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

Inflammasomes as potential mediators of probiotic effects in porcine intestinal immune and epithelial cells

Inaugural-Dissertation
zur Erlangung des Grades eines
Doktors der Veterinärmedizin
an der
Freien Universität Berlin

vorgelegt von
Henriette Loß
Tierärztin aus Schwedt/Oder

Berlin 2019
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Deskriptoren (nach CAB-Thesaurus): pigs, inflammation, probiotics, digestive tract, intestinal mucosa, interleukins, enterotoxigenic *Escherichia coli*, *Enterococcus faecium*

Tag der Promotion: 20.08.2019

Meinen Eltern

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List of abbreviations

AIM2	Absent in melanoma 2
ASC	Apoptosis-associated speck-like protein containing a CARD
ATP	Adenosine triphosphate
CARD	Caspase-recruitment domains
CIITA	MHC class II transactivator
DAMP	Damage-associated molecular patterns
DC	Dendritic cells
E. coli	*Escherichia coli*
E. faecium	*Enterococcus faecium*
ELISA	Enzyme-linked immunosorbent assay
ETEC	Enterotoxigenic *E. coli*
FAO	Food and Agriculture Organization of the United Nations
Fig.	Figure
GALT	Gut-associated lymphoid tissue
GM-CSF	Granulocyte-macrophage colony-stimulating factor
ICE	Interleukin-1β-converting enzyme
IEC	Intestinal epithelial cells
Ig	Immunoglobulin
IL	Interleukin
IPAF	ICE-protease activating factor
LPS	Lipopolysaccharide
LRR	Leucine-rich repeats
MACS	Magnetic-activated cell sorting
M cells	Microfold cells
MHC	Major histocompatibility complex
MoDC	Monocyte-derived DC
NLR	NOD-like receptors
NLRP	NACHT, LRR and PYD domains-containing
NOD	Nucleotide-binding and oligomerization domain
PAMP	Pathogen-associated molecular patterns
PBMC	Peripheral blood mononuclear cells
PRR	Pattern recognition receptors
PRRSV	Porcine reproductive and respiratory syndrome virus
PYD	Pyrin domains
ROS	Reactive oxygen species
RT-qPCR	Real-time quantitative polymerase chain reaction
SLA	Swine leukocyte antigen
TEER	Transepithelial electrical resistance
TGF	Transforming growth factor

TLR	Toll-like receptors
TSLP	Thymic stromal lymphopoietin
WHO	World Health Organization

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Chapter 1: Introduction

At weaning, piglets have to cope with several stressors: the animals are weaned from the milk of the sow onto dry feeding and undergo social and environmental changes, all of which result in deficient feed consumption (Pluske et al. 1997). In addition, the immaturity of the immune system of young pigs contributes to an increased susceptibility to intestinal disorders (Lalles et al. 2004). Weaning-associated alterations in gastrointestinal morphology, such as a restricted villus function, are accompanied by maldigestion and malabsorption, which entails, among others, the proliferation of and colonization with enteropathogenic *Escherichia coli* (*E. coli*) (van Beers-Schreurs et al. 1992). Together with the aforementioned contributing factors, these enteric infections result in post-weaning diarrhea followed by impaired growth rates or mortalities (Fairbrother et al. 2005; Heo et al. 2013).

Over the past few decades, the incidence of antimicrobial resistance, particularly in *E. coli* isolates related to post-weaning diarrhea, has increased, a finding that emphasizes the urgent need for alternative strategies to the use of antibiotics (Fairbrother et al. 2005). Furthermore, this poses a considerable danger for human health because of the potential transfer of resistant bacteria and their resistance genes (Witte 2000; Wegener 2003). This development led to a ban of the usage of in-feed antibiotics as antibiotic growth promoters in the European Union in 2006. In piglet nutrition, probiotic supplementation represents a promising strategic alternative to antibiotic growth promoters; however, evidence regarding its efficacy is often inconsistent, and underlying mechanisms are not well understood (Jensen 1998; Heo et al. 2013; Pluske 2013). The probiotic *Enterococcus faecium* (*E. faecium*) strain NCIMB 10415 is authorized as a feed additive for piglet and sow nutrition in the European Union. Amongst other beneficial effects, feeding trials with *E. faecium* NCIMB 10415 have demonstrated its ability to reduce the incidence and duration of post-weaning diarrhea (Taras et al. 2006), to affect piglet's performance positively (Zeyner and Boldt 2006), and to inhibit intestinal colonization with pathogenic *E. coli* (Scharek et al. 2005).

Being an essential part of the innate system, immune receptors, such as NOD (nucleotide-binding and oligomerization domain)-like receptors (NLR) sense invading gut bacteria as a first-line defense mechanism. Some members of this receptor family are able to form multimeric complexes, called inflammasomes (Tschopp et al. 2003). In the intestinal tract, inflammasome-forming NLR participate in the crosstalk between intestinal epithelial cells (IEC) and underlying cells, including immune cells (Parlato and Yeretssian 2014). Within the latter cell population, dendritic cells (DC) contribute substantially to the orchestration of downstream immune responses (Kelsall and Leon 2005). Apart from the critical role of inflammasomes in host defense against enteropathogens (Chen and Nunez 2011), the inflammasome signaling pathway has been shown to be involved in the pathogenesis of intestinal diseases, such as inflammatory bowel disease (Zaki et al. 2011). Therefore, a better understanding of inflammasome signaling might be helpful for unraveling the coordination of immunological processes by intestinal immune and non-immune cells and for finding new therapeutic approaches for the treatment of intestinal disorders. In contrast to mice and man,

inflammasome research in pigs is still in its infancy. Inflammasome signaling in the context of post-weaning diarrhea and its contribution to probiotic effects of *E. faecium* NCIMB 10415 has not yet been investigated.

The focus of the work presented in the current thesis was the inflammasome response to an enterotoxigenic *E. coli* (ETEC) strain with relevance for post-weaning diarrhea and to probiotic *E. faecium* NCIMB 10415. To this end, inflammasome expression was analyzed in porcine DC and IEC *in vitro* and in various porcine intestinal tissues *ex vivo*. A further aim was the establishment of an *in vitro* intestinal co-culture model to investigate the influence of the bidirectional interplay between IEC and DC on the outcome of these inflammasome analyses.

Chapter 2: Literature review

2.1 The porcine intestinal immune system

Of all mucosa-associated lymphoid tissues, the largest mucosal immune system is found in the gastrointestinal tract (Holmgren et al. 1992). The gut-associated lymphoid tissue (GALT) comprises variously arranged structures: *(i)* Peyer's patches and discrete lymphoid follicles, *(ii)* diffusely distributed immune cells in the *lamina propria*, and *(iii)* intraepithelial lymphocytes of the villous epithelium (Nagura and Sumi 1988). Similar to those of other species of veterinary interest, porcine Peyer's patches are organized as, first, numerous isolated Peyer's patches in the jejunum and, second, a long continuous Peyer's patch in the ileum; these patches differ in their structural und functional characteristics (Chu and Liu 1984; Binns and Licence 1985). Directly after birth, the intestinal mucosa of piglets is equipped with only a small number of immune cells, but during the following weeks, piglets acquire immune competence by developing specific B and T cell populations and other antigen-presenting cells (Rothkotter et al. 1991; Bianchi et al. 1992; Vega-Lopez et al. 1995). Since pigs have an epitheliochorial type of placenta, it is impermeable to macromolecules, such as antibodies and antigens, a feature that in turn contributes to the immunological naivety of newborn pigs (Bailey and Haverson 2006). Moreover, in the early postnatal period, the microbiota colonizing the gut has a significant impact on the maturation of the intestinal immune system (Butler et al. 2000; Bailey et al. 2005). As for other immune organs, innate and adaptive defense mechanisms have to be distinguished within the gut. In the following sections, the former mechanisms will be addressed in more detail.

Microorganisms invading the gut lumen carry specific conserved microbial structures (Janeway 1992) that are known as pathogen-associated molecular patterns (PAMP) and that are detected through germline-encoded pattern recognition receptors (PRR) (Medzhitov and Janeway 1997). In addition to PAMP, PRR also sense damage (or danger)-associated molecular patterns (DAMP) originating from the host (Matzinger 1994). To widen the model that was introduced by Charles Janeway and that allows the differentiation between "infectious non-self" and "non-infectious self", Matzinger's model proposes the consideration of signals derived from injured cells and tissues of the body. Furthermore, the latter model provides an explanation for the identification of indigenous commensals or other non-pathogenic microorganisms as such. In general, PRR can be classified into four main groups that differ in their cellular localization: *(i)* Toll-like receptors (TLR) (location: extracellular), *(ii)* C-type lectin receptors (extracellular), *(iii)* retinoic acid-inducible gene-I-like receptors (intracellular), and *(iv)* NLR (intracellular) (Takeuchi and Akira 2010). In addition to the individual receptor families, a complex interplay occurs, especially between TLR and NLR, with respect to, for example, redundant signaling pathways (Becker and O'Neill 2007). Within the intestinal immune system, PRR-expressing cells are not only immune cells, but also non-immune cells of the gut epithelium (Wells et al. 2010). Studies of the mucosal immunology of pigs have revealed high expression levels of porcine TLR2 and TLR9 in mesenteric lymph nodes and ileal Peyer's

patches, which have been verified in newborn piglets, suggesting a role in defense mechanisms against bacteria in early life (Tohno et al. 2005; Tohno et al. 2006). Moreover, the greatest expression of NOD2 mRNA in neonatal animals has been found in mesenteric lymph nodes and spleen, whereas in adult pigs, the highest NOD2 levels have been detected in ileal Peyer's patches (Tohno et al. 2008).

The structure of the aforementioned NLR includes a NACHT (or NOD) domain in the center that is surrounded by C-terminal leucine-rich repeats (LRR) and N-terminal caspase-recruitment (CARD) or pyrin (PYD) domains (Tschopp et al. 2003). This receptor family can be further divided into four subfamilies based on the phylogenetic studies of their NACHT domains and on functional aspects: the CIITA (major histocompatibility complex [MHC] class II transactivator), NOD, NLRP (NACHT, LRR and PYD domains-containing) (also abbreviated as NALP), and IPAF (ICE [interleukin-1β-converting enzyme]-protease activating factor) subfamilies (Tschopp et al. 2003).

2.1.1 Inflammasomes

Upon their activation, some members of the NLR family form intracellular multiprotein complexes with ASC (apoptosis-associated speck-like protein containing a CARD) adapter proteins and caspases (previously referred to as ICE), which together are then called inflammasomes (Martinon et al. 2002). The term inflammasome is a compound of the word "inflammation" describing its function and the suffix "some" that is used to name molecular complexes, e.g., proteasome or ribosome (Martinon et al. 2009). Various cell types have been ascertained to express inflammasomes, mainly myeloid cells, such as DC (Kummer et al. 2007; Guarda et al. 2011). In addition to immune cells, epithelial cells (Abdul-Sater et al. 2009; Shigeoka et al. 2010; Zaki et al. 2010), keratinocytes (Feldmeyer et al. 2007), and adipocytes (Stienstra et al. 2010) are equipped with functional inflammasomes. The first-discovered inflammasome was the NLRP1 inflammasome (Martinon et al. 2002). The most extensively studied of all inflammasomes in mice and humans is the NLRP3 inflammasome, also known as cryopyrin or NALP3 (Schroder and Tschopp 2010; Lissner and Siegmund 2011; Yeretssian 2012), which is the subject of the following chapter. The NLRP6 inflammasome is of particular relevance in the intestinal tract, because here NLRP6 inflammasome signaling supports intestinal homeostasis and is modulated by the microbial composition (Chen et al. 2011; Elinav et al. 2011; Levy et al. 2015). Other examples of well-defined inflammasomes are the AIM2 (absent in melanoma 2) inflammasome sensing cytosolic DNA (Muruve et al. 2008; Fernandes-Alnemri et al. 2009; Hornung et al. 2009) and the NLRC4 inflammasome responsive to bacterial flagellin and type 3 secretion systems (Mariathasan et al. 2004; Franchi et al. 2006; Miao et al. 2010; Zhao et al. 2011).

The NLR signal is executed *via* caspases. Since caspases are a group of cysteine proteases that are initially generated in the form of inactive zymogens, they need to undergo proteolytic cleavage to be activated (Cohen 1997). Once activated, caspase-1 forms a tetramer comprising two heterodimers of the subunits p20 and p10 (Wilson et al. 1994). During inflammasome activation, enzymatically active caspases, primarily caspase-1, cause the

processing and subsequent secretion of the pro-inflammatory cytokines interleukin (IL)-1β and IL-18 and are therefore called inflammatory caspases (Nadiri et al. 2006). As shown in Figure (Fig.) 1, apart from this cytokine maturation, active caspase-1 can, at least under certain circumstances, lead to cell death. This caspase-1-induced inflammatory cell death, designated as pyroptosis or pyronecrosis, constitutes a level-dependent event (Saleh and Green 2007). The ASC adapter linking the NLR protein to caspase-1 is a fundamental component of the inflammasome, because it is necessary for caspase-1 activation, as first shown in mice (Mariathasan et al. 2004). As mentioned above, the primary outcome of inflammasome activation is the release of bioactive IL-1β and IL-18 after their maturation from precursor molecules (Mariathasan et al. 2006; Sutterwala et al. 2006). In particular, IL-1β acts as a key player in inflammation processes being an endogenous pyrogen produced at sites of infection and injury (Dinarello 1998). On the other hand, IL-18 is known to function as an interferon-γ inducer and to control T helper cell type 1 and T helper cell type 2 responses (Nakanishi et al. 2001).

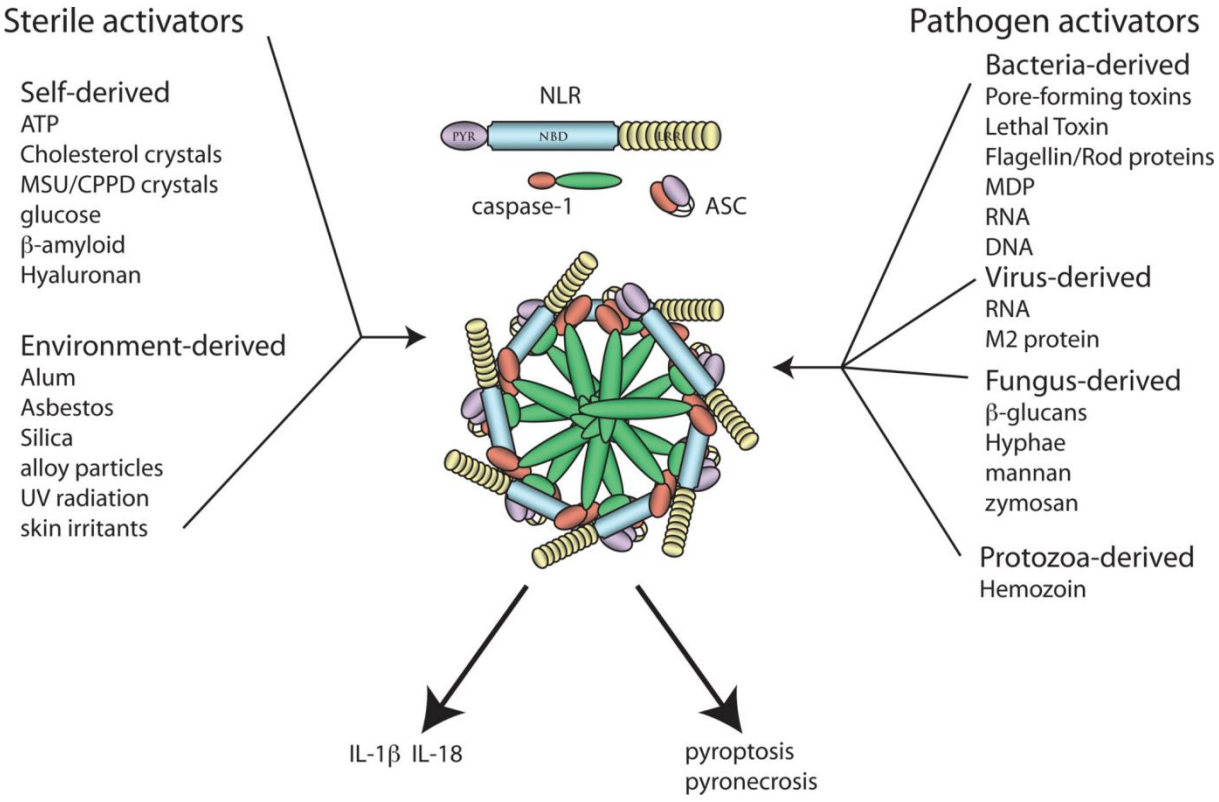

Figure 1: Schematic illustration of inflammasome activation (Davis et al. 2011)
During activation, a three-part complex consisting of the NLR, ASC, and caspase-1 is assembled. This multimerization in turn leads to the enzymatic conversion of pro-IL-1β and pro-IL-18 into their mature forms through caspase-1; they are then released. Furthermore, inflammasome activation can also trigger an inflammatory form of cell death, called pyroptosis or pyronecrosis. A distinction of inflammasome activators is drawn based on their diverse nature. One distinguishes between self- and environment-derived factors, summarized as sterile activators, and pathogen-derived signals originating from bacteria, viruses, fungi, and protozoa (Davis et al. 2011).

In the past few years, apart from this caspase-1-dependent canonical inflammasome activation, a caspase-1-independent non-canonical inflammasome activation pathway has been shown to be present in mice (Kayagaki et al. 2011) and humans (Casson et al. 2015; Vigano et al. 2015). In particular, murine caspase-11 (Kayagaki et al. 2011) and human caspase-4 and -5 (Casson et al. 2015; Vigano et al. 2015) mediate non-canonical inflammasome signaling. However, the underlying mechanisms of non-canonical inflammasome activation in the sense of an upstream (Broz et al. 2012; Ruhl and Broz 2015) or downstream mode of activation for corresponding caspases (Kayagaki et al. 2011; Rathinam et al. 2012) are not yet clarified. In the aforementioned studies, non-canonical inflammasome activation has been verified upon stimulation with Gram-negative bacteria, e.g., *Vibrio cholerae*, *Citrobacter rodentium*, *E. coli*, and *Salmonella* Typhimurium (Kayagaki et al. 2011; Casson et al. 2015). In bovine cells, caspase-13 is considered as the counterpart of human caspase-4 (Koenig et al. 2001). To date, the non-canonical inflammasome has not been described in pigs. In the current thesis, caspase-13 is proposed as a promising candidate targeting non-canonical inflammasome activation in pigs.

Within the classical concept of canonical inflammasome signaling, a wide variety of possible inflammasome activators has been determined and can be divided in two categories: sterile and pathogenic activators (Davis et al. 2011). In the first category, self- and environment-derived signals can be further discriminated (see Fig. 1). Extracellular adenosine triphosphate (ATP) is a well-known stimulus that drives inflammasome activation (Mariathasan et al. 2006; Martinon et al. 2006; Sutterwala et al. 2006; Duncan et al. 2007; Franchi et al. 2009) *via* the purinergic $P2X_7$ receptor evoking a potassium efflux (Perregaux and Gabel 1994). Among other extrinsic activators, silica crystals (Cassel et al. 2008; Dostert et al. 2008; Hornung et al. 2008) and aluminum salts (Eisenbarth et al. 2008; Franchi and Nunez 2008; Kool et al. 2008; Li et al. 2008) represent environmental signals that are capable of inducing inflammasome activation. Since inflammasomes are found intracellularly, pathogens (and other activators) have to gain access to the cytosol before an inflammasome response is initiated. For example, *Staphylococcus aureus* (Mariathasan et al. 2006; Craven et al. 2009; Munoz-Planillo et al. 2009), *Listeria monocytogenes* (Mariathasan et al. 2006; Ozoren et al. 2006; Warren et al. 2008), and *E. coli* (Brereton et al. 2011; Sander et al. 2011; Zhang et al. 2012) have been demonstrated to affect inflammasome signaling. One of the first enteropathogenic bacteria for which inflammasome activation was documented in murine macrophages was *Salmonella* Typhimurium (Mariathasan et al. 2004; Franchi et al. 2006). In swine, *Salmonella* Typhimurium is a serotype of *Salmonella enterica* having a high prevalence in Europe and representing a substantial risk not only for pigs, but also for humans because of its zoonotic potential (Tran et al. 2018). To date, several bacterial exotoxins have been reported to possess the ability to mediate inflammasome assembly and activation by various mechanisms including pore-forming and enzymatic activity (Freche et al. 2007; Greaney et al. 2015). The cell wall component muramyl dipeptide, a naturally occurring degradation product of bacterial peptidoglycan, has also been identified as a potent activator of the inflammasome (Martinon et al. 2004; Pan et al. 2007; Marina-Garcia et al. 2008). In addition, inflammasomes

have previously been shown to be responsive to bacterial and viral RNA or DNA (Kanneganti et al. 2006a; Kanneganti et al. 2006b; Muruve et al. 2008; Hornung et al. 2009). Moreover, inflammasomes have also been implicated in the immune response to protozoa and fungi, e.g., *Candida albicans* (Hise et al. 2009; Joly et al. 2009; Tomalka et al. 2011) and *Aspergillus fumigatus* (Said-Sadier et al. 2010).

In the following, the mechanisms governing inflammasome activation will be described for the NLRP3 inflammasome that was of special interest for the present work.

2.1.1.1 The NLRP3 inflammasome

Recent research has revealed that malfunctioning NLRP3 inflammasome signaling is linked with an increased susceptibility to inflammatory bowel diseases, such as Crohn's disease (Zaki et al. 2011; Liu et al. 2017). Further, evidence suggests the involvement of inflammasomes in auto-inflammatory disorders, e.g., Muckle-Wells syndrome, whose cause is a mutation within the NLRP3 gene (Hoffman et al. 2001). In these patients, the NLRP3 inflammasome assembles spontaneously resulting in an unrestricted overproduction of IL-1β (Agostini et al. 2004).

The activation process of the NLRP3 inflammasome is commonly regarded as a two-step procedure containing a preceding priming step followed by a final activation step (Sutterwala et al. 2014). The priming signal, which can be displayed by several agents, e.g., lipopolysaccharide (LPS) or tumor necrosis factor α (Franchi et al. 2009), activates the nuclear factor NF-κB pathway (Bauernfeind et al. 2009). In response to this ubiquitous transcription factor, a strengthened synthesis of particular inflammasome components is provoked. Whereas the adapter ASC and pro-caspase-1 are considered to be constitutively expressed (Mariathasan and Monack 2007; Schroder et al. 2012), a transcriptional upregulation of pro-IL-1β, pro-IL-18 (Davis et al. 2011; Lissner and Siegmund 2011), and also of NLRP3 itself (Bauernfeind et al. 2009) is initially required. Under steady-state conditions, the last-mentioned is produced in insufficient quantities for inflammasome activation (Bauernfeind et al. 2009).

Subsequent to the priming, a second stimulus induces the assembly of inflammasome components, which finally provokes the autocatalytic cleavage of caspase-1. As previously illustrated, these signals can be of exogenous and endogenous origins. Notably, this multitude of unrelated activators appears to bind not directly to NLRP3, but rather to elicit a series of cellular alterations (see Fig. 2). For example, NLRP3 activation is linked with various ion fluxes, e.g., potassium efflux (Franchi et al. 2007; Petrilli et al. 2007) or calcium influx occurring either from the extracellular space or the endoplasmic reticulum into the cytoplasm (Lee et al. 2012; Murakami et al. 2012; Rossol et al. 2012). According to Sutterwala et al. (2014), uncertainty remains as to whether these cation currents are mandatory for the activation of the NLRP3 inflammasome. Other recognized mechanisms of inflammasome activation are the generation of reactive oxygen species (ROS) by virtually all PAMP and DAMP (Cassel et al. 2008; Dostert et al. 2008) and lysosomal damage attributable to the phagocytosis of crystalline and other particles leading to leakage of lysosomal content associated with the release of cathepsin B (Halle et al. 2008; Hornung et al. 2008). Moreover, the aforementioned $P2X_7$-dependent

potassium channel formation induced by ATP also leads to the insertion of pannexin-1 pores into the membrane enabling PAMP and DAMP to enter the cell (Pelegrin and Surprenant 2006; Kanneganti et al. 2007; Pelegrin and Surprenant 2007).

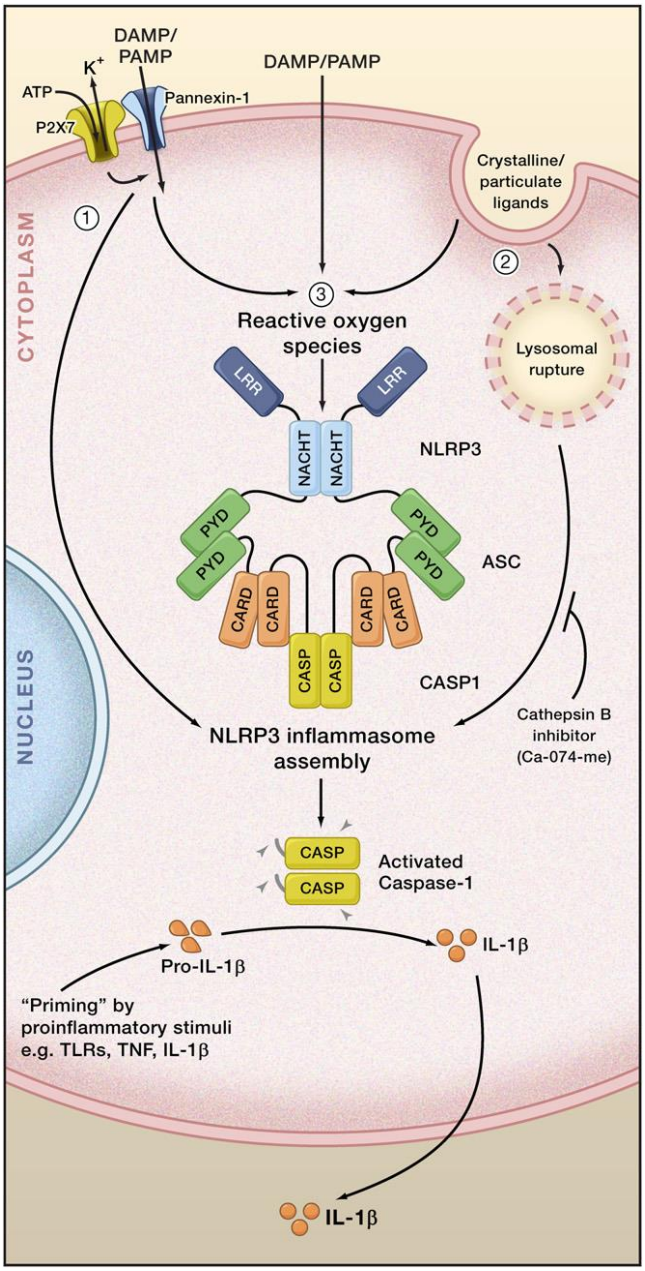

Figure 2: NLRP3 inflammasome activation and its underlying mechanisms (Schroder and Tschopp 2010).

(1) ATP- and $P2X_7$-induced pannexin-1 channeling, which promotes the entry of PAMP and DAMP acting as NLRP3-activating agonists. (2) After phagocytosis of crystalline and particulate compounds, lysosomal vesicles are ruptured resulting in the leakage out of, for example, cathepsin B. (3) The production of ROS also mediates NLRP3 inflammasome activation. Each of these proposed mechanisms finally entails the complex formation of the NLRP3 inflammasome. Following its autoactivation, caspase-1 processes the pro-inflammatory cytokines IL-1β and IL-18, which are subsequently liberated from the cell (Schroder and Tschopp 2010).

Apart from this conventional two-step model, inflammasome activation can also occur when both signals appear at the same time (Juliana et al. 2012; Fernandes-Alnemri et al. 2013; Lin et al. 2014a). Indeed, this fast type of inflammasome activation is organized by a post-translational (therefore transcription-independent) mechanism and involves, for example, IL-1 receptor-associated kinases (Fernandes-Alnemri et al. 2013; Lin et al. 2014a) or the myeloid differentiation primary response 88 pathway (Juliana et al. 2012). In this connection, recent research has revealed the deubiquitination of NLRP3 as an additional route relevant for the regulation of inflammasome activation (Juliana et al. 2012; Lopez-Castejon et al. 2013; Py et al. 2013). Furthermore, other studies have provided evidence for valid inflammasome activation attributable to bacterial infection without preceding priming (Wu et al. 2010; Bouwman et al. 2014). However, LPS priming increases the extent of the inflammatory reaction (Wu et al. 2010; Schroder et al. 2012). Despite such novel insights, many questions remain regarding the actual trigger mechanism of the cytosolic inflammasome sensor (Broz and Dixit 2016; Mathur et al. 2018; Schroder et al. 2018).

Although the majority of the knowledge about inflammasomes and related activation mechanisms has been obtained by using human or murine models, research into porcine inflammasomes is in its infancy (Kim et al. 2014). In general, porcine NLRP3 inflammasome activation has been demonstrated to follow similar patterns as those shown for the NLRP3 inflammasome in humans or mice (Kim et al. 2014). Several research groups have investigated the way in which the inflammasome signaling pathway in pigs is affected by viruses, such as porcine reproductive and respiratory syndrome virus (PRRSV) (Zhang et al. 2013; Bi et al. 2014; Koltes et al. 2015; Wang et al. 2015) and classical swine fever virus (Lin et al. 2014b; Fan et al. 2018a), which represent meaningful pathogenic agents in the pig industry. However, a detailed understanding of inflammasome signaling pathways in pigs is lacking. This seems incomprehensible, since the pig has been evaluated as an appropriate animal model for human research in various aspects including anatomical, genetic, and physiological features (Meurens et al. 2012; Swindle et al. 2012). Dawson et al. (2017) has recently reported greater similarities between the human and porcine immune-related genomes than those between the human and murine genetic constitutions; they have therefore declared the pig a suitable model for immunological investigations. In agreement with this observation, Tohno et al. (2011) have found a closer relationship of the porcine NLRP3 protein with the human counterpart than with murine NLRP3. In addition, murine and human inflammasomes each possess a different receptor and caspase composition and a unique manner of regulation (Schroder et al. 2018). Hence, corresponding verifications for porcine inflammasomes are required.

2.1.2 The intestinal barrier and the role of resident dendritic cells

The intestinal epithelium acts as a cross-linking agent between the GALT and the luminal content comprising a plethora of dietary and microbial antigens (Turner 2009). Although various sections of the intestine possess functional peculiarities, they have in common a polarized epithelial monolayer, a sealed barrier formed by tight junction proteins, a mucus layer covering the gut epithelium, and an interplay with resident immune cells (Rescigno 2011). In

addition to enterocytes, the epithelial barrier also includes mucin-producing goblet cells, Paneth cells generating anti-microbial peptides, and enterochromaffin cells constituting the major neuroendocrine cell population in the gut (Daneman and Rescigno 2009).

Intestinal DC modulate the intestinal equilibrium state and control the mucosal defense and, therefore, provide a sentinel function (Johansson and Kelsall 2005; Kelsall and Leon 2005; Bekiaris et al. 2014). In contrast to intraepithelial lymphocytes, resident DC are able to enter the gut lumen (Rescigno 2011). Mucosal DC cells sample antigens and bacteria that appear in the intestine by projecting their dendrites between IEC into the lumen (Niess et al. 2005; Chieppa et al. 2006). Since DC open enterocytic tight junctions and express tight junction proteins themselves, this is achieved with no disturbance of the barrier integrity (Rescigno et al. 2001). Another mechanism of antigen uptake involves M cells (microfold cells), which are located in the follicle-associated epithelium of Peyer's patches and which allocate antigens for underlying immune cells, including DC, *via* transcytosis across the intestinal epithelial layer (Bockman and Cooper 1973; Owen and Jones 1974; Wolf and Bye 1984). Subsequently, antigen-presenting DC undergo a maturation process and migrate to draining lymphoid tissues, where they initiate B and T cell responses (Banchereau and Steinman 1998). Recruitment sites of such migratory DC are the *lamina propria*, Peyer's patches, and solitary intestinal lymphoid tissues (Worbs et al. 2017). In a study on the phenotype and distribution of mucosal DC in the porcine small intestine, DC have been detected in the subepithelial dome region of Peyer's patches directly adjacent to M cells and a rare subset of *lamina propria* DC located within villi (Bimczok et al. 2006).

Migration of DC occurs constantly, even under steady-state conditions (Pugh et al. 1983; Liu and MacPherson 1993; Worbs et al. 2006; Wilson et al. 2008), whereas in inflammatory states, DC migration rates increase markedly (MacPherson et al. 1995; Yrlid et al. 2006; Vigl et al. 2011). In addition to foreign antigens, some DC are specialized in the transportation of apoptotic IEC to mesenteric lymph nodes, a specialization that probably preserves self-tolerance (Huang et al. 2000). Numerous studies have assigned the chemokine receptor CCR7 a key role in navigating DC to lymph nodes under both non-inflammatory and inflammatory conditions (MartIn-Fontecha et al. 2003; Ohl et al. 2004; Jang et al. 2006; Seth et al. 2011). In the course of maturation, CCR7 expression on DC is upregulated (Dieu et al. 1998; Sallusto et al. 1998) and is linked with an augmented responsiveness towards the two chemoattracting CCR7 ligands CCL19 and CCL21 (Yoshida et al. 1997; Campbell et al. 1998; Yoshida et al. 1998). CCL21 is expressed by stromal cells in T cell areas of lymphatic tissues and by endothelial cells of peripheral lymph vessels (Gunn et al. 1998; Willimann et al. 1998). By contrast, CCL19 can be produced by activated DC themselves (Ngo et al. 1998). The presence of CCL21 and CCL19 has also been documented in porcine intestinal mucosa (Bourges et al. 2007).

To shape their response patterns, IEC "condition" local DC *via* the liberation of soluble mediators (Iliev et al. 2007). Such epithelial-derived factors include the cytokine thymic stromal lymphopoietin (TSLP), which has been shown to direct affected human DC towards a non-inflammatory phenotype *in vitro* (Rimoldi et al. 2005b; Iliev et al. 2009b). *In vivo* investigations

have confirmed the relevance of TSLP for promoting tolerance in murine DC (Zaph et al. 2007). Similarly, transforming growth factor (TGF)-β has been identified as an IEC-delivered modulating agent that induces tolerogenic DC under steady-state conditions in mice (Iliev et al. 2009a). Zeuthen et al. (2008) have demonstrated that human IEC release more TSLP and TGF-β upon stimulation with commensal bacteria indicating the involvement of the gut microbiota in such complex interactions. In addition to TSLP and TGF-β, IEC-derived retinoic acid, a vitamin A metabolite, likewise contributes to the conversion of human (Iliev et al. 2009b) and murine (Iliev et al. 2009a) intestinal DC into a tolerogenic phenotype. Vitamin A is crucial for proper DC functions, e.g., the enhancement of intestinal homing receptors on lymphocytes and antibody class switching to immunoglobulin (Ig) A (Coombes and Powrie 2008). Furthermore, prostaglandins, such as PGE_2, which are *inter alia* produced by IEC, exert diverse impacts on mucosal dendritic and other immune cells (Iliev et al. 2007). Contractor et al. (2007) have highlighted the importance of soluble factors in the intestinal milieu and determined PGE_2, TGF-β, and IL-10 as being regulators controlling interferon production by plasmacytoid DC of murine Peyer's patches.

In summary, IEC and intestinal DC represent two essential cell populations in the gut collaborating to provide an intact intestinal barrier. On the one hand, tolerance towards commensal microbiota has to be ensured, whereas on the other hand, invading pathogens must be combated through adequate immune responses. Although the crosstalk between IEC and underlying immune cells has almost exclusively been studied in human and murine cells, mechanisms supporting IEC/DC interactions in pigs are largely unknown.

2.2 Post-weaning diarrhea and the use of probiotics

In the pig industry, piglets are usually weaned at 3 to 5 weeks (Lalles et al. 2007b), whereas under natural conditions, the weaning process is prolonged until the age of 14 to 17 weeks (Jensen 1986). Thus, the time around weaning is a stressful period associated with a high risk of infectious diseases, primarily diarrhea (Pluske et al. 1997). Factors, such as the change of diet, the separation from the mother sow and litter mates, and the movement into new surroundings, cause a reduction of feed intake resulting in an impaired growth rate (Pluske et al. 1997). This phase of underfeeding is variable in its duration and has an impact on the animal's metabolism, mainly its fat metabolism, and endocrine adaptations (Le Dividich and Seve 2000). In addition, the gut physiology undergoes a variety of morphological changes in the course of weaning, including villus atrophy and a reduced activity of brush border membrane enzymes (Smith 1984; Hampson 1986; Hampson and Kidder 1986). Moreover, the intestinal microbiota is subject to a maturation process, which implies alterations in its composition and diversity shaped by diet and other environmental influences (Wilbur et al. 1960; Jensen 1998; Konstantinov et al. 2004; Inoue et al. 2005). At weaning, the lack of IgA-containing milk from the sow and the exposure to new feed antigens constitute challenges for the development of the mature mucosal immune system (Bailey et al. 2005). Transient allergic reactions to dietary proteins (Miller et al. 1984; Wilson et al. 1989; Li et al. 1990; Bailey et al.

2001) and temporary increases of pro-inflammatory cytokine responses in the intestinal tract (Pie et al. 2004; Stokes et al. 2004) have been described.

As a consequence of these transitions, weaning piglets show a high susceptibility to enteric disorders (Lalles et al. 2007a; Campbell et al. 2013). Above all, dysbiosis of the intestinal microbiota is a pivotal factor for the pathogenesis of post-weaning diarrhea (Lalles et al. 2007a; Gresse et al. 2017). In particular, weaning is followed by a decrease of *lactobacilli* and the occurrence of a less diverse and rather instable microbiota, favoring the expansion of potential pathogenic bacteria (Konstantinov et al. 2006; Gresse et al. 2017). Within the multifactorial etiology of this disease, a rapid proliferation of ETEC strains is the most common agent causing post-weaning diarrhea (Nagy and Fekete 1999; Madec et al. 2000; Fairbrother et al. 2005). The major virulence factors of such strains comprise various fimbrial antigens, especially F18 and F4 (K88), responsible for adhesion to the intestinal mucosa, and heat-labile and heat-stable enterotoxins that induce fluid and electrolyte secretion into the gut lumen (Fairbrother et al. 2005; Dubreuil et al. 2016; Luppi et al. 2016). From an economic perspective, post-weaning diarrhea represents a considerable problem in the pig industry worldwide (Fairbrother et al. 2005).

Since 2006, antibiotic growth promoters have been banned within the European Union (EC Regulation No. 1831/2003) leading to the search for alternative approaches, such as the use of probiotics as feed additives (Allen et al. 2013; Ducatelle et al. 2015). In livestock farming, the most frequently used probiotic genera are *Lactobacillus*, *Enterococcus*, *Bacillus*, *Saccharomyces*, and *Bifidobacterium* (Gaggia et al. 2010). In pigs, the efficacy of probiotic dietary supplements is commonly assessed by means of the following criteria: weight increase, feed conversion ratio, and occurrence of diarrhea in suckling and weaning piglets (Simon et al. 2001). To date, studies on the beneficial effects of porcine probiotics have provided to some extent variable, but nevertheless promising results (Metzler et al. 2005; Roselli et al. 2005; Heo et al. 2013; Ducatelle et al. 2015).

In a feeding trial in weaning piglets, *Bacillus toyoi* and *Bacillus licheniformis* strains have been shown to be suitable for the treatment of post-weaning diarrhea, improving each of the abovementioned efficacy parameters (Kyriakis et al. 1999). Alexopoulos et al. (2001) have observed that supplementation with *Bacillus cereus* spores in pregnant and lactating sows and their litters beneficially affects the health status of the piglets. Suckling and weaning piglets fed with various strains of *Bifidobacterium* spp. and *Lactobacillus* spp. have revealed an enhanced daily weight gain (Abe et al. 1995). Administration of a *Bifidobacterium lactis* strain ameliorates *E. coli*- and rotavirus-induced diarrhea in piglets, augments the feed utilization, and promotes protective immune reactions (Shu et al. 2001). Probiotic treatment with *Lactobacillus sobrius* DSM 16698 leads to decreased ETEC concentrations in the ileum and also to higher increases in the weight of challenged piglets (Konstantinov et al. 2008). Similarly, positive effects on the growth performance of weaning piglets have been demonstrated for the probiotic yeast *Saccharomyces boulardii* (Bontempo et al. 2006).

2.2.1 Probiotics

The concept of probiotics has its origin in the early 20th century, when Metchnikoff (1907) observed health benefits provided by the consumption of *lactobacilli*-containing dairy products. The term probiotics was first mentioned in 1965 by Lilly and Stillwell, who determined that certain microorganisms are able to produce substances that encourage the growth of other species. The Latin ("pro") and Greek ("bios") sources of the word probiotic mean "for life" (Hamilton-Miller et al. 2003). According to guidelines of the Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO), probiotics are now defined as "live microorganisms, which when administered in adequate amounts confer a health benefit on the host" (FAO/WHO 2001).

According to Oelschlaeger (2010), probiotic mode of actions can be categorized into the following three classes:

(i) Probiotics can modulate innate and adaptive immune responses of the host.

(ii) Probiotics can exert a direct influence on other commensal or pathogenic microorganisms.

(iii) Probiotics can interact with substances produced by microorganisms, e.g., toxins, or by the host, e.g., bile salts, and with nutritional components.

Probiotic-triggered immune regulation affects epithelial cells, DC, monocytes or macrophages, and lymphocytes (Ng et al. 2009). On the one hand, with regard to cellular defense mechanisms, immunomodulation has been shown to occur, e.g., *via* enhanced phagocytic activity (Perdigon et al. 1986; Perdigon et al. 1988; Schiffrin et al. 1995). On the other hand, humoral immune mechanisms include the induction of IgA responses (Kaila et al. 1992; Isolauri et al. 1993; Majamaa et al. 1995). More recently, the involvement of NLR, e.g., NOD2, has been detected in immune modulation processes induced by probiotic bacteria (Macho Fernandez et al. 2011; Rice et al. 2016).

The fundamental concept for the use of probiotics includes their ability to modify the intestinal microbiota favorably, although a changed composition of the gut microbiota is not a prerequisite for certain health benefits, such as the modulation of the immune responses (Ouwehand et al. 2002). The principle of competitive exclusion was introduced by Greenberg (1969), who observed an inhibition of *Salmonella* Typhimurium growth by gut microbiota in the maggots of blowflies. Probiotic bacteria not only compete for common mucosal binding sites and nutrients, but also impede the adhesion of enteropathogens by, e.g., biofilm formation or the destruction of certain receptors (Oelschlaeger 2010). Another probiotic mode of action against pathogenic bacteria is the production of antimicrobial substances, such as lactic acid and bacteriocins (Ohland and Macnaughton 2010; Bermudez-Brito et al. 2012). Moreover, several authors have shown that probiotic bacteria strengthen epithelial barrier functions *via* modulation of localization or expression of tight junction proteins (Resta-Lenert and Barrett 2003; Ukena et al. 2007; Zyrek et al. 2007; Ewaschuk et al. 2008; Johnson-Henry et al. 2008).

Among others, important selection criteria for probiotic strains are *(i)* their isolation from the same species as the host, *(ii)* a proven efficacy, *(iii)* non-pathogenic and non-toxic

characteristics, *(iv)* the ability to survive within the intestinal passage, and *(v)* stable formulations with a high cell viability (Collado et al. 2009).

Finally, account has to be taken that probiotic effects are dose-dependent and strain-specific (Sanders 2008; Cho et al. 2011).

2.2.1.1 The probiotic strain *Enterococcus faecium* NCIMB 10415

The commensal bacterium *E. faecium* has a high prevalence in the porcine gut (Devriese et al. 1994). Initially, its probiotic effects on the rates and severity of diarrhea in pigs was described under its former name *Streptococcus faecium* (Underdahl et al. 1982; Underdahl 1983; Ushe and Nagy 1985). The probiotic strain *E. faecium* NCIMB 10415, also referred to as *E. faecium* SF68, is an EU-authorized probiotic for piglet rearing, and its impact on post-weaning diarrhea has previously been assessed in a number of studies.

Taras et al. (2006) demonstrated the reduction of diarrhea incidence and duration in piglets, as previously reported by Manner and Spieler (1997), and a lower occurrence of pathogenicity genes in *E. coli* isolates of fecal samples of pigs supplemented with *E. faecium* NCIMB 10415. Similar findings were obtained in the study of Zeyner and Boldt (2006) in which piglets benefitted from supplementation with *E. faecium* NCIMB 10415 during the period from birth to weaning and exhibited an improved growth performance. In contrast, a feeding trial by Broom et al. (2006) provided no evidence for an ameliorated post-weaning piglet performance after an *E. faecium* NCIMB 10415-containing weaning diet had been fed to piglets.

Scharek et al. (2005) determined lower levels of cytotoxic T lymphocytes in the jejunum epithelium of probiotic-fed piglets; the authors attributed these lower levels to a diminished colonization with pathogenic *E. coli* serovars. An immunostimulatory effect of *E. faecium* NCIMB 10415 was observed by Szabo et al. (2009), who detected higher antibody titers against *Salmonella* Typhimurium in infected weaning piglets; however, in this study, the bacterial burden and clinical symptoms were likewise enhanced. In agreement with these findings, *E. faecium* NCIMB 10415 did not evoke protective immune responses to *Salmonella* Typhimurium, increased the bacterial load in tonsils, and therefore, exerted no advantageous effects during experimental infection of weaning piglets (Kreuzer et al. 2012). In another study, Pollmann et al. (2005) reported a reduced transfer of *Chlamydia* spp. from infected sows occupying carrier status to their offspring following oral administration of *E. faecium* NCIMB 10415. According to Jin et al. (2000), *E. faecium* is capable of inhibiting the attachment of enteropathogens, such as ETEC K88, probably *via* steric hindrance.

Microbiological investigations concerning the effects of *E. faecium* NCIMB 10415-application to sows and their litters on the distribution of other *Enterococcus* species in the gastrointestinal tract of piglets revealed an attenuating influence on the frequency of *Enterococcus faecalis*, but not on total numbers of *E. faecium* (Vahjen et al. 2007). Since the microbial composition of maternal feces was the same before and after probiotic feeding, these modifications were suggested to occur in the piglet's intestine (Vahjen et al. 2007). Starke et al. (2013) postulated individual reactions of mother sows to the feeding of *E. faecium* 10415,

with the altered fecal microbial composition found in responding sows being transferred to their offspring during the suckling period.

Furthermore, Lodemann et al. (2006) have examined epithelial transport processes in the jejunum of piglets *ex vivo* and reported elevated glucose absorption as a result of supplementation with *E. faecium* NCIMB 10415, which in turn might ameliorate diarrhea because of the greater absorption of water. In addition to an increased absorptive capacity, the improved secretory and barrier properties displayed by an enhanced PGE_2-induced chloride secretion and the reduced mannitol flux rates across the jejunal epithelia have been determined in *E. faecium* NCIMB 10415-fed piglets (Klingspor et al. 2013). During an *ex vivo* ETEC challenge, prefeeding with *E. faecium* NCIMB 10415 did not markedly affect the response of the piglet's jejunum, except for the prevented decrease of claudin-4 expression, the latter being a sealing (barrier-enhancing) tight junction protein; this indicates possible barrier-protective effects of the probiotic (Lodemann et al. 2017).

In addition to the aforementioned animal studies, *in vitro* studies with porcine IEC (cell line IPEC-J2) revealed alleviated pro-inflammatory immune response patterns upon ETEC application *via* pre- or coincubation with *E. faecium* NCIMB 10415 (Klingspor et al. 2015; Kern et al. 2017). In these studies, probiotic treatment also provided protection from impairments of epithelial barrier integrity, as measured by the transepithelial electrical resistance (TEER) of IPEC-J2 cells (Klingspor et al. 2015; Kern et al. 2017). Antiviral capacities of *E. faecium* NCIMB 10415 have been identified in a porcine macrophage cell line in response to swine influenza virus, as indicated by suppressed virus replication and altered cytokine expression at distinct time points post-infection (Wang et al. 2013). However, apart from this, relatively few data are available addressing *in vitro* effects of *E. faecium* NCIMB 10415.

Chapter 3: Aims and objectives of this thesis

In recent years, *in vivo* and *in vitro* studies have provided insights into the new research area of inflammasome signaling. Evidence for inflammasome involvement in various disease patterns, e.g., inflammatory bowel disease, suggests that inflammasomes constitute a potential therapeutic target for the treatment of intestinal pathologies. However, inflammasome signaling pathways and mechanisms governing their activation in pigs are mostly unknown.

The main aim proposed as the basis for the work described in the present thesis was to elucidate inflammasome signaling pathways implicated in the immune response to bacterial infection in porcine cells and tissues. The objective was to test the capacity of ETEC IMT4818 and *E. faecium* NCIMB 10415 to provoke inflammasome activation in porcine DC and IEC *in vitro* and in porcine intestinal tissues *ex vivo*. Another crucial goal was to evaluate the impact of the aforementioned bacterial agents on the functions and interactions of IEC and immune cells.

In the first series of the *in vitro* investigations, challenge experiments were carried out on porcine DC derived from blood monocytes. The main objective of this first part was the characterization of inflammasome components and inflammasome-related responses upon stimulation with *E. faecium* NCIMB 10415 or ETEC IMT4818 in porcine monocyte-derived DC (MoDC). The following special issues were further addressed: *(i)* whether probiotic preincubation changes the inflammasome response during an ETEC challenge and *(ii)* to what extent LPS priming affects inflammasome reactions in porcine MoDC.

In the second experimental series, the purpose was to establish a porcine intestinal co-culture model consisting of IEC (cell line IPEC-J2) and the abovementioned MoDC *in vitro* for subsequent bacterial incubation experiments. The central aim was to unravel to which extent the inflammatory response patterns of IEC and DC to the two different types of bacteria were affected by their mutual interplay. Specific issues were: *(i)* in what way inflammasome or IL-8 reactions are altered in IPEC-J2 cells or MoDC upon co-cultivation, *(ii)* whether humoral factors, such as TGF-β and TSLP, mediate the communication between IPEC-J2 cells and MoDC, and *(iii)* whether porcine caspase-13, as a potential candidate targeting non-canonical inflammasome signaling, is modulated in these cell types.

In addition to the *in vitro* studies, the third part of the project involved expression analyses of inflammasome components in small and large intestinal tissues of 29- and 70-day-old pigs by using the Ussing chamber technique. In addition to this systematic screening approach, a further aim was to investigate whether the inflammasome signaling pathway contributes to possible protective effects of *E. faecium ex vivo*. Moreover, the subsequent questions were considered: *(i)* to which extent inflammasome expression in the porcine gut varies in dependence of location and age, *(ii)* as to whether probiotic *E. faecium* supplementation alters inflammasome expression levels in 29-day-old animals in a feeding trial, and *(iii)* whether probiotic preincubation prior to a pathogenic ETEC challenge influences inflammasome activation in porcine jejunum in *ex vivo* experiments.

Chapter 4: Effects of a pathogenic ETEC strain and a probiotic *Enterococcus faecium* strain on the inflammasome response in porcine dendritic cells

This chapter has been published in: Veterinary Immunology and Immunopathology

Volume 203, September 2018, Pages 78-87

Manuscript received at the Journal: December 18th, 2017

Initial review completed: July 18th, 2018

Revision accepted: August 12th, 2018

Authors: Henriette Loss, Jörg R. Aschenbach, Friederike Ebner, Karsten Tedin, Ulrike Lodemann

https://doi.org/10.1016/j.vetimm.2018.08.004

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Chapter 5: The inflammatory response to enterotoxigenic *E. coli* and probiotic *E. faecium* in a coculture model of porcine intestinal epithelial and dendritic cells

This chapter has been published in: Mediators of Inflammation

Volume 2018, Article ID 9368295, 16 pages

Manuscript received at the Journal: August 22nd, 2018

Initial review completed: October 17th, 2018

Revision accepted: October 23rd, 2018

Authors: Henriette Loss, Jörg R. Aschenbach, Friederike Ebner, Karsten Tedin, Ulrike Lodemann

https://doi.org/10.1155/2018/9368295

Hindawi
Mediators of Inflammation
Volume 2018, Article ID 9368295, 16 pages
https://doi.org/10.1155/2018/9368295

Research Article

The Inflammatory Response to Enterotoxigenic *E. coli* and Probiotic *E. faecium* in a Coculture Model of Porcine Intestinal Epithelial and Dendritic Cells

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Received 22 August 2018; Accepted 23 October 2018; Published 20 December 2018

Guest Editor: Renata Sesti-Costa

The gut epithelium constitutes an interface between the intestinal contents and the underlying gut-associated lymphoid tissue (GALT) including dendritic cells (DC). Interactions of intestinal epithelial cells (IEC) and resident DC are characterized by bidirectional crosstalk mediated by various factors, such as transforming growth factor-β (TGF-β) and thymic stromal lymphopoietin (TSLP). In the present study, we aimed (1) to model the interplay of both cell types in a porcine *in vitro* coculture consisting of IEC (cell line IPEC-J2) and monocyte-derived DC (MoDC) and (2) to assess whether immune responses to bacteria are altered because of the interplay between IPEC-J2 cells and MoDC. With regard to the latter, we focused on the inflammasome pathway. Here, we propose caspase-13 as a promising candidate for the noncanonical inflammasome activation in pigs. We conducted challenge experiments with enterotoxigenic *Escherichia coli* (ETEC) and probiotic *Enterococcus faecium* (*E. faecium*) NCIMB 10415. As potential mediators of IEC/DC interactions, TGF-β and TSLP were selected for analyses. Cocultured MoDC showed attenuated ETEC-induced inflammasome-related and proinflammatory interleukin (IL)-8 reactions compared with MoDC monocultures. Caspase-13 was more strongly expressed in IPEC-J2 cells cocultured with MoDC and upon ETEC incubation. We found that IPEC-J2 cells and MoDC were capable of releasing TSLP. The latter cells secreted greater amounts of TSLP when cocultured with IPEC-J2 cells. TGF-β was not modulated under the present experimental conditions in either cell types. We conclude that, in the presence of IPEC-J2 cells, porcine MoDC exhibited a more tolerogenic phenotype, which might be partially regulated by autocrine TSLP production. Noncanonical inflammasome signaling appeared to be modulated in IPEC-J2 cells. Our results indicate that the reciprocal interplay of the intestinal epithelium and GALT is essential for promoting balanced immune responses.

1. Introduction

Intestinal epithelial cells lining the intestinal mucosa are continuously exposed to a variety of potentially harmful antigens and build a physical interface that separates the luminal content from the host milieu [1]. In the gut, DC are found in the *lamina propria*, in the subepithelial dome region of Peyer's patches, and in solitary lymph nodes such as the mesenteric lymph nodes [2–4]. Within the dynamic communication system between enterocytes and mucosal immune cells, IEC direct the function of resident DC by releasing immune mediators, such as the regulatory cytokine TGF-β and TSLP [5, 6]. Intestinal DC and IEC are both pivotal for maintaining normal barrier function as they support the discrimination

between inflammatory and tolerogenic immune responses [7, 8]. Therefore, functional properties of the intestinal epithelium cannot be fully understood by using *in vitro* models in which epithelial cells are solely grown as monocultures [7]. Our objective was to reconstruct the intestinal environment *in vitro* by implementing the presence of MoDC in the subepithelial compartment of a porcine jejunum epithelial cell line grown on cell culture inserts of Transwell systems.

Since luminal microbiota also participate in the crosstalk [9, 10], we hypothesized that the inflammatory response patterns of IEC and immune cells to the different types of bacteria are influenced by the mutual interplay of these cells. Therefore, a pathogenic ETEC strain frequently causing postweaning diarrhea in piglets [11, 12] and an apathogenic *E. faecium* strain were included in the study design. In pigs, the probiotic *E. faecium* NCIMB 10415, which is used as a feed additive for sows and piglets, has previously been demonstrated to exert diverse favorable effects on the immune system and performance parameters both *in vitro* [13–15] and *in vivo* [16–19], especially during the postweaning period.

We aimed to unravel variations in the inflammatory responses of IEC and DC under coculture conditions with a focus on the signaling *via* the inflammasome pathway. Nucleotide oligomerization domain (NOD)-like receptors (NLR) represent a class of intracellular pattern recognition receptors (PRR), some of which are able to form inflammasomes [20]. A well-known member of this inflammasome-forming receptor family is NLRP3 (NLR family, pyrin domain containing 3) [21]. Among other stimuli, the NLRP3 inflammasome can be activated through bacterial infection [22]. Canonical and noncanonical inflammasome activations can be distinguished with regard to the characterization of inflammasome signaling [23]. Upon canonical inflammasome activation, the effector caspase-1 leads to the production and secretion of the proinflammatory cytokines IL-1β and IL-18 [24]. In contrast, noncanonical inflammasome activation requires species-specific inflammatory caspases other than caspase-1, particularly caspase-11 in mice [25] and caspase-4 and -5 in humans [26, 27]. Bovine caspase-13 is presumed to represent the ortholog of human caspase-4 [28]. Based on these findings, we propose that caspase-13 exerts a similar function in pigs. Noncanonical inflammasome activation has been demonstrated for various Gram-negative bacteria, such as *Vibrio cholerae*, *Escherichia coli* (*E. coli*), and *Salmonella* Typhimurium [25, 29]. Most of the inflammasome studies have been carried out in human or mouse models, but a deeper understanding of porcine inflammasome pathways is lacking. In particular, no studies exist regarding noncanonical inflammasome activation in pigs. A further hypothesis tested in the present study was that porcine caspase-13 is involved in noncanonical inflammasome activation in pigs.

2. Material and Methods

2.1. Porcine Intestinal Epithelial Cells. The cell line IPEC-J2 was used as a porcine intestinal epithelial model. The cell line was originally derived from the jejunum of a newborn piglet and was kindly provided by Professor Dr. Anthony Blikslager (North Carolina State University, USA). Cells were cultivated as described elsewhere [15]. Medium was changed 3 times per week. Every 7 days, cells were split at a ratio of 1 : 3. Passages between 73 and 80 were included in the experiments. IPEC-J2 cells were seeded on the top surface of collagenized cell culture inserts of 12-well Transwell systems (12 mm diameter, 1.12 cm^2 growth surface area, 0.4 μm pore size, Costar, Corning BV, Schiphol-Rijk, The Netherlands) at a density of 1×10^5 cells per cell culture insert. Cells were cultivated under a humidified atmosphere of 5% CO_2 at 37°C for 14 to 21 days until reaching confluency.

2.2. Generation of Monocyte-Derived Dendritic Cells. Blood was taken from conventionally reared Danbred × Pietrain pigs (10 to 12 weeks of age) kept at the Institute of Animal Nutrition (Freie Universität Berlin, Germany) or from clinically healthy pigs at a slaughterhouse in Brandenburg, Germany. The blood sample collection procedure was conducted in accordance with the guidelines for animal welfare and was approved by the ethics committee for animal welfare, namely, "Landesamt für Gesundheit und Soziales" (LaGeSo Berlin, no. T0264/15). Blood samples were collected in 9 ml ethylenediamine tetra-acetic acid (EDTA)-coated blood tubes (S-Monovette®, SARSTEDT, Nümbrecht, Germany).

Peripheral blood mononuclear cells (PBMC) were purified by density gradient centrifugation as described by Loss et al. [30] by using Ficoll-Paque™ PLUS (1.077 g/l, GE Healthcare, Uppsala, Sweden). Monocytes were subsequently enriched by magnetic labeling based on their CD14 expression and subsequent cell sorting in a MidiMACS separator and LS separation columns (both from Miltenyi Biotec, Bergisch Gladbach, Germany). $CD14^+$ monocytes were diluted in RPMI-1640 (Biochrom, Berlin, Germany) supplemented with 10% fetal calf serum (FCS, Biochrom), 100 U/ml penicillin, and 100 μg/ml streptomycin (Sigma-Aldrich Chemie GmbH). Cells were seeded at a density of 1.44×10^6 cells/ml and 1 ml per well in 12-well cell culture plates (TPP, Faust Lab, Klettgau, Germany or Eppendorf GmbH, Hamburg, Germany). To differentiate monocytes into MoDC, cells were supplemented with recombinant porcine (rp) granulocyte-macrophage colony-stimulating factor (GM-CSF, 20 ng/ml) and rp IL-4 (50 ng/ml; both from R&D Systems, Minneapolis, MN, USA). Cells were grown at 37°C under a humidified atmosphere of 5% CO_2 for 6 days. After 3 days, cells were fed with another 1 ml of fresh differentiation medium. On day 6, adherent immature MoDC were used for the experiments. In order to ensure successful differentiation, the morphological and phenotypical features of the cells were examined by phase contrast microscopy (Leica DMI 6000 series, Leica Microsystems, Heidelberg, Germany) and flow cytometry. Flow cytometric phenotypical characterization was performed as described elsewhere [30]. Briefly, the monoclonal antibodies anti-human CD14 (clone REA599, isotype IgG1, Miltenyi Biotec), anti-pig CD16 (clone G7, isotype IgG1, Bio-Rad Laboratories GmbH, Munich, Germany), anti-pig CD1 (clone 76-7-4, isotype IgG2$a\kappa$, SouthernBiotech, Cambridge, United Kingdom), and anti-pig swine leukocyte antigen (SLA) II (clone

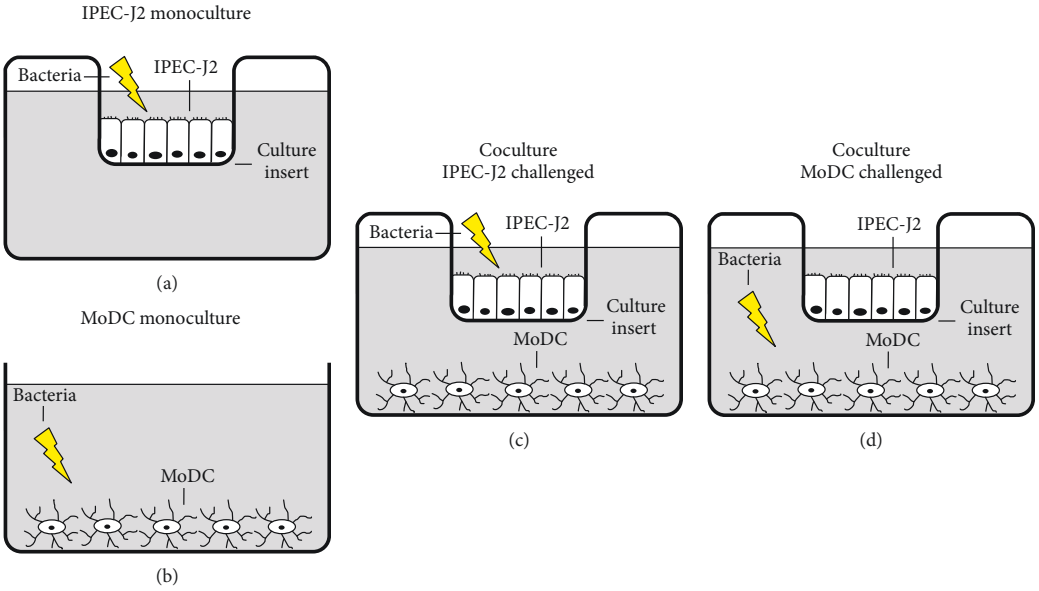

FIGURE 1: Schematic illustration of experimental design. (a) IPEC-J2 monocultures were grown as a monolayer on the top surface of Transwell cell culture inserts. Bacteria were added to the IPEC-J2 compartment. (b) MoDC monocultures were cultivated in 12-well cell culture plates. Bacteria were added to the MoDC compartment. (c)–(d) Cocultures of IPEC-J2 cells grown on Transwell inserts and adherent MoDC located in the bottom compartment. In separate approaches, bacteria were added either (c) to the IPEC-J2 compartment or (d) to the MoDC compartment. The lightning flash indicates the localization of the bacterial challenge with either *E. faecium* or ETEC.

K274.3G8, isotype IgG1, major histocompatibility complex [MHC] II, Bio-Rad Laboratories GmbH) were used. Successful differentiation was considered to have occurred when the cells showed a characteristic DC morphology and were tested as being $CD14^+$ $CD16^+$ $CD1^+$ SLA^+.

2.3. Bacterial Strains. Two different bacterial strains were used for the experiments: the probiotic strain *E. faecium* NCIMB 10415 (Cylactin®, DSM, Kaiseraugst, Switzerland) and enterotoxigenic *E. coli* IMT4818 (isolated from a two-week-old piglet with diarrhea, O149:K91:K88 [F4]). *E. faecium* and ETEC were grown on BHI (brain-heart infusion) and LB (Luria-Bertani) agar plates, respectively. After overnight incubation, *E. faecium* was grown in BHI broth (OXOID GmbH, Wesel, Germany) and ETEC in LB medium containing 10 g/l tryptone, 5 g/l yeast extract, and 10 g/l NaCl, at pH 7.0 (Roth, Karlsruhe, Germany). Each bacterial strain was cultivated at 37°C until the midlog phase was reached. Bacteria were then centrifuged and washed twice with cold PBS. Prior to addition to IPEC-J2 cells, bacteria were diluted in serum- and antibiotic-free IPEC-J2 cell culture medium at a concentration of approximately 10^8 colony-forming units (CFU)/ml. For their application into the MoDC compartment, RPMI-1640 was used for the resuspension of bacterial cells to give a concentration of approximately 10^7 CFU/ml. In order to quantify bacterial concentrations, the optical density (OD) was measured at a wavelength of 600 nm in a Helios™ Epsilon spectrophotometer (Thermo Scientific, Rockford, IL). Additionally, dilution series were made with a subsequent CFU count on LB agar plates.

2.4. Coculture Model and Experimental Design (Figure 1). In the present study, a coculture model comprising IPEC-J2 cells and porcine MoDC was utilized as illustrated in Figures 1(c) and 1(d). To this end, Transwell inserts with confluent IPEC-J2 monolayers grown on their top surface were transferred to the 12-well culture plates containing adherent MoDC on the bottom. On the day prior to the experiments, each cell type was fed with the appropriate cell culture medium. After 24 h in coculture, the cells were challenged with the aforementioned bacterial strains.

Prior to bacterial infection, FCS- and penicillin-streptomycin-supplemented media were removed from the cell cultures and replaced by serum- and antibiotic-free IPEC-J2 or MoDC cell culture medium, respectively, after the appropriate cells had been washed with the aforementioned media.

For the experiments, cells were incubated with either the probiotic *E. faecium* strain or the pathogenic ETEC strain. The number of bacteria differed depending on the cell type infected. IPEC-J2/MoDC cocultures were incubated with bacteria by adding either 1×10^6 CFU per insert to the IPEC-J2 compartment of the cultures or 5.4×10^4 CFU per well to the MoDC compartment (Figures 1(c) and 1(d)). The appropriateness of the applied bacterial concentrations was evaluated in preliminary experiments.

In addition to the IPEC-J2/MoDC cocultures, monocultures of IPEC-J2 cells or MoDC were also included as controls to assess the influence of cocultivation on the reactivity of each cell type (Figures 1(a) and 1(b)).

For the sake of completeness, we examined the immune responses after direct incubation with the bacterial strains

(comparison of mono- *vs.* coculture), as well as after indirect bacterial incubation. In these assays, we additionally assessed the inflammatory responses of cocultured IPEC-J2 cells when MoDC had been challenged and *vice versa*. Thus, an *in vivo*-like situation discriminating between the apical or basolateral occurrence of individual bacteria was simulated. The expected higher bacterial load in the lumen compared with the subepithelial space was also modeled as described above; this has to be taken into account when interpreting the results of the indirect challenge.

In order to prevent bacterial overgrowth, cells were washed with gentamicin-containing medium (150 μg/ml, Biochrom) after 2 h of bacterial incubation. The medium was then replaced with medium supplemented with gentamicin at a final concentration of 50 μg/ml. After this medium change, cells were incubated for further 4 h.

2.5. Transepithelial Electrical Resistance (TEER) Measurements. The transepithelial electrical resistance (TEER) across the IPEC-J2 monolayers was measured in the Transwell systems by using a Millicell-ERS (Electrical Resistance System, Millipore GmbH, Schwalbach, Germany). During the experiments, the TEER was measured every two hours (before bacterial addition and at 2 h, 4 h, and 6 h of incubation). TEER values were corrected against their blank control (cell-free cell culture insert with medium) and against the membrane area. For each experimental condition, three wells were used. Results are reported as [$\Omega \times \text{cm}^2$].

2.6. Real-Time Quantitative Polymerase Chain Reaction (RT-qPCR). To perform mRNA expression analyses, samples for RT-qPCR were collected 6 h after bacterial addition. MoDC and IPEC-J2 cells were washed with cold PBS, harvested by scraping, and stored in RNA*later* RNA stabilization reagent (Qiagen GmbH, Hilden, Germany) at −20°C. Isolation of RNA and its quantitative and qualitative analyses were performed as described by Kern et al. [14]. Samples were used when the RNA integrity number was higher than or equal to 8. An aliquot of 100 ng total RNA was reverse-transcribed into cDNA in a Mastercycler™ Nexus Gradient (Eppendorf GmbH) by using the iScript™ cDNA Synthesis Kit (Bio-Rad Laboratories GmbH). All primers for RT-qPCR were synthesized by Eurofins MWG Synthesis GmbH (Ebersberg, Germany). In preliminary experiments, various reference genes were validated for each cell line by using ge-Norm® software. Three reference genes were selected for normalization (MoDC: TATA-binding protein [TBP], tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta [YWHAZ], and beta-2-microglobulin [B2M]; IPEC-J2: TBP, YWHAZ, and glyceraldehyde-3-phosphate dehydrogenase [GAPDH]). Primer information regarding the target and reference genes is given in Table 1. RT-qPCR was conducted in an iCycler iQ™ Real-Time PCR Detection System (Bio-Rad Laboratories GmbH) by using SYBR Green I detection. Samples were run in triplicate. The final volume of the reaction (20 μl) was composed of iQ SYBR Green Supermix (Bio-Rad Laboratories GmbH), primers (0.38 μl of 20 pmol/μl each), and 5 μl cDNA. An inter-run calibration sample was used to correct for run-to-run variations. To check for possible genomic DNA contamination, minus-reverse transcriptase controls were included in the experiments. The software iQ5 (Bio-Rad Laboratories GmbH) was utilized to calculate the relative expression of target genes by using the ΔΔCt method.

2.7. Enzyme-Linked Immunosorbent Assay (ELISA). For the analysis of cytokine release from MoDC or IPEC-J2 cells, cell-free cell culture supernatants were collected 6 h after bacterial addition, centrifuged (6000 rpm for 5 min), and stored at −80°C until used. IL-1β, IL-8, TGF-β, and TSLP concentrations were determined by using the following ELISA kits according to the manufacturer's instructions: porcine IL-1β ELISA (Quantikine ELISA, Porcine IL-1β/IL-1F2 Immunoassay, R&D Systems), porcine IL-8 ELISA (Invitrogen ELISA Kit, Swine IL-8, Invitrogen Life Technologies GmbH), porcine TGF-β ELISA (Quantikine ELISA, Porcine TGF-β1 Immunoassay, R&D Systems), and porcine TSLP ELISA (Porcine TSLP ELISA kit, BlueGene, Shanghai, China). A microplate reader (EnSpire Multimode Plate Reader, Perkin Elmer, Rodgau, Germany) was employed to measure the absorbance values and to calculate the OD-specific sample concentrations from a standard curve by using a four-parameter logistic curve fit. Results are reported as (pg/ml).

2.8. Statistical Analysis. Statistical analyses and the creation of graphs were performed by using SigmaPlot 11.0 for Windows (Systat Software Inc., San Jose, CA, USA). Statistical significance of differences between the various treatment groups was assessed by two-way repeated measures analysis of variance (ANOVA) for the factors "bacteria" ("control", "*E. faecium*", and "ETEC") and "culture" ("IPEC-J2 monoculture"/"MoDC monoculture", "coculture - IPEC-J2 challenged", and "coculture - MoDC challenged"). In addition to the analysis of these two main effects, we also tested for possible interactions between the two factors. If interactions occurred, comparisons among the different treatment groups of the factor "bacteria" were made for each "culture" condition and *vice versa*. Findings were considered to be significant when $P \leq 0.05$. When overall analysis of the data of each cell type and a certain parameter (TEER, mRNA, or protein expression) showed a statistical difference between treatment groups (including interactions), the Fisher least significant difference *post hoc* test was carried out. In the figures, results are presented as means ± standard error of the means (SEM).

3. Results

3.1. TEER. During the course of experiments, TEER values of the IPEC-J2 monolayers were measured at four time points in order to monitor the barrier integrity (Figure 2). In addition to the cocultures, TEER was also determined in corresponding IPEC-J2 monocultures. As shown in Figure 2(a), initial TEER values of the cocultures did not differ from those of IPEC-J2 monocultures before the bacterial challenge.

In IPEC-J2 monocultures, ETEC significantly reduced the TEER after 2 h ($P \leq 0.05$) (Figure 2(b)) and after 4 h ($P \leq 0.05$) of incubation (Figure 2(c)). In cocultures with

Table 1: Oligonucleotide primers and amplicon length of PCR products.

Gene information	Primer sequence	Amplicon length	Accession number	Reference
IL1B1 (interleukin-1, beta 1, *Sus scrofa*)	(S) 5′- CCT CCT CCC AGG CCT TCT GT -3′ (AS) 5′- GGG CCA GCC AGCA CTA GAG A -3′	178 bp		[31]
IL-18 (interleukin-18, *Sus scrofa*)	(S) 5′- ACG ATG AAG ACC TGG AAT CG -3′ (AS) 5′- GCC AGA CCT CTA GTG AGG CTA -3′	205 bp	AF191088.1	
NLRP3 (NLR family, pyrin domain containing 3, *Sus scrofa*)	(S) 5′- AGC AGA TTC CAG TGC ATC AAA G -3′ (AS) 5′- CCT GGT GAA GCG TTT GTT GAG-3′	75 bp	NM_001256770.2	[32]
NLRC4 (NLR family, CARD domain containing 4, *Sus scrofa*)	(S) 5′- TGC TCT GAA ACA CCT TGC AT -3′ (AS) 5′- GCA TAG ATT CCT GCC TCC AG -3′	92 bp	XM_013987922.1	
CASP13 (caspase-13, apoptosis-related cysteine peptidase, *Sus scrofa*)	(S) 5′- GTG CTA CAG AAA CGC CAT GA -3′ (AS) 5′- AGG GCA AAG CTT GAG GGT AT-3′	150 bp	XM_003129812.6	
CASP1 (caspase-1, apoptosis-related cysteine peptidase, *Sus scrofa*)	(S) 5′- CTC TCC ACA GGT TCA CAA TC -3′ (AS) 5′- GAA GAC GCA GGC TTA ACT GG -3′	116 bp	NM_214162	[33]
ASC (LOC100522011) (apoptosis-associated speck-like protein containing a CARD, *Sus scrofa*)	(S) 5′- CCG ACG AGC TCA AGA AGT TT -3′ (AS) 5′- AGC TCA GCG CTG TAC TCC TC -3′	154 bp	XM_003124468.4	
IL-8 (interleukin-8, *Sus scrofa*)	(S) 5′- GGC AGT TTT CCT GCT TTC T -3′ (AS) 5′- CAG TGG GGT CCA CTC TCA AT -3′	154 bp	X61151	[34]
TLR4 (toll-like receptor 4, *Sus scrofa*)	(S) 5′- AGA ACT GCA GGT GCT GGA TT -3′ (AS) 5′- AGG TTT GTC TCA ACG GCA AC -3′	180 bp	AB188301	
TGF-β (transforming growth factor beta, *Sus scrofa*)	(S) 5′- TGA CCC GCA GAG AGG CTA TA -3′ (AS) 5′- CAT GAG GAG CAG GAA GGG C -3′	164 bp	NM_214015.2	
TBP (TATA box binding protein, *Sus scrofa*)	(S) 5′- GAT GGA CGT TCG GTT TAG G -3′ (AS) 5′- AGC AGC ACA GTA CGA GCA A -3′	124 bp	DQ178129	[35]
YWHAZ (tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide, *Sus scrofa*)	(S) 5′- ATG CAA CCA ACA CAT CCT ATC -3′ (AS) 5′- GCA TTA TTA GCG TGC TGT CTT -3′	178 bp	DQ178130	[35]
B2M (beta-2-microglobulin, *Sus scrofa*)	(S) 5′- AAA CGG AAA GCC AAA TTA CC -3′ (AS) 5′- ATC CAC AGC GTT AGG AGT GA -3′	178 bp	DQ178123	[35]
GAPDH (glyceraldehyde-3-phosphate dehydrogenase, *Sus scrofa)*	(S) 5′- ACT CAC TCT TCT ACC TTT GAT GCT -3′ (AS) 5′- TGT TGC TGT AGC CAA ATT CA -3′	100 bp	DQ178124	[35]

challenged IPEC-J2 cells, this ETEC effect was only present at 2 h after bacterial infection ($P \leq 0.05$) (Figure 2(b)). Bacterial infection of cocultured MoDC revealed no ETEC-induced drop of TEER of IPEC-J2 monolayers at each considered time point. Unlike ETEC, *E. faecium* treatment led to no modifications in the TEER throughout the experimental period in each experimental setup.

3.2. Expression of Inflammation-Related Genes in IPEC-J2 Cells. The mRNA expression of various inflammation-related genes was analyzed in IPEC-J2 cells (and porcine MoDC—see next section) after 6 h of bacterial stimulation. Cells were incubated with either probiotic *E. faecium* or pathogenic ETEC. Samples were obtained from cocultures or from the corresponding monocultures.

To gain insight into the potential involvement of the inflammasome pathway, IL-1β, IL-18, and NLRP3 were selected for the analysis of the inflammasome response to the applied bacterial strains. As shown in Figure 3(a), mRNA expression levels of IL-1β in IPEC-J2 cells remained rather stable independent of the cultivation method (cocultures or monocultures) and the bacterial challenge. However, the IL-18 mRNA expression of IPEC-J2 cells was generally higher in the coculture setup when MoDC had been challenged compared with IPEC-J2 monocultures ($P \leq 0.05$) (Figure 3(b)). This effect was, as a trend, mainly based on greater values in cocultures challenged with ETEC. Incubation with the pathogenic ETEC strain also provoked an upregulation of NLRP3 mRNA expression in IPEC-J2 cells in comparison with the control and the *E. faecium* group ($P \leq 0.05$) (Figure 3(c)).

We hypothesized that caspase-13 would be a promising candidate targeting noncanonical inflammasome activation in pigs. As indicated in Figure 3(d), caspase-13 mRNA expression was strongly enhanced in ETEC-infected IPEC-J2 cells under either cultivation methods ($P \leq 0.05$). Notably,

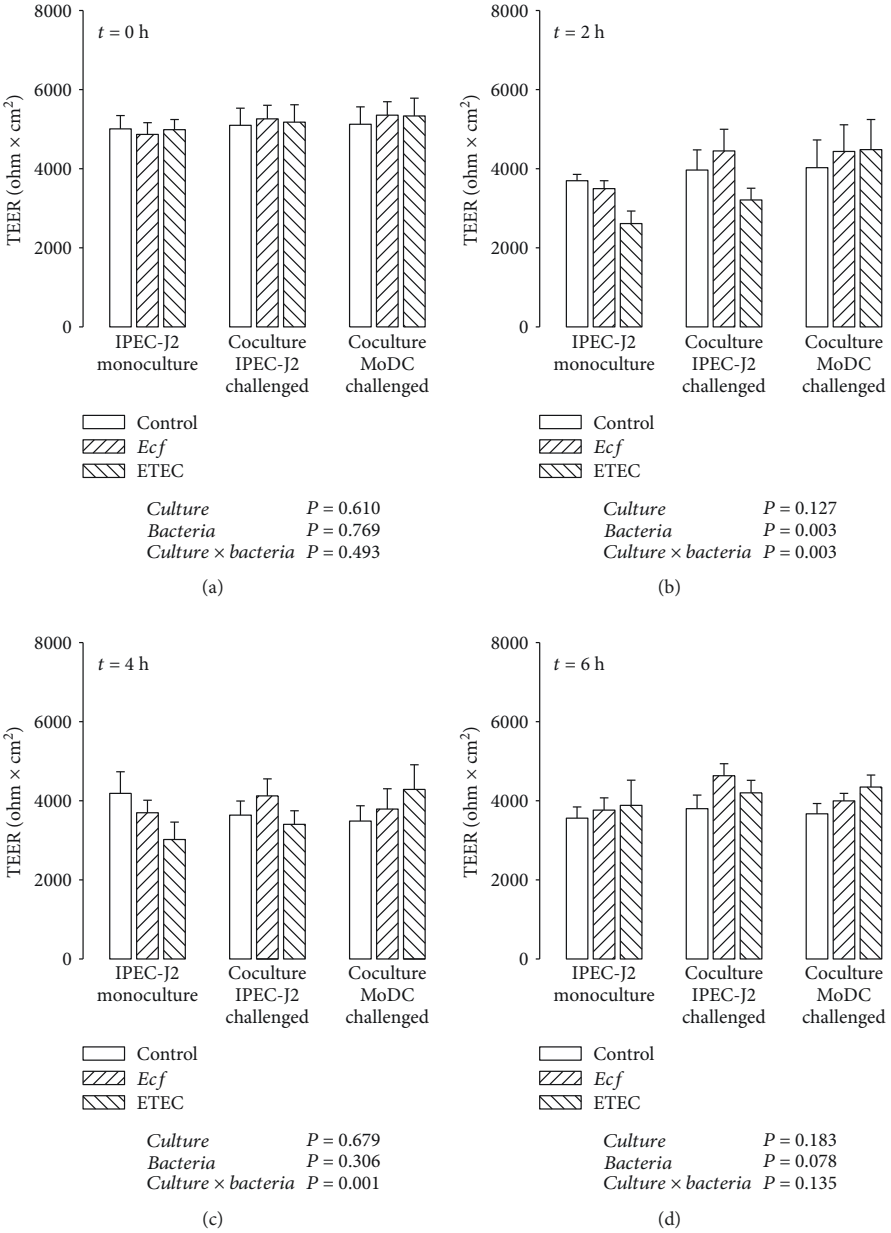

Figure 2: Transepithelial electrical resistance (TEER, in $\Omega \times cm^2$) of IPEC-J2 monolayers after stimulation with either *E. faecium* (*Ecf*) or ETEC. In IPEC-J2/MoDC cocultures, *Ecf* or ETEC was added either to the apical side of IPEC-J2 cells or to the MoDC compartment. In IPEC-J2 monocultures, the bacteria were added to the apical compartment. TEER values were measured at 0 h, 2 h, 4 h, and 6 h (a)-(d). Data are expressed as means ± SEM. $N = 6$ independent experiments per bar. Results of the ANOVA are indicated below each graph. Results of *post hoc* tests are presented in Supplementary Table 1.

the observed upregulation was more evident in cocultured IPEC-J2 cells than in IPEC-J2 monocultures ($P \leq 0.05$). An interesting additional finding was that the cocultivation of IPEC-J2 with MoDC (irrespective of infection) was followed by a higher caspase-13 mRNA expression in IPEC-J2 cells ($P \leq 0.05$).

To further illuminate the noncanonical inflammasome signaling pathway in IPEC-J2 cells, we additionally included the following genes in our analyses: inflammasome-forming NLRC4 (NLR family, CARD domain containing 4), the adapter ASC (apoptosis-associated speck-like protein containing a CARD), caspase-1, and toll-like receptor (TLR) 4 (Supplementary Tables 6 and 7). We found a lack of NLRC4 mRNA in IPEC-J2 cells (Supplementary Table 6). Whilst ASC mRNA expression was not regulated, caspase-1 mRNA expression was upregulated in cocultured IPEC-J2 cells

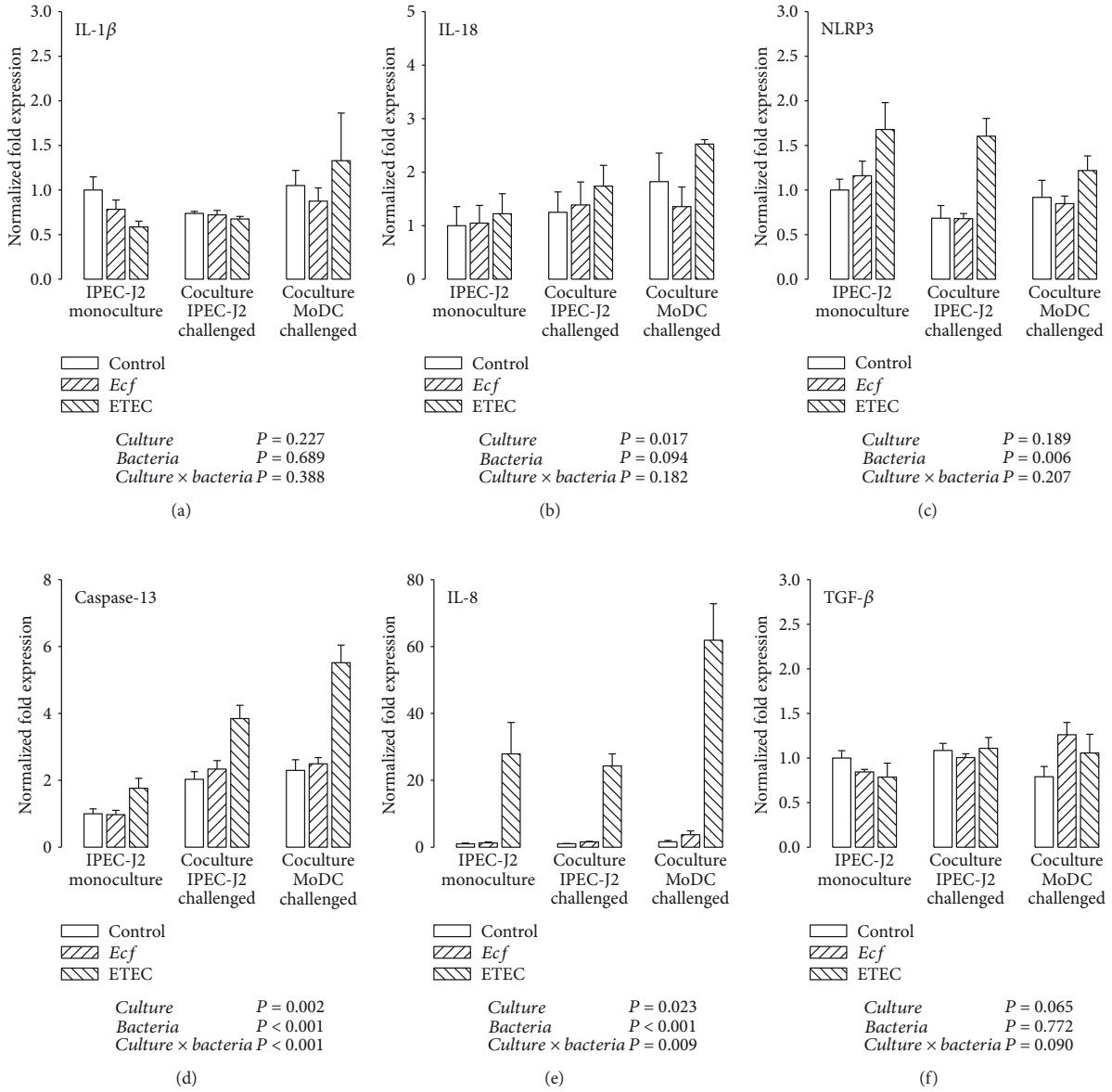

Figure 3: mRNA expression of (a) IL-1β, (b) IL-18, (c) NLRP3, (d) caspase-13, (e) IL-8, and (f) TGF-β in IPEC-J2 cells after stimulation with either *E. faecium* (*Ecf*) or ETEC. In IPEC-J2/MoDC cocultures, *Ecf* or ETEC was added either to the apical side of IPEC-J2 cells or to the MoDC compartment. In IPEC-J2 monocultures, the bacteria were added to the apical compartment. Samples were taken at 6 h after addition of bacteria (means ± SEM). $N = 4$ independent experiments per bar. Normalized fold expression was calculated by the ΔΔCt method. Results of the ANOVA are indicated below each graph. Results of *post hoc* tests are presented in Supplementary Table 2.

($P \leq 0.05$). TLR4 mRNA levels were higher in the ETEC-incubated treatment groups ($P \leq 0.05$).

The mRNA expression of the proinflammatory chemokine IL-8 in IPEC-J2 cells was markedly augmented by incubation with ETEC under each culture condition ($P \leq 0.05$) (Figure 3(e)). As with caspase-13, IPEC-J2 cells from the setting in which cocultured MoDC was treated with the bacteria exhibited the largest ETEC response ($P \leq 0.05$).

In contrast, *E. faecium* treatment did not alter the mRNA expression of the considered genes within the experimental design and showed expression levels similar to those of the unchallenged controls.

As a regulatory cytokine, we also investigated the expression of TGF-β, which was affected neither by the cultivation method nor by bacterial incubation in IPEC-J2 cells (Figure 3(f)).

3.3. Expression of Inflammation-Related Genes in Porcine MoDC. Similarly, mRNA expression was studied in porcine MoDC. Expression levels were compared between

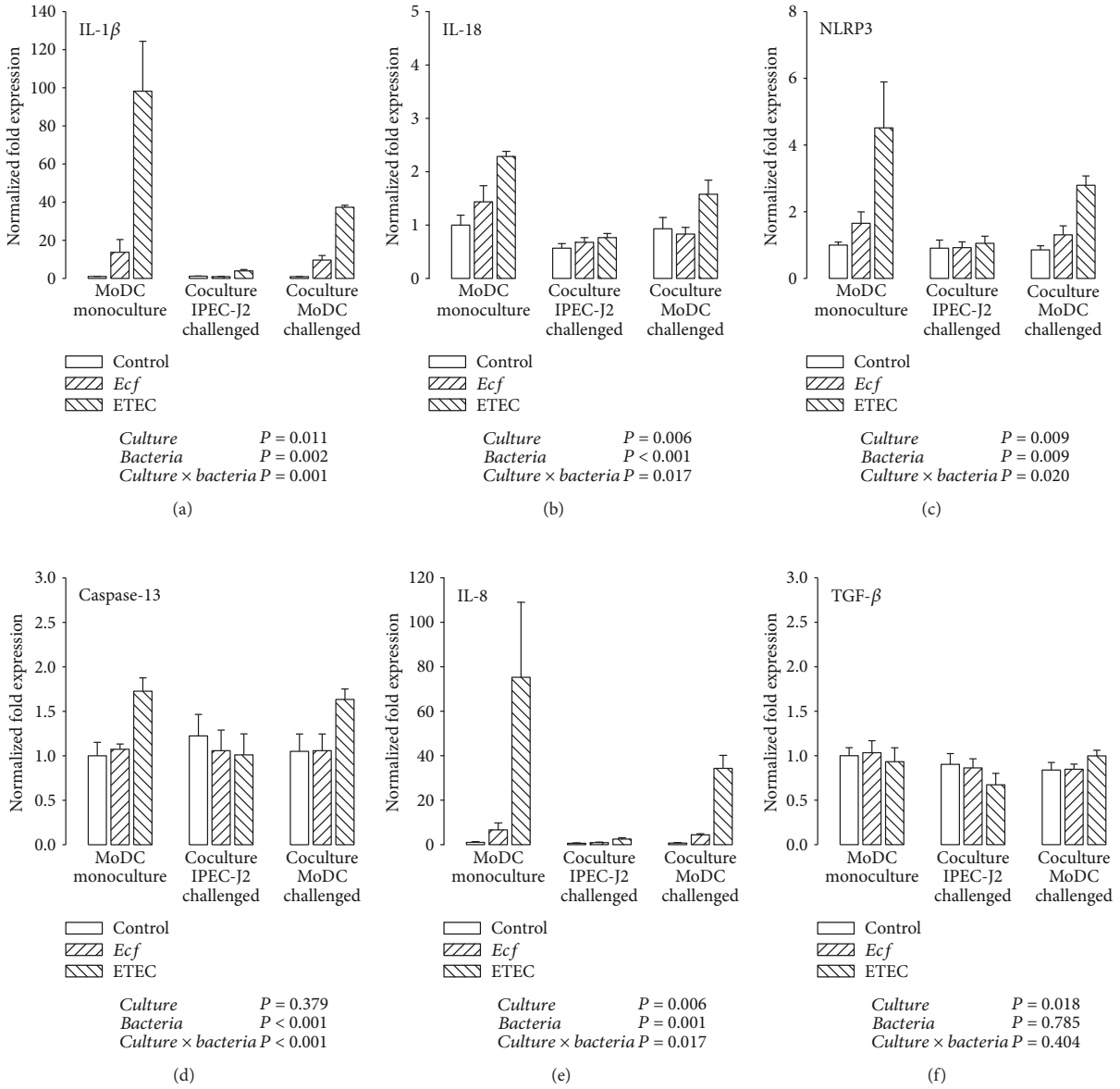

Figure 4: mRNA expression of (a) IL-1β, (b) IL-18, (c) NLRP3, (d) caspase-13, (e) IL-8, and (f) TGF-β in porcine MoDC after stimulation with either *E. faecium* (*Ecf*) or ETEC. In IPEC-J2/MoDC cocultures, *Ecf* or ETEC was added either to the apical side of IPEC-J2 cells or to the MoDC compartment. In MoDC monocultures, the bacteria were added to the basolateral compartment. Samples were taken at 6 h after addition of bacteria (means ± SEM). $N = 4$ independent experiments per bar. Normalized fold expression was calculated by the ΔΔCt method. Results of the ANOVA are indicated below each graph. Results of *post hoc* tests are presented in Supplementary Table 3.

cocultured MoDC (challenged with bacteria directly or indirectly by infection of IPEC-J2 cells) and MoDC originating from monocultures.

The analysis of inflammasome-linked genes (IL-1β, IL-18, and NLRP3) revealed an upregulation by ETEC in MoDC cultivated alone ($P \leq 0.05$) (Figures 4(a)–4(c)). To a significantly lesser extent, ETEC enhanced the mRNA expression of IL-1β, IL-18, and NLRP3 in the challenged MoDC of the cocultures ($P \leq 0.05$). In addition, cocultured MoDC remained relatively unaffected by the bacterial challenge of IPEC-J2 cells. Likewise, ETEC caused an enlarged caspase-13 transcription when MoDC were challenged in mono- and cocultures ($P \leq 0.05$) (Figure 4(d)). In contrast to genes associated with canonical inflammasome activation, the induced caspase-13 mRNA increase in cocultured MoDC was as great as in MoDC monocultures.

Expression patterns of IL-8 resembled those of IL-1β and NLRP3 (Figure 4(e)). The highest response to ETEC was

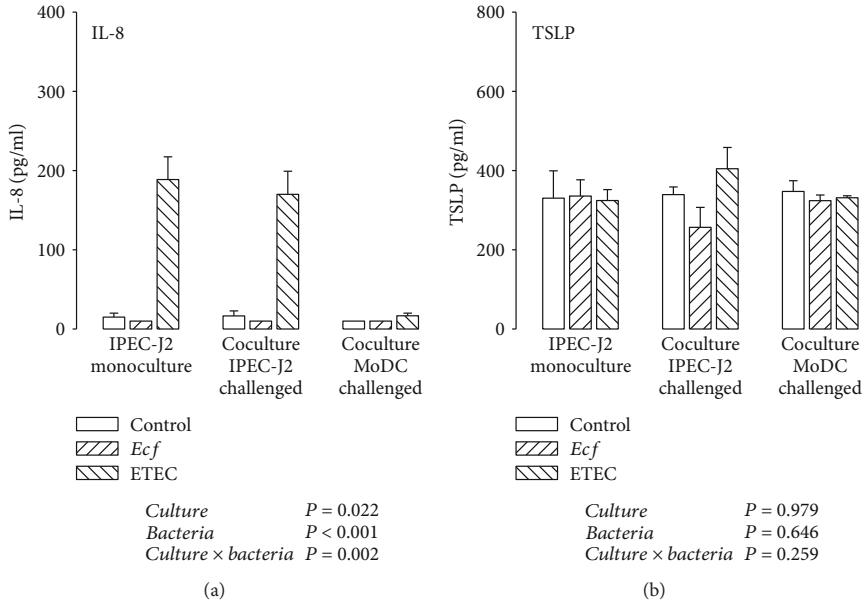

Figure 5: Protein expression (in pg/ml) of (a) IL-8 and (b) TSLP detected by ELISA in supernatants of IPEC-J2 cells after stimulation with either *E. faecium* (*Ecf*) or ETEC. In IPEC-J2/MoDC cocultures, *Ecf* or ETEC was added either to the apical side of IPEC-J2 cells or to the MoDC compartment. In IPEC-J2 monocultures, the bacteria were added to the apical compartment. Samples were taken at 6 h after addition of bacteria (means ± SEM). $N = 3$ independent experiments per bar. Results of the ANOVA are indicated below each graph. Results of *post hoc* tests are presented in Supplementary Table 4.

detected in MoDC monocultures, whereas a weaker ETEC-triggered amplification of IL-8 mRNA occurred in cocultured MoDC ($P \leq 0.05$).

Exposure to the probiotic *E. faecium* strain resulted in only a slight increase of IL-18 mRNA expression in MoDC that were cultivated alone ($P \leq 0.05$) (Figure 4(b)). Similar tendencies were recognized for IL-1β, NLRP3, and IL-8 mRNA expressions without reaching statistical significance (Figures 4(a), 4(c), and 4(e)).

Similar to that of IPEC-J2 cells, the mRNA expression of anti-inflammatory TGF-β in MoDC showed no clear effects in the context of bacterial treatment or the cultivation technique (Figure 4(f)). On average, the smallest expression level was detected in IPEC-J2/MoDC cocultures in which IPEC-J2 cells had been bacterially challenged ($P \leq 0.05$); this was attributable to a numerical ETEC-induced decrease.

3.4. Cytokine Secretion by IPEC-J2 Cells. The protein secretion of IL-8, IL-1β, TGF-β, and TSLP into cell culture supernatants of IPEC-J2 cells and porcine MoDC (see next section) was determined by ELISA. For the analysis of the selected cytokines, samples were collected 6 h after bacterial addition.

In challenged IPEC-J2 cells of mono- and cocultures, a strong secretion of IL-8 attributable to ETEC infection could be observed ($P \leq 0.05$) (Figure 5(a)). These results corresponded with those of the qPCR analysis. Interestingly, the results after bacterial addition to the MoDC compartment varied considerably from the mRNA to the protein level. The high upregulation of IL-8 mRNA expression could not be verified at the protein level.

IPEC-J2 cells secreted TSLP, which we proposed as being a promising candidate mediating the interactive IEC/DC crosstalk in addition to TGF-β, at levels of around 300 pg/ml, but the detected levels did not show significant variations attributable to different cultivation variants and bacterial stimulation (Figure 5(b)).

IL-1β and TGF-β concentrations in the tested IPEC-J2 supernatant samples were mostly below the minimum detection level of the ELISA kits used (6.7 and 4.6 pg/ml, respectively; data not shown).

3.5. Cytokine Secretion by Porcine MoDC. In supernatants of mono- and cocultured MoDC, direct ETEC incubation caused an IL-1β accumulation ($P \leq 0.05$) with a tendency of lower IL-1β concentrations in the presence of IPEC-J2 cells (Figure 6(a)).

The IL-8 release of ETEC-infected MoDC was greater in MoDC monocultures than in cocultures with IPEC-J2 cells ($P \leq 0.05$) (Figure 6(b)). This was in agreement with results obtained at the mRNA level. Furthermore, incubation with probiotic *E. faecium* also elicited a higher IL-8 protein level in directly challenged MoDC monocultures (compared with *E. faecium* responses under the remaining culture conditions), but this was lower than the ETEC-induced increases ($P \leq 0.05$) (Figure 6(b)).

Porcine MoDC secreted low amounts of TGF-β into the respective supernatants, which tended to be increased in *E. faecium*-incubated cells ($P = 0.052$) (Figure 6(c)).

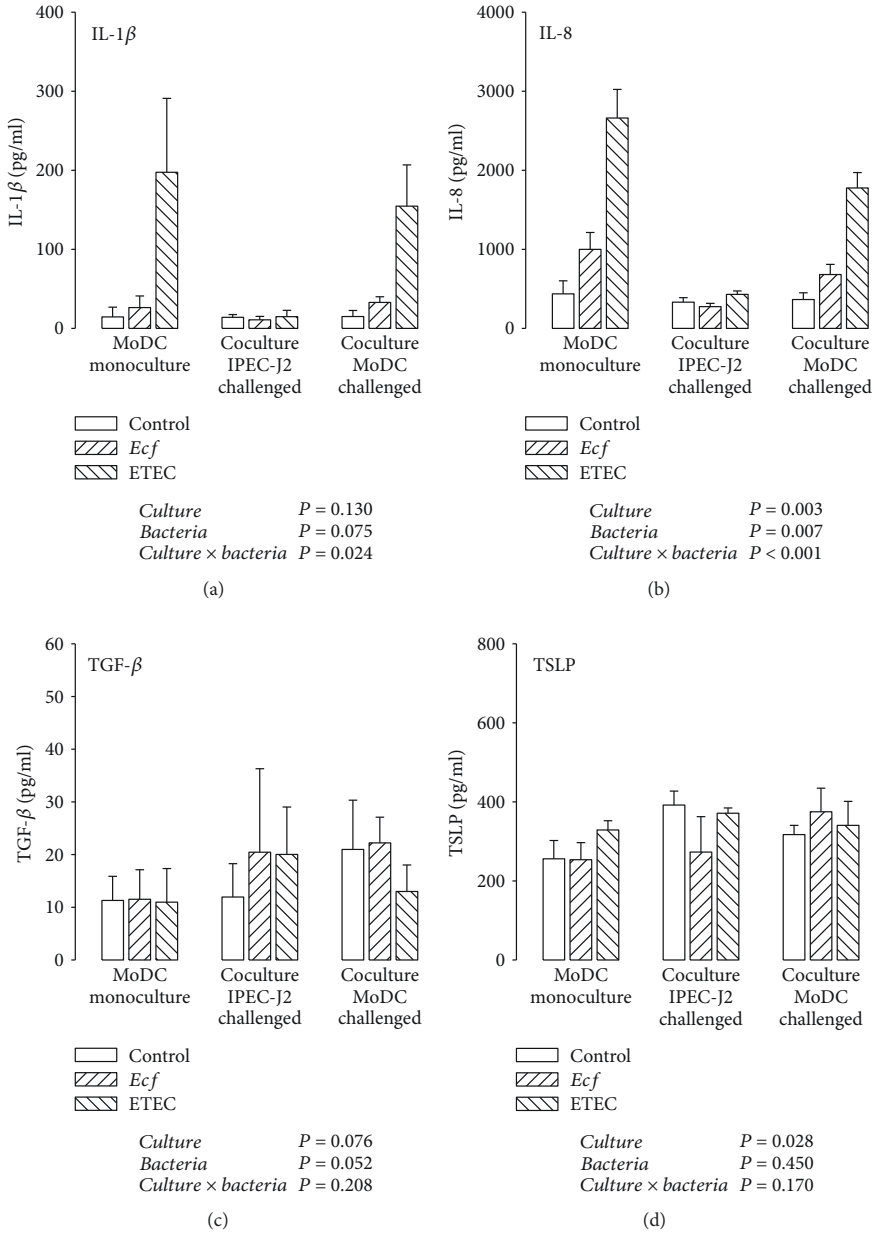

Figure 6: Protein expression (in pg/ml) of (a) IL-1β, (b) IL-8, (c) TGF-β, and (d) TSLP detected by ELISA in supernatants of porcine MoDC after stimulation with either *E. faecium* (*Ecf*) or ETEC. In IPEC-J2/MoDC cocultures, *Ecf* or ETEC was added either to the apical side of IPEC-J2 cells or to the MoDC compartment. In MoDC monocultures, the bacteria were added to the basolateral compartment. Samples were taken at 6 h after addition of bacteria (means ± SEM). $N = 3 - 4$ independent experiments per bar. Results of the ANOVA are indicated below each graph. Results of *post hoc* tests are presented in Supplementary Table 5.

Surprisingly, we also detected TSLP expression at the protein level in MoDC samples (Figure 6(d)). The quantities of MoDC-derived TSLP were comparable with those measured in IPEC-J2 cells. The supernatant of MoDC cultivated in the presence of IPEC-J2 cells contained more TSLP than that of monocultures, regardless of the bacterial treatment ($P \leq 0.05$).

4. Discussion

In the present study, the main objective was to determine whether the inflammatory response to a bacterial challenge in porcine MoDC and IPEC-J2 cells is changed by their mutual interference in an *in vitro* coculture model. As encountered enteric bacteria can be of different types, we conducted

challenge experiments with an apathogenic *E. faecium* strain and a pathogenic *E. coli* strain, the latter having disease relevance for pigs, especially in the postweaning period.

4.1. TEER. The analysis of TEER values of IPEC-J2/MoDC cocultures and their corresponding IPEC-J2 monocultures revealed that the cocultivation of IPEC-J2 cells with MoDC per se did not have an effect on the TEER of IPEC-J2 monolayers. Similar findings were achieved with human intestinal models consisting of human IEC and MoDC [36, 37].

Previous studies have shown that ETEC is capable of altering the barrier function of apically infected IPEC-J2 monolayers adversely in a dose- and time-dependent manner [15, 38–40]. In the present study, ETEC effects on the barrier integrity were predominantly detectable after 2 h of incubation. Apically challenged IPEC-J2 monocultures showed a significantly lowered TEER even after 4 h, suggesting that IPEC-J2 monocultures were slightly more sensitive to ETEC-induced impairments of the epithelial barrier function than cocultures. Surprisingly, basolateral bacterial infection had no influence on TEER at any time point. The latter might be attributable to the lower number of pathogenic bacteria added to cocultured MoDC on the basolateral side of IPEC-J2 cells resulting in a lower bacteria : IPEC-J2 cell ratio. When adding the same bacterial concentration, other research groups have shown that the basolateral infection of human IEC (cell line T84) monocultures with pathogenic bacteria, such as adherent-invasive *E. coli* [41] or *Campylobacter jejuni* [42], resulted in a considerable TEER drop, which was greater after basolateral application compared with apical application. Nonetheless, the lower number of ETEC applied to the basolateral compartment of IPEC-J2 cells in the current study induced an evident proinflammatory response (see next section).

4.2. The Inflammatory Response in IPEC-J2 Cells. In the present study, we provide evidence that inflammasome activation following a pathogenic ETEC challenge occurred in both cell types examined. In IPEC-J2 cells, this was particularly validated by an upregulation of NLRP3 mRNA expression. In addition to the main cell wall component lipopolysaccharide (LPS), other inflammasome-stimulating components of pathogenic *E. coli* include toxins, such as enterohemolysin and heat-labile enterotoxin [43, 44], or bacterial RNA [45, 46]. The role of NLRP3 in intestinal inflammation and homeostasis is controversial [47–49]. Lissner and Siegmund [50] have underlined that the outcome depends on the affected cell type. Within the epithelium, NLRP3 performs regulatory functions, e.g., by promoting enterocyte proliferation, whereas disproportionate NLRP3 activation by *lamina propria* immune cells provokes detrimental effects [50]. A protective role of the NLRP3 inflammasome in IEC has been postulated by Song-Zhao et al. [51] and Zaki et al. [52]. In a recent study, Fan et al. [53] addressed inflammasome activation in IPEC-J2 cells upon stimulation with the mycotoxin zearalenone and reported evidence supporting regulatory functions of the NLRP3 inflammasome within the gut [53].

We further studied NLRC4 mRNA expression in IPEC-J2 cells as the NRLC4 inflammasome is another well-characterized inflammasome beyond NLRP3. We found no NLRC4 mRNA in these cells. We and others had previously reported similar findings for different porcine cells and tissues, suggesting that a functional NLRC4 gene is missing in pigs [30, 54, 55].

Whilst IEC are the main source for IL-18 being especially important for epithelial regeneration [52, 56, 57], the ability of IEC to produce IL-1β is a matter of debate [58]. Based on our results, IL-1β played a negligible role in IPEC-J2 cells, whereas IL-18 mRNA expression tended to follow similar expression patterns as determined for caspase-13, indicating that there might be a correlation between caspase-13 and IL-18.

Based on the assumption that caspase-13 is the porcine counterpart to murine caspase-11, the striking upregulation of caspase-13 mRNA expression in IPEC-J2 cells upon ETEC exposure suggests that ETEC could primarily trigger noncanonical inflammasome activation in IPEC-J2 cells. In addition, the caspase-13 induction as a result of the pathogenic ETEC challenge was more evident in cocultured IPEC-J2 cells than in IPEC-J2 monocultures. In human and murine IEC, Knodler et al. [59] have observed noncanonical inflammasome activation *via* caspase-4 and caspase-11, respectively, in response to enteropathogens. Recent research has assigned the murine ortholog caspase-11 guard functions within the gastrointestinal tract in inflammatory states [60–62]. For example, caspase-11-deficient mice revealed a hypersensitivity to dextran sulfate sodium-induced colitis associated with an impeded IL-18 production [60, 61], suggesting an ameliorating effect of caspase-11 during intestinal inflammation [63]. To date, it is unknown how inflammasome signaling by IEC is cross-linked with other defense mechanisms that ultimately coordinate the recruitment of neighboring immune cells [58].

Some authors have demonstrated that caspase-11 activation acts upstream of caspase-1-dependent canonical inflammasome formation [64, 65], whereas others have reported that caspase-11 forms a noncanonical inflammasome complex itself [25, 66]. Caspase-1, in contrast to caspase-4, -5, and -11, is capable of processing interleukins [67]. Analysis of caspase-1 mRNA expression in IPEC-J2 cells revealed an increase upon cocultivation, which had likewise been detected at the level of caspase-13 in IPEC-J2 cells, indicating a possible link between caspase-13 and caspase-1. Resembling results have been obtained in murine cocultures consisting of preadipocytes and muscle cells or fibroblasts, in which the mRNA expression of certain caspases (caspase-3, -7, and -9) was in some cases enhanced as an effect of coculturing [68, 69]. To our knowledge, similar investigations for caspases associated with noncanonical inflammasome signaling have not yet been carried out.

Furthermore, several authors have shown that the signaling pathway for caspase-11 activation includes TLR4 (and the TLR adapter TRIF [TIR domain containing adaptor inducing interferon-β]), which senses extracellular LPS [66, 70]. In IPEC-J2 cells, we could verify ETEC-associated upregulations of TLR4 mRNA expression, which might indicate that this signal cascade is likewise involved in porcine noncanonical inflammasome activation.

The transcriptional control differs between the caspases of different species, e.g., it has been established for murine caspase-11 but not for human caspase-4, which is constitutively expressed [71]. Summarizing the observations of the current study, it was suggested that the porcine ortholog caspase-13 responds similarly to the murine counterpart. In this respect, the verification in future studies as to whether caspase-13 constitutes the porcine equivalent to the aforementioned caspases of the noncanonical inflammasome pathway would be intriguing. Collectively, we can conclude that the DC-driven regulation of neighboring IPEC-J2 cells was mainly evidenced by caspase-13 modulation. This caspase-13 modulation might be one possible explanation for the altered TEER response observed after apical ETEC infection of IPEC-J2 mono- *vs.* cocultures and for the lack of a TEER response after basolateral ETEC infection of IPEC-J2 cells.

4.3. The Inflammatory Response in Porcine MoDC. Investigations into the inflammatory response in porcine MoDC revealed that MoDC from IPEC-J2/MoDC cocultures reacted more moderately to the pathogenic ETEC challenge than did monocultured MoDC; the expression of IL-1β, IL-18, and NLRP3 was attenuated at the mRNA level and of IL-1β, as a trend, also at the protein level. For the proinflammatory cytokine IL-8, a similar pattern was noted at both the mRNA and protein levels. In contrast to IEC, DC are known to express NLRP3 abundantly and to generate high IL-1β levels [72]. An exaggerated production of cytokines, such as IL-1β and IL-8, can lead to the development of intestinal pathologies linked with a disruption of the intestinal barrier, such as inflammatory bowel disease [73, 74]. Hence, our findings concerning inflammasome and IL-8 reactions support the hypothesis that IEC act beneficially to adapt the proinflammatory responsiveness of MoDC to invading enteropathogens. We propose an inflammation-restricting effect of adjacent IPEC-J2 cells on porcine MoDC in the present study. Other research groups have provided evidence that IEC are able to suppress proinflammatory responses of cocultured immune cells [37, 75]. In a human model of the intestinal epithelium, DC cultivated in direct contact with IEC were less sensitive to LPS and exhibited a reduced proinflammatory response [37].

Of note, the caspase-13 mRNA expression in MoDC did not appear to be influenced following cocultivation with IPEC-J2 cells; and the ETEC-induced caspase-13 upregulation was reduced compared with those detected in IPEC-J2 cells. We presume that the transcriptional induction of caspase-13 plays a rather minor role in porcine MoDC, at least, within our experimental design. This underlines the observation that different cell types fulfil a unique contribution to the development of immune responses, particularly in the gut [58].

The apathogenic *E. faecium* strain used in this study had only a minor impact on certain proinflammatory markers (IL-18, IL-1β, and IL-8) in MoDC monocultures. Comparable proinflammatory responses to different *E. faecium* strains have been documented by several working groups that recorded a strain-specific and dose-dependent induction of, for example, IL-1β, IL-8, IL-6, and tumor necrosis factor-α, in human DC or murine macrophages [76–79]. TGF-β was suggested to contribute to probiotic-triggered immunoregulatory mechanisms [80–82]. Accordingly, a tendency for an *E. faecium*-induced increase of TGF-β secretion by MoDC was also noted in our experiments.

4.4. Potential Mediators of Crosstalk between IPEC-J2 Cells and Porcine MoDC. As porcine MoDC revealed an attenuated inflammatory ETEC response when cocultured with IPEC-J2 cells, we aimed to look more closely at underlying IPEC-J2/MoDC interactions. In our experimental design, MoDC had no direct contact with neighboring IPEC-J2 cells. Hence, the modulation of the immune cells was assumed to occur through cell-derived humoral signals capable of crossing the filter membrane. In our analyses, we included TSLP and TGF-β, which we considered as potential mediators in this bidirectional crosstalk.

Consistent with the idea that soluble factors are likely to be responsible for the regulation of DC responses, Rimoldi et al. [8] demonstrated that human DC conditioned by supernatants of IEC displayed a downregulated IL-1β secretion after *Salmonella* infection. In their study, IEC-derived TSLP was identified as the controlling agent [8]. Although TSLP is commonly regarded as an epithelial-derived cytokine, it has previously been detected in murine [83] and human DC [84, 85], where it was released in an autocrine manner in response to pathogenic and allergenic agents. In the present study, we observed TSLP expression by both IPEC-J2 and porcine DC. Unexpectedly, MoDC-derived TSLP appeared to contribute to an autocrine regulation under coculture conditions.

An autocrine regulation mechanism of MoDC has likewise been proposed on the basis of TGF-β secretion [37]. Butler et al. [37] observed a higher TGF-β release by human DC cocultured in direct contact with IEC; this was accompanied by weaker inflammatory reactions to pathogenic stimuli, as stated earlier. However, this effect was absent in a separated coculture setup that was more similar to our IPEC-J2/MoDC cocultures [37]. According to our results, no clear impact of cocultivation on TGF-β expression in porcine MoDC was present, either at the mRNA or at the protein level.

Since it is unclear whether IPEC-J2 cells are capable of producing TGF-β [86], we measured TGF-β at the mRNA level. We verified TGF-β mRNA expression in IPEC-J2 cells but which was, however, not regulated in the different treatment groups. Consistent with our data, Butler et al. [37] detected only very small amounts of TGF-β liberated by IEC, so that TGF-β could not be identified as a modulating IEC-derived mediator in the present experimental design.

Future studies are needed to obtain knowledge as to the extent to which results may be different when a cocultivation technique is used that allows direct contact between the cocultured cell types. Here, we provide evidence supporting a possible involvement of TSLP derived by porcine MoDC in the communication between IPEC-J2 cells and MoDC.

5. Conclusions

In the present study, we established a porcine intestinal model consisting of IPEC-J2 cells and MoDC. We

investigated inflammatory reactions to selected bacterial agents and found a more tolerogenic phenotype of MoDC cocultured with IEC. This conclusion was supported by a downregulation of inflammasome-related and other proinflammatory cytokines in comparison with MoDC cultivated alone. We further provide the first evidence that porcine caspase-13 is regulated in IPEC-J2 cells and porcine MoDC in response to bacterial infection. In IPEC-J2 cells, the possibly related noncanonical inflammasome pathway appeared to be induced not only by ETEC infection but also by the presence of MoDC. Finally, we demonstrated the ability of IPEC-J2 cells and MoDC to secrete TSLP, whereby an autocrine adaptation of cocultured MoDC was indicated. Our results suggest that the control of inflammatory responses by IEC is of critical importance to prevent unrestricted cytokine production by resident immune cells. More research is needed to unravel further the soluble factors that are implicated in IEC/DC interactions and to verify the functional aspects of porcine caspase-13 in noncanonical inflammasome signaling. We suggest the presented *in vitro* coculture model is a promising tool for studying such interactions in future.

Abbreviations

ANOVA: Analysis of variance
ASC: Apoptosis-associated speck-like protein containing a CARD
BHI: Brain-heart infusion
B2M: Beta-2-microglobulin
CFU: Colony-forming unit
DC: Dendritic cells
E. coli: *Escherichia coli*
EDTA: Ethylenediamine tetra-acetic acid
E. faecium: *Enterococcus faecium*
ELISA: Enzyme-linked immunosorbent assay
ETEC: Enterotoxigenic *Escherichia coli*
FCS: Fetal calf serum
GALT: Gut-associated lymphoid tissue
GAPDH: Glyceraldehyde-3-phosphate dehydrogenase
GM-CSF: Granulocyte-macrophage colony-stimulating factor
IEC: Intestinal epithelial cells
IL: Interleukin
LB: Luria-Bertani
LPS: Lipopolysaccharide
MHC: Major histocompatibility complex
MoDC: Monocyte-derived DC
NLR: NOD-like receptor
NLRC4: NLR family, CARD domain containing 4
NLRP3: NLR family, pyrin domain containing 3
NOD: Nucleotide oligomerization domain
OD: Optical density
PBMC: Peripheral blood mononuclear cells
PBS: Phosphate-buffered saline
PRR: Pattern recognition receptors
rp: Recombinant porcine
RT-qPCR: Real-time quantitative polymerase chain reaction
SEM: Standard error of the means
SLA: Swine leukocyte antigen
TBP: TATA-binding protein
TEER: Transepithelial electrical resistance
TGF-β: Transforming growth factor-β
TLR: Toll-like receptor
TSLP: Thymic stromal lymphopoietin
YWHAZ: Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta.

Data Availability

The TEER, ELISA, and qPCR data used to support the findings of this study are included within the supplementary information file.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this article.

Acknowledgments

The authors thank K. Söllig whose assistance is greatly appreciated. We are grateful to P. Schwerk for technical support with the bacterial strains and J. Schulte for providing the TGF-β primers. This research was financially supported by the German Research Foundation (grant number LO 2058/1-1) and by an Elsa Neumann grant to Henriette Loss.

Supplementary Materials

In Supplementary Table 1, the results of the post hoc tests of the TEER data are presented. Supplementary Tables 2, 3, 6, and 7 show the results of the post hoc tests of the mRNA expression analyses in IPEC-J2 cells and MoDC. In the Supplementary Tables 4 and 5, those results are presented for the protein expression analyses in IPEC-J2 cells and MoDC. *(Supplementary Materials)*

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Chapter 6: Characterization of inflammasome components in pig intestine and analysis of the influence of probiotic *Enterococcus faecium* during an *Escherichia coli* challenge

This chapter has been published in: Immunological Investigations

Volume 46, 2017 - Issue 7, Pages 742-757

Manuscript received at the Journal: February 27th, 2017

Initial review completed: April 30th, 2017

Revision accepted: July 04th, 2017

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https://doi.org/10.1080/08820139.2017.1360341

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Chapter 7: Discussion

Enterotoxigenic *E. coli* and the NLRP3 inflammasome response

Inflammasomes are recognized as an integral part of defense mechanisms against intestinal pathogens (Chen and Nunez 2011; Opipari and Franchi 2015). Other than *Salmonella* Typhimurium, leading causes of diarrhea in pigs are pathogenic *E. coli* bacteria, predominantly ETEC (Dubreuil et al. 2016; Sun and Kim 2017). At the time around weaning, certain circumstances, such as the withdrawal of the sow's milk, permit an augmented replication and colonization of toxin-producing *E. coli* bacteria in the piglet's intestine (van Beers-Schreurs et al. 1992). As outlined in the Literature review (**Chapter 2**), inflammasome research in pigs is still in its infancy. In a recent review, Vrentas et al. (2018) have outlined current developments in this young field of research in species of veterinary interest, highlighting the discrepancies seen in these species compared with our understanding of inflammasomes in humans and rodents. In the present thesis, the focus of the investigation was on the impact of a pathogenic ETEC strain frequently causing post-weaning diarrhea in piglets on inflammasome responses and other inflammation parameters in porcine cells and tissues.

The aim of the first study of the present thesis (**Chapter 4**) was to characterize inflammasome activation in porcine MoDC. To assess whether LPS priming is required for inflammasome activation upon bacterial stimulation or to what extent it alters the respective reactions in pigs, inflammasome responses were evaluated in primed and unprimed cells. As a priming signal, *E. coli*-derived LPS and a priming period of 3 h were used. Upon LPS incubation, qPCR analyses revealed increased mRNA expression levels of several inflammasome components, in particular NLRP3, IL-1β, IL-18, and caspase-1. As described in the Literature review (**Chapter 2**), an amplified transcription of NLRP3, IL-1β, and IL-18 is an expected outcome of LPS priming in mice and man. Given the results, LPS appeared to elicit comparable effects in porcine cells. Regarding the necessity of caspase-1 upregulation for inflammasome activation, evidence obtained in murine or rat macrophages is contradictory (Bauernfeind et al. 2011; Xie et al. 2014; Luo et al. 2017). In the study of Bauernfeind et al. (2011), inflammasome activation in murine macrophages was provoked without requiring a prior induction of caspase-1 synthesis.

Corresponding to the elevated IL-1β mRNA expression measured in primed MoDC, the protein secretion of IL-1β was also increased by LPS priming. This is in line with findings achieved in human MoDC, in which LPS addition also elicited IL-1β liberation (Aiba et al. 2003).

According to the present results, NLRP3 inflammasome activation upon ETEC infection occurred in primed and unprimed MoDC with earlier responses being detected in LPS-preincubated cells. At the mRNA level, the stimulatory ETEC effect was mainly evidenced by augmented NLRP3 and IL-1β expression levels with a variable extent over time, whereas such effects on IL-18 mRNA expression were only clearly recognizable in unprimed MoDC. In a study of Zhang et al. (2013), the authors verified NLRP3 inflammasome activation by PRRSV infection in the presence and absence of LPS priming and reported a lower extent of

inflammasome responses without LPS pretreatment compared with that of primed macrophages. In human macrophages, mucosa-associated *E. coli* from patients with inflammatory bowel disease promoted NLRP3 inflammasome activation without additional preconditioning (De la Fuente et al. 2014). As previously elucidated in the Literature review (**Chapter 2**), bacterial stimulation can trigger both steps of inflammasome activation independently, and therefore, a preceding priming signal appears to be dispensable.

Similar to the uncertainty concerning caspase-1 inducibility *via* priming, the effects on caspase-1 mRNA expression upon ETEC exposure were inconclusive in both primed and unprimed MoDC. Since Xue et al. (2017) determined caspase-1 upregulation by Shiga toxin-producing *E. coli* O157:H7 at the protein level, such verification should also have been carried out in the present study. However, under the given circumstances, caspase-1 appeared to be sufficiently available to initiate a subsequent ETEC-triggered IL-1β protein release, even without a previous transcriptional amplification of caspase-1.

In agreement with our own gene expression analyses, the ETEC-induced protein secretion of pro-inflammatory IL-1β was provoked at later stages in unprimed MoDC than in LPS-primed cells. With regard to possible modes of action, the aforementioned *E. coli* serotype O157:H7 has been demonstrated to promote inflammasome-dependent IL-1β liberation by human macrophages, mainly *via* its pore-forming toxin enterohemolysin (Zhang et al. 2012). In murine macrophages, *E. coli*-derived heat-labile enterotoxin has been identified as the agent responsible for IL-1β release associated with NLRP3 inflammasome activation (Li et al. 2014). Apart from these exotoxins, *E. coli* RNA (Kanneganti et al. 2006b; Eigenbrod and Dalpke 2015; Wang et al. 2016) and ROS generated upon *E. coli* infection (Xue et al. 2017) are also relevant for inflammasome activation. Furthermore, Gram-negative bacteria, such as *E. coli*, contain LPS in their outer membrane, which also drives inflammasome activation. It has to be taken into account that inflammasome activation processes are strain-specific and dependent on the host species, as previously shown for murine and human macrophages infected with uropathogenic *E. coli* strains (Schaale et al. 2016). As in this study, most of the insights into inflammasome activation mechanisms have been gained by using immune cells; however, Yen et al. (2016) have also considered it important to evaluate such *E. coli*-associated modes of action in IEC.

In the second study of the thesis (**Chapter 5**), inflammasome responses were investigated in an *in vitro* co-culture model consisting of porcine IEC (cell line IPEC-J2) and MoDC. By using such an intestinal co-culture model, the study aimed to simulate the natural co-existence of both cell types. An assessment was made as to whether inflammatory response patterns of IPEC-J2 cells and porcine MoDC to a bacterial challenge were altered by the interplay between these cells.

The extent to which the detected inflammasome responses were modified by co-cultivation will be discussed in one of the following paragraphs. In general, the pathogenic ETEC challenge induced inflammasome activation in porcine MoDC, as previously reported in the first study (**Chapter 4**). Interestingly, IL-18 mRNA expression in porcine MoDC was less highly regulated in response to bacterial stimulation than IL-1β mRNA expression. In

agreement with this, some authors have stated that IL-18, in contrast to IL-1β, is constitutively expressed, and that, thus, an amplification of its transcription is not mandatory for valid inflammasome activation (Ghonime et al. 2014; Sutterwala et al. 2014). Moreover, the applied ETEC strain was also capable of triggering an inflammasome response in the jejunal epithelial cell line IPEC-J2, as primarily evidenced by an increased NLRP3 mRNA expression. The inflammasome pathway in IPEC-J2 cells has recently been examined by Fan et al. (2018b), who stimulated cells with the mycotoxin zearalenone and found elevated mRNA levels of caspase-1, the ASC adapter, IL-1β, and IL-18, indicating inflammasome activation. Other than these data, no studies exist regarding inflammasome activation in IPEC-J2 cells. To date, the significance of the NLRP3 inflammasome in the inflammatory and homeostatic conditions of the intestine remains a matter of debate (Sellin et al. 2015; Pellegrini et al. 2017; Rathinam and Chan 2018). According to Lissner and Siegmund (2011), the specific cell type determines whether inflammasome signaling acts in a regulatory or disruptive manner. The former is postulated for inflammasome activation in epithelial cells where it induces a renewal of the epithelial layer, whereas in gut-associated immune cells, inflammasome responses represent potentially harmful events (Lissner and Siegmund 2011). The use of murine models of inflammatory bowel disease confirmed that the NLRP3 inflammasome of the intestinal epithelium participates in gut homeostasis (Zaki et al. 2010; Song-Zhao et al. 2014). Based on this understanding, the role of IL-18 is likewise considered as two-sided, possessing a guard function within IEC and having an inflammation-promoting effect within mononuclear cells of the *lamina propria* (Siegmund 2010).

To examine *ex vivo* effects of ETEC on inflammasome signaling, challenge experiments were performed by using porcine jejunal tissue from 80-day-old animals (**Chapter 6**). For this purpose, the jejunal epithelia were incubated with pathogenic ETEC bacteria for 3 h by applying the Ussing chamber technique. At the protein level, ETEC induced IL-1β liberation into the incubation medium, whereas IL-1β mRNA expression (and the mRNA expression of other inflammasome-related genes) remained unaffected. This observation indicated that a preceding transcription of IL-1β was not necessarily required for the assembly of inflammasome components and an associated IL-1β release. Barada et al. (2015) have investigated the time course of IL-1 responses upon electrocautery-induced colitis in rats and found time-dependent IL-1 transcription and secretion in small intestinal tissues, implying a biphasic IL-1 protein response. Likewise, time-dependent inflammasome responses of variable degrees have been documented by Wu et al. (2015) who monitored *E. coli*-triggered IL-1β, IL-18, NLRP3, caspase-1, and ASC expression levels up to 24 h post-infection in a bovine mastitis model. Thus, the sampling time is likely to have an impact on the outcome of such analyses.

Probiotic *Enterococcus faecium* NCIMB 10415 and the NLRP3 inflammasome response

Strong evidence suggests that probiotic species are capable of modulating inflammasome signaling pathways (Llewellyn and Foey 2017). In porcine GALT, probiotic *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Lactobacillus gasseri* have been demonstrated to induce

NLRP3 mRNA expression in neonatal and adult pigs (Tohno et al. 2011). In the present work, probiotic *E. faecium* NCIMB 10415 was selected for its well-known positive effects on enteric infections in piglets (Scharek et al. 2005; Taras et al. 2006; Zeyner and Boldt 2006). The aim was to verify the ability of this probiotic strain to evoke inflammasome activation in the various cell types and intestinal tissues of porcine origin and to test the hypothesis that inflammasome signaling is involved in the probiotic effects of *E. faecium* NCIMB 10415.

In the first study (**Chapter 4**), the influence of *E. faecium* NCIMB 10415 on the inflammasome signaling pathway in porcine MoDC was analyzed *in vitro*. In both primed and unprimed MoDC, probiotic incubation provoked no variations in inflammasome expression levels, either at the mRNA level or at the protein level. With regard to caspase-1 mRNA expression, a slight increase in primed MoDC upon *E. faecium* treatment could be observed. Similarly, caspase-1 activation by *Enterococcus faecalis* was found in a human macrophage cell line, in which the probiotic bacterium in turn caused caspase-1-induced IL-1β transcription and secretion (Yang et al. 2014). Conversely, the latter finding could not be confirmed in the present study. However, in the second study described in the current thesis (**Chapter 5**), *E. faecium* NCIMB 10415 had a weak impact on the inflammasome-linked cytokines IL-18 and IL-1β in MoDC monocultures at both the transcriptomic and the protein levels. The immunomodulatory capacity of various *E. faecium* strains has been documented in former studies in which strain- and dose-dependent IL-1β responses were measured in murine macrophages (Choi et al. 2012; Li et al. 2012) and human MoDC (Mansour et al. 2014).

The first study described in the present thesis further aimed at testing whether preincubation with the probiotic strain altered the inflammasome response upon the subsequent ETEC challenge in porcine MoDC (**Chapter 4**). To this end, MoDC were pretreated with *E. faecium* for 1 h prior to the ETEC incubation. As indicated above, *E. faecium* monoincubation did not affect or only marginally influenced the NLRP3 inflammasome signaling pathway in these cells. In agreement with this finding, probiotic preincubation did not modify the ETEC response. The synthesis and release of the considered inflammasome components (NLRP3, IL-1β, and IL-18) were equally enhanced in the coincubation setup and after ETEC monoincubation. Hence, no NLRP3 inflammasome-dependent ameliorating effects by the probiotic on ETEC infection could be detected. In contrast to these results, Okada et al. (2009) demonstrated that *Bifidobacterium* species reduced *E. coli* LPS-induced IL-1β mRNA upregulation in a murine macrophage cell line. Likewise, probiotic *Lactobacillus rhamnosus* GR-1 revealed its protective potential during an *E. coli* O111:K58 challenge in bovine mammary epithelial cells attributable to a diminished NLRP3 inflammasome activation (Wu et al. 2015). As a possible mechanism for inflammasome activation by Gram-positive (and Gram-negative) bacteria, the cell wall component muramyl dipeptide has been identified (Martinon et al. 2004). In human monocytic cells (cell line THP-1), *Lactobacillus plantarum* lipoteichoic acid alleviated IL-1β responses induced by peptidoglycan derived from *Shigella flexneri* (Kim et al. 2011). The ambiguous results achieved in these different studies emphasize the functional inhomogeneity of probiotic agents.

Referring to one central hypothesis of the present thesis, the observations obtained in the two different experimental designs do not support the assumption that the beneficial effects of *E. faecium* involve the NLRP3 inflammasome signaling pathway in porcine DC.

In the second study detailed in the present thesis (**Chapter 5**), the capacity of the applied *E. faecium* strain to elicit inflammasome activation in IPEC-J2 cells was likewise investigated, with the result that the probiotic did not affect inflammasome expression in IPEC-J2 cells, either in the mono- or in the co-culture systems. In contrast, probiotic *E. coli* Nissle 1917 has been demonstrated to stimulate the NLRP3 inflammasome in human IEC (Caco-2) (Becker et al. 2014). Moreover, previous studies have revealed that probiotic bacterial strains and their products are capable of suppressing pathogen-driven pro-inflammatory responses in IEC (Roselli et al. 2006; Finamore et al. 2014; Ksonzekova et al. 2016). For example, *Lactobacillus reuteri*-derived exopolysaccharides have been shown to attenuate the ETEC-triggered IL-1β response in IPEC-1 cells after preincubation (Ksonzekova et al. 2016). In the human intestinal cell line Caco-2, probiotic species, such as *Lactobacillus amylovorus* and *Lactobacillus rhamnosus*, were able to inhibit the IL-1β and IL-8 reactions caused by ETEC K88 when the probiotic bacteria or their cell-free supernatants were added simultaneously with the enteropathogen (Roselli et al. 2006; Finamore et al. 2014). Since a coincubation of the probiotic and the pathogenic strain was not included in the present study design, no statement regarding *E. faecium*-associated modifications during a pathogenic ETEC challenge in IPEC-J2 cells can be made. However, preliminary experiments revealed no inflammasome-related beneficial potential of *E. faecium* upon ETEC infection in IPEC-J2 cells (unpublished data). Apart from this signaling pathway, our working group has previously found evidence for the capacity of *E. faecium* NCIMB 10415 to protect IPEC-J2 cells against ETEC-induced pro-inflammatory alterations, such as the reduction of heat-shock-protein stress and the IL-8 response (Klingspor et al. 2015) or the prevention of barrier disruption in IPEC-J2 monolayers (Kern et al. 2017).

The impact of *E. faecium* NCIMB 10415 was also investigated in porcine jejunum *ex vivo*, and, additionally, the influence of probiotic preincubation followed by ETEC application was addressed (**Chapter 6**). In the coincubation setup, jejunal tissues were preincubated with *E. faecium* for 1 h as in the *in vitro* co-cultures and subsequently challenged with ETEC for 3 h. The duration of monoincubations was 3 h in this experimental design. Interestingly, the results obtained in this *ex vivo* approach differed markedly from those achieved *in vitro*. In particular, the pretreatment of jejunal epithelia with probiotic *E. faecium* abolished the ETEC-triggered IL-1β protein release. In contrast to the *in vitro* results (**Chapter 4**), this indicates that *E. faecium* is capable of modifying inflammasome signaling. Similarly, in a feeding trial, *Lactobacillus plantarum* strains reduced the expression levels of genes linked to inflammasome signaling in the small intestine of diabetic rats (Vilahur et al. 2015).

In that third study (**Chapter 6**), a further focus was on the effects of probiotic *E. faecium* supplementation to 29-day-old piglets on the mRNA expression of various inflammasome components. The main finding of this feeding trial was a stimulatory effect of *E. faecium* on the ASC expression in the jejunum and ileum in comparison with the control group. However, the

expression profiles of IL-1β, IL-18, NLRP3, and caspase-1 were unchanged by probiotic supplementation. The latter findings were in agreement with those achieved in probiotic-fed dogs, in which *E. faecium* had no influence on the expression of IL-1β, IL-18, and NLRP3 in the duodenum and colon (Schmitz et al. 2015).

In summary, the observations concerning the probiotic effects of *E. faecium* are to some extent contradictory in the present studies and differ between *ex vivo* and *in vitro* approaches. As illustrated earlier, probiotic effects are often related to a specific strain and dependent on a certain dose. In the various studies described in the current thesis, the applied doses of *E. faecium* and ETEC varied depending on the experimental design, considering the appropriateness of the bacterial concentrations for the employed cells and tissues. In particular, whole intestinal tissues were less vulnerable than the used cell cultures. In a recent review of the beneficial effects of probiotic bacteria on ETEC-induced diarrhea in pigs, Dubreuil (2017) has underlined the high variability of results from cell culture *versus* animal studies addressing such probiotic effects. Thus, *in vitro* intestinal co-culture models probably do not fully allow the modeling of the complexity of whole tissues. The suitability of the various models applied in the present work will be discussed in one of the following sections.

Non-canonical inflammasome activation

One hypothesis examined in the current thesis was that porcine caspase-13 represents a promising candidate mediating non-canonical inflammasome signaling in pigs (**Chapter 5**). Caspase-13 mRNA levels increased upon ETEC incubation in porcine MoDC and more clearly in IPEC-J2 cells. These results indicate that ETEC drives non-canonical inflammasome activation in these cells. As outlined in the Literature review (**Chapter 2**), non-canonical inflammasome activation is regulated by caspase-4 in human and by caspase-11 in murine cells. An amplified transcription upon stimulation has been reported for murine caspase-11, whereas human caspase-4 is constitutively expressed (Russo et al. 2018). In this regard, porcine caspase-13 appeared to share similar characteristics as murine caspase-11. Interestingly, IEC-related caspase-11 has been observed to possess protective functions during experimental colitis in mice, as verified by a hypersensitivity to dextran-sulfate-sodium-induced intestinal inflammation in caspase-11-deficient mice associated with impeded IL-18 production (Oficjalska et al. 2015; Williams et al. 2015). Furthermore, non-canonical inflammasome activation has been postulated in human IEC *via Salmonella* Typhimurium and enteropathogenic *E. coli* mediated by human caspase-4 (Kobayashi et al. 2013; Knodler et al. 2014). With regard to underlying modes of action, TLR4-dependent TIR-domain-containing adaptor-inducing interferon-β has been identified to regulate caspase-11 expression and activation by mediating type I interferon signaling in response to Gram-negative bacteria, such as enteropathogenic *E. coli* (Broz et al. 2012; Gurung et al. 2012; Rathinam et al. 2012). Since caspase-13 expression was considerably changed in IPEC-J2 cells, TLR4 mRNA expression levels were studied in these cells. The detected ETEC-induced upregulation indicated an involvement of TLR4 in the signaling pathway for caspase-13 activation in IPEC-J2 cells. Shi et al. (2014) have further verified that, during non-canonical inflammasome activation, murine

caspase-11 and human caspase-4/-5 directly bind to LPS. In a murine model, Lupfer et al. (2014) have demonstrated that ROS drive caspase-11-linked non-canonical inflammasome signaling in mice infected with enteropathogenic *Citrobacter rodentium*. Likewise, bacterial secretion systems have been shown to contribute to caspase-11 activation in murine macrophages (Casson et al. 2013). As the non-canonical inflammasome pathway has not yet been described in pigs, such investigations are lacking in this species.

Analysis of the caspase-13 mRNA expression in porcine MoDC indicated that the impact of ETEC on caspase-13 expression was weaker compared with that found in IPEC-J2 cells. Moreover, caspase-4 and -11 have been found to be abundantly expressed in IEC and intestinal tissues, underlining the significance of non-canonical inflammasome signaling for the epithelial host defense in the intestine (Kang et al. 2004; Demon et al. 2014; Knodler et al. 2014). Therefore, the transcriptional control of caspase-13 in porcine MoDC is assumed to be of minor importance, at least under the given experimental conditions. Future studies are required to verify whether caspase-13 constitutes the porcine functional equivalent to the aforementioned caspases of the non-canonical inflammasome pathway.

The NLRP6 and NLRC4 inflammasome

In addition to the NLRP3 inflammasome, the NLRP6 and NLRC4 inflammasomes represent two well-characterized inflammasomes. The first study presented in the current thesis (**Chapter 4**) not only investigated NLRP3 inflammasome signaling in porcine DC, but also aimed at screening other members of the inflammasome-forming NLR receptor family in these cells. In the second study (**Chapter 5**), the NLRC4 gene was included in the expression analyses in IPEC-J2 cells. In the third study (**Chapter 6**), tissue-specific NLRP6 mRNA expression levels were further examined in the jejunum, ileum, and colon of piglets of different ages (29 and 70 days).

In porcine MoDC, NLRP6 mRNA could not be detected, whereas NLRP6 expression was found in variable quantities in porcine intestinal tissues from different locations, corresponding to the assumption that NLRP6 is primarily present in cells of the intestinal epithelium (Levy et al. 2017). In the intestinal segments, the amount of NLRP6 mRNA differed in dependence on location and age, with declining expression levels from the jejunum to the colon. With regard to the age of the tested animals, it was observed that the detected differences became smaller with increasing age. In terms of these expression patterns, the porcine intestinal mucosa appeared to be more similar to the human counterpart in which NLRP6 is more strongly expressed in the small intestine than in the colon (Gremel et al. 2015). In the murine gut, high NLRP6 expression is found in the epithelial cells of the small and large intestine (Elinav et al. 2011; Normand et al. 2011). Therefore, pigs have been suggested to be a suitable model for such immunological studies on inflammasome signaling pathways in preference to mice. As previously mentioned, the NLRP6 inflammasome is mainly involved in inflammasome signaling in the intestinal epithelium, but in addition to IEC, murine immune cells, including DC, have also been shown to express NLRP6 (Normand et al. 2011). Although Dawson et al. (2017) have stated that the structure of NLRP6 is similar in humans, mice, and

pigs, the findings of the present studies appear to show inconsistencies with regard to the porcine counterparts, e.g., concerning the lack of NLRP6 in porcine MoDC.

NLRC4 mRNA expression was also addressed in porcine MoDC (**Chapter 4**) and IPEC-J2 cells (**Chapter 5**). In the applied experimental designs, porcine MoDC and IPEC-J2 cells were both negative for NLRC4 mRNA. In agreement with this finding, Sakuma et al. (2017) and Dawson et al. (2017) also ascertained no NLRC4 gene expression in pigs, a result that implies the absence of a functional NLRC4 inflammasome in this species. Interestingly, Ahn et al. (2018) have recently reported that the well-known NLRC4 trigger flagellin induces IL-1β secretion by porcine peripheral blood mononuclear cells (PBMC), leading to the conclusion that the NLRC4 inflammasome works properly in these cells. In the comparative study of Dawson et al. (2017), the authors emphasize that alternatives to the NLRC4 inflammasome pathway are probably involved in the immune response to flagellin and type 3 secretion systems derived from *E. coli* in pigs. In murine macrophages, redundant roles are thought to account for the NLRP3 and NLRC4 inflammasomes in responses against *Salmonella* Typhimurium (Broz et al. 2010). In this regard, Ahn et al. (2018) have declared that they doubt whether their determined IL-1β release was evoked by the activation of the NLRC4 inflammasome or by the stimulation of other inflammasomes, such as NLRP3.

The inflammatory response in an *in vitro* co-culture model of porcine intestinal and dendritic cells

In the second study described in the present thesis (**Chapter 5**), pro-inflammatory inflammasome and cytokine responses of IPEC-J2 cells and porcine MoDC were revealed to be influenced by their mutual interplay to different extents.

Porcine MoDC displayed a more tolerogenic phenotype in the presence of IPEC-J2 cells. Compared with MoDC monocultures, the response of co-cultured MoDC to the pathogenic ETEC challenge was attenuated with a lower upregulation of IL-1β, IL-18, and NLRP3 at the mRNA level and, as a trend, of IL-1β at the protein level. Similarly, pro-inflammatory IL-8 reactions were also lower at the mRNA and protein levels in MoDC from IPEC-J2/MoDC co-cultures.

Immune cells, such as DC, have been shown to express NLRP3 abundantly; this enables a large production of IL-1β (Kummer et al. 2007). However, in patients suffering from inflammatory bowel disease, the generation of pro-inflammatory cytokines including IL-1β and IL-8 occurs in an unrestricted and destructive manner (Nakamura et al. 1992; Grimm et al. 1996). In the current study, porcine IEC appeared to alleviate enteropathogen-associated pro-inflammatory reactions in neighboring MoDC. In previous studies, similar beneficial effects of human and murine IEC on the pro-inflammatory responsiveness of adjacent immune cells have been documented (Butler et al. 2006; Chen et al. 2006). In the study of Butler et al. (2006), the pro-inflammatory LPS response of human MoDC was downregulated when they were co-cultivated in direct contact with human IEC (cell line Caco-2). Likewise, murine Peyer's patch lymphocytes have been shown to secrete smaller quantities of pro-inflammatory IL-6 upon LPS challenge in the presence of IEC (Chen et al. 2006).

In contrast to porcine MoDC, the modifications in IPEC-J2 cells attributable to interactions with MoDC were less intense and primarily reflected by caspase-13 expression. As mentioned above, ETEC application induced an upregulation of caspase-13 at the mRNA level. This induction was more pronounced in IPEC-J2 cells co-cultured with porcine MoDC than in IPEC-J2 monocultures. In addition, it was observed that caspase-13 expression in IPEC-J2 cells was also augmented merely as a result of the co-cultivation with MoDC (without any bacterial treatment). To illuminate this finding further, caspase-1 expression was examined in IPEC-J2 cells, and an increase of caspase-1 mRNA was likewise detected in co-cultured IPEC-J2 cells. These similarities indicate a correlation between caspase-13 and caspase-1 expression. In murine co-cultures containing preadipocytes and muscle cells or fibroblasts, the mRNA expression levels of caspase-3, -7 or -9 were in some cases upregulated as an effect of co-culturing (Pandurangan et al. 2012; Subramaniyan et al. 2016). To date, comparable studies on caspases targeting non-canonical inflammasome activation are lacking. Unlike caspase-4, -5, and -11, caspase-1 is able to process interleukins (Ding and Shao 2017). IEC are recognized as the main source of IL-18, which has a pivotal role in the regeneration of the intestinal epithelium (Zaki et al. 2010; Harrison et al. 2015), whereas uncertainty persists regarding the ability of IEC to produce IL-1β (Crowley et al. 2017). Our own observations confirmed the minor production of IL-1β in IPEC-J2 cells. Since IPEC-J2-related IL-18 mRNA expression patterns tended to resemble those found for caspase-13, this indicates a link between caspase-13 and IL-18. Underlying mechanisms of non-canonical inflammasome activation are as yet incompletely understood; however, this signaling pathway appears to reveal differences between IEC and myeloid cells (Crowley et al. 2017). Contrary to the previous assumption that inflammasome signaling is restricted to cells of myeloid origin, recent research has indicated that non-canonical inflammasome signaling in IEC, in particular, plays a key role in immune defenses against enteropathogens (Knodler et al. 2014; Crowley et al. 2017). In conclusion, the obtained results indicate that non-canonical inflammasome signaling prevailing under co-culture conditions is modulated in IPEC-J2 cells.

A further aim was to elucidate the underlying mechanisms of the bidirectional crosstalk between IPEC-J2 cells and MoDC (**Chapter 5**). In the applied Transwell co-culture systems, the two cell types had no direct contact with each other. Consequently, cell-delivered humoral signals, which were able to pass through the filter membrane, were suggested as being involved in IPEC-J2/MoDC interactions. As potential candidates, TSLP and TGF-β were included in the analyses.

In the present study, IPEC-J2 cells and porcine MoDC were both found to be capable of releasing TSLP. TSLP is considered to be an epithelial-derived cytokine, but it can also be produced by immune cells such as DC (Kashyap et al. 2011; Elder et al. 2013; Elder et al. 2016). In these former studies, an autocrine TSLP secretion by DC was demonstrated upon pathogenic or allergenic exposure (Kashyap et al. 2011; Elder et al. 2013; Elder et al. 2016).

Rimoldi et al. (2005b) conditioned human DC with cell-free supernatants of IEC followed by a *Salmonella* challenge in order to establish that soluble factors mediate IEC/DC interactions. Pretreated DC secreted lower amounts of pro-inflammatory cytokines, e.g., IL-1β,

which the authors attributed to conditioning by IEC-delivered TSLP (Rimoldi et al. 2005b). The present data suggest an autocrine regulation mechanism in co-cultured MoDC, as indicated by the elevated TSLP secretion in the presence of IPEC-J2 cells.

With respect to TGF-β, its expression was detectable in both cell types (mRNA and protein levels) under the present experimental conditions, and the expression levels were unaffected by the various treatments. In the aforementioned human intestinal model of Butler et al. (2006), the TGF-β release by IEC was measured and found to be very low in these cells; therefore, TGF-β could not be pinpointed as the IEC-derived modulating agent in their study. Similar to TSLP, TGF-β secretion by human DC appeared to follow autocrine reaction patterns with higher TGF-β levels being detected in DC co-cultured in a direct contact system with Caco-2 cells (Butler et al. 2006). Notably, this observation could not be verified in a second separated co-culture setup (Butler et al. 2006), which resembled the present IPEC-J2/MoDC co-cultures.

In future studies, the utilization of co-culture systems that allow direct contacts between the co-cultured cell types should broaden previous insights. Moreover, other soluble mediators should be addressed to further our understanding of the communication between IEC and underlying immune cells.

Screening of inflammasome expression in porcine small and large intestinal tissues

The third study of the current thesis focused on a functional screening of inflammasome expression along the gut, whereby intestinal tissues were systematically analyzed for various inflammasome components (NLRP3, IL-1β, IL-18, caspase-1, and ASC) (**Chapter 6**). The expression levels between the following intestinal regions were compared: jejunum, ileum, and colon. No data concerning the longitudinal distribution of inflammasomes in porcine intestinal tissues had been previously available. In addition to location effects, two different age groups were included in the experimental design: 29-day-old piglets and 70-day-old growing pigs.

Evaluation of caspase-1 and ASC revealed decreasing mRNA expression levels from the jejunum to the ileum to the colon. This tendency could not be verified with regard to IL-1β. However, in the control group of the feeding trial with *E. faecium* (**Chapter 6**), a trend for decreasing IL-1β mRNA expression levels was noted along the various intestinal tissues of 29-day-old piglets. In a similar study, regional differences of immune genes were investigated in porcine intestinal tissues of piglets of the same age under steady-state conditions (Collado-Romero et al. 2010). In that study, the authors detected higher IL-1β mRNA expression in the ileum than in the colon (Collado-Romero et al. 2010). IL-18 mRNA expression exhibited no clear region-dependent differences in the present study. In comparison, Munoz et al. (2015) determined higher IL-18 expression in murine duodenum compared with jejunum and ileum, whereas no marked differences between the latter two locations were observed.

Apart from that, differences in the distribution of caspase-1 and ASC along the gut were more pronounced in 29-day-old piglets compared with 70-day-old growing pigs. During the development of the porcine immune system, the number and dissemination of intestinal immune cells changed with a greater number of immune cells in the jejunum of 25-day-old

piglets than in the ileum, whereas this gap narrowed in older animals, possibly because of, for example, the increased number of $CD2^+$ cells in the ileum (Brown et al. 2006). Since inflammasomes are expressed in immune cells and also in epithelial cells (see Literature review, **Chapter 2**), the levels of inflammasome-associated genes might be affected by the number of these cell types. However, no age-related effects were noted with regard to IL-1β and IL-18 mRNA expression in the present study. In agreement with this, a comparison between two age groups revealed no age-dependent variations in the IL-1β mRNA level of human ileum (Man et al. 2015).

Models for microbial investigations

In the current work, various bacterial challenge experiments were carried out by employing *in vitro* and *ex vivo* techniques. In the first study, immunological analyses were performed in *in vitro* monocultures of porcine MoDC (**Chapter 4**), whereas in the second part, this approach was broadened by establishing an *in vitro* co-culture model consisting of porcine IEC and MoDC (**Chapter 5**). To draw further comparisons, diverse porcine intestinal tissues were studied *ex vivo* by means of the Ussing chamber technique (**Chapter 6**).

Since knowledge concerning inflammasome pathways in pigs is sparce, the first focus was on analyses of inflammasome responses to bacterial incubation in porcine immune cells. DC were selected as this cell type represents a unique immune cell population crucial for the control of appropriate immune responses in the gut (see Literature review, **Chapter 2**). The generation of blood-derived DC is a widely used technique in various species. Prior to the bacterial challenge experiments, the utilized protocol for the generation of porcine MoDC was established in our laboratory. After the isolation of PBMC by density gradient centrifugation, porcine $CD14^+$ monocytes can be purified by using antibodies against CD14 followed by magnetic-activated cell sorting (MACS) (Wang et al. 2007; Park et al. 2008). Like their human and murine counterparts, porcine MoDC can be differentiated from blood monocytes by the addition of the cytokines IL-4 and granulocyte-macrophage colony-stimulating factor (GM-CSF) (Carrasco et al. 2001; Paillot et al. 2001). The advantages of this procedure are, for example, the ready availability of blood samples and the possibility of repeated blood samplings without the killing of pigs (Summerfield and McCullough 2009). However, this method requires a large number of PBMC (Nedumpun et al. 2016), but this was not limiting in the current studies. Porcine DC are well-characterized and have been shown to be valuable for immunological investigations, e.g., upon pathogenic exposure (McCullough et al. 2009; Summerfield and McCullough 2009; Facci et al. 2010). Moreover, DC are well suited for such studies because of their ability to produce a large quantity of cytokines upon stimulation (Kleiveland 2015). In the present studies (**Chapters 4** and **5**), flow cytometric analyses were performed to confirm the successful generation of porcine MoDC. The surface markers CD14, CD16, CD1, and swine leukocyte antigen (SLA) II were examined before and after differentiation (day 0 and day 6). Determination of CD14 expression allowed the effectiveness of the separation technique after the positive selection of $CD14^+$ cells to be assessed. In addition, viability was monitored during the differentiation process by using propidium iodide

as a viability dye. Flow cytometric phenotypical characterization revealed a high purity of MACS-separated $CD14^+$ monocytes. Following differentiation, the cells were considered as porcine MoDC when tested as being $CD14^+$ $CD16^+$ $CD1^+$ SLA^+ cells.

As initially reviewed (**Chapter 2**), gut-associated DC maintain close contact with the intestinal epithelium. The interplay between porcine MoDC and porcine IEC was the key focus of the second study of the current thesis (**Chapter 5**). To this end, the non-cancerous and non-transformed continuous cell line IPEC-J2 was selected as a porcine small intestinal epithelial model. This cell line is valued as a useful tool for microbiological examination, which may also deliver results transferable to human intestinal infections (Schierack et al. 2006; Brosnahan and Brown 2012). However, *in vitro* intestinal models including IEC merely as monocultures do not adequately reflect the *in vivo* characteristics of functional intestinal epithelia (Bermudez-Brito et al. 2013). Therefore, the aim was to model the intestinal environment *in vitro* by reconstructing the presence of MoDC in the subepithelial compartment of IPEC-J2 cells grown on culture inserts of Transwell systems. The inclusion of IEC into the study design allowed barrier integrity during bacterial challenges to be monitored by TEER measurements, which represent an important readout for the possible pathogen-associated impairment of the epithelial barrier. In a number of differently designed studies, Transwell co-culture systems of human IEC and immune cells were utilized to model inflammatory states of the gut and to assess the impact of various apathogenic and pathogenic bacteria (Haller et al. 2000; Parlesak et al. 2004; Nurmi et al. 2005; Rimoldi et al. 2005a; Mileti et al. 2009; Fang et al. 2010; Pozo-Rubio et al. 2011; Bermudez-Brito et al. 2015). Only a few studies have been carried out in porcine IEC/immune cell co-cultures, in which the focus was on the immunomodulatory capacity of probiotic *Lactobacilli* strains (Nissen et al. 2009; Hosoya et al. 2011; Suda et al. 2014). In none of these human and porcine intestinal co-culture models have inflammasome signaling pathways been addressed. The outcome of the present co-culture study indicates that the reactivity patterns of DC are strongly affected by the interplay with co-existing IEC. The results demonstrate that inflammasome and other inflammatory responses are modified by co-culture. Those co-culture systems are advantageous as they allow the examination of interactions between IEC and underlying immune cells, although they are time-consuming (Kleiveland 2015). Moreover, the systems are cultured under static conditions and, therefore, the experiments are restricted in their duration (Benam et al. 2015). Our own preliminary experiments have revealed that the limiting factors are *(i)* the increasingly detrimental effects of the bacteria over time, a problem that requires the adjustment of bacterial concentrations depending on the experimental setup, and *(ii)* the negative influence of repeated rinsing resulting in enhanced cell detachment.

To expand the findings achieved *in vitro*, an *ex vivo* study was conducted that enabled to capture the entire complexity of the intestinal mucosa (**Chapter 6**). On the one hand, the expression levels of the various inflammasome components were determined in three different segments of the gut (jejunum, ileum, and colon). The data were compared between 29-day- and 70-day-old animals, and in addition, the effect of probiotic supplementation with *E. faecium* NCIMB 10415 was analyzed. On the other hand, Ussing chamber experiments were performed

with jejunal tissues from 80-day-old piglets. In these *ex vivo* experiments, the epithelia were directly incubated with the probiotic *E. faecium* and/or the pathogenic ETEC strain.

The Ussing chamber technique, named after Hans Ussing who introduced this method to study ion transport processes across the epithelium of frog skin (Ussing and Zerahn 1951), involves epithelial tissues (or epithelial cell monolayers grown on permeable cell culture inserts) being mounted in a chamber such that they separate the chamber into an apical and a basolateral compartment; thus, the differentiation between the mucosal and serosal side is allowed. Each half of the system contains buffer solution, which is constantly perfused with gas (95% O_2 and 5% CO_2, also known as carbogen). The Ussing chamber technique has been applied not only to intestinal epithelial tissues, but also, for example, to epithelia originating from the reproductive or respiratory tract, the epithelia of the exocrine and endocrine glands, the choroid plexus, and the eye (Clarke 2009). A major benefit compared with the aforementioned *in vitro* approaches is that whole tissues and not mere single cell types can be evaluated. By using the Ussing chamber method, mucosal permeability can be assessed, in addition to transport and barrier properties and immune responses being analyzed with regard to regional differences along the gut. Consequently, more information is provided compared with investigations involving *in vitro* cell cultures. However, similar to the Transwell co-culture systems, the throughput of this method is limited (Westerhout et al. 2015). The disadvantages of the Ussing chamber technique are the restricted viability of the tissue samples and the often great variations between individual animals (Westerhout et al. 2015). Moreover, mechanical effects attributable to intestinal peristalsis cannot be considered in the analysis of enteropathogen-associated alterations (Benam et al. 2015).

In summary, the work of the current thesis, which was divided into three parts in order to carry out inflammasome-related investigations at various experimental levels, provides insights into porcine inflammasome signaling pathways, which is still a largely unexplored area of research. The effects observed after probiotic *E. faecium* and pathogenic ETEC treatment vary partially between *in vitro* and *ex vivo* experiments, underlining the urgent need for verification at both levels.

Conclusion

The inflammasome pathway represents a relatively newly discovered immune signaling pathway responding to bacteria appearing in the gastrointestinal tract. In the three studies described in the current thesis, inflammasome responses to various bacterial agents were examined in porcine *in vitro* cell culture models and in *ex vivo* experiments with diverse intestinal tissues of pigs.

The bacterial challenge with diarrhea-causing ETEC IMT4818 stimulated the NLRP3 inflammasome *in vitro* and *ex vivo*, whereas probiotic *E. faecium* NCIMB 10415 did not promote inflammasome activation. Given the *in vitro* results, *E. faecium* appeared not to exert its beneficial effects during ETEC infection through NLRP3 inflammasome signaling in porcine DC and the porcine jejunal cell line IPEC-J2. In contrast, during an *ex vivo* ETEC challenge, the probiotic *E. faecium* strain was able to abolish ETEC-induced IL-1β protein liberation from jejunal epithelia, indicating an inflammasome-dependent protective effect of *E. faecium*. With regard to caspase-13, first indications were obtained suggesting its involvement in the non-canonical inflammasome signaling pathway in porcine cells; however, functional considerations require further clarification. The systematic analysis of inflammasome expression in porcine intestinal tissues (jejunum, ileum, and colon) revealed decreasing levels of distinct inflammasome components (NLRP6, ASC, and caspase-1) in an oral to aboral direction resembling the human counterparts.

Furthermore, a porcine *in vitro* co-culture model of IPEC-J2 cells and MoDC was established. Using this intestinal model, a more tolerogenic phenotype of MoDC was determined in response to ETEC when IEC were present. Although the evidence suggests TSLP as a potential mediator between both cell types, mechanisms ensuring the IPEC-J2/MoDC crosstalk should be elucidated in future studies.

The results detailed in the current thesis emphasize the pig as a suitable model for immunological investigations involving inflammasome pathways. Since the findings regarding the positive effects of *E. faecium* are ambiguous, the assessment of such scientific issues at various experimental levels has emerged as being of particular importance. In addition, the porcine intestinal co-culture model appears to be a valuable tool for investigating IEC/immune cell interactions *in vitro*.

Chapter 8: Summary

Summary of the PhD thesis:

Inflammasomes as potential mediators of probiotic effects in porcine intestinal immune and epithelial cells

Post-weaning diarrhea is a widespread problem in pig rearing. Common causative agents are enterotoxigenic *E. coli* bacteria. Since post-weaning diarrhea is associated with a high morbidity and mortality rate, innovative prevention and therapy strategies are required. The probiotic strain *E. faecium* NCIMB 10415 has been demonstrated to be a promising tool to counter this disease. With regard to immunological events, host-pathogen interactions include recognition *via* innate immune receptors, such as NLR, some of which form inflammasomes. These multiprotein complexes license caspase-1 to process the pro-inflammatory cytokines IL-1β and IL-18. Little is known about the involvement of inflammasome signaling pathways in post-weaning diarrhea in piglets or about the role of the NLRP3 inflammasome in promoting probiotic effects.

The objective of the present work was to analyze inflammasome responses to pathogenic ETEC IMT4818 with relevance for post-weaning diarrhea and probiotic *E. faecium* NCIMB 10415, an authorized feed additive for sows and piglets, in *in vitro* and *ex vivo* experiments in various porcine cells and tissues. Since myeloid cells are well suited for investigations into the functions of inflammasomes, the first study of the current thesis was carried out on porcine DC derived from blood monocytes. To determine whether inflammasome signaling contributes to probiotic effects of *E. faecium* NCIMB 10415, porcine MoDC were pretreated with the probiotic prior to a pathogenic ETEC challenge. Moreover, inflammasome activation processes were further monitored in the presence and absence of a priming signal displayed by LPS. LPS priming induced the transcription of inflammasome components, a characteristic of the first step of inflammasome activation. Inflammasome stimulation occurred upon incubation with ETEC, but not with *E. faecium*. As compared with LPS-preincubated cells, the observed ETEC effects appeared at later time points when the MoDC were left unprimed. In the applied experimental setup, preincubation with probiotic *E. faecium* did not mediate protective effects during a pathogenic ETEC challenge *via* the NLRP3 inflammasome in porcine DC.

In the second study, a porcine intestinal co-culture model consisting of IEC (cell line IPEC-J2) and immune cells (MoDC) was established in order to mimic the bidirectional interplay between these two cell types. The aim was to unravel any alterations in the immune response patterns of IPEC-J2 cells and DC to the added bacteria attributable to mutual IEC/immune cell interactions. In addition, the question was addressed as to which soluble factors mediate this communication. Furthermore, the expression of caspase-13 was analyzed, as it has been suggested as a potential candidate driving non-canonical

inflammasome activation in pigs. MoDC revealed a more tolerogenic phenotype in the presence of IPEC-J2 cells showing attenuated inflammasome and IL-8 responses. Porcine caspase-13 was affected by bacterial incubation in each cell type. In the cell line IPEC-J2, non-canonical inflammasome signaling appeared to be initiated by ETEC infection and by co-cultivation with DC. With regard to possible mediators of IPEC-J2/MoDC crosstalk, evidence was found for TSLP secretion by IPEC-J2 cells and MoDC. The detected tolerogenic activity of co-cultured MoDC might be partly explained by an autocrine TSLP regulation in these cells.

The third part of the present thesis comprises a systematic investigation in which inflammasome components have been examined in tissues from the small and large intestine of pigs at two different ages. A feeding trial was aimed at testing the impact of probiotic *E. faecium* on inflammasome expression in piglets. To verify the results obtained *in vitro*, porcine jejunal epithelia were incubated *ex vivo* with the aforementioned bacterial strains in mono- and coincubation setups (probiotic preincubation and a subsequent challenge with ETEC) employing the Ussing chamber technique. The systematic analysis showed that, similar to their human counterparts, the expression of certain inflammasome components (particularly NLRP6, ASC, and caspase-1) decreased gradually from the jejunum to the colon. Probiotic supplementation had only a weak impact on inflammasome expression levels. However, *E. faecium* was capable of reducing ETEC-triggered IL-1β protein liberation in the experiments challenging jejunal tissues. In contrast to the *in vitro* results, this indicated the involvement of the inflammasome pathway in probiotic effects of *E. faecium* NCIMB 10415.

In conclusion, infection with ETEC IMT4818 caused inflammasome activation *in vitro* and *ex vivo*. The results of probiotic treatment with *E. faecium* NCIMB 10415 varied between *in vitro* and *ex vivo* approaches, with inflammasome-related advantageous effects being detected solely in the *ex vivo* study involving the ETEC challenge of porcine jejunum. In the established IPEC-J2/MoDC co-culture model, the presence of IPEC-J2 cells induced a tolerogenic phenotype in co-cultured MoDC indicating that reciprocal interactions between IEC and underlying immune cells orchestrate immunological responses.

Zusammenfassung der Dissertation:

Das Inflammasom als potentieller Vermittler probiotischer Effekte in porzinen Darmimmun- und Darmepithelzellen

Durchfallerkrankungen beim Absatzferkel sind ein weitverbreitetes Problem in der Schweineaufzucht. Häufige verursachende Erreger sind enterotoxische *E. coli*-Bakterien. Da Durchfallerkrankungen beim Absatzferkel mit einer hohen Morbiditäts- und Mortalitätsrate assoziiert ist, sind innovative Präventions- und Therapiestrategien erforderlich. Es wurde gezeigt, dass der probiotische Stamm *E. faecium* NCIMB 10415 ein vielversprechendes Werkzeug darstellt, diesem Erkrankungskomplex zu begegnen. Hinsichtlich der immunologischen Vorgänge kommt es im Zuge der Wechselwirkungen zwischen Wirt und Pathogen zur Erkennung durch angeborene Immunrezeptoren, wie NLR, von denen einige Inflammasome bilden. Diese Multiproteinkomplexe ermöglichen, dass Caspase-1 die pro-inflammatorischen Zytokine IL-1β und IL-18 prozessiert. Es ist wenig über die Beteiligung von Inflammasom-Signalwegen bei Durchfallerkrankungen der Absatzferkel sowie über die Rolle des NLRP3-Inflammasoms bei der Vermittlung probiotischer Effekte bekannt.

Ziel der vorliegenden Arbeit war es, in *in-vitro*- und *ex-vivo*-Versuchen mit verschiedenen porzinen Zellen und Geweben die Inflammasom-Antworten auf den pathogenen Stamm ETEC IMT4818, der bei Durchfallerkrankungen der Absatzferkel eine Rolle spielt, und das Probiotikum *E. faecium* NCIMB 10415, welches als Futterzusatzstoff für Sauen und Ferkel zugelassen ist, zu analysieren. Aufgrund dessen, dass sich myeloide Zellen sehr gut dazu eignen, Funktionen des Inflammasoms zu untersuchen, wurden in der ersten Studie der vorliegenden Dissertation porzine dendritische Zellen verwendet, die aus Blutmonozyten differenziert wurden. Um zu bestimmen, ob der Inflammasom-Signalweg zu den probiotischen Effekten von *E. faecium* NCIMB 10415 beiträgt, wurden porzine Monozyten-abgeleitete dendritische Zellen (MoDC) vor der pathogenen ETEC-Challenge mit dem Probiotikum vorbehandelt. Zudem wurde die Aktivierung des Inflammasoms in der An- und Abwesenheit eines Priming-Signals, welches durch LPS vermittelt wurde, betrachtet. Das Priming mittels LPS induzierte die Transkription von Inflammasom-Komponenten, was den ersten Schritt der Inflammasom-Aktivierung darstellt. Als Folge einer Inkubation mit ETEC, nicht aber durch *E. faecium*, kam es zur Aktivierung des Inflammasoms. Im Vergleich zu mit LPS vorbehandelten Zellen traten die beobachteten ETEC-Effekte zu späteren Zeitpunkten auf, wenn die MoDC keinem Priming unterzogen wurden. In porzinen dendritischen Zellen vermittelte die Präinkubation mit probiotischen *E. faecium*-Bakterien im Rahmen der pathogenen ETEC-Challenge keine protektiven Effekte über das NLRP3-Inflammasom.

In der zweiten Studie wurde ein porzines intestinales Kokultur-Modell aus intestinalen Epithelzellen (Zelllinie IPEC-J2) und Immunzellen (MoDC) etabliert, um das bidirektionale Wechselspiel zwischen diesen beiden Zelltypen nachzuahmen. Das Ziel war es, Veränderungen der immunologischen Reaktionsmuster von IPEC-J2- und dendritischen

Zellen auf die zugegebenen Bakterien aufzudecken, zu denen es infolge der gegenseitigen Beeinflussung kam. Zusätzlich wurde der Fragestellung nachgegangen, welche löslichen Faktoren diese Kommunikation vermitteln. Weiterhin wurde die Expression von Caspase-13 untersucht, welche als potentieller Kandidat für die Steuerung der nicht-kanonischen Inflammasom-Aktivierung im Schwein vorgeschlagen wurde. In Anwesenheit von IPEC-J2-Zellen zeigten die MoDC einen toleranteren Phänotyp, indem sie abgeschwächte Inflammasom- und IL-8-Reaktionen aufwiesen. In beiden Zelltypen wurde die porzine Caspase-13 durch die bakterielle Inkubation beeinflusst. In der Zelllinie IPEC-J2 schien der nicht-kanonische Inflammasom-Signalweg sowohl durch eine Infektion mit ETEC, als auch durch die Kokultivierung mit dendritischen Zellen angestoßen zu werden. In Bezug auf mögliche Mediatoren der Kommunikation zwischen IPEC-J2-Zellen und MoDC wurden Hinweise für eine TSLP-Sekretion durch beide Zelltypen gefunden. Dabei könnte die Toleranzinduktion der kokultivierten MoDC zum Teil auf eine autokrine TSLP-Regulation in diesen Zellen zurückzuführen sein.

Im dritten Teil der vorliegenden Arbeit wurde eine systematische Untersuchung von Inflammasom-Komponenten in Geweben des Dünn- und Dickdarms von Schweinen unterschiedlichen Alters durchgeführt. Ein Fütterungsversuch zielte darauf, den Einfluss des Probiotikums *E. faecium* auf die Inflammasom-Expression in Ferkeln zu prüfen. Um die *in-vitro*-Ergebnisse zu verifizieren, wurden porzine jejunale Epithelien *ex vivo* unter Verwendung der Ussing-Kammer-Technik mit den zuvor genannten Bakterienstämmen in Mono- und Koinkubationsansätzen (Präinkubation mit dem Probiotikum und anschließende ETEC-Challenge) inkubiert. Die systematische Analyse ergab, dass die Expression bestimmter Inflammasom-Komponenten (namentlich NLRP6, ASC und Caspase-1) ähnlich zu ihren humanen Pendants schrittweise vom Jejunum zum Kolon sank. Die Supplementierung mit dem Probiotikum hatte nur einen geringen Einfluss auf das Inflammasom-assoziierte Expressionsniveau. Jedoch waren die *E. faecium*-Bakterien im Rahmen der Challenge-Versuche mit Jejunumgeweben in der Lage, die ETEC-induzierte IL-1β-Proteinsekretion zu reduzieren. Im Gegensatz zu den *in-vitro*-Ergebnissen deutete dies an, dass der Inflammasom-Signalweg an den probiotischen Effekten von *E. faecium* NCIMB 10415 beteiligt ist.

Als Schlussfolgerung ergab sich, dass die Infektion mit ETEC IMT4818 sowohl *in vitro* als auch *ex vivo* zur Inflammasom-Aktivierung führte. Die Resultate der probiotischen Behandlung mit *E. faecium* NCIMB 10415 variierten zwischen *in-vitro*- und *ex-vivo*-Ansätzen insofern, dass vorteilhafte Effekte in Verbindung mit dem Inflammasom-Weg ausschließlich in der *ex-vivo*-Studie gezeigt werden konnten, bei der porzines Jejunum einer ETEC-Challenge unterzogen wurde. Im entwickelten Kokultur-Modell aus IPEC-J2-Zellen und MoDC zeigte sich, dass die Anwesenheit von IPEC-J2-Zellen einen toleranten Phänotyp in kokultivierten MoDC hervorrief, was andeutete, dass reziproke Wechselwirkungen zwischen Darmepithel- und darunter liegenden Immunzellen immunologische Antworten orchestrieren.

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2018

Loss H, Aschenbach JR, Ebner F, Tedin K, Lodemann U
The inflammatory response to enterotoxigenic *E. coli* and probiotic *E. faecium* in a coculture model of porcine intestinal epithelial and dendritic cells
Mediators of Inflammation
DOI: 10.1155/2018/9368295

Loss H, Aschenbach JR, Ebner F, Tedin K, Lodemann U
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2017

Kern M, Aschenbach JR, Tedin K, Pieper R, **Loss H**, Lodemann U
Characterization of inflammasome components in pig intestine and analysis of the influence of probiotic *Enterococcus faecium* during an *Escherichia coli* challenge
Immunological Investigations 46 (7), 742-757
DOI: 10.1080/08820139.2017.1360341

Abstracts in proceedings & participation in conferences

2019

Loss H, Aschenbach JR, Tedin K, Lodemann U
Analysis of the inflammasome signaling pathway in a porcine intestinal co-culture model exposed to probiotic *E. faecium* and enterotoxigenic *E. coli*
73. Jahrestagung der Gesellschaft für Ernährungsphysiologie (GfE) Göttingen – 13.-15.03.2019.
Proceedings of the Society of Nutrition Physiology – Gesellschaft für Ernährungsphysiologie 28, p. 86, ISBN: 978-3-7690-4112-5

2018

Loss H, Aschenbach JR, Tedin K, Lodemann U
Inflammasome signaling in porcine monocyte-derived dendritic cells after stimulation with probiotic *E. faecium* and enterotoxigenic *E. coli*
72. Jahrestagung der Gesellschaft für Ernährungsphysiologie (GfE) Göttingen – 13.-15.03.2018.
Proceedings of the Society of Nutrition Physiology – Gesellschaft für Ernährungsphysiologie 27, p. 123, ISBN: 978-3-7690-4111-8

2017

Loss H, Aschenbach JR, Ebner F, Lodemann U
The inflammatory response of porcine monocyte-derived dendritic cells to treatment with probiotic *E. faecium* and pathogenic *E. coli* strains
71. Jahrestagung der Gesellschaft für Ernährungsphysiologie (GfE) Göttingen – 14.-16.03.2017.
Proceedings of the Society of Nutrition Physiology – Gesellschaft für Ernährungsphysiologie 26, p. 102, ISBN: 978-3-7690-4110-1

2016

Loss H, Aschenbach JR, Lodemann U
Generating porcine monocyte-derived dendritic cells and establishing a co-culture model with porcine intestinal epithelial cells
5. Symposium der Jungen Physiologen Jülich – 22.-23.09.2016.
Abstracts p. 53

Danksagung

Zunächst möchte ich mich bei **Prof. Dr. Jörg Aschenbach** für die Überlassung des spannenden Promotionsthemas bedanken. Ich danke Ihnen dafür, dass Sie mich hinsichtlich der Entscheidung, die Dahlem Research School zu besuchen, bestärkt haben. Den Einblick in die verschiedenen Forschungsbereiche sowie den Austausch mit anderen jungen Wissenschaftlern betrachte ich rückblickend als große Bereicherung. Vielen Dank auch für die konstruktiven Ratschläge und die stetige Unterstützung sowie insbesondere die Förderung während des letzten halben Jahres der Dissertation. Nur dadurch war es mir möglich, diese Arbeit abzuschließen.

Mein größter Dank gilt **PD Dr. Ulrike Lodemann**, mit der mir eine Betreuerin zur Seite stand, die ich mir nicht hätte besser vorstellen können. Du warst immer als Ansprechpartnerin für mich da – selbst nach Aufnahme deiner neuen beruflichen Tätigkeit. Als du mir deine Entscheidung mitteiltest, das Institut zu verlassen, war ich unheimlich traurig (ich wüsste niemanden, der mit mehr Herzblut für die Physiologie dabei ist als du). Dennoch wusste ich, dass ich mich trotzdem jederzeit auf deine Unterstützung verlassen konnte. Das war nicht selbstverständlich, was ich sehr zu schätzen weiß. Vielen Dank für deine unermüdliche Hilfe im Labor, deine vielen Ideen und deine engagierte Art.

Ich möchte mich zudem bei dem dritten Mitglied meines Betreuungsteams bedanken: **Prof. Dr. Robert Klopfleisch**. Ich danke Ihnen dafür, dass Sie sich bereit erklärt haben, mein Mentoren-Team zu vervollständigen sowie für Ihre produktive Kritik und die hilfreichen Anregungen.

Einen großen Dank richte ich an **Katharina Söllig**, die mich im Labor stets großartig unterstützt hat und mich mit ihrem unerschütterlichen Optimismus und ihrer herzlichen Art oftmals vor dem Verzweifeln bewahrte, wenn mal wieder etwas nicht so funktionierte wie geplant.

Darüber hinaus danke ich der **Elsa-Neumann-Stiftung** für die finanzielle Förderung, die mir das Anfertigen der Dissertation überhaupt erst ermöglicht hat.

Außerdem möchte ich mich bei meiner tollen Büro-Kollegin bedanken. Liebe **Judith**, ich habe unsere gemeinsame Zeit am Institut sehr genossen. Ich wünsche mir, dass unsere Freundschaft auch über diese Zeit hinaus bestehen bleibt.

Zu guter Letzt gilt mein ganz besonderer Dank meiner **Familie**, insbesondere meinen Eltern, und **Andy Fester**, die ich in allen Lebenslagen unterstützend an meiner Seite weiß. Danke für den Rückhalt, den ihr mir gebt und dafür, dass ihr immer an mich glaubt.

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 20. August 2019

Henriette Loß

Druck: Mensch und Buch Verlag Berlin