine such as the jejunum and ileum (Andersen *et al.,* 1999). Structurally, three main areas of PPs can be distinguished: the follicular area, the interfollicular area, and the follicle-associated epithelium (FAE; Jung *et al.,* 2010). The first two mentioned contain B-lymphocytes, macrophages, and follicular dendritic cells. The subepithelial dome, also called the corona, surrounds the PP follicles and includes B- and T-lymphocytes, dendritic cells, and macrophages (Cesta, 2006) The FAE covers the PP and forms the border between the underlying lymphatic tissue and the luminal contents. The structural and functional composition of cells that form the FAE differ from the neighboring villus epithelium (VE): the FAE mainly consists of enterocyte-like cells and microfold cells (M cells), and the production of mucus is low (Ermund *et al.,* 2013). The specialized M cells can present antigens to the underlying lymphoid follicles *via* transcytosis and therefore play a crucial role in the induction of immune responses (Mabbott *et al.,* 2013). An illustration of PPs can be seen in **Figure 5**.

Because of the important immunological role of FAE, its epithelial barrier function is substantially altered. Compared with adjacent VE, PP tissue specimens show an adjusted claudin composition and exhibit notably higher TER values and lower paracellular permeability (Markov *et al.,* 2016). Furthermore, a major sealing function of claudin-4 in porcine PPs has been discovered, underlining the important function of PPs as a first intestinal immunological defense (Radloff *et al.,* 2017b). Hence, the limitation of the paracellular pathway in PP FAE contributes to the mandatory function of the transcellular route for a controlled uptake and presentation of antigens to the underlying lymphoid follicles (Markov *et al.,* 2016).

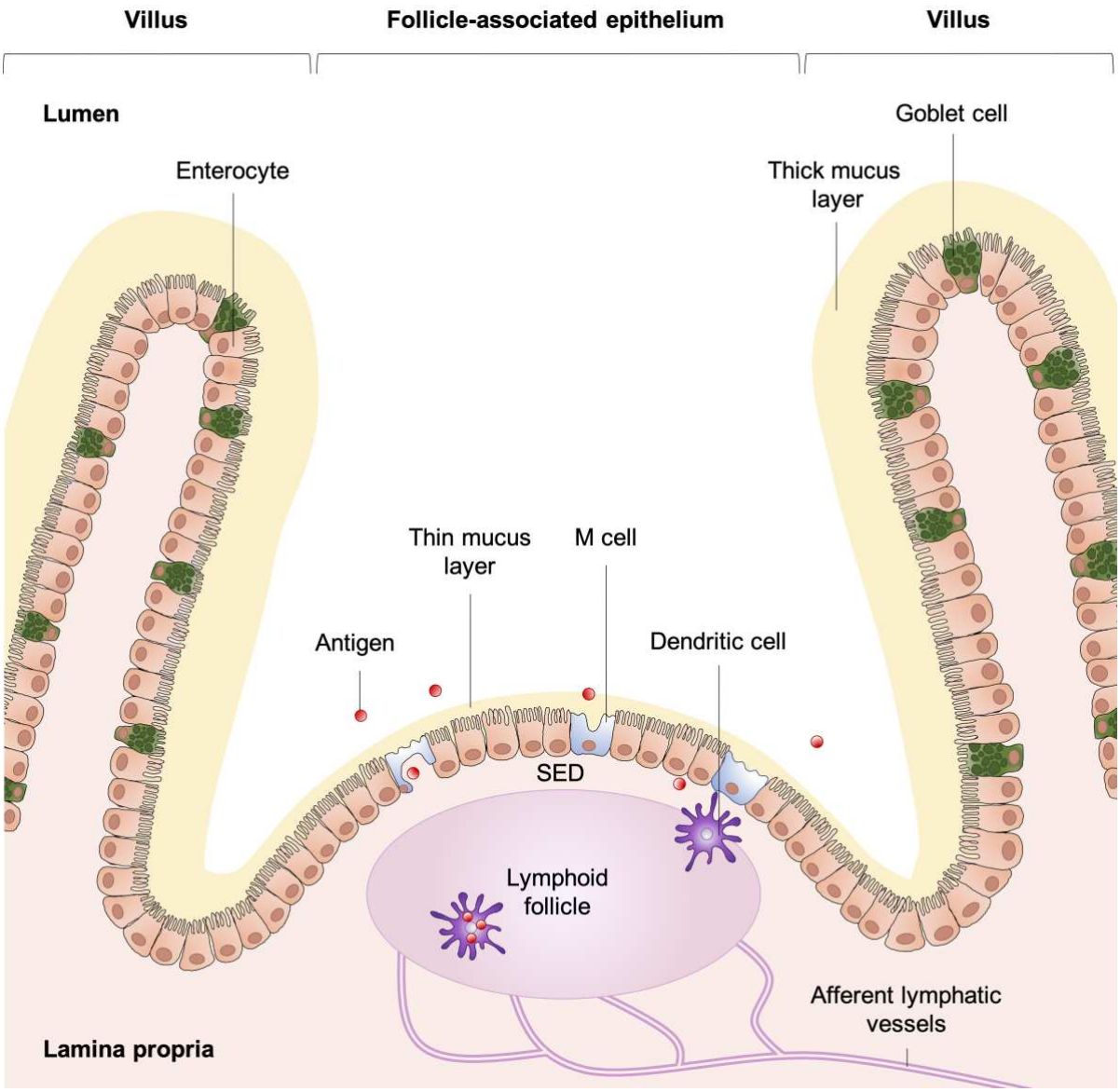

Figure 5. Illustration of Peyer's patches and surrounding villus epithelium. The follicle-associated epithelium (FAE) covers lymphoid follicles belonging to Peyer's patches (PPs) and consists of enterocytes and specialized M cells that regulate the sampling and presentation of antigens from the gut lumen. After transcytosis of antigens to the subepithelial dome (SED), dendritic cells present the antigens to T cells within the lymphoid follicles. Subsequently, the gut-associated lymphatic tissue (GALT) is activated *via* the afferent lymphatic vessels (Radloff *et al.,* 2017b). Whereas the neighboring villus epithelium (VE) contains many goblet cells that are specialized for mucus production, only a thin mucus layer can be observed in the area of PP FAE (Ermund *et al.,* 2013).

Chapter 3: Aims and objectives of this thesis

Intestinal inflammatory diseases have gained increasing interest in recent years. Because TNF is one of the major cytokines involved in diseases such as IBDs, special attention is being given to the examination of the pathophysiological actions of this pro-inflammatory cytokine. With a view to the establishment of potential therapeutic agents for the treatment or prevention of intestinal inflammations, barrier-perturbing mechanisms need to be precisely analyzed. However, the signaling pathways and detailed effects of TNF on epithelial barrier function are not completely understood as yet.

The main aim of the work described here has been to elucidate the functional and molecular effects of TNF on porcine intestinal epithelial barrier function. Therefore, the focus of this research has been to explore TNF effects on two different experimental models:

1. In vitro on the porcine intestinal epithelial cell line IPEC-J2

2. *Ex vivo* on porcine Peyer's patches compared with the surrounding villus epithelium

Both models were used to analyze epithelial barrier function, during incubation with TNF, by employing TER and [^{3}H]-D-Mannitol flux measurements. Effects of incubation with TNF on the composition of TJ proteins and the receptor density of the specific cell surface receptors TNFR-1 and -2 were carried out and subsequently analyzed. At the end of each experiment, both cells and tissue specimens were therefore further processed for detailed functional and structural analysis by Western blot analysis and immunostaining. Moreover, as another aspect of the project was to research the signaling cascade of TNF-induced changes in epithelial barrier function, further experiments using a specific blocker against MLCK were conducted.

For the *ex vivo* Ussing chamber experiments, an optimized experimental setup was established to analyze the long-term effects of TNF on various parts of the porcine small intestine for a time period of up to 10 hours. Furthermore, the functional and molecular differences of PP compared with neighboring VE during incubation with TNF was analyzed, emphasizing the importance of the immunological FAE during inflammatory conditions.

Chapter 4: Tumor Necrosis Factor Alpha Effects on the Porcine Intestinal Epithelial Barrier Include Enhanced Expression of TNF Receptor 1

This chapter has been published in: International Journal of Molecular Sciences

Manuscript received at the Journal: July 6th, 2021

Initial review completed: July 23rd, 2021

Revision accepted: August 11th, 2021

Authors: Linda Droessler, Valeria Cornelius, Alexander G. Markov, Salah Amasheh

https://doi.org/10.3390/ijms22168746

Author contribution:

As stated in this publication, I have conceptualized and performed the experiments and evaluated the data. Western blot analyses and immunostaining was carried out by me, personally. I performed statistical data analysis, designed the figures, and wrote the article.

International Journal of
Molecular Sciences

MDPI

Article

Tumor Necrosis Factor Alpha Effects on the Porcine Intestinal Epithelial Barrier Include Enhanced Expression of TNF Receptor 1

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Abstract: Tumor necrosis factor alpha (TNFα) has been shown to impair the intestinal barrier, inducing and maintaining inflammatory states of the intestine. The aim of the current study was to analyze functional, molecular and regulatory effects of TNFα in a newly established non-transformed jejunal enterocyte model, namely IPEC-J2 monolayers. Incubation with 1000 U/mL TNFα induced a marked decrease in transepithelial electrical resistance (TEER), and an increase in permeability for the paracellular flux marker [^{3}H]-D-mannitol compared to controls. Immunoblots revealed a significant decrease in tight junction (TJ) proteins occludin, claudin-1 and claudin-3. Moreover, a dose-dependent increase in the TNF receptor (TNFR)-1 was detected, explaining the exponential nature of pro-inflammatory effects, while TNFR-2 remained unchanged. Recovery experiments revealed reversible effects after the removal of the cytokine, excluding apoptosis as a reason for the observed changes. Furthermore, TNFα signaling could be inhibited by the specific myosin light chain kinase (MLCK) blocker ML-7. Results of confocal laser scanning immunofluorescence microscopy were in accordance with all quantitative changes. This study explains the self-enhancing effects of TNFα mediated by MLCK, leading to a differential regulation of TJ proteins resulting in barrier impairment in the intestinal epithelium.

Keywords: claudins; epithelial barrier; IPEC-J2; ML-7; tight junction; TNFα; TNFR-1

Citation: Droessler, L.; Cornelius, V.; Markov, A.G.; Amasheh, S. Tumor Necrosis Factor Alpha Effects on the Porcine Intestinal Epithelial Barrier Include Enhanced Expression of TNF Receptor 1. *Int. J. Mol. Sci.* **2021**, *22*, 8746. https://doi.org/10.3390/ijms22168746

Academic Editors: Maria Luisa Balestrieri and Gautam Sethi

Received: 6 July 2021
Accepted: 11 August 2021
Published: 14 August 2021

1. Introduction

The intestinal epithelial paracellular barrier is essential since it prevents the immune system from being overly induced by a wide variety of exogenous antigens and microorganisms. Tight junctions (TJs), which are formed in strands and located at the apicolateral membrane of adjacent cells, represent the functional correlate of the epithelial paracellular barrier function. These TJ strands are defined by the common presence of several integral membrane proteins, such as occludin [1], junctional adhesion molecule (JAM) [2] or the claudin protein family, from which at least 27 members are known [3]. Along the longitudinal axis of the gastrointestinal tract, a strong correlation of TJ protein expression and barrier properties has been demonstrated [4]. In inflammatory bowel disease (IBD), increased paracellular permeability due to a lack or disruption of TJ proteins results in intestinal inflammation and may cause diarrhea [5].

The proinflammatory cytokine tumor necrosis factor α (TNFα) plays a key role in the pathogenesis of IBD, and thus has been shown to be increased in patients with Crohn's disease (CD) or ulcerative colitis (UC) [6,7]. One potential proinflammatory action of TNFα is the ability to alter the intestinal epithelial TJ composition, leading to an increase in paracellular permeability and a decrease in transepithelial electrical resistance (TEER) [8,9]. Sealing TJ proteins, such as claudin-1, -7 or occludin, have been reported to be a primary

target affected by incubation with TNFα [10], and the molecular changes in TJ composition cause a disturbance in intestinal epithelial barrier function and provoke diarrhea [11,12]. Although the exact mechanisms of TNFα on TJ composition have not been analyzed in detail, the myosin light chain kinase (MLCK) appears to be involved in intestinal barrier disturbance, as increased MLCK production can be observed after TNFα treatment [13–15]. One function of MLCK is to phosphorylate the myosin light chain (MLC), resulting in a contraction of actin and therefore rearranging the cytoskeleton [16,17]. In several inflammatory diseases, such as IBD, an enhanced expression of MLCK has been observed, leading to impaired epithelial barrier function [16]. The activation of MLCK by TNFα leads to a reorganization of TJ proteins and therefore affects the paracellular barrier [18].

The permanent porcine intestinal epithelial cell line IPEC-J2, a non-transformed cell line obtained from jejunal epithelia of an unsuckled piglet, has been characterized as a suitable in vitro intestinal model [19]. Due to its capacity to grow as a monolayer and form an apical and a basolateral compartment, as well as apicolateral junctional complexes, it can be useful for the analysis of the paracellular barrier and permeability. By culturing this cell line under species-specific conditions, using porcine serum (PS) instead of conventional serum, IPEC-J2 cells constitute an improved model for the analysis of porcine jejunal barrier function in vitro [20]. On account of the great similarities between the anatomy and physiology of swine and humans, the pig provides an extremely valuable model for studies compared to other animal models [21], as it is in accordance with human nutritional and digestive effects and mechanisms [22].

The barrier-weakening effect of TNFα has been evaluated on various cell culture models, such as the rat ileal intestinal epithelial cell line IEC-18 [23] or the human carcinogenic cell lines Caco-2 [24,25] and HT-29/B6 [10,26,27], but not in detail on the non-transformed porcine intestinal epithelial cell line IPEC-J2 so far. Therefore, our study aimed to analyze the effect of TNFα on the barrier function as well as the TJ composition of IPEC-J2 cells. This was assessed by the measurement of TEER and [^{3}H]-D-Mannitol flux during treatment with the cytokine. Subsequent to the incubation experiments, the TJ composition was analyzed in detail and the impact of TNFα on the density of tumor necrosis factor receptor 1 and 2 (TNFR-1, TNFR-2) was investigated. As MLCK has been discussed in context with barrier perturbation in a series of studies, further experiments were carried out using a specific blocker against MLCK to analyze the signaling cascade of TNFα on IPEC-J2 cells. This provides new experimental data, showing that (i) the non-transformed cell line IPEC-J2 represents a useful model for the exploration of inflammation-induced changes in porcine intestinal epithelial barrier function and (ii) explains the self-enhancing effects of TNFα resulting in TJ impairment in the intestinal epithelium on a functional, molecular and regulatory level.

2. Results

2.1. Effects of TNFα on Epithelial Barrier Function of IPEC-J2

To examine the effects of TNFα on the transepithelial barrier function of the porcine jejunal cell line IPEC-J2, concentration-dependent effects of the cytokine were analyzed by addition to the basolateral compartment of the cells, and TEER and [^{3}H]-D-Mannitol flux were measured. After 48 h, 1000 U/mL and 5000 U/mL TNFα showed a marked decrease in TEER compared to the control group (one-way ANOVA: $F(5,109) = 9.22$, $p < 0.0001$, $n = 14$–22; ctrl: 101.06 ± 2.89%; 1000 U/mL: 88.45 ± 2.56%, $p = 0.0113$; 5000 U/mL: 78.74 ± 3.19%, $p < 0.0001$, Figure 1A). With lower concentrations of TNFα, TEER remained unchanged over the whole incubation period (50 U/mL: 97.47 ± 1.93%, $p = 0.91$; 100 U/mL: 102.21 ± 5.41%, $p = 0.99$; 500 U/mL: 98.49 ± 2.35%, $p = 0.96$). Hence, the following experiments were carried out using concentrations of 1000 U/mL TNFα. After 24 h, 1000 U/mL did not have significant effects on TEER (ctrl: 93.81 ± 1.81%; 1000 U/mL: 92.96 ± 3.60%, $p = 0.99$, $n = 23$; Figure 1B).

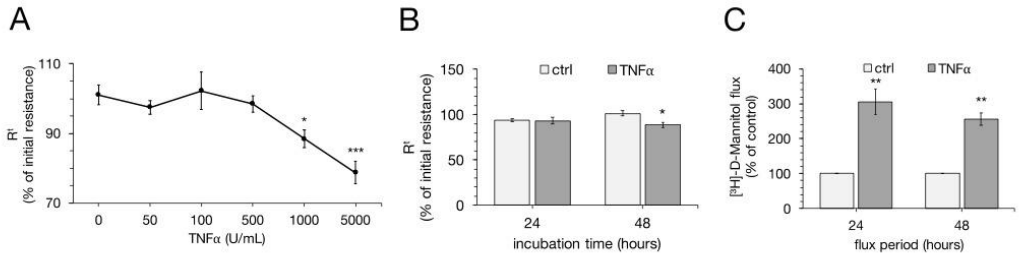

Figure 1. (**A**) To examine the dose-dependent effect of tumor necrosis factor α (TNFα) on IPEC-J2 cells, increasing concentrations were added basolateral to the monolayers and transepithelial electrical resistance (TEER) was measured for 48 h. Concentrations of 1000 U/mL and 5000 U/mL revealed a decrease in TEER in IPEC-J2 cells (one-way ANOVA, * $p < 0.05$, *** $p < 0.001$; $n = 14$–22). Therefore, following experiments were carried out using 1000 U/mL. (**B**) No significant changes in TEER could be observed after 24 h with 1000 U/mL TNFα. (**C**) Flux measurements using [^{3}H]-D-Mannitol revealed an increased paracellular flux after 24 as well as 48 h (unpaired *t*-test, ** $p < 0.01$, $n = 6$). Resistance values are expressed as percentage of initial resistance and compared to controls, respectively. For analysis of paracellular flux, controls were set to 100% and values were compared to this, respectively. All data shown are presented as means ± SEM.

For permeability studies, unidirectional paracellular tracer flux from the apical to the basolateral side of the cell culture filters was measured using [^{3}H]-D-Mannitol. The control groups were set to 100% and flux measurements of TNFα-incubated cell filters were compared to control groups, respectively. For the first flux period of 24 h, 1000 U/mL TNFα indicated a stronger paracellular permeability compared to the control groups (ctrl: 100%; TNFα: 306.18 ± 59.76%, $p = 0.006$, $n = 6$). The same effect could be observed in the second flux period from 24 to 48 h (ctrl: 100%; TNFα: 256.32 ± 40.91%, $p = 0.003$, $n = 6$; Figure 1C).

2.2. Western Blot and Densitometry of TJ Proteins

Following electrophysiological measurements, protein samples of the cells were used for immunoblotting, analyzing TJ proteins claudin-1, -3, -4, -7, occludin and ZO-1. For densitometry, the protein levels of the specific bands were normalized on total protein amount. The protein expression of untreated cells was normalized to 100% and the TNFα-treated groups were compared to controls, respectively. Densitometric analysis of immunoblots revealed a significant decrease in claudin-1 expression in IPEC-J2 cells after treatment with TNFα for 48 h (51.94 ± 4.52%, $p < 0.0001$, $n = 6$). The same effect could be observed for claudin-3 (52.51 ± 16.09%, $p = 0.012$, $n = 6$), as well as for occludin expression (65.57 ± 12.31%, $p = 0.019$, $n = 6$). The expression of the TJ proteins claudin-4, -7 and ZO-1 did not significantly change during TNFα treatment (claudin-4: 87.39 ± 10.37%, $p = 0.25$; claudin-7: 82.99 ± 7.77%, $p = 0.054$; ZO-1: 99.36 ± 20.82%, $p = 0.98$; $n = 6$, Figure 2A,B).

2.3. Immunohistochemistry of TJ Proteins

As the incubation with TNFα led to changes in the expression levels of claudin-1, -3 and occludin in IPEC-J2 cells, confocal laser scanning immunofluorescence was performed to examine the localization of these TJ proteins. After 48 h incubation with TNFα, the claudin-1 signal (green, Figure 3A) no longer appeared as fine paracellular lines as seen for the controls, but rather as washed-out lines with a weaker signal. The ZO-1 signal remained mostly unaltered over the whole incubation period (red, Figure 3A). In Figure 3B, a co-localization of claudin-3 (green) and occludin (red), resulting in a yellow signal in merged confocal images, could be shown by double staining. TNFα caused an evident loss of specific claudin-3 and occludin immunofluorescence signals from the apicolateral membrane (Figure 3B), which correlates with the changes in the expression levels of claudin-3 and occludin.

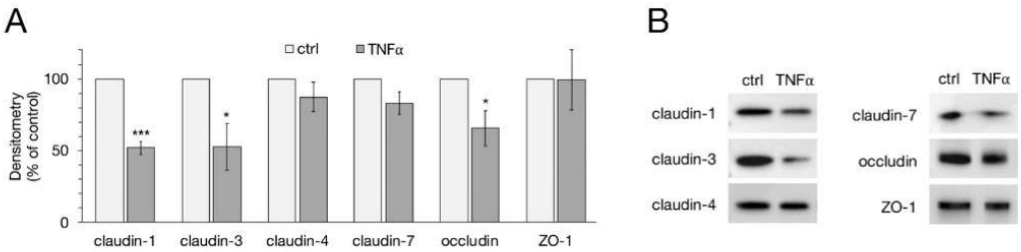

Figure 2. (**A**) Densitometry and (**B**) representative Western blot bands of tight junction (TJ) proteins after TNFα incubation revealed significantly weaker expression of claudin-1, -3 and occludin compared to controls, while claudin-4, -7 and ZO-1 remained unaffected (unpaired *t*-test, * $p < 0.05$, *** $p < 0.001$; $n = 6$). For Western blot analysis, the specific bands were normalized on total protein amount. All data presented are stated in means ± SEM.

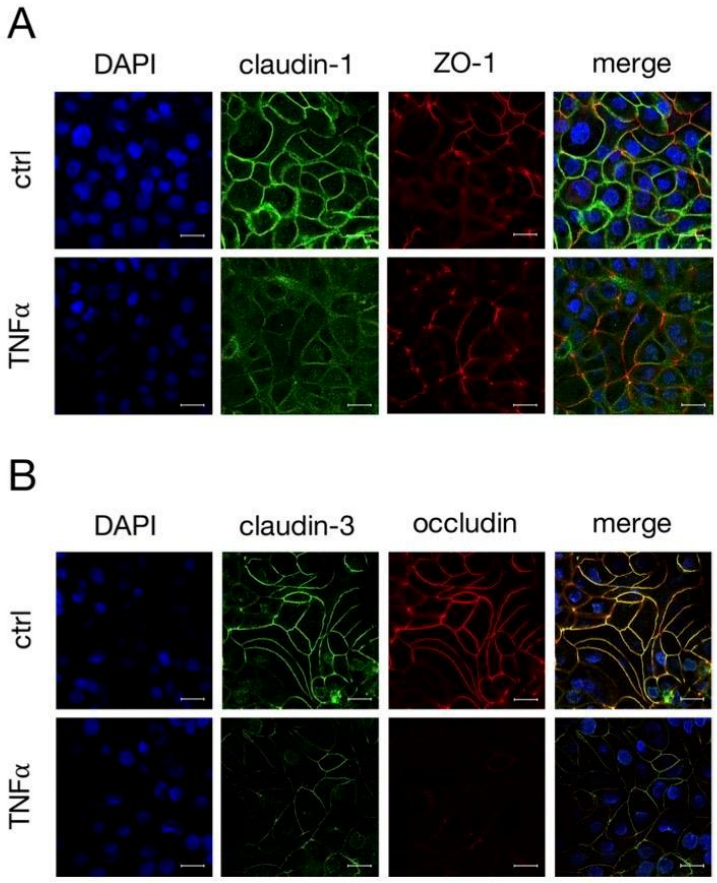

Figure 3. Confocal laser scanning immunofluorescence microscopy was performed to analyze the localization of (**A**) claudin-1 (green) and ZO-1 (red), or (**B**) claudin-3 (green) and occludin (red) after incubation with TNFα for 48 h. Nuclei were stained in blue (DAPI). Claudin-3 shows a strong colocalization with occludin (merge, yellow signal, B) (scale bar: 20 µm; $n = 4$; representative pictures).

2.4. TNFR-1 and TNFR-2 Expression Level

Western blot analysis was performed to investigate whether the incubation with TNFα has any effect on the expression of TNFR-1 or -2. After normalization on total protein amount, the control groups were set to 100% and the TNFα-treated groups were compared to them, respectively. After 48 h with 500, 1000 and 5000 U/mL TNFα, a marked up-regulation of TNFR-1 expression could be observed (one-way ANOVA: F (3,12) = 9.70, $p = 0.0016$, $n = 4$; 500 U/mL: 168.34 ± 7.77%, $p = 0.0036$; 1000 U/mL: 159.08 ± 14.90%, $p = 0.0098$; 5000 U/mL: 182.35 ± 16.10%, $p = 0.0008$; Figure 4). Conversely, the densitometric analysis of TNFR-2 did not show any significant changes due to TNFα treatment (one-way ANOVA: F (3,12) = 0.46, $p = 0.71$, $n = 4$; 500 U/mL: 113.23 ± 24.21%, $p = 0.87$; 1000 U/mL: 88.00 ± 13.26%, $p = 0.89$; 5000 U/mL: 101.89 ± 12.57%, $p = 0.99$).

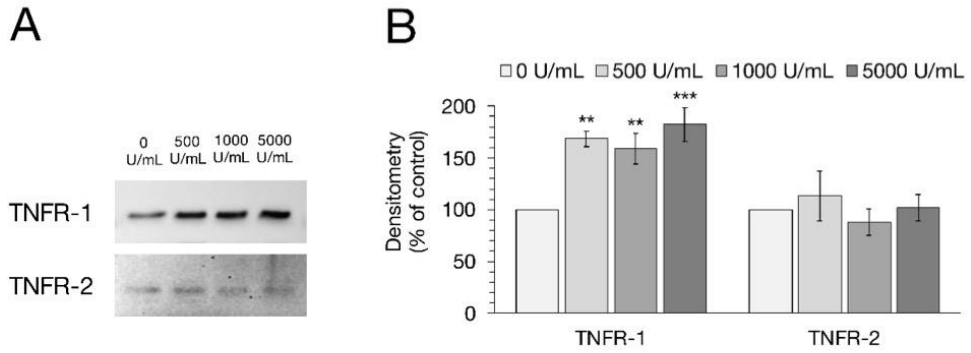

Figure 4. (**A**) Representative Western blot bands and (**B**) Densitometry of TNFR-1 and TNFR-2 after 48 h incubation with rising TNFα concentrations (500, 1000, 5000 U/mL). After specific protein bands were normalized on total protein amount, the protein expression of untreated cells was normalized to 100% and groups treated with TNFα were compared to this, respectively. Incubation with the cytokine led to a clear increase in TNFR-1 expression, while TNFR-2 remained unchanged. Data are shown in mean ± SEM (one-way ANOVA, ** $p < 0.01$, *** $p < 0.001$; $n = 4$).

2.5. Confocal Laser Scanning Immunofluorescence Microscopy of TNFR-1

In addition to changes on the expression of TNFR-1, we aimed to analyze whether TNFα also leads to an altered localization of TNFR-1 in IPEC-J2. Therefore, cells were stained with TNFR-1 and ITGβ1, an integral membrane protein that is located at the basolateral membrane of cells [28]. With rising TNFα concentrations, a stronger signal for TNFR-1 (green, Figure 5A) could be observed, while the signal for ITGβ1 (red, Figure 5A) remained unchanged due to the incubation with TNFα. The co-localization of TNFR-1 with ITGβ1, resulting in the basolateral localization of TNFR-1, can be seen as a yellow signal in the merged pictures. Additionally, Z-stack images were performed to investigate the localization of TNFR-1 more precisely. In Figure 5B, the weak TNFR-1 signal (green) is solely orientated to the basal and basolateral membrane, which is pointed out by white arrows. After incubation with 5000 U/mL TNFα, the TNFR-1 signal appears entirely stronger and a localization, not only on the basolateral membranes of the cells, but also on the apical membrane, can be observed (Figure 5C).

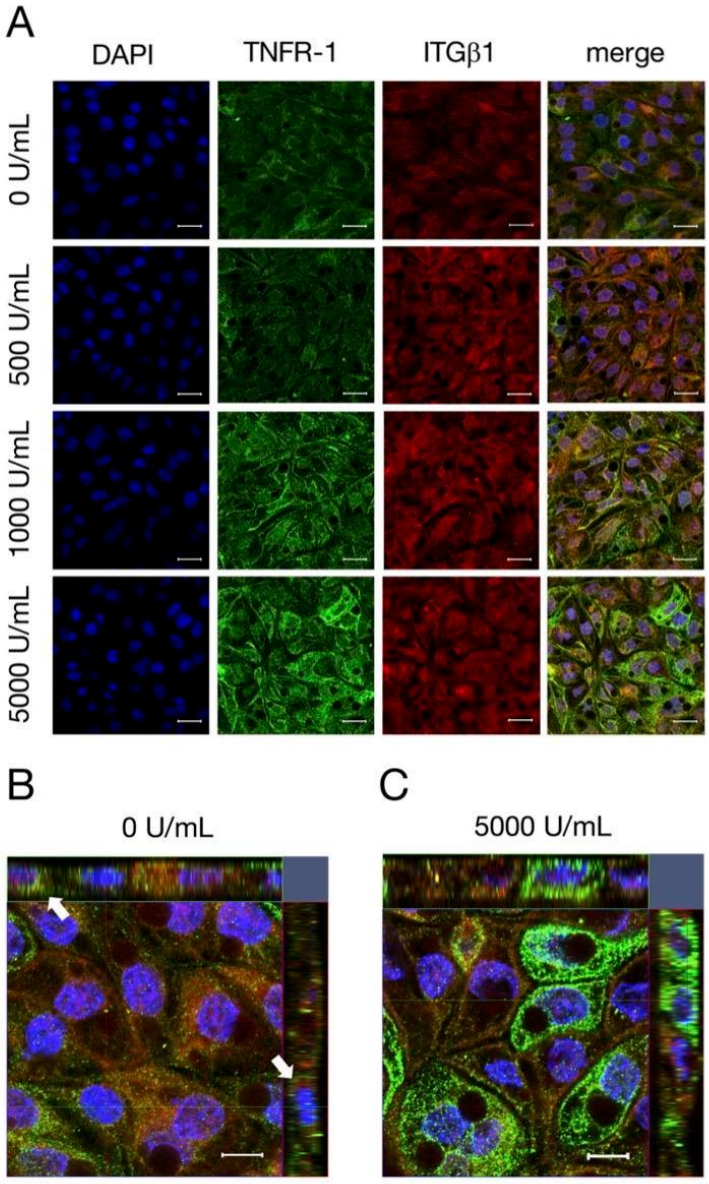

Figure 5. (**A**) To analyze the localization of TNFR-1 after incubation with different concentrations of TNFα (500, 1000, 5000 U/mL) for 48 h, confocal laser scanning immunofluorescence microscopy of TNFR-1 (green) and ITGβ1 (red, A) was performed. The colocalization of TNFR-1 with ITGβ1 can be seen as a yellow signal in the merged pictures. Nuclei were stained in blue (DAPI). In addition, Z-stack images with (**C**) or without (**B**) TNFα were recorded for a more detailed analysis of the localization of TNFR-1. (**B**) White arrows point out the basal side of the cells (scale bar: A 20 µm; B,C 40 µm; $n = 3$; representative images).

2.6. Signaling Experiments with Specific MLCK Blocker

We used ML-7, a specific MLCK blocker, to analyze the signaling of TNFα-induced changes in epithelial barrier function in more detail. After 48 h, cells treated with TNFα together with ML-7 showed a significant difference compared to cells treated solely with TNFα (one-way ANOVA: F (2,41) = 15.37, $p < 0.0001$, n = 7–29; TNFα: 92.38 ± 2.66%; TNFα + ML-7: 113.76 ± 5.69%, $p = 0.0053$). Comparable results could be seen between cells treated with TNFα and control groups (TNFα: 92.38 ± 2.66%; ctrl: 126.50 ± 8.61%; $p < 0.0001$; Figure 6A). To investigate whether these changes are in connection with the altered expression of TJ proteins, immunoblot analysis was subsequently performed (Figure 6B,C). Densitometric analysis revealed that ML-7 almost completely averted the decrease in claudin-3 expression after treatment with TNFα (Kruskal–Wallis test: H (2) = 9.37, $p = 0.009$, $n = 4$; TNFα: 69.18 ± 10.86%; TNFα + ML-7: 95.58 ± 1.68%, $p = 0.04$). While the expression of claudin-1 was also notably higher with TNFα and ML-7 compared to cells treated only with TNFα, just a tendency towards significance could be shown (one-way ANOVA: F (2,9) = 5.30, $p = 0.03$, $n = 4$; TNFα: 69.34 ± 5.08%; TNFα + ML-7: 94.31 ± 11.17%, $p = 0.079$). Surprisingly, treatment with the MLCK blocker did not affect the expression of occludin (Kruskal–Wallis test: H (2) = 7.65, $p = 0.02$, $n = 4$; TNFα: 50.76 ± 10.04%; TNFα + ML-7: 50.28 ± 12.91%; $p = 1.00$). The strong increase in TNFR-1 due to TNFα incubation was also inhibited by ML-7 (Kruskal–Wallis test: H (2) = 9.09, $p = 0.01$, $n = 4$; TNFα: 160.70 ± 12.04%; TNFα + ML-7: 117.57 ± 11.32%; $p = 0.026$).

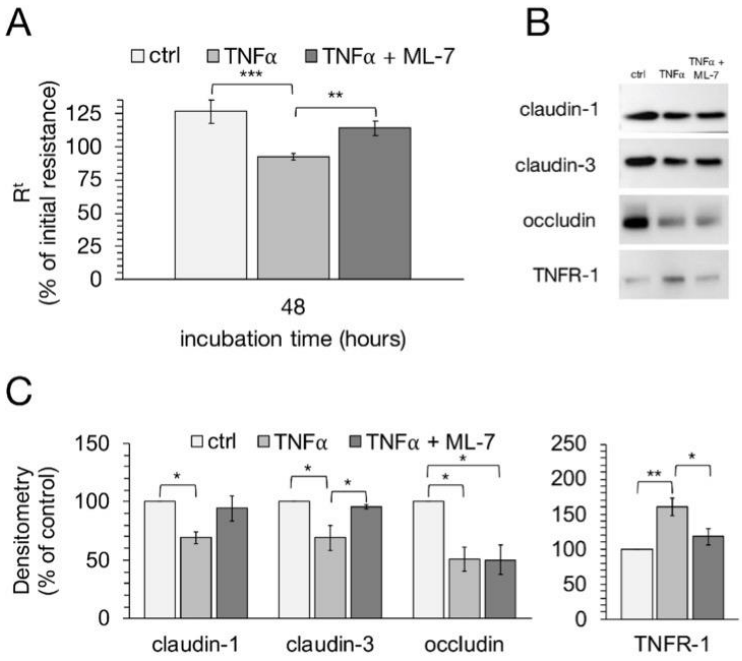

Figure 6. Effects of ML-7, a specific MLCK blocker, were studied after TNFα incubation using TEER measurements and Western blot analysis. (**A**) After 48 h, the groups treated solely with TNFα showed a significant difference in TEER compared to controls and cells treated with TNFα and ML-7 (one-way ANOVA, ** $p < 0.01$, *** $p < 0.001$; n = 7–29). (**B**) Representative Western blot bands and (**C**) densitometry after 48 h with TNFα in the presence or absence of ML-7 revealed that the MLCK blocker prevented the TNFα-induced decrease in claudin-1 and -3, while the decrease in occludin was not affected. Furthermore, the TNFα-induced increase in TNFR-1 is also inhibited by ML-7. Data are presented as mean ± SEM (* $p < 0.05$, ** $p < 0.01$; $n = 4$).

Subsequently, confocal laser scanning immunofluorescence microscopy was carried out after 48 h to see whether ML-7 is also capable of inhibiting the changed localization of TJ proteins caused by TNFα. As shown in Figure 7A, the treatment with TNFα led to a loss of claudin-1 signal (green), in accordance with our earlier observations. ML-7 prevented the TNFα-induced changes since claudin-1 was detected as fine paracellular lines comparable to the controls. The paracellular signal of claudin-3 (green, Figure 7B) appeared to be mitigated compared to claudin-1 after treatment with the cytokine, while an increased intracellular signal was also observed. Furthermore, after TNFα-treatment, the occludin signal (red) appeared rather weak. Both can be partly prevented by ML-7, as claudin-3 and occludin were detected as fine, paracellular lines equivalent to the controls.

2.7. Recovery Experiment

Because TNFα has apoptotic potential [29,30], we performed further experiments to see whether IPEC-J2 cells can recover from incubation with the cytokine. Therefore, a medium exchange was carried out after 48 h, and cells were further incubated with or without TNFα. As shown in Figure 8A, cells with TNFα removal seem to recover from 48 h incubation with TNFα, since the TEER values showed a strong tendency to increase again, while the cells treated without cytokine removal decreased further on. However, the increase in TEER from cells with TNFα removal was not sufficient enough for a significant difference to the cells without the removal of the cytokine, but was close to being statistically significant (one-way ANOVA: F (2,26) = 6.64, $p = 0.0047$, $n = 7–13$; TNFα w/o Recovery: 77.36 ± 11.41%; TNFα w/ Recovery: 115.88 ± 15.65%; $p = 0.076$). The control groups showed a strong significant difference compared to the cells treated with TNFα for 96 h, (ctrl: 129.75 ± 7.82%; TNFα w/o Recovery: 77.36 ± 11.40; $p = 0.0036$). To analyze the effects a recovery period from TNFα might have on the changes in TJ proteins and TNFR-1, Western blot analysis and confocal laser scanning immunofluorescence microscopy were performed subsequently. A recovery period of 48 h after incubation with the cytokine led to a marked increase in claudin-1 expression in IPEC-J2 cells (one-way ANOVA: F (2,6) = 61.35, $p = 0.0001$, $n = 3$; TNFα w/o Recovery: 38.11 ± 2.34%; TNFα w/ Recovery: 63.20 ± 6.47%; $p = 0.01$; Figure 8B,C). Though, there was still a significant difference between controls and cells treated with TNFα recovery (Ctrl: 100%; TNFα w/ Recovery: 63.20 ± 6.47%; $p = 0.002$). Densitometric analysis of claudin-3 also revealed a rise after the removal of the cytokine, yet not to the same extent as claudin-1 (one-way ANOVA: F (2,6) = 5.84, $p = 0.039$, $n = 3$; TNFα w/o Recovery: 69.77 ± 8.10%; TNFα w/ Recovery: 95.08 ± 8.33%; $p = 0.08$). For occludin expression, the recovery period led to a strong increase compared to the cells treated with TNFα for 96 h (one-way ANOVA: F (2,6) = 16.24, $p = 0.004$, $n = 3$; TNFα w/o Recovery: 58.32 ± 8.40%; TNFα w/ Recovery: 81.70 ± 3.18%; $p = 0.04$). The increased expression of TNFR-1 due to TNFα incubation was not affected by the recovery period (Kruskal–Wallis test: H (2) = 4.78, $p = 0.09$, $n = 3$).

Moreover, confocal laser scanning immunofluorescence microscopy revealed that the paracellular TJ strands seemed to reconstitute after a recovery period of 48 h, as the claudin-1 signal appeared to be integrated again in the lateral membrane after a loss due to TNFα incubation (Figure 9A). Forty-eight hours after the removal of the cytokine, the signal for claudin-3 appeared to be as strong as detected in controls. The occludin signal also appeared to recover from the incubation with TNFα, but still showed a weaker signal compared to the controls, which was also reflected by a weaker yellow signal in the merged pictures (Figure 9B). Furthermore, a marked internalization of claudin-3 to sub-junctional compartments was observed when cells were incubated with TNFα for 96 h.

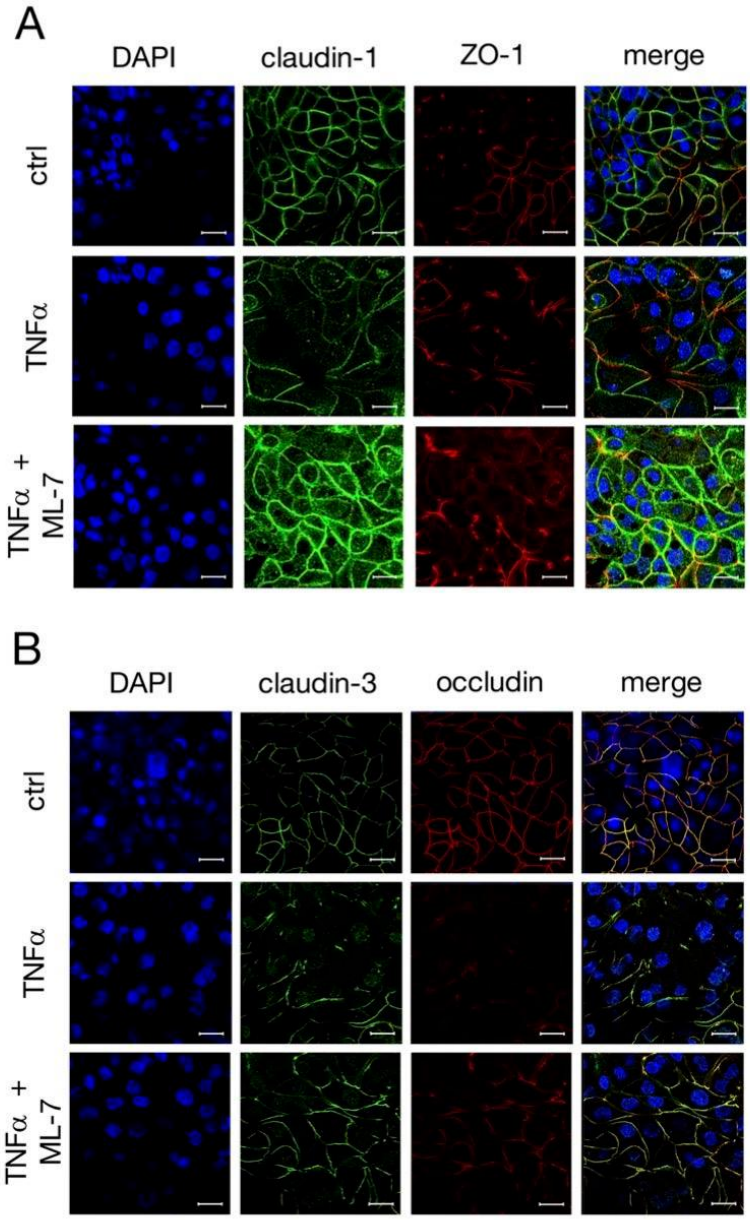

Figure 7. Confocal laser scanning immunofluorescence microscopy of (**A**) claudin-1 (green) and ZO-1 (red), as well as (**B**) claudin-3 (green) and occludin (red), nuclei were stained in blue (DAPI). IPEC-J2 monolayers were treated for 48 h with TNFα in the presence or absence of the specific MLCK blocker ML-7. (**B**) The yellow signal in the merged pictures represents the colocalization of claudin-3 and occludin (scale bar: 20 µm; $n = 4$; representative images).

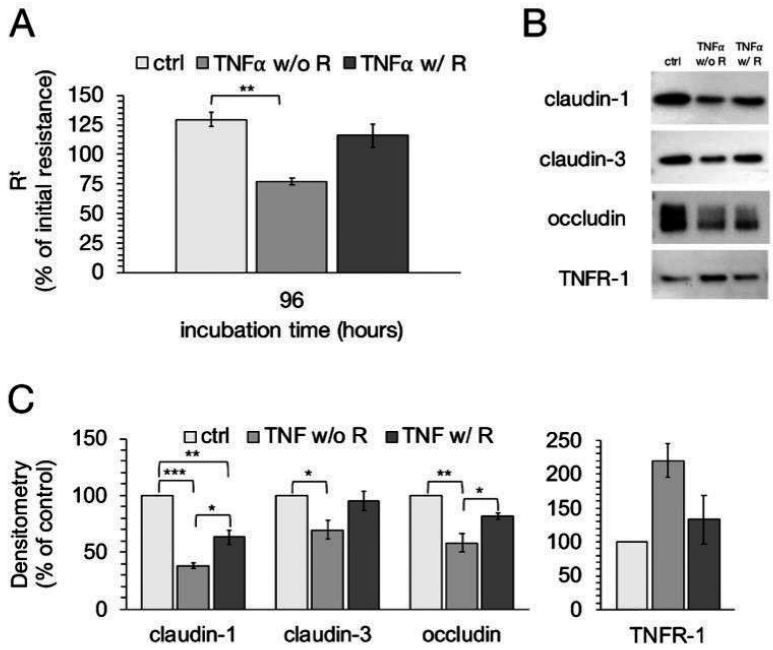

Figure 8. After IPEC-J2 cells were treated for 48 h with 1000 U/mL TNFα, a medium exchange was carried out and cells were either further incubated with the cytokine (TNFα without recovery; w/o R) or incubated with normal medium without TNFα (TNFα with recovery; w/R) for another 48 h. (**A**) TEER values of groups treated with TNFα removal were compared to cells treated without removal of the cytokine, respectively (one-way ANOVA, ** $p < 0.01$; $n = 7–13$). (**B**) Representative Western blot bands and (**C**) Densitometry of IPEC-J2 cells with or without recovery after 48 h incubation with TNFα revealed an increase in claudin-1 and occludin after removal of the cytokine, while claudin-3 expression did not show significant changes. The expression level of TNFR-1 also did not seem to be affected by TNFα removal. The shown data are presented as mean ± SEM (one-way ANOVA, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; $n = 3$).

2.8. ApoTox-Glo Assay

In addition to the recovery experiments, an ApoTox-Glo assay was performed and the apoptotic potential of TNFα was further analyzed to ensure that the changes in barrier function are not due to the induction of apoptosis by the cytokine. Despite all this, neither the apoptotic or the cytotoxic rate nor the viability of IPEC-J2 cells was significantly changed after treatment with 1000 U/mL TNFα for 48 h. Hence, apoptosis could be eliminated as a cause for the decrease in transepithelial resistance in IPEC-J2 cells (data not shown).

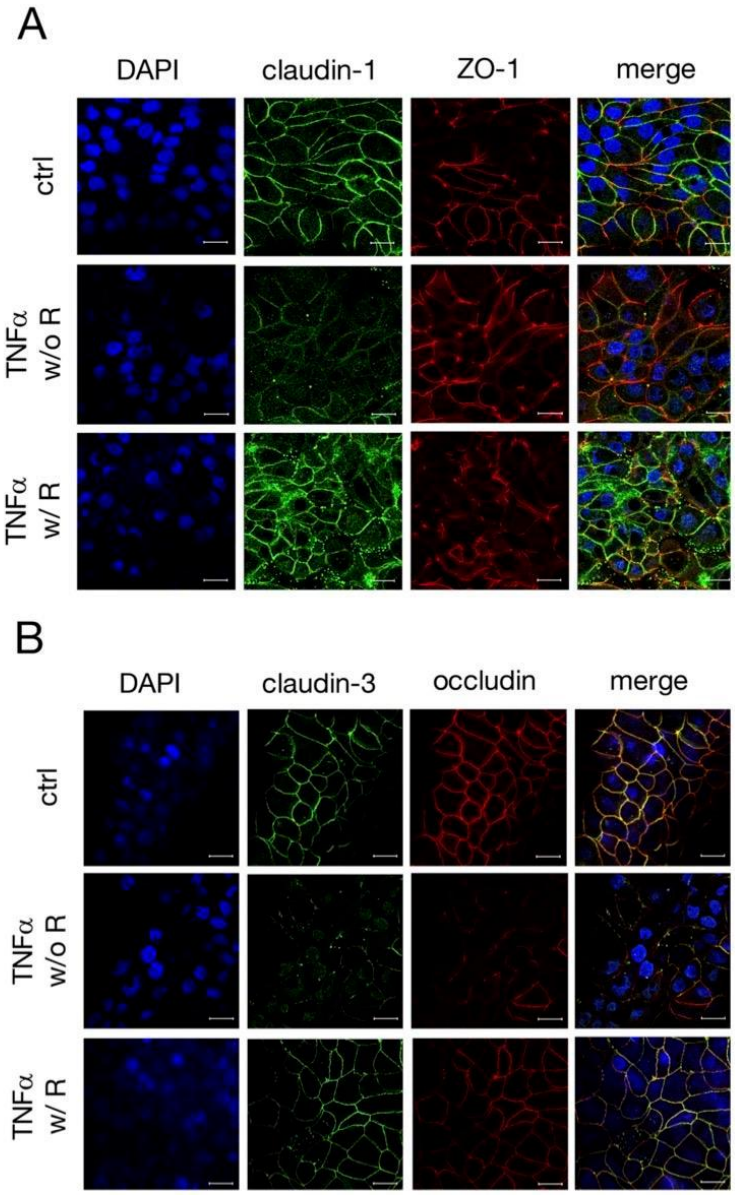

Figure 9. To analyze whether a recovery period from TNFα may have effects on the localization of changed TJ proteins due to treatment with the cytokine, confocal laser scanning immunofluorescence microscopy was performed subsequent to experiments. (**A**) Claudin-1 (green) and ZO-1 (red), and (**B**) claudin-3 (green) and occludin (red) were stained. Nuclei were stained in blue (DAPI). The yellow signal in the merged pictures constitutes a colocalization of the stained TJ proteins (scale bar: 20 µm; $n = 3$; representative pictures).

3. Discussion

The intestinal mucosa provides one of the most important barriers to the outside environment, and an intact barrier is maintained by TJs linking adjacent epithelial cells and the immune system [31,32]. One of the main cytokines mediating between immune cells and barrier regulation is TNFα, a pro-inflammatory cytokine produced mainly by activated macrophages, monocytes and T-cells [33]. An increased, persistent production of TNFα has been shown to cause mucosal inflammation, leading to the destruction of the intestinal barrier with increased permeability due to the reduced function of the TJs, but also to the apoptosis of intestinal epithelial cells [34].

In our current work, we analyzed the effects of TNFα on the intestinal epithelial barrier function of the non-transformed porcine intestinal epithelial cell line IPEC-J2. Although this cell line has been described as a suitable in vitro model so far [19], the central effect of TNFα on the barrier function of IPEC-J2, in particular on the expression and localization of TJ proteins, has not been analyzed in detail yet.

As already assumed, the use of TNFα showed a highly significant decrease in TEER, as well as an elevation in paracellular permeability to [^{3}H]-D-Mannitol, both representing epithelial barrier function. To analyze this barrier-weakening effect more precisely, immunoblots and confocal laser scanning immunofluorescence microscopy of TJ proteins including claudin-1, -3, -4, -7, occludin and ZO-1 were subsequently carried out. In our study, strong yet differential effects of the cytokine on different TJ proteins were observed. Hence, the participation of the sealing TJ proteins claudin-1, as well as claudin-3 and occludin, on the disruption of epithelial barrier function could be shown, as the decrease in these integral membrane proteins after incubation with TNFα was in accordance with functional changes. In addition, not only a marked reduction in protein expression could be shown, but also a disruption of those TJ proteins, as the signal of claudin-1, -3 and occludin was either much reduced, disintegrated or located more intracellularly than in the cells treated without the cytokine.

Because TNFα can bind two specific receptors, namely TNFR-1 and TNFR-2, we aimed to analyze if the observed changes are linked to the altered density of these receptors. Hence, we were able to detect a dose-dependent increase in TNFR-1 due to the incubation with TNFα, while TNFR-2 remained unchanged. One reason for this might be the expression of TNFR-1 in most tissues, while TNFR-2 appears to be found mostly in the lymphoid system. TNFR-1 is also the key signaling receptor for TNFα, as it is activated by both soluble and membrane-bound TNFα and mediates the signaling of apoptosis and inflammation [35,36]. Furthermore, TNFR-2 can only be fully activated by membrane-bound but not soluble TNFα [29].

To exclude the possibility that the observed changes are associated with an activation of apoptosis by TNFα [29,30], we performed recovery experiments as well as an ApoToxGlo assay. As the ApoTox-Glo assay did not show any significant changes in caspase-3/-7 activity, and the previously mentioned alterations in TEER and TJ proteins were reversible after the removal of the cytokine, we were able to eliminate apoptosis as a reason for the observed changes.

Because the disruption of TJ proteins has been explained to be in accordance with an increased expression of MLCK [13–15,37], we analyzed if the TNFα-induced effects could be prevented by ML-7, a specific MLCK blocker [38,39]. TNFα can operate as an activator for MLCK, in turn leading to the increased permeability of the paracellular barrier [18]. Regarding a changed TJ barrier function, different routes can be involved, in particular the low-capacity leak and the high-capacity pore pathways [40,41]. The low-capacity leak pathway allows macromolecules to cross the TJ barrier, and is involved when TNFα stimulates MLCK [18]. To understand what role MLCK plays in our observed changes in TJ composition, cells were incubated with TNFα in the presence or absence of ML-7. TEER was recorded and the protein expression and distribution of claudin-1, -3, occludin and TNFR-1 were analyzed, indicating MLCK as the major pathway component of TNFα signaling.

Incubation with TNFα in the presence of the specific MLCK blocker ML-7 prevented the decrease in TEER and claudin-3 expression. However, in our experiments, occludin expression was not significantly altered. Whether the effects of MLCK are related to a role for myosin transport of the affected proteins, or if MLCK might have other, e.g., structural roles [42,43], could not be addressed within the scope of the current study in more detail, but might be of interest for ongoing research. However, as a possible major MLCK substrate, myosin light chain II (MLC-2) has been shown to mediate alterations of paracellular permeability in gastrointestinal disorders [14].

In our current study, the pharmacological perturbation of MLCK was performed by the small molecule inhibitor ML-7. Although additional results such as (i) MLCK knockdown during TNFα treatment and (ii) MLCK overexpression in the absence of TNFα might even have strengthened the study, the blocking experiment has proven reliable for a convincing conclusion on the role of MLCK. Other additional mechanisms and signaling events might be still involved, though.

In a publication from Xiao et al., IPEC-J2 cells were stimulated with TNFα to analyze whether TGF-β1 may have protective effects. After TNFα challenge, a reduction in occludin and ZO-1 of IPEC-J2 monolayers was observed, while the TJ protein claudin-1 remained unaffected [44]. This is in contrast with the alteration of TJ proteins in our work, as in our experiments, the TJ proteins claudin-1, -3 and occludin were markedly reduced after incubation with TNFα. Thus, our findings demonstrate once again that the cell line IPEC-J2, cultured under species-specific conditions using porcine serum instead of conventional serum [20], shows an improved model for the analysis of porcine jejunal epithelium, as the changes in TJ composition are more commensurate to those in other models after TNFα challenge [45,46].

An effect of TNFα not only on sealing but also on pore-forming tight junction proteins has been described previously [11]. In different models [47–49], incubation with TNFα led to a significant increase in claudin-2, a paracellular channel for small cations and water [50]. Due to the lack of IPEC-J2 regarding the expression of pore-forming TJ proteins [20], an examination of the effect on these particular proteins could not be performed in our current approach. However, with a lack of claudin-2 expression, the TJ expression pattern of IPEC-J2 appears to be very close to Peyer's patches follicle-associated epithelium, therefore representing the main relevant epithelium involved in immune surveillance in the intestine [51,52]. Currently, many studies demonstrate the susceptibility and reliability of the non-malignant porcine epithelial cell line IPEC-J2 regarding a wide variety of mechanisms in native tissue of different species in vitro [19,53].

4. Material and Methods

4.1. Cell Culturing and TNFα Treatment

Confluent monolayers of the porcine jejunal cell line IPEC-J2 (DSMZ, Braunschweig, Germany) were grown in 25 cm^2 culture flasks in Dulbecco's MEM/Ham's F-12 (Biochrom, Berlin, Germany) containing 3.15 g/L glucose, 2 mM stable glutamine, 10% porcine serum (Sigma Aldrich, Munich, Germany) and 1% penicillin/streptomycin (Sigma Aldrich, Munich, Germany). Cells were cultured at 37 °C in a humified 5% CO_2 atmosphere. The medium was changed every 2–3 days and cells were split once a week at a ratio of 1:3. For electrophysiological measurements, cells were seeded at a density of 2×10^5 cells/mL on semipermeable cell culture inserts with a diameter of 12 mm and a pore size of 0.45 µm (Millipore, Darmstadt, Germany) and placed into multi-well plates. Routinely, 500 µL of media was added to the apical compartment, and the basolateral compartment was filled with 1 mL of media to guarantee an equal hydrostatic pressure, as specified by the manufacturer. Experiments were performed after cells reached similar R^t values (~14 days) to ensure a functional barrier. Therefore, recombinant human TNFα (peprotech, Hamburg, Germany) was added in various concentrations (50, 100, 500, 1000 and 5000 U/mL) to the basolateral side of the cell culture inserts, and transepithelial resistance was monitored for 48 h. The cells were further processed and used for immunoblotting and immunohisto-

chemistry, as described below. Resistance values were corrected with the blank values of the filters and the media used in the experiments. Only cells with passages between 7 and 12 were used for experimental purposes.

4.2. TEER and Flux Measurements

Transepithelial electrical resistance (TEER) measurements, presenting the epithelial barrier function, were assessed by using a chopstick electrode and an Epithelial Volt/Ohm Meter (EVOM, World Precision Instruments, Sarasota, FL, USA). Permeability studies were carried out using [^{3}H]-D-Mannitol to examine the unidirectional paracellular tracer flux from the apical to the basolateral side of the cell filters. Cells were seeded on semipermeable cell culture inserts as described previously. A total of 0.18 µCi of [^{3}H]-D-Mannitol (PerkinElmer, Waltham, MA, USA) was added to the apical side of the filters, and samples of 50 µL were taken directly and 48 h after addition of the tracer. Subsequently, the specific activity of the tracer was calculated using Equation (1).

$$\text{specific activity } [\text{nmol}] = \frac{\text{mean}(\text{counts}_{\text{donor side}})}{\text{concentration}_{\text{donor side}} \times \text{volume}_{\text{donor side}}} \quad (1)$$

For permeability measurements, samples of 300 µL were taken every 24 h during the incubation with the cytokine from the basolateral side, resulting in two flux periods. The removed media were replaced with fresh media containing the corresponding TNFα concentration. Following the sampling, Aquasafe 300plus liquid scintillation cocktail (Zinsser Analytic, Frankfurt, Germany) was added and each sample was examined using TriCarb 4910TR liquid scintillation counter (PerkinElmer, Waltham, MA, USA). By using Equation (2), the resulting paracellular flux was calculated.

$$J\left[\text{nmol} \times \text{cm}^{-2} \times \text{h}^{-1}\right] = \frac{\text{counts}_t \times \frac{V_{\text{chamber}}}{V_{\text{sample}}} - \text{counts}_{t-1} \times \frac{V_{\text{chamber}} - V_{\text{dilution}}}{V_{\text{sample}}}}{\text{specific activity} \times \text{area} \times \text{time}} \quad (2)$$

4.3. Protein Extraction and Quantification

After incubation of the cells with TNFα, IPEC-J2 monolayers were washed in PBS with calcium and magnesium and lysed in RIPA buffer, containing 25 µM HEPES pH 7.6, 25 µM NaF, 2 µM EDTA, 1% Sodium Dodecyl Sulfate (10%), H2O and enzymatic protease inhibitors (Complete EDTA-free, Boehringer, Mannheim, Germany). Cells were then scraped off the permeable supports and the suspension was transferred into Eppendorf tubes. Samples were homogenized after incubation on ice for 30 min. Protein quantification was performed by using Bio-Rad DC Protein Assay (Bio-Rad Laboratories GmbH, Munich, Germany) as prescribed by the manufacturer. For the detection, EnSpire Multimode Plate Reader (Perkin Elmer, Waltham, MA, USA) was used.

4.4. Immunoblotting and Densitometry

Western blot analysis was performed to identify the TJ protein expression after TNFα treatment. Protein (20 µg) and Laemmli buffer (Bio-Rad Laboratories GmbH, Munich, Germany) were mixed and loaded onto 10% TGX Stain-Free FastCast gels (Bio-Rad Laboratories GmbH, Munich, Germany). Electrophoresis was carried out for 60 min at 150 V. Subsequently, samples were transferred onto a PVDF membrane for 90 min at 100 V and blocked for 60 min in 5% milk (in Tris-buffered saline with 0.1% Tween 20). Membranes were then incubated with specific antibodies raised against TJ proteins (all from Thermo Fisher Scientific) claudin-1 (cat. #51-9000), -3 (cat. #34-1700), -4 (cat. #32-9400), -7 (cat. #34-9100), occludin (cat. #33-1500) and ZO-1 (cat. #33-9100) following the manufacturer's instructions at 4 °C overnight, respectively. To bind the primary antibodies, horseradish-peroxidase-conjugated goat anti-mouse or goat anti-rabbit antibodies were used for 1 h at room temperature. Specificity was shown in detail previously [19,51,52]. For visualization of the protein bands, Clarity Western ECL Blotting Substrate (Bio-Rad Laboratories GmbH,

Munich, Germany) was used after the total protein amount was detected with the Chemi-Doc MP Luminescence imager (ChemiDoc MP, Munich, Germany). Later, the density of the specific bands was quantified and analyzed using the imager-associated software Image Lab. For densitometry, bands were normalized on total protein amount and compared to the control groups, respectively.

4.5. Immunohistochemistry

Confocal laser scanning immunofluorescence microscopy was performed for the detection and localization of TJ proteins after TNFα treatment. Therefore, cells were washed twice with PBS and fixated in ice-cold methanol for 10 min at −20 °C. Permeabilization was carried out using Triton X-100 for 10 min at room temperature. Cells were then blocked for 60 min in PBS containing 1% bovine serum albumin and 5% goat serum and subsequently incubated with primary antibodies raised against claudin-1, -3, occludin and ZO-1 for 60 min at 37 °C (Thermo Fisher Scientific; claudin-1 (cat. #51-9000), -3 (cat. #34-1700), occludin (cat. #33-1500) and ZO-1 (cat. #33-9100)). Again, cells were washed with PBS and then incubated with secondary goat anti-rabbit Alexa Fluor-488 (1:1000, Thermo Fisher Scientific, cat. #A-11008) or goat anti-mouse Alexa Fluor-594 (cat. #A-11005) for 60 min at 37 °C. Nuclei were stained with DAPI (1:2000) for 5 min at room temperature. Following this, filters were mounted with ProTaqs Mount Fluor (Biocyc, Luckenwalde, Germany) and slides were analyzed by using Zeiss 710 confocal microscope (Zeiss, Oberkochen, Germany).

4.6. Investigation of Dose-Dependent Changes in the Expression Level and Localization of Tumor Necrosis Factor Receptor 1 or 2 after Treatment with TNFα

To examine whether the treatment with TNFα affects the expression level of tumor necrosis factor receptors 1 and 2 (TNFR-1, TNFR-2) in IPEC-J2 cells, Western blot analysis was carried out as mentioned above. Membranes were incubated with rabbit polyclonal antibodies raised against TNFR-1 (abcam, cat. #ab1939) and TNFR-2 (antibodies-online, cat. #ABIN2789622). After detection, specific bands were normalized on total protein amount and analyzed compared to the untreated groups, respectively. Moreover, immunohistochemistry of IPEC-J2 cells, treated with rising TNFα concentrations, was performed for examination of expression level and localization of TNFR-1. Therefore, a staining using specific antibodies raised against TNFR-1 together with a marker for the basolateral membrane, namely Integrin beta-1 (ITGβ1; Thermo Fisher, Rockford, IL, USA), was carried out as mentioned above. To analyze the location of TNFR-1 in more detail, Z-stacks were performed additionally.

4.7. Specific Myosin Light Chain Kinase (MLCK) Blocker in TNFα-Induced Changes in Epithelial Barrier Function

For a more detailed characterization of the signaling in TNFα-induced barrier changes, ML-7 (Sigma Aldrich, Munich, Germany), a specific blocker against MLCK, was used. Therefore, IPEC-J2 cells were incubated with TNFα (1000 U/mL) in the presence or absence of ML-7 (10 μM). Stock solutions of ML-7, dissolved in dimethyl sulfoxide (DMSO; Sigma Aldrich, Munich, Germany), were diluted in medium and added simultaneously with TNFα to the apical and basolateral compartment of the cell filters. Respectively, 0.1% of DMSO was added to controls as well as to cell filters incubated with TNFα without ML-7, to exclude a DMSO-dependent effect on IPEC-J2 cells. TEER measurements were carried out before addition and after 48 h. Subsequent to TEER measurements, cells were either fixated for immunohistochemistry or proteins were extracted and further processed for immunoblotting, analyzing localization and expression of TJ proteins after experiments with or without ML-7.

4.8. Recovery Experiments in IPEC-J2 after Removal of TNFα

Because TNFα has been described to have apoptotic potential, we wanted to investigate if the IPEC-J2 cells can recover from the TEER decrease after a TNFα-challenge.

Therefore, cells were incubated with 1000 U/mL TNFα for 48 h as described above. Subsequently, a medium exchange was carried out and fresh media without TNFα were added to the cells that were treated with the cytokine before. Following TEER measurements, possible alterations of TJ proteins were examined using Western blot technique or immunohistochemistry as described above.

4.9. ApoTox-Glo Assay

IPEC-J2 cells were seeded as triplicates at a density of 2×10^5 cells/mL on PET cell culture inserts of a 24-well Transwell system with 6.5 mm membrane diameter and a 0.4 µm pore size (Costar, Corning Incorporated, Kennebunk, ME, USA). After a cultivation period of 14 to 16 days, cells were treated with TNFα (1000 U/mL) and further incubated for 48 h. To measure the viability, cytotoxicity and apoptosis of the IPEC-J2 cells, the ApoTox-Glo™ Triplex Assay (Promega GmbH, Walldorf, Germany) was carried out according to the manufacturer's specifications. After the incubation period with TNFα, the viability/cytotoxicity reagent, containing both the bis-AAF-R110 substrate and GF-AFC substrate, was added to each well and mixed for 30 s on an orbital shaker. After an incubation of 30 min at 37 °C, the fluorescence was measured at 400EX/505EM for viability and 485EX/520EM for cytotoxicity. Following this, the apoptosis reagent, containing the Caspase-Glo 3/7 substrate, was added to each well, mixed gently and incubated for another 30 min at constant room temperature. Luminescence was measured to detect cell apoptosis. All measurements were carried out using EnSpire Multimode Plate Reader (Perkin Elmer, Waltham, MA, USA).

4.10. Statistical Analysis

Data are always expressed in means and standard error of the mean (SEM). N represents the number of cell culture inserts used unless stated otherwise. For TEER measurements, statistical analysis was performed using one-way ANOVA and Dunnett's test for the correction of multiple testing. For comparison between two groups, unpaired Student's *t*-test was used. Statistical analysis for densitometry of Western blots was performed with one-way ANOVA for normally distributed data and with Kruskal–Wallis test for non-normally distributed data. For the signaling experiments, Tukey–Kramer method was used as post hoc test for pairwise comparisons. Values of $p < 0.05$ were considered to be statistically significant, being presented as * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

5. Conclusions

Employing the non-transformed intestinal epithelial cell line IPEC-J2, our study demonstrates, for the first time, that the exponential nature of barrier impairment by TNFα in porcine intestinal inflammatory processes can be explained by the finding that its own receptor, TNFR-1, is upregulated by TNFα itself.

Author Contributions: Conceptualization, S.A. and L.D.; methodology, S.A., L.D., V.C. and A.G.M.; investigation, L.D. and V.C.; writing—original draft preparation, L.D. and S.A.; writing—review and editing, L.D., S.A., A.G.M. and V.C.; supervision, S.A.; funding acquisition, S.A. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by a grant of the German Research Foundation, DFG grant no. AM141/11-2. The publication of this article was funded by Freie Universität Berlin.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Data are contained within the article. The datasets analyzed during the current study are available from the corresponding author upon reasonable request.

Acknowledgments: We thank Katharina Söllig, Susanne Trappe and Martin Grunau for excellent technical assistance.

Conflicts of Interest: The authors declare no competing or financial interests.

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Chapter 5: Barrier Perturbation in Porcine Peyer's Patches by Tumor Necrosis Factor is Associated With a Dysregulation of Claudins

This chapter has been published in: Frontiers in Physiology, Gastrointestinal Sciences

Manuscript received at the Journal: March 4th, 2022

Initial review completed: April 20th, 2022

Revision accepted: April 26th, 2022

Authors: Linda Droessler, Valeria Cornelius, Elisa Boehm, Laura Stein, Nora Brunner, Salah Amasheh

https://doi.org/10.3389/fphys.2022.889552

Author contribution:

As stated in this publication, I have planned and performed the experiments. Western blot analyses and immunostaining, as well as data evaluation and statistical analyses was performed by me, personally. I designed the figures and wrote the manuscript.

frontiers | Frontiers in Physiology

ORIGINAL RESEARCH
published: 30 May 2022
doi: 10.3389/fphys.2022.889552

Barrier Perturbation in Porcine Peyer's Patches by Tumor Necrosis Factor is Associated With a Dysregulation of Claudins

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The proinflammatory cytokine tumor necrosis factor (TNF) has been described as one of the main mediators of intestinal inflammatory diseases, affecting the composition of tight junction (TJ) proteins and leading to a disruption of the epithelial barrier. An intact intestinal barrier is mandatory, because the follicle-associated epithelium of Peyer's patches represents the first defense line of the intestinal immune system and ensures a controlled uptake of antigens from the gut lumen. In the current study, we have analyzed the detailed effects of TNF on the follicle-associated epithelium of porcine Peyer's patches by applying the Ussing chamber technique. Epithelial tissue specimens of Peyer's patches and the surrounding villus epithelium were mounted into conventional Ussing chambers and incubated with TNF for 10 h. The transepithelial resistance, representing epithelial barrier function of the tissue, was recorded. A reduction of transepithelial resistance was detected after 8 h in Peyer's patch tissue specimens, whereas the villus epithelium was not significantly affected by TNF. Subsequent molecular analysis of TJ protein expression revealed a marked decrease of claudin-1 and -4, and an increase of claudin-2. In neighboring villus epithelium, no significant changes in the expression of TJ proteins could be shown. A strong increase of TNF receptor-2 (TNFR-2) could also be detected in Peyer's patches, in agreement with the major role of this receptor in Peyer's patches. Our findings were in accordance with changes detected by confocal laser scanning immunofluorescence microscopy. The regulation of TNF effects via myosin light chain kinase (MLCK) was analyzed in blocking experiments. Our detailed analysis is the first to show that TNF affects the barrier function of the follicle-associated epithelium of porcine Peyer's patches but has no effects on the villus epithelium. These findings reveal not only the basic differences of epithelial barrier function between the two structures, but also the significance of Peyer's patches as a primary mucosal immune defense.

Keywords: epithelial barrier, claudins, inflammation, peyer's patch, tight junction, TNF, TNFR, ussing chamber

OPEN ACCESS

Edited by:
Elizabeth S. Fernandes, Pelé Pequeno Príncipe Research Institute, Brazil

Reviewed by:
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Specialty section:
This article was submitted to Gastrointestinal Sciences, a section of the journal Frontiers in Physiology

Received: *04 March 2022*
Accepted: *26 April 2022*
Published: *30 May 2022*

Citation:
Droessler L, Cornelius V, Boehm E, Stein L, Brunner N and Amasheh S (2022) Barrier Perturbation in Porcine Peyer's Patches by Tumor Necrosis Factor is Associated With a Dysregulation of Claudins. Front. Physiol. 13:889552. doi: 10.3389/fphys.2022.889552

Abbreviations: FAE, follicle-associated epithelium; GALT, gut-associated lymphatic tissue; IFNγ, Interferon gamma; $I_{SC,}$ short-circuit current; PFA, paraformaldehyde; PP, Peyer's patch; TER, transepithelial resistance; TJ, tight junction; TNF, tumor necrosis factor; TNFR, tumor necrosis factor receptor; VE, villus epithelium.

INTRODUCTION

The intestine is exposed to a multitude of exogenous substances, and therefore an efficient epithelial barrier is mandatory for limiting paracellular permeability. As a major part of the gut-associated lymphatic tissue (GALT), Peyer's patches (PP) are located in the distal small intestine and hence represent a first line of immunological defense (Neutra et al., 2001). It consists of three main components: the follicular and interfollicular area, containing the germinal center with macrophages, dendritic cells or proliferating B-lymphocytes, and the follicle-associated epithelium (FAE) (Jung et al., 2010). The FAE, which covers PP, is the main component building a functional barrier of the lymphatic tissue against the gut lumen. In comparison with the surrounding villus epithelium (VE), the FAE lacks mucin-secreting goblet cells but contains M cells, which are responsible for the uptake and presentation of specific antigens to the lymphoid follicle (Sakhon et al., 2015). Furthermore, the FAE also differs from the VE with regard to the expression of tight junction (TJ) proteins, which enable a more restrictive sealing of the paracellular pathway in accordance with the specific immunological role of Peyer's patches (Markov et al., 2016; Radloff et al., 2017).

Tumor necrosis factor (TNF) is released by macrophages and can subsequently mediate the production of other cytokines, which is the reason that it is described as a proinflammatory cytokine (Ruder et al., 2019). It plays a crucial role in the pathogenesis of infections, inflammation, and apoptosis, and is one of the main components in the pathogenesis of inflammatory bowel diseases (IBDs) (Barbara et al., 1996; Gibson, 2004; Schulzke et al., 2009). By disturbing TJ proteins, TNF is able to affect the epithelial barrier function, resulting in a decreased transepithelial resistance and an increased permeability to solutes as initially shown in cultured renal epithelial cells (Mullin and Snock, 1990). The decreased expression of tightening TJ proteins and an increased expression of the pore-forming TJ protein claudin-2 can be observed in intestinal inflammatory diseases, such as Crohn's disease or ulcerative colitis (Zeissig et al., 2007; Oshima et al., 2008; Amasheh S. et al., 2009). In light of general immunological questions, the study of porcine models has recently gained major attention. Moreover, a closer look at porcine gut pathophysiology is of major interest, as intestinal diseases have been shown to lead to drastic economic consequences. For example, post weaning diarrhea (PWD) is a common disease in the swine industry, often leading to sudden death within 2 weeks after weaning (Fairbrother et al., 2005). Recent studies have revealed a major function of TNF in the pathogenesis of PWD, as increased expression levels have been observed both *in vivo* and *in vitro* (Tang et al., 2020; Wu et al., 2021). However, the effects of TNF on the FAE of porcine PP have not been analyzed as yet.

In our current study, we hypothesized the functional and molecular difference of PP compared to VE under inflammatory conditions. Based on our previous findings (Droessler et al., 2021), we have aimed at analyzing the effect of TNF on paracellular barrier function in the porcine PP over 10 h. Therefore, an optimized Ussing chamber protocol has been applied to ensure the vitality of the tissue over time. The Ussing chamber technique is widely used for measurements of ion or drug transport in intestinal epithelia and provides an essential method for electrophysiological analysis (Amasheh et al., 2004; Westerhout et al., 2015). Tissue samples have been further processed for the analysis of TJ composition and regulation.

MATERIALS AND METHODS

Tissue Preparation

Tissue specimens from the small intestine with and without PP from 28 adult pigs were taken immediately after slaughter at a slaughterhouse (Lehr-und Versuchsanstalt für Tierzucht und Tierhaltung, Ruhlsdorf, Germany). The tunica serosa and muscularis externa were stripped off the mucosa, and the remaining tissue was rinsed with 0.9% NaCl to remove chyme and mucus. The tissue samples were transported to the laboratory in ice-cold buffer containing (in mmol/L): Na^+ (149.4), Cl^- (128.8), K^+ (5), Ca^{2+} (1.2), Mg^{2+} (1.2), HCO_3^- (25), $H_2PO_4^-$ (0.6), HPO_4^{2-} (2.4), D-Glucose (10) (all from Carl Roth GmbH, Karlsruhe, Germany) and Enrofloxacin (30 µmol/L; MP Biomedicals, Eschwege, Germany). The buffer was gassed with 95% O_2 and 5% CO_2, and the pH was adjusted to 7.4 before transport.

Ussing Chamber Experiments and TER Measurements

PP and VE of the tissue specimens were differentiated visually and mounted into conventional Ussing chambers equipped with gas lifts. The bathing solution was equivalent to the transport buffer and was constantly gassed with 95% O_2 and 5% CO_2 at a pH of 7.4. The area of the tissue exposed to the buffer was 0.96 cm^2. After an equilibration period of 60 min, TNF was added at three different concentrations (1000, 5000, or 10,000 U/ml) to the serosal side of the tissue, and TER was recorded permanently for 10 h. A buffer exchange was carried out after 3, 6, and 8 h to ensure the viability of the tissue (**Figure 1**).

Permeability Measurements

Unidirectional paracellular tracer flux measurements from the mucosal to the serosal side were carried out under voltage-clamp conditions for the higher TNF concentrations (5000, 10,000 U/ml). After 8 h of incubation with the cytokine, 2 µCi [^{3}H]-D-Mannitol (182 Da; PerkinElmer, Waltham, MA, United States) was added to the mucosal side, and samples were taken directly after addition of the tracer and at the end of the flux periods from the same side. Samples of 600 µl were taken from the basolateral side every 40 min, giving three flux periods over 2 h. After removal of the samples, the absent volume was refilled with 600 µl fresh buffer containing the corresponding TNF concentration. Subsequently, Aquasafe 300 plus liquid scintillation cocktail (Zinsser Analytics, Frankfurt, Germany) was added, and each sample was analyzed using a TriCarb 4910 TR liquid scintillation counter (PerkinElmer, Waltham,

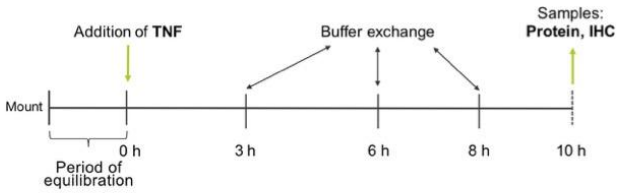

FIGURE 1 | Experimental setup.

MA, United States). Calculation of the specific activity and paracellular flux of the tracer was carried out as described previously (Droessler et al., 2021).

Fixation and Processing of Tissue Samples

Following the Ussing chamber experiments, tissue samples were fixed at room temperature in 4% paraformaldehyde (PFA) in PBS containing Mg^{2+} and Ca^{2+} (PBS +/+) for 4 h. Subsequently, the samples were washed once in PBS +/+ and transferred into 25 mM glycine in PBS +/+ containing 0.1% NaN_3. Tissue samples were then stored at 4°C until the staining step. For dehydration, the samples were first transferred into increasing alcohol concentrations (from 70 to 100%), then into xylol, and finally into paraffin for embedding.

Hematoxylin-Eosin Staining

For analysis of tissue quality, samples of VE and PP were taken after 10 h of incubation with or without TNF. Cross-sectioning, deparaffinization, and rehydration were carried out prior to staining. Samples were stained for 3 min with hematoxylin solution according to Harris (Carl Roth GmbH, Karlsruhe, Germany), rinsed in 0.1% hydrochloric acid, and differentiated under flowing water for 3–5 min. The sections were then stained with eosin Y solution (1%; Carl Roth GmbH, Karlsruhe, Germany) for another 3 min, washed with water, and again transferred into increasing alcohol concentrations with a final step of xylol as explained above. Slides were subsequently embedded with ProTaqs paramount (Biocyc, Luckenwalde, Germany).

Protein Extraction and Quantification

Subsequent to the Ussing chamber experiments, tissue samples were frozen in liquid nitrogen and stored at −80°C. For protein extraction, the specimens were homogenized in RIPA buffer containing 25 µM HEPES pH 7.6, 25 µM NaF, 2 µM EDTA, 1% SDS (10%), H_2O, and enzymatic protease inhibitors (Complete EDTA-free, Boehringer, Mannheim, Germany). The samples were then centrifuged for 1 min at 16,000 × g, and the supernatant was left for 30 min on ice for further lysis. A second centrifugation step for 15 min at 15,000 × g at 4°C (sigma 3–30 ks, Sigma-Aldrich, Munich, Germany) was carried out, and the supernatant was transferred into Eppendorf tubes. The Bio-Rad DC Protein Assay (Bio-Rad Laboratories GmbH, Munich, Germany) was used to quantify the proteins, which were detected by an EnSpire Multimode Plate Reader (Perkin Elmer, Waltham, MA, United States).

Immunoblotting and Densitometry

TJ proteins in FAE of porcine PPs after incubation with TNF in Ussing chambers were analyzed by primary antibodies raised against claudin-1, -2, -3, -4, -7, and occludin (Life Technologies, Carlsbad, California, United States). For the detection of claudin-2, urea (9 mol/L, Carl Roth GmbH, Karlsruhe, Germany) was added, and samples were denatured at 55°C for 8 min to ensure the unlocking of the hydrogen bonds. Expression of the specific receptors in VE and PP subsequent to incubation with TNF was examined using primary antibodies raised against TNFR-1 (abcam, Berlin, Germany) and TNFR-2 (antibodies-online GmbH, Aachen, Germany). Immunoblotting was performed as described previously (Droessler et al., 2021). Densitometry was carried out by normalizing protein bands to the amount of total protein and TNF-treated groups were compared with controls, respectively.

Immunohistochemistry

For immunohistological staining, tissue samples were first rehydrated in reverse order as described in Fixation and Processing of Tissue Samples. Subsequently, epitopes were exposed by boiling the samples in EDTA buffer (pH 8) for 45 min, followed by a permeabilization step in Triton X-100 in PBS +/+ for 5 min at room temperature. The tissue sections were framed using a PAP pen (Kisker Biotech GmbH & Co. KG, Steinfurt, Germany) to provide a hydrophobic barrier and were blocked afterwards for 30 min at room temperature in PBS containing 5% goat serum and 1% bovine serum albumin. Samples were then incubated with primary antibodies raised against claudin-1, -2, -4, occludin, ZO-1 (Thermo Fisher Scientific), TNFR-1 (abcam, Berlin, Germany), and TNFR-2 (antibodies-online GmbH, Aachen, Germany) for 1 h at 37°C, followed by four washing steps in blocking solution. Subsequently, secondary goat anti-rabbit Alexa Fluor-488, goat anti-mouse Alexa Fluor-594, and DAPI for the staining of nuclei were added to the samples, which were incubated again for 1 h at 37°C. Another four washing steps in blocking solution were carried out, followed by one wash in distilled water, and finally the sections were mounted in ProTaqs Mount Fluor (Biocyc, Luckenwalde, Germany). Microscopic analysis was carried out using a Zeiss 710 confocal microscope (Zeiss, Oberkochen, Germany).

Signaling Experiments With ML-7

Experimental approaches with ML-7, a specific blocker of the myosin light chain kinase (MLCK), were subsequently carried out to determine whether the observed changes in TER were related to MLCK signaling. PP tissue samples from eight animals were processed as described in Tissue Preparation. TNF was added at a concentration of 5000 U/ml to the serosal side of the tissue. ML-7 (Sigma Aldrich, Munich, Germany) was dissolved in dimethyl sulfoxide (DMSO; Sigma Aldrich, Munich, Germany), corresponding to a final concentration of 7.5 μM, and was added simultaneously with TNF to the mucosal and serosal sides. Controls were treated with the respective amount of DMSO. Again, TER was recorded for 10 h, and buffer exchanges were carried out as described previously.

Testing of Vitality

After 10 h in the Ussing chamber, the vitality of the tissues was examined. Therefore, 4 ml buffer was removed from both sides of the Ussing chamber and replaced with 4 ml of fresh buffer containing theophylline (8 mM; Sigma Aldrich, Munich, Germany). The theophylline challenge was carried out for 10 animals, respectively.

Cell Culture Experiments

We used IPEC-J2 cells (DSMZ, Braunschweig, Germany), a non-transformed cell line that stems from porcine jejunal epithelia, to analyze whether the changes in epithelial barrier function in PP tissue also occur in a cell culture model. Cells were seeded on semi-permeable cell culture inserts (Millipore, Darmstadt, Germany) at 10^5 cells per filter. Dulbecco's MEM/Ham's F12 with 3.15 g/L glucose and 2 mM stable glutamine (Biochrom, Berlin, Germany) was used and supplemented with 10% porcine serum and 1% penicillin/streptomycin (Sigma Aldrich, Munich, Germany). Medium was changed every 2–3 days, and filters were filled with 500 μl apically and 1 ml basolaterally. By using a chopstick electrode and an epithelial Volt/Ohm Meter (EVOM, World Precision Instruments, Sarasota, FL, United States), we measured the TER immediately before each media exchange, and values were corrected with the media and blank values used in our experimental settings. Once the cells had built up a confluent monolayer, resulting in consistent TER values, the incubation experiments were started. We added 5000 U/ml TNF (PeproTech, Hamburg, Germany) to the basolateral side of the cell filters, and TER was recorded for up to 10 h. Cell passages between 8 and 13 were used for experiments.

Statistical Analysis

The data are expressed as means and standard error of the mean (SEM). For *in vitro* experiments, n is the number of cell filters, whereas for *ex vivo* experiments, n represents the number of animals used. For TER measurements, statistical analysis was performed using one-way ANOVA and Dunnett's test for the correction of multiple testing for normally distributed data and Kruskal Wallis test for non-normally distributed data. Student's *t*-test was used for the statistical analysis of the immunoblotting densitometry of the TNF-treated groups compared with controls. Values of $p < 0.05$ were considered to be statistically significant and are presented as $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$.

RESULTS

Hematoxylin-Eosin Staining

To check the quality of tissue specimens after incubation for 10 h in Ussing chambers, we performed hematoxylin-eosin staining subsequent to the experiments. Both TNF-treated and untreated tissue samples showed no significant reduction of villus length compared with specimens fixed without incubation, and incubation with the cytokine did also not lead to significant changes in villus length compared with the control groups (ctrl: 0.63 ± 0.05 mm; TNF: 0.58 ± 0.05 mm; $p = 0.57$; unpaired *t*-test; **Figure 2**).

Impact of TNF on Intestinal Epithelial Barrier Function of Porcine Peyer's Patches and Villus Epithelium (Resistance and Permeability)

Ussing chamber experiments were carried out to examine the effect of the cytokine on immunologically active PP tissues compared with that on the surrounding VE. In PP tissue specimens, 1000 U/ml TNF caused no significant changes, whereas 5000 U/ml and 10,000 U/ml led to a decrease of TER after 8 h in the Ussing chamber compared with the control groups. All the following experiments were therefore performed with 5000 U/ml TNF. For clarity of presentation, only 5000 U/ml is presented in **Figure 3A** and is given as "TNF". TER values at all concentrations are shown in **Supplementary Table S1**. The TER values of TNF-treated VE remained unaltered throughout the incubation (**Figure 3B**). The increasing trend of TER for both, controls and TNF-treated samples, could be a result of VE tissue being less robust during the time course of the experiment, although still vital. To investigate whether incubation with TNF led to altered paracellular permeability, unidirectional paracellular tracer flux measurements were carried out using 182 Da [^{3}H]-D-Mannitol. Three flux periods of 40 min duration (P1—P3) were monitored. In specimens from PP tissues, neither 5000 U/ml nor 10,000 U/ml TNF led to significant changes in the apparent permeability during all three flux periods (**Figure 4A**). Furthermore, no significant changes in paracellular permeability were observed in neighboring VE tissue (**Figure 4B**). The flux rates of [^{3}H]-D-Mannitol and the respective statistical analysis are shown in **Supplementary Table S2**.

Changes in the Expression of TJ Proteins in PP and VE Attributable to TNF Treatment

To determine whether the observed changes in the TER of PP tissues were attributable to the altered expression of TJ proteins, immunoblotting was performed subsequent to the Ussing chamber experiments (**Figure 5**). Densitometric analysis of Western blot bands revealed a remarkable decrease of

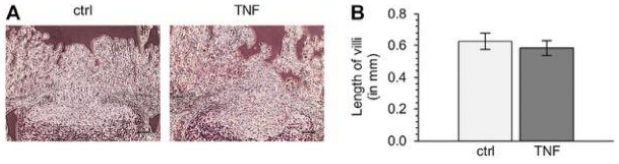

FIGURE 2 | (A) Representative images of hematoxylin-eosin-stained tissues **(B)** Analysis of villus length of Peyer's patch (PP)-surrounding villus epithelium (VE) after 10 h in the Ussing chamber with or without TNF (scale bar: 0.1 mm, $n = 6$).

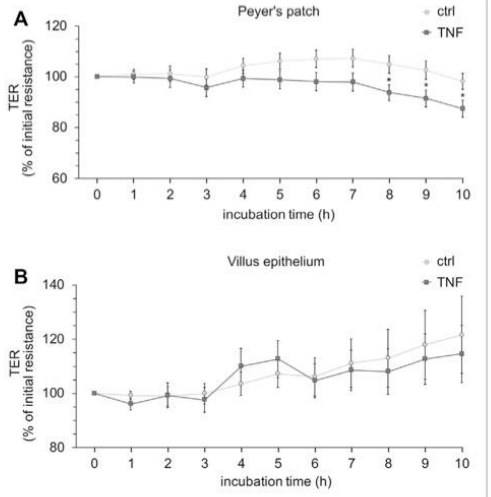

FIGURE 3 | Time-dependent effect of TNF (5000 U/ml) on PP **(A)** or VE **(B)**. The cytokine was added to the serosal side of the tissues, and TER-values were recorded for up to 10 h. Data are shown as the percentage of initial resistance ± SEM ($n = 8$–23, $^*p < 0.05$).

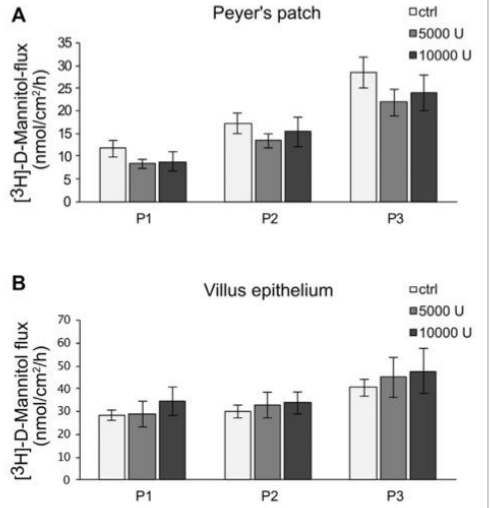

FIGURE 4 | Paracellular permeability of PP **(A)** and VE **(B)** by using [^{3}H]-D-Mannitol flux measurements. After an incubation of 8 h with TNF (5,000, 10,000 U/mL), flux measurements were carried out for 2 h, resulting in three flux periods of 40 min each. Data are presented as mean ± SEM ($n = 7$).

claudin-1 after 10 h (52.40 ± 13.28%, $p = 0.012$, $n = 4$; unpaired t-test). In addition, we detected a major reduction of claudin-4 signal (55.84 ± 5.81%, $p = 0.0003$, $n = 4$). On the other hand, the pore-forming TJ protein claudin-2 had increased after 10 h (149.25 ± 16.77%, $p < 0.026$, $n = 4$). Treatment with TNF did not affect the expression of claudin-3 (82.40 ± 26.49%, $p = 0.29$, $n = 5$), claudin-7 (118.10 ± 24.12%, $p = 0.47$, $n = 5$), or occludin (110.47 ± 8.91%, $p = 0.28$, $n = 4$). In VE tissue, none of the above-mentioned TJ proteins showed any changes after treatment with the cytokine (data not shown).

Confocal Laser Scanning Immunofluorescence Microscopy

Tissue samples were stained with antibodies raised against specific TJ proteins that had shown alterations in their expression when analyzed by Western blot. In the control tissue specimens after 10 h in the Ussing chamber, we observed a weak but specific claudin-2 signal (green, **Figure 6A**). Incubation with TNF strongly enhanced the expression of claudin-2, as the whole FAE of PP showed an intense signal for this TJ protein. The staining cells inside the FAE might be subjunctionally located signals of claudin-2, which were caused during protein biosynthesis. The same epithelial preparation revealed decreased signals and a disruption of claudin-1 after incubation with the cytokine, as the signal for claudin-1 was solely located in the basal membrane after TNF treatment. In untreated PPs, a strong claudin-1 signal was located in the apicolateral membrane (red, **Figure 6A**). Incubation with TNF also disturbed the strong paracellular signal of claudin-4. In contrast to claudin-1, the restricted claudin-4 signal was enriched

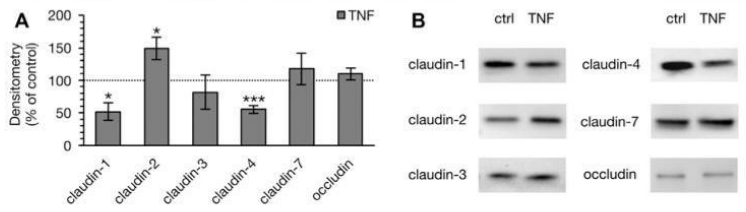

FIGURE 5 | Densitometry **(A)** and representative Western blots **(B)** of TJ proteins in PP tissue after 10 h of incubation with 5000 U/ml TNF. A marked lower expression of claudin-1 and -4 was observed, whereas the expression of claudin-2 was stronger ($n = 4$–6, $^*p < 0.05$, $^{***}p < 0.001$). The dotted line indicates the controls, which were set to 100% and compared to the TNF treated groups, respectively.

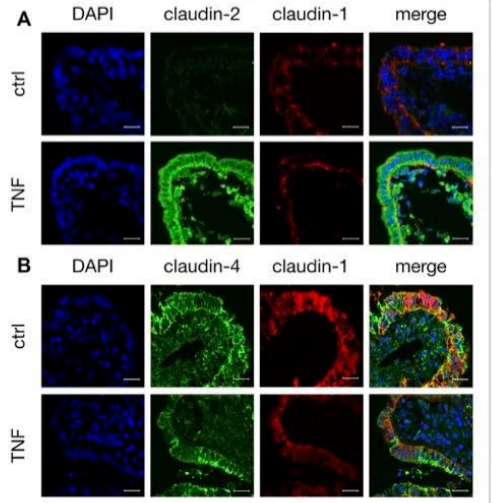

FIGURE 6 | Immunohistological staining of affected TJ proteins after TNF incubation **(A)** Confocal laser scanning immunofluorescence microscopy of claudin-2 (green) and claudin-1 (red) after 10 h incubation of tissue in the Ussing chamber with or without 5000 U/ml TNF **(B)** Staining of PP FAE for claudin-4 (green) and claudin-1 (red). Cell nuclei were stained blue by DAPI (scale bar: 20 µm, $n = 3$, representative images).

in fragments at the apical region of the cells. The different orientations of the protein fragments of claudin-1 and -4 within the cells can be seen most clearly in the merged picture of the TNF-treated tissue in **Figure 6B** in which their colocalization is diminished.

Expression Level of Specific TNF Receptors TNFR-1 and -2

Because TNF can bind two specific receptors, namely TNFR-1 and -2, we examined which of these receptors mediates the TNF-induced changes in PP tissues. Western blot analysis was carried out with specific antibodies raised against TNFR-1 and -2. For densitometry, the Western blot bands were normalized to the total amount of protein, and controls were set to 100%. In FAE of PP, an increase of TNFR-2 was detected (TNF: 127.39 ± 4.48%; $p < 0.0001$, $n = 7$; unpaired t-test), whereas the expression of TNFR-1 was not significantly altered (TNF: 81.77 ± 10.13%; $p = 0.12$, $n = 4$, **Figure 7**). In VE, neither TNFR-1 (TNF: 88.38 ± 10.84%; $p = 0.3$, $n = 7$), nor TNFR-2 (TNF: 91.45 ± 11.44%; $p = 0.47$, $n = 6$), showed a significantly altered expression after exposure to TNF for 10 h. Confocal laser scanning immunofluorescence microscopy of PP tissue samples did not show any specific signals for TNFR-1, regardless of incubation with TNF (green, **Figure 8A**). In accordance with the results from the Western blot analysis, incubation with TNF did not alter the localization or signal intensity of occludin (red, **Figure 8A**). Only single cells of the FAE of untreated PP tissue specimens showed a specific signal for TNFR-2, which was only located in the basolateral membrane (green, white arrows, **Figure 8B**). After incubation with 5000 U/ml TNF for 10 h, almost all of the cells showed a strong signal for TNFR-2, which was located not only to the basolateral part, as seen for the control tissues, but also to the apical membrane. The signal for ZO-1 was not altered by incubation with the cytokine (red, **Figure 8B**).

Signaling Experiments With ML-7

PP tissues were incubated with either TNF (5000 U/ml) or TNF + ML-7 (5000 U/ml + 7.5 µM), whereas controls were incubated with neither. The point at which the substances were added, TER values were set to 100% and recorded for 10 h. The TER of the TNF-treated group decreased to 72.12 ± 7.46%. Control groups exhibited TER values of 85.32 ± 6.68%, and the group treated with TNF + ML-7 showed resistance values of 83.32 ± 9.28% after 10 h incubation. All values were compared with initial resistance. Although a trend could be observed (**Supplementary Figure S1**), it did not reach significance ($p = 0.46$, $n = 8$; one-way ANOVA).

Theophylline Challenge

By using theophylline, chloride secretion is stimulated in epithelial cells *via* cAMP, and therefore leads to an increased short-circuit current (I_{sc}) indicating vitality of the tissue (Markov et al., 2014). An incubation with TNF did not influence the vitality of VE (ctrl: 56.25 ±

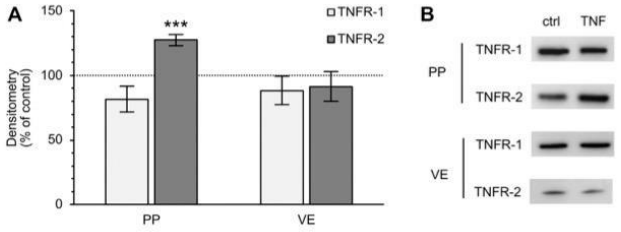

FIGURE 7 | Densitometry **(A)** and representative Western blot bands **(B)** of TNFR-1 and -2 after 10 h incubation of PP FAE and VE tissue in the Ussing chamber with or without TNF. The expression of TNFR-2 increased in PP FAE tissue specimens after incubation with the cytokine, whereas the expression of TNFR-1 was not significantly altered ($n = 4–7$, $^{***}p < 0.001$). In VE samples, neither of the two receptors showed significant changes after exposure to TNF ($n = 6–7$). The controls were set to 100% and are depicted by the dotted line.

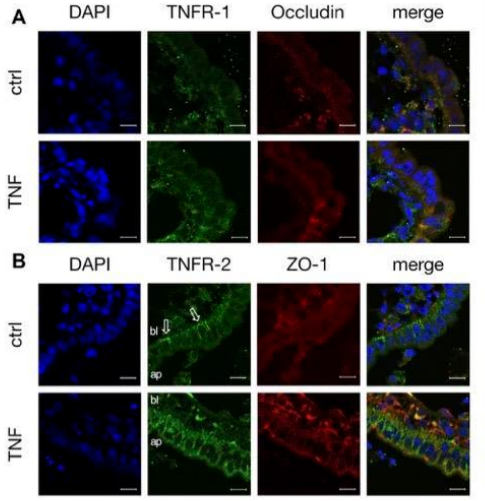

FIGURE 8 | Immunohistological staining of specific TNF receptors **(A)** TNFR-1 (green) and occludin (red) after 10 h incubation of tissue with or without TNF **(B)** TNFR-2 (green) and ZO-1 (red) were stained for confocal laser scanning immunofluorescence microscopy. For better orientation, the basolateral side of FAE is labelled "bl", and the apical part "ap". White arrows indicate the basolateral signal for TNFR-2 in control tissues. DAPI was used for the staining of cell nuclei (scale bar: 40 µm, $n = 3$, representative images).

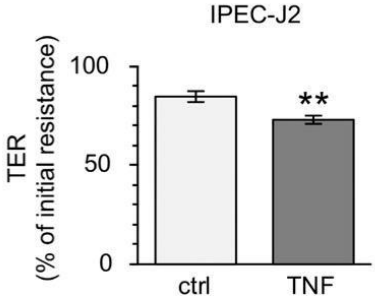

FIGURE 9 | Incubation of IPEC-J2 cells with 5000 U/ml TNF for 10 h. The cytokine was added to the basolateral compartment of the cell inserts. A marked decrease in TER, comparable with results seen in PP tissue specimens, was observed ($n = 23$, $^{**}p < 0.01$).

5.46 µA/cm^2; 1000 U/ml: 58.63 ± 7.68 µA/cm^2; 5000 U/ml: 55.38 ± 5.75 µA/cm^2; 10,000 U/ml: 59 ± 8.07 µA/cm^2, $p = 0.98$, $n = 8$; one-way ANOVA) and PP tissues (ctrl: 1.63 ± 0.75 µA/cm^2; 1000 U/ml: 2 ± 1 µA/cm^2; 5000 U/ml: 1.13 ± 0.77 µA/cm^2; 10,000 U/ml: 2.25 ± 1.03 µA/cm^2, $p = 0.79$, $n = 8$; Kruskal–Wallis test, **Supplementary Figure S2**), though. Regarding the outcome of the vitality tests carried out, a cut-off TER for vital tissue was determined for the following experiments, indicated by respective VE values representative for both tissues of one preparation step.

Incubation of Porcine Intestinal Epithelial Cell Line With TNF

Because the incubation of PP tissues with TNF led to notable changes in epithelial barrier function, further analyses with the non-transformed cell line IPEC-J2 were performed. After incubation with 5000 U/ml for 10 h, the TER of the confluent cell monolayers was reduced comparable to the changes seen in Ussing chamber trials (ctrl: 84.63 ± 2.79%; TNF: 73.09 ± 2.21%, $p = 0.002$, $n = 23$; unpaired t-test; **Figure 9**).

DISCUSSION

Previous studies have focused on the effects of TNF on the intestinal epithelial barrier, and, in particular, on the disturbance of TJ proteins (Barmeyer et al., 2017). Nevertheless, the impact that TNF might have, especially on the barrier properties of the FAE of porcine PP, remains unknown.

The porcine PP contributes to the GALT and represents one of the first lines of intestinal immune defense (Radloff et al., 2017). By presenting luminal antigens to the immune cells, the FAE, which covers PPs, strictly narrows and regulates the contact of the immune system with exogenous substances and plays a key role in the regulation of immune processes (Gebert et al., 1996). An exposure of PPs to cytotoxic substances resulted in an increased expression of the proinflammatory cytokine TNF (Andreev-Andrievskiy et al., 2021; Takasu et al., 2021). Therefore, the barrier function of PPs and its regulation is profoundly biomedically significant, as the GALT controls immune processes, such as food allergies, cancer development or intestinal inflammation (Ishii et al., 2010; Nakajima-Adachi et al., 2014; Fujimoto et al., 2015).

Claudin-1 has been described as a major sealing TJ protein in a variety of tissues including intestine, kidney, pleura, skin, and the blood-brain barrier (Amasheh et al., 2011; Rosenthal et al., 2012; Markov and Amasheh, 2014). In several publications, enhanced TNF levels have been shown to decrease the expression of claudin-1, explaining the disturbed barrier function after exposure to the cytokine (Bruewer et al., 2003; Epple et al., 2009). However, differential effects have been described: incubation with TNF can also lead to the increased expression of claudin-1 (Amasheh et al., 2010; Shiozaki et al., 2012; Bhat et al., 2016). One explanation for this apparently paradoxical effect of claudin-1 might be that, even although an increased expression of claudin-1 occurs, its functional contribution remains questionable if it is not localized in apicolateral TJs (Poritz et al., 2011). The effect might be the result of a counter-regulatory induction effect, with claudin-1 not being properly trafficked into the functionally relevant TJ complexes. However, in our current study, a decrease of claudin-1 was observed after incubation with TNF, which is in accordance with the disrupted barrier function in porcine PP FAE. Therefore, this effect might explain barrier disruption in many inflammatory processes.

In contrast to the sealing function of claudin-1, claudin-2 is a pore-forming TJ protein allowing the paracellular passage of water and cations that are smaller than 182 Da (Amasheh et al., 2002; Rosenthal et al., 2010). In several gastrointestinal disorders, such as IBDs or gastrointestinal carcinomas, the expression of claudin-2 is increased (Aung et al., 2006; Mankertz and Schulzke, 2007). An increase of claudin-2 in the intestine results in a drop in TER and in increased paracellular permeability (Ahmad et al., 2014; Wang et al., 2017; Markov et al., 2019). This is in accordance with our findings in the current study, as we have detected an increase of claudin-2 after 10 h incubation with TNF.

Another TJ protein with a major sealing function is claudin-4. Radloff *et al.* have shown higher expression of claudin-4 in the porcine PPs compared with the surrounding VE, in agreement with the stronger barrier properties of the FAE (Radloff et al., 2017). Therefore, the decrease of claudin-4 in our study contributes to the disturbed sealing function in FAE after the TNF challenge, representing a weakened barrier in PPs during intestinal inflammation. The expression of claudin-3, -7, and occludin, which are also barrier-forming TJ proteins, was not significantly changed by TNF in our study.

In contrast to the strong decrease of TER, the flux of [^{3}H]-D-Mannitol, representing the paracellular permeability, remained unchanged. Because of the differential regulation of permeability pathways, it is not unusual that the paracellular permeability is unaltered, whereas TER has changed (Schoultz and Keita, 2020). The underlying reason is the activation of barrier permeability by two different TJ pathways, namely the pore and leak pathway (Turner, 2009). The high-capacity pore pathway, which is size- and charge-selective, enables solutes smaller than 4 Å in radius to cross the paracellular route (Van Itallie et al., 2008). Molecules larger than 4 Å might pass the paracellular barrier *via* the low-capacity leak pathway. The knockdown of ZO-1 or occludin and disrupted cell-to-cell contacts have been reported to increase the leak pathway (Anderson and Van Itallie, 2009). Furthermore, with cytokines such as TNF, the paracellular flux through the leak pathway seems to be increased. In our experiments, the incubation of porcine PP with TNF led to a decrease of TER after 8 h, whereas the paracellular flux was not significantly changed. This might be attributable to the molecular weight of [^{3}H]-D-Mannitol (182 Da), which is slightly too big to pass the claudin-2-formed pore (Amasheh et al., 2002). The results are also in accordance with the functional properties of claudin-4 sealing the paracellular pathway against the passage of ions (Van Itallie et al., 2001). Non-significant effects on mannitol permeability could also reflect an early or initial TNF effect, as in different epithelial cell models, permeability changes were seen after incubation with TNF for at least 24 h (Ma et al., 2004; Poritz et al., 2011).

An induced leak to Na^+ and Cl^- would represent a higher permeability of the high-capacity pore pathway, and our model most likely represents an early pathomechanism of barrier perturbation by TNF, i) affecting the secretory mechanisms associated with diarrhea and ii) initiating to stronger barrier perturbation in synergism with other proinflammatory cytokines, e.g., Interferon γ (IFNγ; Wang et al., 2006). In accordance, TNF only affecting the ion permeability has been observed previously (Marano et al., 1998).

In previous studies of TNF effects, a pre- or co-incubation with IFNγ has been carried out (Wang et al., 2005; Amasheh M. et al., 2009; Cao et al., 2013). The reason for this step is the ability of IFNγ to increase the expression of specific TNF-receptors (TNFR), hence leading to a faster effect of TNF (Wang et al., 2006). Though, whether the observed changes are a pure effect of TNF or whether it is linked to the incubation with IFNγ remains unclear. Here, however, we have analyzed the single (and direct) effect of TNF, as in our recent publication, a long term-effect of TNF on the non-transformed porcine cell line IPEC-J2 has been shown without prior or simultaneous IFNγ incubation, representing the selective effect of TNF (Droessler et al., 2021).

The TNF effect can be induced by activation of the specific TNF receptors TNFR-1 or -2. Therefore, we carried out additional approaches in order to determine whether incubation with TNF leads to changes in the expression of TNF receptors in PP FAE in comparison with VE. Our study has revealed the main relevance of TNFR-2 in FAE, whereas in VE no significant effects on TNF receptors were observed.

Although TNFR-1 is constitutively found in most tissues, TNFR-2 plays a major role in cells of the immune system (Wajant et al., 2003). TNFR-2 has been described as being involved particularly in immune modulation and tissue regeneration, whereas TNFR-1 on the contrary is mostly associated with inflammation. Another major difference between the two receptors is the ability of TNFR-1 to become activated by soluble and membrane-bound TNF, while TNFR-2 can only be activated by membrane-bound TNF (Fischer et al., 2015). Selective targeting of TNFR-1 and -2 is currently of great interest for therapeutic approaches, as the employment of anti-TNF drugs might cause severe side-effects. Although the genetic deletion of TNFR-1 in animal models leads to an absence of TNF-induced diseases, the deletion of TNFR-2 worsens the disorders (Fischer et al., 2020). The findings that the effects of TNF in porcine PP are mediated by TNFR-2 emphasizes the importance of FAE as being the first step of the intestinal immune response. The predominant TNF-signaling *via* TNFR-2 might also be the reason for the lack of an effect of the inhibitor ML-7 on porcine PP, as previous studies have shown that the blocking of MLCK prevents the strong increase of TNFR-1 after TNF-incubation (Droessler et al., 2021).

To strengthen our findings further, additional experiments were carried out using the non-transformed cell-line IPEC-J2. This porcine jejunal cell-line has recently been used for analysis of TNF effects on the intestinal barrier function. Significant effects on transepithelial resistance and paracellular flux of [^{3}H]-D-Mannitol having been seen after incubation of the cells with 1000 U/ml TNF for 48 h (Droessler et al., 2021). In the present study, incubation of these cells with 5000 U/ml for 10 h has revealed changes in transepithelial resistance, in agreement with our findings in the Ussing chamber experiments. Although IPEC-J2 cells are a suitable model for analysis of small intestine and these cells represent many aspects of the VE, the different outcome of IPEC-J2 cells and VE in our manuscript indicates that this cell model needs to be compared carefully in different experimental approaches. Therefore, experiments with IPEC-J2 in our current study were performed under the same conditions regarding concentration and time course, to allow a better comparison of the results with previous approaches.

Our aim has been to analyze PP and neighboring VE *ex vivo*, as the tissue samples are more comparable with the intestine *in vivo*. Moreover, the PP samples allow us to investigate the direct effect of TNF on FAE. By employing the Ussing chamber technique, we performed a detailed analysis of the effects of TNF on TER for up to 10 h. Subsequently, tissues were stained with hematoxylin-eosin, and the size of PPs and the length of the surrounding villi were measured and compared between the control and TNF-treated groups. Apoptosis could be excluded as a reason for the observed changes, as the tissue samples still responded to vitality tests carried out using theophylline. Because no significant alterations in tissue quality after incubation with TNF were observed, and no evidence for apoptosis could be detected, other mechanisms for the drop in TER needed to be explored, with special attention being given to barrier perturbation, as described in detail above. It cannot be ruled out, that a longer incubation with TNF might also affect the functional barrier properties of VE additional to changes in PP FAE, as intestinal inflammation like in PWD go also in accordance with a disturbed morphology of VE in porcine (Chen et al., 2020).

In our current study, we have been able to confirm the functional and molecular differences of PP and VE under intestinal inflammatory conditions. The importance of the porcine FAE as a first step in intestinal immunological defense has been established, as only PP tissue specimens reacted to the addition of the proinflammatory cytokine TNF with changes in barrier function, TJ protein composition, and the expression of TNFR-2, whereas VE did not respond. The outcome of our current study might allow further approaches focusing on the identification and analysis of counter-regulatory, beneficial, and therefore possibly therapeutic and preventative compounds for administration under inflammatory intestinal conditions.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusion of this article will be made available by the authors on request.

AUTHOR CONTRIBUTIONS

LD, VC, EB, LS, and NB performed the experiments, SA conceptualized and supervised the study, LD prepared the figures and wrote the initial draft. LD, VC, and SA analyzed the data, and all authors reviewed the manuscript.

FUNDING

This study was funded by the German Research Foundation (DFG), Grant No. AM141/11-2.

ACKNOWLEDGMENTS

Our thanks go equally to Julius Dahl, Martin Grunau, Katharina Söllig and Susanne Trappe for outstanding technical assistance.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2022.889552/full#supplementary-material

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Chapter 6: Discussion

Barrier impairment of IPEC-J2 cells after incubation with TNF

By playing a crucial role in the pathogenesis of various inflammatory diseases, the pro-inflammatory cytokine TNF is currently of primary interest and being extensively researched. The negative impact of TNF on epithelial barrier function *via* the disturbance of TJ proteins has been proposed to be one of the main pro-inflammatory mechanisms in IBD (Pagnini and Cominelli, 2021). As described in the literature review (**Chapter 2**), a better understanding of the barrier disturbance induced by TNF together with alterations in the density of its specific receptor and the expression of TJ proteins might aid future therapeutical approaches.

The TNF-mediated disturbance of barrier function was first described by using the porcine renal cell line LLC-PK1 (Mullin and Snock, 1990). Subsequently, the effects of TNF on the epithelial barrier function have been analyzed in various cell culture models, e.g., Caco-2 (Ma *et al.,* 2005; Cui *et al.,* 2010), HT29/B6 (Gitter *et al.,* 2000a; Mankertz *et al.,* 2009), T84 cells (Li *et al.,* 2008), and IEC-18 cells (Poritz *et al.,* 2011). As the use of cancerogenic cell lines, such as Caco-2 or HT29/B6, is questionable with regard to their comparability with *in vivo* conditions, the experiments in the present work were carried out by using the nontumorigenic cell line IPEC-J2. This non-transformed cell line, which grows as a monolayer and lacks malignant properties, has been characterized as a suitable *in vitro* model for analyzing porcine epithelial functionality and bacterial interactions (Schierack *et al.,* 2006; Zakrzewski *et al.,* 2013). Because of their appropriate comparability with *in vivo* conditions, IPEC-J2 cells have recently been used to investigate the effects of various feed additives on epithelial barrier function (Spitzer *et al.,* 2016; Granica *et al.,* 2020; Cornelius *et al.,* 2022). However, a shortage of data exists concerning the effects of inflammatory cytokines such as TNF on epithelial barrier function in the porcine cell line IPEC-J2.

In the first study presented in this thesis (**Chapter 4**), the focus of the research was the molecular and functional analysis of barrier properties in IPEC-J2 cells *in vitro* during incubation experiments with TNF. Epithelial barrier disturbance after incubation with TNF for 48 h was confirmed, represented by (i) a significant decrease in TER and (ii) an increase of paracellular permeability to [^{3}H]-D-Mannitol. The disturbing effects on barrier integrity after TNF challenge in IPEC-J2 cells were in accordance with findings from other working groups (Xiao *et al.,* 2017). However, in addition to its pro-inflammatory functions, TNF can activate an apoptotic signaling cascade (Wajant *et al.,* 2003), which might also result in a decrease in TER because of the loss of cell cohesion (Gitter *et al.,* 2000b). Therefore, an ApoTox-Glo assay

was performed following incubation experiments with TNF in order to analyze the apoptotic and cytotoxic potentials of TNF and the viability of IPEC-J2 cells. None of these three aspects showed a significant alteration after incubation with TNF. To strengthen these findings further, the recovery ability of IPEC-J2 cells after incubation with TNF was analyzed for another 48 h. Whereas the cells with TNF showed a further decrease in TER, cells having had the cytokine removed increased their TER values to values similar those of control groups. The outcome of these two unbiased experiments together suggested that apoptosis could be eliminated as a reason for the TER decrease after incubation with TNF.

Although cell lines are commonly used for the analysis of (patho-)physiological gastrointestinal functions, a few limitations need to be mentioned with regard to its usage. The physiological intestinal epithelium *in vivo* contains a plurality of cell types, such as enterocytes, goblet cells, or enteroendocrine cells (Joshi *et al.,* 2022). As epithelial cell culture models often only consist of a single cell type, one disadvantage of these models is the strong difference from the intestinal environment *in vivo* (Rahman *et al.,* 2021). Under inflammatory conditions *in vivo*, intestinal cells are also in close interaction with immunological cells that are absent *in vitro* (Joshi *et al.,* 2022). Furthermore, the gastrointestinal tract is covered with a mucus layer that acts as a first line of defense against exogen stimuli (Pelaseyed *et al.,* 2014). Although IPEC-J2 cells cultured with porcine serum instead of fetal bovine serum show increased mucus production, it is not comparable with the situation *in vivo* (Vergauwen*,* 2015).

Current studies are, therefore, increasingly focusing on other *in vitro* models containing more than one cell type. One such approach is that of co-culture models comprising intestinal cells cultivated together with cells from the immune system (Leonard *et al.,* 2010; Kämpfer *et al.,* 2017; Loss *et al.,* 2020), hence providing additional experimental information from more than one cell type. Other novel approaches include those with specific organoids that display all cell types that are present in the intestinal epithelium (Hoffmann *et al.,* 2021). In this method, adult stem cells derived from native tissue start to differentiate into three-dimensional organotypic structures (Kim *et al.,* 2020). They provide some great benefits compared with other *in vitro* models, such as their fast establishment, good representation of organ physiology, and stability over a long time (Kim *et al.,* 2020). Recently, intestinal organoids have even been used for scientific research on the pathophysiology of human infectious diseases such as SARS-CoV-2 (Lamers *et al.,* 2020). However, the establishment of organoids is relatively new to the field and also exhibits a few limitations, such as high costs and non-standardized protocols that restrict their usage (Kim *et al.,* 2020).

Effects of TNF on barrier properties of PP FAE and adjacent VE

In the second study described in the present thesis (**Chapter 5**), tissue samples of both immunologically active PP and neighboring VE were taken from adult pigs and mounted into a conventional Ussing chamber setup for *ex vivo* analysis. As previously outlined in the literature review (**Chapter 2**), the Ussing chamber technique provides a well-established *ex vivo* model for investigating epithelial barrier function under experimental conditions.

The effects of the pro-inflammatory cytokine TNF on barrier properties of porcine PP were analyzed and compared with those of the adjacent VE. Incubation with TNF led to a significant decrease in TER in PP tissue samples after 8 h in the Ussing chamber. In contrast, VE tissue specimens did not respond to the addition of TNF during the entire incubation period. Unidirectional paracellular tracer flux measurements were carried out with [^{3}H]-D-Mannitol in order to investigate the influences of TNF on paracellular permeability. Neither PP nor VE showed altered paracellular permeability after incubation with TNF. This is unlike the results shown in the first study of this work (**Chapter 4**) in which TNF led to a significant alteration of both TER and paracellular flux in IPEC-J2 cells.

However, differential measurements representing the epithelial barrier function frequently provide divergent outcomes: Whereas the TER refers to the movement of ions and solutes, the paracellular flux describes the movement of tracer molecules of different size and charge across the epithelium (Schoultz and Keita, 2020). A similar outcome has been observed by Marano *et al.* (1998) who have found that the incubation of Caco-2 cells with TNF also affects the TER, whereas the flux of mannitol is unaltered. This might be attributable to the influence of paracellular permeability in TJs by distinct pathways. As depicted in the literature review, the paracellular transport can occur *via* either the leak pathway or the pore pathway (**Chapter 2.2.1**). The low-capacity leak pathway is independent of charge and allows the passage of large ions and molecules, whereas the high-capacity pore pathway is charge-selective and only allows the flux of small ions and uncharged molecules (Shen *et al.,* 2011). During the TER measurements, the passage of all ions across the epithelium, mainly represented by Na^+ and Cl^-, is measured. As these small ions do not differentiate between the pore or the leak pathway, a reduced TER could indicate an alteration of permeability *via* both pathways (Shen *et al.,* 2011). By using a series of noncharged polyethylene glycols on different cell lines and porcine ileum, Van Itallie *et al.* (2008) have been able to identify the size selectivity of the pore pathway that excludes the passage of molecules larger than 4 Å in radius. In contrast, the low-capacity leak pathway allows the flux of large molecules such as proteins or bacterial lipopolysaccharides and has been shown to be increased when cell-cell contacts are disrupted (Anderson and Van Itallie, 2009). Several groups have shown an alteration in TER associated

with changes in claudin composition, whereas the flux for noncharged solutes such as mannitol is not affected (Van Itallie *et al.,* 2001; Amasheh *et al.,* 2002). Furthermore, paracellular flux *via* the leak pathway, e.g., induced by pro-inflammatory cytokines such as Interferon gamma (IFNγ), can also increase in the absence of an altered pore pathway (Watson *et al.,* 2005). Hence, the analysis of several parameters of epithelial barrier function is always necessary if precise statements are to be made.
In the present work, the effect of TNF on TER in combination with an unaltered paracellular flux in PP tissue samples might have several explanations. One might be the induction of the high-capacity pore pathway by TNF, representing an increased flux of Na^+ and Cl^- and, hence, early secretory mechanisms in diarrhea. Furthermore, with an experimental time of 10 h in the Ussing chamber, only an initial TNF effect might occur, whereas in other models, changes in mannitol flux induced by TNF have been examined after at least 24 h (Ma *et al.,* 2004).

The application of *ex vivo* models for analyzing gastrointestinal barrier function provides a cost-effective and high-throughput model. However, a few limitations need to be considered when working with *ex vivo* models: Observations made in *ex vivo* experiments only represent the behavior of the respective tissue sample. During situations *in vivo*, the natural immune system contributes an additional influencing factor, as specific cells can be transported to the respective tissue during immune responses (Shi *et al.,* 2019). This disadvantage should certainly be considered because the extracted tissue samples in *ex vivo* experiments lack a blood circulation and a natural immune system. Another essential restriction might be the short lifespan of native tissue samples, which makes the Ussing chamber model mostly impractical for long-term studies (Pearce *et al.,* 2018). Therefore, an optimized Ussing chamber protocol was established during the second study (**Chapter 5**) in order to monitor cytokine-dependent effects on epithelial barrier function for up to 10 h. To ensure the vitality of the respective tissue samples, buffer exchanges were carried out, and vitality was tested by using theophylline. By stimulating Cl^- secretion *via* cAMP leading to an increased I_{SC} (Markov *et al.,* 2014), only vital tissue showed a response after the addition of theophylline. Moreover, after experimental periods of 10 h in the Ussing chamber, tissue samples of both PP and VE were stained with hematoxylin-eosin in order to determine the size of PPs and length of surrounding villi; the data thus obtained excluded morphological changes as being a reason for the observed disturbance in barrier function.

TNF effects on claudin composition in IPEC-J2 cells and PP FAE

After the significant impact of TNF on the barrier function of IPEC-J2 cells and PP tissue samples had been explored, an analysis of TJ proteins was executed to investigate barrier disturbance by the cytokine in more detail. Immunoblotting and confocal laser scanning immunofluorescence microscopy was therefore conducted after experiments to gain insight into molecular and functional changes of TJ proteins.

In the *in vitro* studies on IPEC-J2 cells (**Chapter 4**), incubation with TNF significantly decreased sealing TJ proteins claudin-1, -3, and occludin. Other TJ proteins present in the porcine intestine, such as claudin-4, -7, and ZO-1, were not affected by incubation with the cytokine. These observations agreed with the functional changes of TJ proteins seen in confocal laser scanning immunofluorescence microscopy. All TJ proteins that showed decreased expression levels in Western blot analysis after TNF incubation also exhibited altered signals in immunocytochemistry. Compared with controls, signals for claudin-1, -3, and occludin appeared reduced and were no longer functionally located to the apicolateral membrane of the IPEC-J2 cells. Hence, TNF led to a disturbance of TJ proteins on a molecular and a functional level, as the integral membrane proteins showed a distinct disruption of their physiological localization.

An impact of TNF on the TJ composition of porcine FAE *ex vivo* was evaluated in the second study (**Chapter 5**). Western blot analysis revealed reduced expression levels of the sealing TJ proteins claudin-1 and -4 after treatment with TNF for 10 h. Moreover, treatment with TNF for 10 h led to a significant increase of the pore-forming TJ protein claudin-2 in PP tissue specimens. Other TJ proteins investigated, namely claudin-3, -7, and occludin, were not significantly altered. Immunohistochemistry emphasized the outcome of the Western blot analysis, as the signals for claudin-1 and -4 were redistributed away from the TJ and were no longer functionally located to the apicolateral membrane of the FAE after treatment with TNF. In contrast, a strongly enhanced claudin-2 signal after TNF incubation could be observed in PP FAE. As stated earlier in this chapter, the barrier function of VE, as indicated by TER and flux measurements, was not impaired by TNF. This is consistent with the outcome of the Western blot analysis and immunohistochemistry, as none of the observed TJ proteins were affected by treatment with the cytokine.

Both the *in vitro* and *ex vivo* experiments revealed an effect of TNF on the expression of claudin-1. Claudin-1 is expressed in the small intestine and belongs to the sealing TJ proteins (Amasheh *et al.,* 2011). One would therefore assume that a decrease in claudin-1 is consistent with an impaired barrier function. This corresponds to the results in the present work, as TNF led to disturbed barrier integrity in terms of a down-regulation of claudin-1 in both the *in vitro*

and *ex vivo* experiments. Similar effects have been observed under inflammatory conditions before (Amasheh *et al.,* 2009a; Mennigen *et al.,* 2009), and Bertiaux-Vandaële *et al.* (2011) have shown an altered distribution of claudin-1 in IBS patients. Furthermore, a decrease in claudin-1 attributable to TNF, as demonstrated in the present project, has previously been shown in various models (Poritz *et al.,* 2004; Baker *et al.,* 2008).
In several inflammatory conditions, however, a paradoxical effect of claudin-1 has been observed, as a disturbed barrier function seemed to be associated with an up-regulation of claudin-1 (Weber *et al.,* 2008; Poritz *et al.,* 2011). Moreover, an increase in claudin-1 has been shown in numerous neoplastic diseases (Dhawan *et al.,* 2005; Gröne *et al.,* 2007) and is considered to be a positive prognostic indicator for cancer (Resnick *et al.,* 2005). One possible explanation for this paradoxical effect might be the involvement of claudin-1 in tumorigenesis (Bhat *et al.,* 2016). Another reason might be the counter-regulatory mechanism of claudin-1 in the context of disturbed barrier function. However, an increase of claudin-1 expression by TNF has been postulated not to contribute to improved barrier function, as it is not adequately localized to its functional junctional position (Poritz *et al.,* 2011).

Claudin-2 belongs to the group of pore-forming TJ proteins and is mainly localized to villus and crypt cells of the small intestine (Lameris *et al.,* 2013). In FAE, claudin-2 is only expressed weakly in the crypt region compared with the surrounding VE (Tamagawa *et al.,* 2003). Under inflammatory conditions, an increase of TNF occurs concomitantly with an up-regulation of claudin-2 (Weber *et al.,* 2008; Mankertz *et al.,* 2009). This finding is consistent with the results in the present work and might explain the strongly enhanced signal for claudin-2 as observed in FAE after incubation with TNF (**Chapter 5**). Claudin-2 builds channels for cations and water, and therefore, its increased expression influences the tightness of epithelia and results in a decreased TER (Amasheh *et al.,* 2002; Rosenthal *et al.,* 2010). Thus, an up-regulation of claudin-2 induced by TNF might be a functionally important and early mechanism, while sealing TJ proteins are relocated away from the apicolateral membrane in mechanisms associated with intestinal inflammatory conditions (Luettig *et al.,* 2015). A leaky barrier because of an increased expression of claudin-2 in the inflamed intestine, furthermore, contributes to diarrhea *via* leak-flux mechanisms (Luettig *et al.,* 2015). An explanation for this might be the increased paracellular flux of water, which follows increased permeability to cations, such as Na^+, through the claudin-2 channel (Luettig *et al.,* 2015). However, claudin-2 restricts the passage of anions and molecules larger than 182 Da (Amasheh *et al.,* 2002); this might also explain the absence of effects on paracellular permeability to mannitol *via* the high-capacity pore pathway during the *ex vivo* Ussing chamber studies (**Chapter 5**). The leakiness of epithelia is not only attributable to an increase in pore-forming and less tightening claudins, but might also be in accordance with an increased breakage of TJ strands. However,

TJ strand breaks would also lead to an increased flux of macromolecules, whereas an up-regulation of pore-forming claudins would lead to increased permeability only for a certain group of ions (Anderson *et al.,* 2004). Hence, an increased uptake of bacteria or antigens during inflammation is less likely to be the result of an increased expression in claudin-2, but rather arises because of exaggerated TJ strand breaks during inflammation (Zeissig *et al.,* 2007). However, claudin-2 is just one of many TJ proteins involved in inflamed barrier dysfunction, and changes in other TJ proteins should always be considered.
Claudin-3 contributes to the barrier-building TJ proteins (Milatz *et al.,* 2010) and is present in IPEC-J2 cells (Zakrzewski *et al.,* 2013) and porcine FAE (Radloff *et al.,* 2017b). A down-regulation of claudin-3 after incubation with TNF has recently been observed in various cell culture models (Mei *et al.,* 2015; Haines *et al.,* 2016), a finding consistent with the results seen in IPEC-J2 cells. In contrast, no significant effect of TNF on claudin-3 expression in PP FAE has been observed. This suggests that other claudins sustain major barrier properties in the FAE, as discussed in the following.

Another barrier-building TJ protein is claudin-4, which is preferably expressed in the dome region of the FAE (Tamagawa *et al.,* 2003). An increased expression of claudin-4 in porcine PP FAE compared with VE has been shown by Radloff *et al.* (2017b), explaining the improved selectivity of the transcellular pathway in FAE and indicating the major paracellular sealing function of claudin-4. Thus, claudin-4 plays a pivotal role in intestinal TJs, as it is diminished in intestinal pathophysiological conditions (Prasad *et al.,* 2005) but can also be affected by barrier-strengthening compounds (Amasheh *et al.,* 2008; Noda *et al.,* 2013). An up-regulated expression of claudin-4 leads to an increase in TER associated with a selective decrease of permeability to Na^+, whereas Cl^- is unaffected (Van Itallie *et al.,* 2001). The enhanced ion permeability associated with a reduction of claudin-4 is consistent with the results discussed earlier in this chapter, as the high-capacity pore pathway might be the reason for the barrier-disturbing effects of TNF on PP FAE *ex vivo.*

A down-regulation of occludin by TNF has also been observed in cell-culture models, such as HT29/B6 cells (Mankertz *et al.,* 2000) and Caco-2 cells (Kuo *et al.,* 2019; Al-Sadi *et al.,* 2011). Treatment with TNF, furthermore, leads to the endocytosis of occludin and results in its redistribution from the TJ structure (Clayburgh *et al.,* 2005). Reduced levels of occludin are associated with decreased levels of CASP3, limiting apoptosis and thus preventing mucosal damage in response to inflammation (Kuo *et al.,* 2019). Moreover, Al-Sadi *et al.* (2011) have found an increased flux of macromolecules associated with a knockdown of occludin. Both findings are consistent with the effects of TNF on IPEC-J2 cells, as a barrier disturbance by

TNF results in an increased flux to mannitol, which is linked to a reduction of occludin in the absence of apoptosis (**Chapter 4**).

The differential outcomes of TNF effects on TJ proteins *in vitro* and *ex vivo* might have various reasons. Whereas in both studies, a significant decrease has been observed in claudin-1 expression, the impact on claudin-2, -3, -4, and occludin differs.
One reason for the variable outcomes might be that different periods of time and concentrations were investigated in the two experimental conditions. Whereas the effect of TNF on IPEC-J2 cells was examined for up to 48 h with 1000 U/mL TNF, examination of PP tissue samples in the Ussing chamber was performed with 5000 U/mL TNF and monitored for only 10 h. This was because of the short lifespan of the native tissue samples, as discussed earlier in this chapter.
The effect on claudin-4 expression in FAE might represent an early and initial effect of TNF attributable to the prominent occurrence of claudin-4 at the top of the PP dome (Tamagawa *et al.,* 2003). As claudin-3 and occludin are also located throughout the FAE, but not preferentially at the tip of the dome, as is claudin-4 (Tamagawa *et al.,* 2003), the missing effect might be related to the localization of the TJ proteins and might instead represent a long-time effect of TNF, such as is seen in IPEC-J2 cells after 48 h. However, differing alterations in claudin composition during inflammatory diseases have previously been seen in numerous studies. The contradictory effects on claudin-3 and -4 in IBD have been summarized in a review by Lu *et al.* (2013): Some studies only show an effect on claudin-3 in the absence of changes in claudin-4 (Zeissig *et al.,* 2007) and *vice versa* (Oshima *et al.,* 2008).
Another explanation for the differential alteration of claudins might be the missing expression of pore-forming TJ proteins in IPEC-J2 cells (Zakrzewski *et al.,* 2013). Although these cells constitute a suitable model for porcine enterocytes, they do not express the TJ protein claudin-2, which is present in the porcine intestine (Radloff *et al.,* 2017b). Hence, this might be one disadvantage when comparing results in IPEC-J2 cells with TNF effects on porcine tissue *ex vivo* and gives rise to the necessity for complementary approaches.

TNF-signaling within epithelial barrier function

As the pro-inflammatory cytokine TNF is a regulatory component of the immune system, it is involved in the development of numerous pathophysiological conditions. Therefore, knowledge about the signaling pathways of TNF is necessary for preventing and treating inflammatory diseases. Currently, TNF-neutralizing antibodies are approved and used to medicate several autoimmune disorders such as rheumatoid arthritis, IBD, or psoriasis (Fischer *et al.,* 2020).

However, the use of anti-TNF medication is limited because of the lack of responsiveness or the occurrence of severe side effects (Peyrin-Biroulet *et al.,* 2021).

The effects of TNF can be achieved by activating its specific receptors, namely TNFR-1 or TNFR-2. As outlined in the literature review (**Chapter 2**), both receptors can mediate multiple functions and fulfill opposing roles in the signaling of TNF. Therefore, the targeting of specific TNF-receptors is of great interest in current research projects.
During the first study of the present thesis (**Chapter 4**), an increase in TNFR-1 density was observed *in vitro*, dose-dependently to TNF. The expression of TNFR-2 in IPEC-J2 cells was unaffected by incubation with TNF. This was in contrast to the effects on the density of TNFR-1 and -2 in Ussing chamber experiments *ex vivo* (**Chapter 5**): When PP tissue samples were incubated with TNF for up to 10 h, a significant increase in the density of TNFR-2 was observed, whereas TNFR-1 remained unaffected. In VE, neither TNFR-1 nor TNFR-2 showed any alterations after incubation with TNF, consistent with the results on barrier function discussed earlier in this chapter. The different findings of incubating IPEC-J2 cells and PP FAE with TNF might be related to the physiological distribution of TNF receptors. Whereas TNFR-1 is present in most nucleated cells, TNFR-2 is preferentially expressed in cells of the immune system and plays a considerable role in the signaling of the lymphoid system (Wajant *et al.,* 2003). Therefore, the major response of TNFR-2 in PP samples emphasizes the immunological importance of FAE during inflammatory conditions. Moreover, the self-enhancing effect of TNF on its receptors, observable during both *in vitro* and *ex vivo* experiments, underlines the exponential inflammatory effect of TNF in intestinal epithelia.

TNFR-1 represents the crucial mediator for most of the pro-inflammatory actions by TNF and can be activated by both tmTNF and sTNF (Richter *et al.,* 2021). TNFR-2, in contrast, can only be stably activated by tmTNF, and its effects mostly involve tissue regeneration and protection from programmed cell death (Fischer *et al.,* 2011). Hence, tmTNF is mainly associated with the remedy of inflammation (Canault *et al.,* 2004) and the maintenance of immunity to several pathogens (Fremond *et al.,* 2005; Torres *et al.,* 2005). Inhibition of both TNF forms, which occurs in conventional approved anti-TNF medications, might have counterproductive side effects (Richter *et al.,* 2021). Current research, therefore, aims selectively to activate TNFR-2 or specifically to inhibit sTNF and TNFR-1 (Richter *et al.,* 2021).
A selective inhibitor of sTNF was first shown to be able successfully to inhibit inflammation in a mouse arthritis model (Zalevsky *et al.,* 2007). In the following years, the inhibition of sTNF has been evaluated further as a promising candidate for many disorders, such as Parkinson's disease (McCoy *et al.,* 2008), ischemic stroke (Clausen *et al.,* 2014), and Alzheimer's disease (MacPherson *et al.,* 2017).

In a study by Williams *et al.* (2014), the selective targeting of TNFR-1 and -2 has been used with antagonistic antibodies to investigate their influences in an animal model of multiple sclerosis. Whereas treatment with a TNFR-2 antibody leads to an increased severity of symptoms, anti-TNFR-1 treatment ameliorates the disease (Williams *et al.,* 2014). Thus, a few selective TNFR-1 inhibitors are of primary interest to current research and are the subject of clinical studies (Steeland *et al.,* 2017; Richter *et al.,* 2019).
In summary, combinations of sTNF and TNFR-1 antagonists, even with TNFR-2 agonists, constitute promising therapy strategies superior to conventional anti-TNF drugs (Fischer *et al.,* 2020). The findings of the present work confirm this assumption, as TNF has been observed to have an effect on the expression density of TNFR-2 in FAE. This might represent an initial response of the immune system to the stimulation by TNF and reveals the importance of the FAE as an essential part of the GALT.

TNF initiates numerous different signaling pathways that are not fully understood yet, because of the complexity of the actions of this molecule. The exact mechanism by which TNF exacerbates intestinal barrier damage has, for the same reason, not been completely elucidated. However, an increased barrier permeability by TNF might be related to the activation of myosin light chain kinase (MLCK; Cunningham and Turner, 2012). An increased expression of MLCK leads to the phosphorylation of the myosin II light chain (MLC) and induces a contraction of the perijunctional actomyosin ring (He *et al.,* 2020). This contraction in turn leads to a re-organization of epithelial TJ proteins and is associated with occludin endocytosis, which might be a crucial step for the TNF-induced barrier dysfunction (Marchiando *et al.,* 2010). As many inflammatory diseases are associated with an abnormal expression of MLCK, the potential use of anti-MLCK agents as therapeutics is currently under discussion (Xiong *et al.,* 2017).
To evaluate the role of MLCK in the barrier disturbance seen to be produced by TNF in the present work, IPEC-J2 cells and PP tissue samples were incubated with a specific MLCK blocker, namely ML-7. In IPEC-J2 cells (**Chapter 4**), the co-incubation of TNF and ML-7 prevented both the decrease in TER and the increase in the expression density of TNFR-1. Western blot analysis revealed that treatment with the MLCK blocker also significantly inhibited the TNF-induced loss of claudin-3, whereas the expression of occludin was not affected. Immunofluorescence microscopy demonstrated, however, that treatment with ML-7 led to an increased and restored occludin signal after TNF treatment. In PP FAE (**Chapter 5**), no significant effects on barrier function after incubation with ML-7 could be detected, although a trend was visible.
The activation of MLCK by inflammatory cytokines allows the passage of large molecules through the paracellular barrier, which is associated with the activation of the low-capacity leak

pathway (Ma *et al.,* 2005). As an involvement of MLCK in TNF-induced effects in IPEC-J2 cells has been shown, this would also explain the enhancement of the paracellular permeability to mannitol. Furthermore, the missing effect of ML-7 in the Ussing chamber experiments verifies the contribution of the high-capacity pore pathway in FAE.

Several studies have examined the barrier-disturbing effects of TNF in combination with IFNγ. Similar to TNF, IFNγ is a cytokine that plays a critical role in mediating cell immunity (Kak *et al.,* 2018). It can recognize and eliminate pathogens and is involved in the pathogenesis of infectious and autoimmune diseases (Kak *et al.,* 2018). The reason that incubation experiments with TNF are often performed in combination with IFNγ is that co-incubation with IFNγ leads to the increased expression of TNF receptors, therefore explaining the rapid onset of TNF effects (Wang *et al.,* 2006). However, differential regulation pathways have been shown for IFNγ and TNF. First, IFNγ signals through a different receptor from that of TNF, namely through the IFNγ receptor (Castro *et al.,* 2018). Second, although both cytokines lead to MLC phosphorylation in epithelial cells, they signal through two independent pathways. Whereas TNF activates the MLCK *via* NF-κB, IFNγ increases the expression of Rho-associated kinase, which leads to the phosphorylation of the MLC (Capaldo and Nusrat, 2009). Furthermore, in several studies, the pure effect of IFNγ on epithelial barrier function has been examined and has been shown partly to differ from the effect of incubation with TNF (Capaldo and Nusrat, 2009). In a study by Zeissig *et al.* (2007), for example, incubation with IFNγ even led to the decreased expression of the pore-forming claudin-2, whereas exposure to TNF strongly enhanced this expression.
The work described in the present thesis has therefore focused on the single effect of TNF on epithelial barrier function without (pre-)incubation with IFNγ in order to analyze the unadulterated impact on claudin composition.

Importance of TNF research in human and veterinary medicine

As outlined in the present thesis, an increased expression of TNF leads to changes in claudin composition that result in severe barrier disturbance. The TJ discontinuity during inflammatory conditions results in increased uptake of bacterial toxins, antigens, and substances usually retained by the physiological epithelial barrier (Lu *et al.,* 2013).
However, not only an increased antigen uptake but also a dysbiosis following perturbed barrier function can lead to multiple inflammatory diseases (Toor *et al.,* 2019). Hence, the gut microbiome and the gut-brain axis are the subjects of many current studies. Dysbiosis, associated with increased cytokine levels and an altered epithelial barrier function, has been

demonstrated to cause not only diseases originating in the gastrointestinal tract, but also neurological disorders such as depression, Alzheimer's disease, or epilepsy (Mitrea *et al.,* 2022). Chronically increased stress levels, on the other hand, have been shown to disturb the gut microbiome, possibly resulting in intestinal inflammatory diseases such as IBD (Mitrea *et al.,* 2022). A recent review by Souders *et al.* (2022) has summarized the interplay of TNF and the gut microbiota, outlining the importance of TNF in mediating gut dysbiosis and inflammation.

In conclusion, a healthy microbiome can bias the progression of numerous diseases. It is therefore of major interest with regard to not only therapy those disorders, but also to prevent their development by the establishment of a healthy gut microbiome. Therefore, the effects of secondary plant compounds on the gut microbiome and intestinal inflammation are the subject of ongoing research. Recently, beneficial effects of plant compounds, such as curcumin (Ohno *et al.,* 2017) or caffeic acids (Zhang *et al.,* 2016), have been shown on the gut microbiota. However, the interpretation of such results requires detailed and precise knowledge of the effects of cytokines, such as TNF, on epithelial barrier function.

As previously been outlined in the literature review (**Chapter 2**), the effects of TNF on intestinal health also play a critical role in veterinary medicine, as PWD leads to enormous economic losses worldwide and is associated with suffering in pigs (Fairbrother *et al.,* 2005). Although the etiology of PWD is multifactorial, it is generally associated with *Escherichia coli* and is therefore treated with antibiotics. Because of the growing bacterial resistance to a broad range of commonly used antibiotics, suitable alternatives for medicating diarrheal diseases in pigs are urgently required (Rhouma *et al.,* 2017). However, in-depth knowledge of the impact of TNF on the pathomechanism of multifactorial PWD is prerequisite for the investigation of alternative treatment options (Girard and Bee, 2020).

Therefore, the use of porcine models for analyzing gastrointestinal barrier dysfunction caused by inflammation shows versatility, not only as a model for human gastrointestinal physiology, but also for detailed research of porcine intestinal health.

Conclusion

In both studies described in the present thesis, a weakening effect of TNF on the porcine intestinal epithelial barrier function has been shown, even though the pathways of barrier disturbance differ in the two models.

Incubation of IPEC-J2 cells with TNF led to barrier disruption by (i) the decreased expression of sealing TJ proteins claudin-1, -3, and occludin, and (ii) the up-regulation of the specific receptor TNFR-1. Both could mostly be prevented by co-incubation with ML-7, a specific MLCK blocker. The activation of MLCK under inflammatory conditions causes barrier impairment *via* the low-capacity leak pathway, leading to an increased flux of macromolecules, as seen in IPEC-J2 cells after incubation with the cytokine. Furthermore, the exponential nature of inflammation in porcine intestinal epithelium was demonstrated because TNF caused a significantly increased expression of its specific receptor TNFR-1.

The second study has also revealed a significant effect of TNF on porcine intestinal barrier function *ex vivo* and has further confirmed the molecular and functional differences between VE and PP under inflammatory conditions. Whereas VE tissue samples did not respond to the addition of TNF, PP FAE showed a dysfunctional barrier represented by a decreased TER and an altered TJ composition. However, in contrast to *in vitro* experiments, no effect on [^{3}H]-D-Mannitol flux has been observed, most likely because of an activation of the high-capacity pore pathway in FAE. Furthermore, an increased expression of TNFR-2 has been determined in PP samples, emphasizing the immunological importance of this structure.

Despite the limitations and differences between the *in vitro* and *ex vivo* experiments described in the present thesis, both the models are highly suitable for inflammatory investigations. These findings are essential for further studies of potentially harmful or beneficial substances that might trigger or prevent intestinal inflammation and pave the way for future approaches for preventing and treating intestinal inflammatory diseases in humans and pigs.

Chapter 7: Summary

Summary of the thesis:

Effects of the pro-inflammatory cytokine TNF on functional and molecular barrier properties of porcine intestinal epithelial models

One of the major proteins involved in the pathogenesis of inflammatory diseases of the intestine is tumor necrosis factor (TNF), a pro-inflammatory cytokine that can disturb epithelial barrier function by altering the composition of tight junction (TJ) proteins, such as claudins (Mullin and Snock, 1990). A disbalance of sealing and pore-forming claudins in impaired intestinal barrier function attributable to TNF is often characterized by an uncontrolled permeability to toxins and bacteria, resulting in diarrhea. Nevertheless, the detailed functional and molecular mechanisms of TNF-induced barrier disturbance remain the focus of current research in order to provide improved preventive and therapeutical approaches in the future.
Because of the morphological and nutritional similarities between the gastrointestinal tract of pigs and humans, porcine models are suitable for analyzing both pig and human intestinal diseases. However, to date, only limited information regarding TNF effects on porcine epithelial barrier function is available.
Therefore, the objective of the present work has been to analyze the functional and molecular impact of the pro-inflammatory cytokine on epithelial barrier function in porcine models. Subsequent to experiments, the effects of TNF on barrier function have been examined more closely, by paying special attention to TJ proteins and TNF signaling. These aspects have been assessed by using two distinct models as explained in the following.

1. The non-transformed cell line IPEC-J2

During the first study described in the present thesis, barrier-disturbing effects of TNF on the porcine intestinal epithelium were examined *in vitro* by using IPEC-J2 cells. The IPEC-J2 cell line, which originated from the jejunal epithelium of a neonatal piglet, constitutes a well-established model for the analysis of gastrointestinal barrier function (Schierack *et al.,* 2006). Incubation with TNF led to a considerable disturbance of the epithelial barrier, represented by a decreased transepithelial resistance (TER) and an increased paracellular permeability to [^{3}H]-D-Mannitol. This barrier damage was accompanied by a significant reduction of the sealing TJ proteins claudin-1, -3, and occludin. Furthermore, incubation of IPEC-J2 cells with TNF led to an increase in the expression of specific TNF receptor-1

(TNFR-1), verifying the self-enhancing inflammatory effect under TNF treatment. Use of ML-7, a specific blocker of the myosin light chain kinase (MLCK), significantly prevented effects on TER and alterations in claudin-3 and TNFR-1 expression. Moreover, cells recovered from TNF-induced barrier loss, as 48 h after removal of the cytokine the TJ proteins claudin-1 and occludin were integrated once again at the functional lateral membrane of the cells. In addition, no significant changes in an ApoTox-Glo assay were observed, excluding an induction of apoptosis by TNF in IPEC-J2 cells.

The obtained data from this study explain (i) the barrier-disturbing effect of TNF on TJ composition, (ii) the involvement of MLCK in TNF-induced signaling in IPEC-J2, and (iii) the up-regulation of the specific TNFR-1, underlining the exponential nature of inflammation.

2. The follicle-associated epithelium of porcine Peyer's patches

The second part of the present work examined the effects of TNF on porcine intestinal tissue samples *ex vivo* by using the Ussing chamber technique. The experiments were conducted by using two different types of tissue, one being the immunological active Peyer's patch (PP), and the other one being the neighboring villus epithelium (VE), both obtained from adult pigs at a slaughterhouse. The PP is covered by the follicle-associated epithelium (FAE), whose primary purpose is to limit the uptake of antigens from the intestine, and which therefore requires an efficient epithelial barrier (Jung *et al.,* 2010). As one major restriction of the Ussing chamber technique is the experimental time limitation (Pearce *et al.,* 2018), an improved setup was established to analyze the effects of TNF on PP and VE for up to 10 h. An incubation of PP with TNF led to a significant decrease in TER after 8 h, whereas the paracellular flux to [^{3}H]-D-Mannitol was unaltered. Western blot analysis and immunostaining revealed a decrease of sealing claudin-1 and -4, whereas the pore-forming claudin-2 was significantly up-regulated and functionally integrated into the TJ structure. In contrast to *in vitro* experiments, a significant increase of TNFR-2 could be observed in PP FAE after addition of TNF. In adjacent VE, neither TER alterations, nor effects on TJ proteins or TNF receptors could be observed after incubation with the cytokine. This highlights the important role of FAE in intestinal mucosal defense.

Zusammenfassung der Dissertation:

Auswirkungen des proinflammatorischen Zytokins TNF auf die funktionellen und molekularen Barriereeigenschaften von porzinen intestinalen epithelialen Modellen

Eines der wichtigsten Proteine, das an der Pathogenese entzündlicher Darmerkrankungen beteiligt ist, ist der Tumornekrosefaktor (TNF), ein proinflammatorisches Zytokin welches die Barrierefunktion stören kann indem es die Zusammensetzung von Tight Junction (TJ) Proteinen wie Claudinen verändert (Mullin und Snock, 1990). Ein Ungleichgewicht der abdichtenden und porenbildenden Claudine bei einer gestörten intestinalen Barrierefunktion aufgrund von TNF ist häufig durch eine unkontrollierte Permeabilität für Toxine und Bakterien gekennzeichnet, was zu Durchfall führt. Um zukünftige präventive und therapeutische Ansätze zu verbessern, stehen die detaillierten funktionellen und molekularen Mechanismen der TNF-induzierten Barrierestörung im Mittelpunkt der aktuellen Forschung.
Aufgrund der morphologischen und nutritiven Ähnlichkeiten zwischen dem Magen-Darm-Trakt von Schweinen und Menschen eignen sich Schweinemodelle gut für die Untersuchung von sowohl menschlichen, als auch porzinen Darmerkrankungen. Bislang liegen allerdings nur wenige Informationen über die Auswirkungen von TNF auf die epitheliale Barrierefunktion von Schweinen vor.
Ziel der vorliegenden Arbeit war es, die funktionellen und molekularen Effekte des proinflammatorischen Zytokins auf die epitheliale Barrierefunktion von porzinen Modellen zu analysieren. Im Anschluss an die Experimente wurden die genauen Auswirkungen von TNF auf die Barrierefunktion untersucht, mit besonderem Augenmerk auf die TJ Proteine und den TNF Signalweg. Dies wurde anhand von zwei verschiedenen Modellen untersucht, welche im Folgenden erläutert werden.

1. Die nicht-transformierte Zelllinie IPEC-J2

Während der ersten Studie, die in der vorliegenden Arbeit beschrieben wird, wurden die barrierestörenden Effekte von TNF auf das porzine intestinale Epithel *in vitro* durch die Verwendung von IPEC-J2 Zellen untersucht. Die Zelllinie IPEC-J2 stammt aus dem jejunalen Epithel eines neugeborenen Ferkels und stellt ein gut etabliertes Modell für die Analyse der gastrointestinalen Barrierefunktion dar (Schierack *et al.,* 2006). Eine Inkubation mit TNF führte zu einer erheblichen Störung der epithelialen Barriere, die sich in einem verringerten transepithelialen Widerstand (TER), sowie einer erhöhten parazellulären Permeabilität von [^{3}H]-D-Mannitol zeigte. Diese Barriereschädigung ging mit einer signifikanten Abnahme der abdichtenden TJ Proteine Claudin-1, -3 und Occludin einher. Darüber hinaus führte die

Inkubation von IPEC-J2 Zellen mit TNF zu einem Anstieg der Expression des spezifischen TNF Rezeptors-1 (TNFR-1), was die selbstverstärkende Wirkung von Entzündungsreaktionen unter TNF bestätigt. Durch die Verwendung von ML-7, einem spezifischen Blocker der Myosin-leichte-Ketten-Kinase (MLKK), konnten die Auswirkungen auf den TER, sowie die veränderte Expression von Claudin-3 und TNFR-1 verhindert werden. Des Weiteren waren die Zellen in der Lage, sich von der TNF-induzierten Barrierestörung zu erholen, indem 48 Stunden nach Entfernung des Zytokins die TJ Proteine Claudin-1 und Occludin wieder in die funktionelle laterale Zellmembran integriert wurden. Darüber hinaus konnten keine signifikanten Veränderungen in einem ApoTox-Glo-Assay beobachtet wurden, weshalb eine Apoptoseinduktion in den IPEC-J2-Zellen durch TNF ausgeschlossen werden konnte.
Die in dieser Studie gewonnenen Daten erklären (i) die barrierestörende Wirkung von TNF auf die TJ Zusammensetzung, (ii) die Beteiligung der MLKK an dem TNF-induzierten Signalweg in IPEC-J2 Zellen und (iii) die Hochregulierung des spezifischen TNFR-1, was die exponentielle Natur von Entzündungen verdeutlicht.

2. Das follikel-assoziierte Epithel der porzinen Peyer‘schen Plaques

Im zweiten Teil der vorliegenden Dissertation wurden die Auswirkungen von TNF auf porzine Darmgewebeproben *ex vivo* mithilfe der Ussing-Kammer Technik untersucht. Dazu wurden zwei verschiedene Gewebearten verwendet, zum einen die immunologisch aktiven Peyer'schen Plaques (PP) und zum anderen das benachbarte Villusepithel (VE), welche beide von erwachsenen Schweinen aus einem Schlachthof entnommen wurden. Die PP sind von dem Follikel-assoziierten Epithel (FAE) bedeckt, dessen Hauptaufgabe es ist, die Aufnahme von Antigenen aus dem Darm einzuschränken und dementsprechend eine effiziente epitheliale Barriere erfordert (Jung *et al.,* 2010). Eine wesentliche Beschränkung der Ussing-Kammer Technik ist die zeitliche Begrenzung der Experimente (Pearce et al., 2018), weshalb ein verbessertes Protokoll etabliert wurde um die Auswirkungen von TNF auf PP und VE für bis zu 10 Stunden zu analysieren. Eine Inkubation von PP mit TNF führte zu einer signifikanten Abnahme des TER nach 8 Stunden, während der parazelluläre Flux von [^{3}H]-D-Mannitol unverändert blieb. Western Blot Analysen und Immunfärbungen zeigten eine Abnahme der abdichtenden Claudine-1 und -4, während das porenbildende Claudin-2 signifikant hochreguliert und funktionell in die TJ Struktur integriert wurde. Im Gegensatz zu den *in vitro* Experimenten konnte im FAE nach Zugabe von TNF ein signifikanter Anstieg von TNFR-2 beobachtet werden. Im angrenzenden VE konnten nach Inkubation mit dem Zytokin weder Änderungen des TERs, noch Auswirkungen auf TJ Proteine oder TNF Rezeptoren beobachtet werden. Dies unterstreicht die bedeutende Rolle des FAEs für die intestinale mukosale Abwehr.

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List of publications

Publications (peer reviewed)

2022

Droessler L, Cornelius V, Boehm E, Stein L, Brunner N, Amasheh S

Barrier Perturbation in Porcine Peyer's Patches by Tumor Necrosis Factor is Associated With a Dysregulation of Claudins

Frontiers in Physiology, 13:889552

Doi: 10.3389/fphys.2022.889552

Cornelius V, Droessler L, Boehm E, Amasheh S

Concerted action of berberine in the porcine intestinal epithelial model IPEC-J2: Effects on tight junctions and apoptosis

Physiological Reports, 10(7):e15237

Doi: 10.14814/phy2.15237

2021

Bekusova V, Droessler L, Amasheh S, Markov AG

Effects of 1,2-Dimethylhydrazine on Barrier Properties of Rat Large Intestine and IPEC-J2 Cells

International Journal of Molecular Sciences, 22(19):10278

Doi: 10.3390/ijms221910278

Droessler L, Cornelius V, Markov AG, Amasheh S

Tumor Necrosis Factor Alpha Effects on the Porcine Intestinal Epithelial Barrier Include Enhanced Expression of TNF Receptor 1

International Journal of Molecular Sciences, 22(16):8746.

Doi: 10.3390/ijms22168746

Abstracts in proceedings & participation in conferences

2023

Boehm E, Droessler L, Amasheh S

Effects of TNF and Cannabidiol on porcine intestinal IPEC-J2 cells

77. Tagung der Gesellschaft für Ernährungsphysiologie, Göttingen – 07.03. – 09.03.2023

In: Proceedings of the Society of Nutrition Physiology – Gesellschaft für Ernährungsphysiologie (Hrsg.) 32, p. 85, ISBN: 978-3-7690-4116-3

2022

Boehm E, Droessler L, Amasheh S

Analysis of cannabidiol effects on the epithelial barrier of porcine intestinal IPEC-J2 cells

DVG Fachgruppentagung Physiologie und Biochemie Gießen – 24. – 26.06.2022

2021

Droessler L, Cornelius V, Markov AG, Amasheh S

TNFa induces epithelial barrier perturbation by a self-enhancing effect on TNFR-1 in porcine jejunal epithelial cells

100th Meeting of the German Physiological Society, Frankfurt am Main 30.09. – 02.10.2021

In: 100th annual meeting of the German Physiological Society – Deutsche Physiologische Gesellschaft (Hrsg.) p. 450

Droessler L, Cornelius V, Amasheh S

TNFα-effects on tight junction proteins in IPEC-J2 cells are associated with changes in TNFR-1 and MLCK

4th International Tight Junction Conference, Berlin 27. – 29.09.2021

Droessler L, Cornelius V, Amasheh S

TNFα induced changes in the composition of tight junction proteins in follicle-associated epithelium of porcine Peyer's Patches

75. digitale Tagung der Gesellschaft für Ernährungsphysiologie, Berlin – 16.03.-18.03.2021

In: Proceedings of the Society of Nutrition Physiology – Gesellschaft für Ernährungsphysiologie (Hrsg.) 30, p. 110, ISBN: 978-3-7690-4114-9

2020

Droessler L, Cornelius V, Amasheh S

IPEC-J2 cells as a model to investigate the effects of TNFα on porcine intestinal epithelial barrier function

74. Tagung der Gesellschaft für Ernährungsphysiologie, Göttingen – 03.03.-05.03.2020

In: Proceedings of the Society of Nutrition Physiology – Gesellschaft für Ernährungsphysiologie (Hrsg.) 29, p. 119, ISBN: 978-3-7690-4113-2

Danksagung

Ich möchte mich bei **Prof. Dr. Salah Amasheh** für die Möglichkeit zur Promotion am Institut für Veterinär-Physiologie bedanken. Durch dich habe ich gelernt kleine Hürden und Probleme nicht als solche zu sehen, sondern diese mit Leichtigkeit zu bewältigen und daran zu wachsen. Vielen Dank insbesondere für dein entgegengebrachtes Vertrauen und deine wissenschaftliche Betreuung während meiner Dissertation.

Bei **Prof. Dr. Alexander G. Markov** möchte ich mich für die Möglichkeit zur internationalen Kooperation bedanken. Die wertvolle Unterstützung während der Bearbeitung meines Promotionsthemas war sehr hilfreich für mich.

Vielen Dank an **Katharina Söllig** für die fachliche und mentale Unterstützung im Labor, an **Susanne Trappe** für die herzliche Zusammenarbeit in der Zellkultur und an **Martin Grunau** für die unermüdliche Hilfe während stundenlangen Versuchen in der Ussing Kammer. Ohne eure Geduld, sowie eurer fachlichen Expertise, wäre die Erarbeitung meiner Dissertation nicht möglich gewesen.

Danke auch an meine Arbeitsgruppen Kolleginnen **Elisa**, **Laura**, **Nora** und **Valeria** für den tollen fachlichen als auch privaten Austausch, sowie die gemeinsamen Kuchen-Pausen. Des Weiteren möchte ich meinen KollegInnen aus dem **Institut für Veterinär-Physiologie** für die herzliche Aufnahme, die netten Gespräche und die schönen Feiern danken. Ich hätte mir kein besseres Institut für eine Promotion vorstellen können.

Ganz besonders möchte ich mich bei meiner Familie für ihre Unterstützung bedanken. Vielen Dank an meine Eltern **Mona** und **Andreas**, die immer an mich geglaubt und mir das Tiermedizin-Studium ermöglicht haben. Darüber hinaus möchte ich **Marcus** für die liebevolle Unterstützung und den Rückhalt, den du mir während des Studiums und der Promotion gegeben hast, bedanken. Besonders in schweren Zeiten wart ihr immer unterstützend an meiner Seite und habt mich nie meinen Humor verlieren lassen, wofür ich euch von ganzem Herzen dankbar bin.

Finanzierungsquellen

Die vorliegende Arbeit wurde durch die Förderung der Deutschen Forschungsgemeinschaft (DFG) finanziert, Förderungsnummer: AM141/11-2.

Interessenkonflikte

Im Rahmen dieser Arbeit bestehen keine finanziellen oder kommerziellen Interessenkonflikte.

Selbstständigkeitserklärung

Hiermit bestätige ich, dass ich die vorliegende Arbeit selbstständig angefertigt habe. Ich versichere, dass ich ausschließlich die angegebenen Quellen und Hilfen in Anspruch genommen habe.

Berlin, den 02.03.2023

Linda Drößler