University of Alberta

Studying the effects of mouse prion protein on mice and rat colon antibacterial gene expression profiling

by

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ABSTRACT

The objectives of this thesis research are: 1) to evaluate whether there is interspecies translocation of mouse recombinant prion protein (moPrP) through colon tissue of rats; 2) to investigate whether lipopoplysaccharide (LPS) from *Escherichia coli* influences permeability of moPrP through the colon tissue of mice; 3) to assess whether mouse (mo)PrP affects antibacterial gene expression profile in the colon of rats and mice; 4) to test whether combinations of moPrP with LPS or detoxified LPS (D-LPS) are able to affect genes related to antibacterial response; 5) to examine whether presence of LPS on the mucosal or serosal side of the colon tissues will affect permeability of moPrP and gene expression profiles in the colon. Overall, moPrP alone or in combination with LPS or D-LPS did not translocate through the intact colon tissues of rats or mice. moPrP alone or combined with LPS or D-LPS on the mucosal or serosal sides of diffusion chamber affected expression of various genes related to inflammation and antibacterial response.

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LIST OF ABBREVIATION

APC	Antigen-presenting cell
BBB	Blood brain barrier
Birc3	Baculoviral IAP repeat-containing 3
BPI	Bactericidal permeability-increasing protein
BSE	Bovine spongiform encephalopathy
c-IAP2	Cell inhibitor of apoptosis protein 2
CLR	C-type lectin receptor
CNS	Central nervous system
CVO	Circumventricular organ
CWD	Chronic wasting disease
DC	Dendritic cell
EC	Endothelial cell
ECL	Enzyme chemiluminescence
GPI	Glycosyl-phosphatidylinositol
HSPG	Heparan sulfate proteoglycan
IRAK	IL-1 receptor-associated kinase
ITF	Intestinal trefoil factor
JNK	c-Jun amino terminal kinase
KDO	2-keto-3-deoxyoctonic acid
LBP	LPS-binding protein
LPS	Lipopolysaccharide
LTA	Lipoteichoic acid
Mal	MyD88-adaptor-like protein
MAPK	Mitogen-activated protein kinase

MBM	Meat and bone meal
moPrP	Mouse prion protein
MPLA	Monophosphoryl lipid A
MyD88	Myeloid differentiation primary-response protein 88
NF-kappaB	Nuclear factor kappaB
NLR	Nod-like receptor
NO	Nitric oxide
PAMP	Pathogen-associated molecular pattern
PGN	Peptidoglycan
pIgR	Polymeric Ig receptor
PK	Proteinase K
PMCA	Protein misfolding cyclic amplification
POPG	1-palmitoyl-2-oleoylphosphatidylglycerol
PrP	Prion protein
PrP^{C}	Cellular prion protein
PrP^{Sc}	Scrapie prion protein
PRR	Pattern recognition receptor
PVDF	Polyvinylidene difluoride
RLR	RIG-I-like receptor
rPrP-res	PK-resistant recPrP
$ShaPrP^{Sc}$	Hamster scrapie prion protein
SLPI	Secretory leukocyte protease inhibitor
TLR	Toll-like receptor
TRAM	TRIF-related adaptor molecule
TRIF	TIR-domain-containing adaptor protein inducing IFN-beta

TSE	Transmissible spongiform encephalopathies
GALT	Gut-associated lymphoid tissue

CHAPTER 1: Literature review

1.1 The prion protein: Cellular prion protein (PrP^{C}) and scrapie prion protein (PrP^{Sc})

The causative agent of transmissible spongiform encephalopathy (TSE) is believed to be a protein known as prion, a proteinaceous infectious particle that lacks nucleic acid content (Prusiner, 1998). Accumulated evidence over the past years has supported this hypothesis (Castilla et al., 2005).

1.1.1 Structure of prion protein (PrP)

Prion proteins exist in two different conformations at the level of secondary structure PrP^{C} and PrP^{Sc}. The celluar prion protein (PrP^{C}) is the non-pathogenic form of prions, which is encoded by the *prnp* gene located on chromosome 20 in humans (Robakis et al., 1986). In Syrian hamster, PrP^{C} is composed of 254 amino acids in its unprocessed form. Human PrP has 253 amino acids and show 90% similarity to hamster PrP (Kretzschmar et al., 1986a). PrP^{C} consists of two structural domains; an unordered N-terminal fragment up to residue 128, which contains the octapeptide repeats, and a globular C-terminal domain composed of three α-helices and two small β-strand regions (Soto, 2006). No differences in amino acid sequence between PrP^{C} and PrP^{Sc} have been identified. However, PrP^{C} and PrP^{Sc} differ in their structural characteristics. PrP^{C} is rich in α-helical structure while PrP^{Sc} has substantially more β-sheet structure. In their biochemical properties, while PrP^{C} is monomeric and protease sensitive, PrP^{Sc} is protease-resistant and detergent-insoluble.

1.1.2 Expression of PrP^C

PrP^C is present in various organs and tissues in at least 27 mammalian species, birds and reptiles (Gains and LeBlanc, 2007). PrP^C is highly expressed in the brain, but has also been detected in other non-neuronal tissues. In fact, PrP^C is especially abundant in the central nervous system (CNS; Velayos et al., 2010; Bendheim et al., 1992) but its expression content may change depending on brain regions, cell types, and neurons with distinct neurochemical phenotypes. Immune cellular components in the blood, bone marrow, and peripheral tissues also have high amounts of PrP^C (Linden et al., 2008). Eventually, PrP^C was found to be localised in many other tissues and organs such as gastrointestinal tract (Fournier et al., 1998), mammary gland (Didier et al., 2006), spleen, lymph nodes, kidney, heart, salivary gland, adrenal gland (Horiuchi et al., 1995), thymus, liver (Kubosaki et al., 2001), testes, lungs (Ford et al., 2002), and muscle (Gohel et al., 1999). PrP^C is highly but not exclusively expressed by neuronal cells (Kretzschmar et al., 1986b). PrP^C is also present in immune cells, including neutrophils (Mariante et al., 2012), T cells, dendritic cells, and macrophages. In addition, some authors suggest that the expression of PrP^C may vary with age (Williams et al., 2004) and physiological state (Lasmezas, 2003). The expression pattern of PrP^C suggests a wide-range and conserved function of the protein and supports a fundamental role for PrP^C.

1.1.3 Biological functions of PrP^C

The normal function of the PrP^C in healthy tissue is still unknown. It has been suggested that PrP^C is required for copper metabolism, synaptic transmission,

signal transduction and neuroprotective signaling (Brown et al., 1997; Hodak et al., 2009; Millhauser, 2007; Encalada et al., 2008; Spielhaupter and Schatzl, 2001; Chiarini et al., 2002). PrP^{C} may also play a role in cell survival and differentiation (Chen et al., 2003; Santuccione et al., 2005)

Recently, antimicrobial activities of PrP^{C} have been demonstrated. PrP^{C} has been shown to harbour an exposed cationic and heparin-binding N-terminus (Pan et al., 2002) which can exert antimicrobial activities (Pasupuleti et al., 2009). In addition to this feature, PrP^{C}, a cell-surface protein, is expressed in many tissues and organs and an increase in prion protein expression has been demonstrated during bacterial infection (Konturek et al., 2005) and inflammation (Pammer et al., 2000). Therefore, it has been suggested that antimicrobial activities could be one of the potential functions of prion protein. In fact, the prion protein was shown to have antimicrobial activity against some bacteria comprising Gram-negative *Escherichia coli* and the fungus *Candida parapsiliosis* at normal (pH 7.4) and low pH (pH 5.5) (Pasupuleti et al., 2009). A N-terminal intact form of PrP^{C} (containing amino acids 23-231) bound LPS, a cell wall component of Gram-negative bacteria, at normal as well as low pH (Pasupuleti et al., 2009).

1.2 Conversion of PrP^{C} to PrP^{Sc}

Conversion of PrP^{C} into a pathogenic form, PrP^{Sc}, is the central event in the pathogenesis of transmissible prion diseases. In the most accredited model of prion formation and replication, a physical interaction between the pathogenic PrP^{Sc} template and the endogenous PrP^{C} substrate is a precondition to the

structural changes, thus the pathogenic conversion of PrP (Prusiner, 1998). This notion was supported by an experiment using mice devoid of PrP gene (Bueler et al., 1993). These knockout mice were resistant to prion disease and unable to generate new infectious particles. Mixing purified PrP^{C} with PrP^{Sc} also generates a protease-resistant PrP^{Sc}-like form (PrP-res) *in vitro* by using protein misfolding cyclic amplification (PMCA) system (Saborio et al., 2001). More than 30-fold increases in the amount of PrP-res over that provided in the infected brain extract were reported when mixing detergent extracts of TSE-infected brain homogenate and vast excesses of similar extracts of PrP^{C}-containing normal brain tissue. However, no data have been published so far to indicate whether or not the newly formed PrP-res is infectious.

According to the template-assisted conversion model, the PrP^{C} exists in equilibrium with a transient conformational intermediate (named PrP*), which after interaction with a cellular chaperone coined as "protein X", is able to make a heterodimer with PrP^{Sc} (Cohen and Prusiner, 1998). Spontaneously, this heterodimer is converted into a PrP^{Sc} homodimer consisting of the old and newly formed PrP^{Sc} molecules. "Protein X" was termed to refer the cofactor of the PrP^{C} → PrP^{Sc} conversion (Cohen and Prusiner, 1998). However, there is no formal evidence that the accessory molecule is indeed a protein. The existence of conversion factors also has been suggested by Saborio et al. (2001). In this PMCA studies of PrPC → PrP^{Sc} conversion, purified hamster PrP^{C} was not converted when mixed with highly purified PrP^{Sc}; however, conversion was restored when the complete brain homogenate was added to the sample (Saborio et al., 2001).

The results suggested that unknown factors present in the brain homogenate are essential for prion conversion.

Many investigations aimed at the identification of the unknown cofactor which is thought to be essential for the prion pathogenic conformational conversion and replication. Several studies reported that various different polyanionic compounds comprising proteoglycans molecules, lipids and host-like encoded RNA may bind to PrP^{C} and act as prion conversion factors (Taylor et al., 2009; Wang et al., 2010; Deleault et al., 2003; Deleault et al., 2005). However, treatments that eliminate nucleic acids, proteins or lipids do not prevent prion replication *in vitro* (Abid et al., 2010).

Recent data have demonstrated that PrP^{C} can exert antimicrobial activity and binds to LPS, a cell-wall component of Gram-negative bacteria at normal as well as low pH (Pasupuleti et al., 2009). Therefore, it has been hypothesized that LPS is able to interact with PrP and to trigger the conversion of PrP^{C} to PK resistant β-sheet-rich isoform. In fact, Ametaj et al., (2010) reported that LPS is able to immediately bind PrP^{C} and to catalytically induce the β-rich conformation of PrP, which is resistant to PK under normal pH. Using sonication-free PMCA experiments, the researchers found that the PrP^{β}-seed was generated by adding LPS to rPrP, inducing the prion conversion. Moreover, the pre-form LPS-induced PrP^{β} may play a role as a template in processing the prion conversion and its presence was required and accounted for 100% effective sonication-free PMCA (Ametaj et al., 2010). This investigation also shows that the product of the

conversion (PrP^{β}) has many characteristics of PrP^{Sc} comprising PK-resistance, high beta-rich content, and the propensity to form fibrils.

1.3 Absorption of prions from GI tract

PrP^{Sc} has been considered as a biomaker of infectivity. This protease resistant form of prion protein plays a key role in diagnostics and it is used as a tracking device for investigating pathways of the TSE infectious agent spread within the body (Shmakov and Ghosh, 2001). An early presence of prion infectivity after oral exposure to TSE-infested material is observed in the ileum and tonsil (Keulen et al., 2008). This applies to several species but the most extensively investigations were done in sheep and deer (Heggebo et al., 2003; Sigurdson et al., 2002). PrP^{Sc} accumulation has been found in the intestinal epithelium and in the ganglia of enteric nervous system (ENS) in orally TSE-challenged animals before invading the CNS, indicating this might be the entry pathway of PrP^{Sc} through intestinal mucosa (Beekes and McBride, 2000).

In the oral transmission pathway, there may be the existence of several biological barriers that PrP^{Sc} should overcome. When the pathological agent penetrates the intestinal epithelial barrier, it would initially be subjected to digestion in the gastrointestinal tract. It is worth noting that whereas normal proteins derived from foods are degraded into digested proteins by various peptic secretions in the gastrointestinal tract then incorporated through the absorptive epithelial cells, PrP^{Sc} can withstand the digestive process due to its rich β-sheet content (Ano et al., 2009). Although Jeffrey et al. (2006) reported that PrP^{Sc} are digested by alimentary fluids, trace levels of PrP^{Sc} could be detected by Western blotting in

this experiment, suggesting the ability of PrP^{Sc} to overcome the degradation of gastric-intestinal enzymes. The resistance of this natural barrier may also explain why the efficiency of oral infection in animal models is generally very low that 10 times more agent needs to be given orally compared to direct intra-cerebral inoculation (Prusiner et al., 1985). Once the infectious agent escapes from the digestion in the gastrointestinal tract, the agent would reach the next obstacle, the intestinal epithelium, before invading ENS and CNS.

Lining the vast mucosal surface of the gastrointestinal tract, the epithelia serve as delicate interfaces between external environments, rich in foreign antigens and microbial pathogens, and the internal environment of the mucosa (Neutra et al., 1996). It is not clear how prions cross the intestinal mucosa. However, several routes of entry are conceivable: First, prions may invade the host via M cells (Heppner et al., 2001; Ghosh, 2002), an epithelial cell type that is situated in the follicle-associated epithelium covering organized mucosa-associated lymphoid tissue and is able to translocate particulate matter from the lumen (Neutra et al., 1996). The translocated prions may subsequently be taken up by macrophages or dendritic cells (DCs) and transported to adjacent lymphoid tissues (Beekes and McBride, 2000; Huang et al., 2002). Second, subepithelial DCs may capture prions directly from the lumen of the digestive tract via their dendrites that they extend outside the epithelium and may transport the infectious matter to adjacent lymphoid tissues (Rescigno et al., 2001). Third, prions may become partially degraded by digestive or microbial proteases (Scherbel et al., 2006). The trimmed, yet still infectious, prions may then be translocated by mucosal epithelial lining

cells either directly or after complexation with intraluminal carrier proteins, such as ferritin (Mishra et al., 2004). Subepithelial DCs and macrophages may pick up the trimmed prions and transport them to adjacent lymphoid tissues. Fourth, because of the fact that only a few percent of animals in a herd of cattle exposed to the same feed develop BSE, additional factors (e.g., lesions in the mucosa of the gastrointestinal tract), which may have contributed to or are necessary for the prion uptake, are considered (Weissmann et al., 2002). Prions may exploit microlesions in the epithelial layer induced by stress or microbial infections to cross the epithelium. The broken-through prions may be cleared by subepithelial DCs and macrophages and transported to adjacent lymphoid tissues.

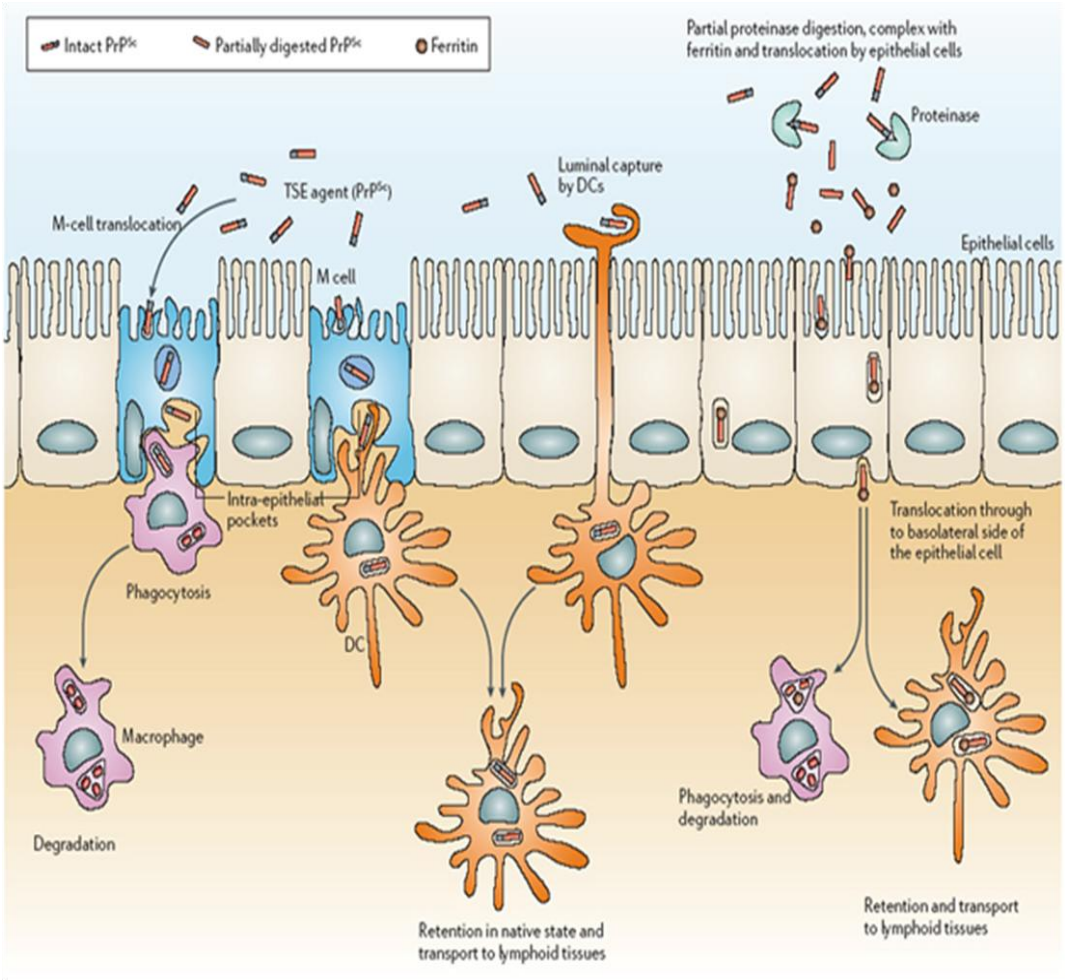

Figure 1.1 Potential mechanisms of transmissible spongiform encephalopathy (TSE) agent translocation across the intestinal epithelium**.** Figure copied with permission from Mabbott and MacPherson (2006)

1.4 Putative link between LPS and BSE origin from meat and bone meal

Although different hypotheses have been suggested, regarding the origins of BSE, clearly the BSE infective agents may spread among the cattle population through ruminant feeding of rendered ruminant meat and bone meal (Smith and Bradley, 2003). Meat and bone meal (MBM) originates from animal rendered by-products including bone, meat, exclusive of any added blood, hair, hoof, horn, hide, manure, stomach, and ruminal contents. In fact, the slaughterhouse-discarded

materials are converted into two products: tallow (fat) and defatted mixture of MBM. The process usually involves mixing, heating with steam, milling and extraction with hydrocarbon organic compounds that act as fat solvents. However, the solvent extraction procedure required a high energy input and fuel prices increased during the 1970s. Thus, to reduce the price of processing MBM, the solvent extraction practice was largely discontinued during the late 1970s and early 1980s (Taylor and Woodgate, 2003). In addition, rendering temperature was lowered compared to the temperature used before 1970. These events are concurrent with the postulated first transmission of BSE to cattle. Therefore, it is plausible that the changes in the rendering practice have resulted in lower inactivation of the infectious PrP^{Sc}, allowing it to concentrate through recycling of infected animals in MBM (Horn et al., 2001).

It is worth noting that two Scottish renderers that contributed the highest percentage of the MBM used in Scotland continued to use heptane and hexane as extraction solvents (Wilesmith et al., 1991). Surprisingly, the incidence of BSE in Scotland was low and the incidence later increased due to the acquisition of subclinically BSE-infected cattle from England. Therefore, it has been suggested that solvent extraction practice was able to diminish the titers of BSE or scrapie infectivity. Nevertheless, an evaluation of the solvent extraction processes used by renderers in the past reported that they could not significantly inactivate the scrapie and BSE agents (Taylor et al., 1997). Why the incidence of BSE in Scotland was low at that time? It must be taken into account that animal rendered by-products are frequently contaminated with bacteria. Whereas the cooking

process may kill bacteria, it does not eliminate the endotoxins from Gram-negative bacteria released when during their death. Noticeably, although endotoxins are resistant to heat, they are neutralized by solvent extraction. Furthermore, recent data support the hypothesis that LPS may be a cofactor for the conversion of PrP^{C} to PrP^{Sc} because it is able to bind PrP^{C} and to catalytically induce a β-rich conformation of PrP which is resistant to proteinase K (Ametaj et al., 2010). Therefore, the capacity to neutralize LPS of solvent extraction applied in Scotland at that time may be a possible reason for the low incidience of BSE in Scotland compared to that in England and it can be proposed that LPS may play a role in TSE infectivity.

1.5 Relationship between inflammatory conditions and prions

It was thought that the neuropathology of prion disease was not associated with an inflammatory response. However, this viewpoint has now been modified with evidence that the inflammatory response in prion diseases is dominated by microglial activation (Perry et al., 2002). It is recently known that the resident macrophages of the CNS, the microglia, may secrete molecules that cause neuronal dysfunction or degeneration when they are activated (Perry et al., 2002). A number of studies have shown that microglia are activated in prion diseases including murine prion diseases (Betmouni et al., 1996), bovine spongiform encephalopathy (Manuelidis et al., 1997), and Creutzfeldt-Jakob disease (Baker et al., 1999).

Gastrointestinal infections caused by viruses, bacteria, and parasites as well as idiopathic inflammatory diseases, are common in animals and humans, and their

contribution to susceptibility to prion infection has been demonstrated. Sigurdson et al. (2009) reported that moderate colitis caused by an attenuated *Salmonella* strain more than doubled the susceptibility of mice to oral prion infection and modestly accelerated the development of disease after prion challenge. The prion protein was up-regulated in intestines and mesenteric lymph nodes of mice with colitis, providing a possible mechanism for the effect of colitis on the pathogenesis of prion disease. Therefore, moderate intestinal inflammation at the time of prion exposure may constitute one of the elusive risk factors underlying the development of TSE.

Prion shedding routes have been shown to be modified by inflammation in excretory organs such as kidney (Seeger et al., 2005) and mammary gland (Ligios et al., 2011). Ligios et al. (2011) reported that sheep with scrapie and lentiviral mastitis secrete prions into the milk and infect nearly 90% of suckling naive lambs.

1.6 Lipopolysaccharide

1.6.1 Structure of LPS

Endotoxin, otherwise known as lipopolysaccharide (LPS), is a major constituent of the outer membrane of the cell wall of Gram-negative bacteria. Single bacterial cell contains approximately 3.5×10^{6} LPS molecules (Rietschel et al., 1994), which are essential for the growth and stability of the bacterium. Endotoxin is released when bacteria grow or when bacteria cell wall is damaged. Endotoxin is encountered on feedstuffs and other sources of animal feed.

A full length LPS molecule consists of four different parts: a lipid A moiety, an inner core, an outer core, and an O-antigen. The lipid A moiety of LPS is composed of two phosphorylated glucosamine saccharides linking at least six fatty acids. This part is the toxic component of the molecule, since injection of a chemically synthesized lipid A induces effects *in vivo* similar to an injection with a full length LPS molecule (Kotani et al., 1985). The two phosphate groups attached to the saccharides are essential for the toxic activity of lipid A. Removal of one phosphate group results in the formation of monophosphoryl lipid A (MPLA), which has a significant reduced bioactivity compared to lipid A (Schromm et al., 2000).

The inner core is the second part of the LPS molecule consisting of two or three 2-keto-3-deoxyoctonic acid (KDO) sugars and two or three heptose sugars. The minimal LPS structure that is produced by bacteria, which is required for growth and stability, consists of one KDO sugar and the lipid A moiety (Raetz, 1990).

The outer core contains common sugars with more structural diversity than the inner core. It is composed of three sugars with one or more sugars covalently bound as side chains.

The fourth part of the LPS molecule is the O-antigen, which is composed of a polymer of oligosaccharides with repeating units of three sugars. The structure of the repeating unit is characteristic for a bacterial strain within a serotype, which results in an important antigen. Lipopolysaccharide molecules containing the O-antigen are denoted as smooth-LPS (S-LPS), since presence of this part results in a smooth appearance of a bacterial colony on a plate. Bacteria expressing LPS

without an O-antigen have a rough (R) appearance when grown on plate; hence the LPS molecule in this case is called R-LPS.

1.6.2 Functions of LPS in Gram-negative bacteria

Lipopolysacchaide is an essential membrane molecule of bacterial cells and has several functions. First, it forms a permeability barrier that is impermeable to large molecules and hydrophobic compounds from the environment and allows only low molecular weight, hydrophilic molecules. This prevents penetration of bacteria by bile salts and other molecules from the GI tract such as lysozyme and antimicrobial peptides. Second, it may impede destruction of the bacterial cells by plasma components and phagocytic cells. Third, LPS serves as an important surface structure in the interaction of the pathogen with its host. For instance, LPS may be involved in adherence, resistance to phagocytosis, or antigenic shifts that determine the course and the outcome of the infection. Thus, the presence of LPS in the bacteria benefits bacterial persistence in host mucosa while recognizing it by host allows the host to indentify and kill invading bacteria in subepithelial tissues and prevent dissemination.

1.7 Mucosal immune responses to lipopolysaccharide in GI tract

Mucosal surface integrates a complex array of mechanisms to prevent the infection by luminal organisms such as attachment or invasion of pathogens and damage by the toxic products. Epithelial barrier is quite vulnerable and its integrity is maintained by mucosal immune system that provides specialized innate and adaptive immunity.

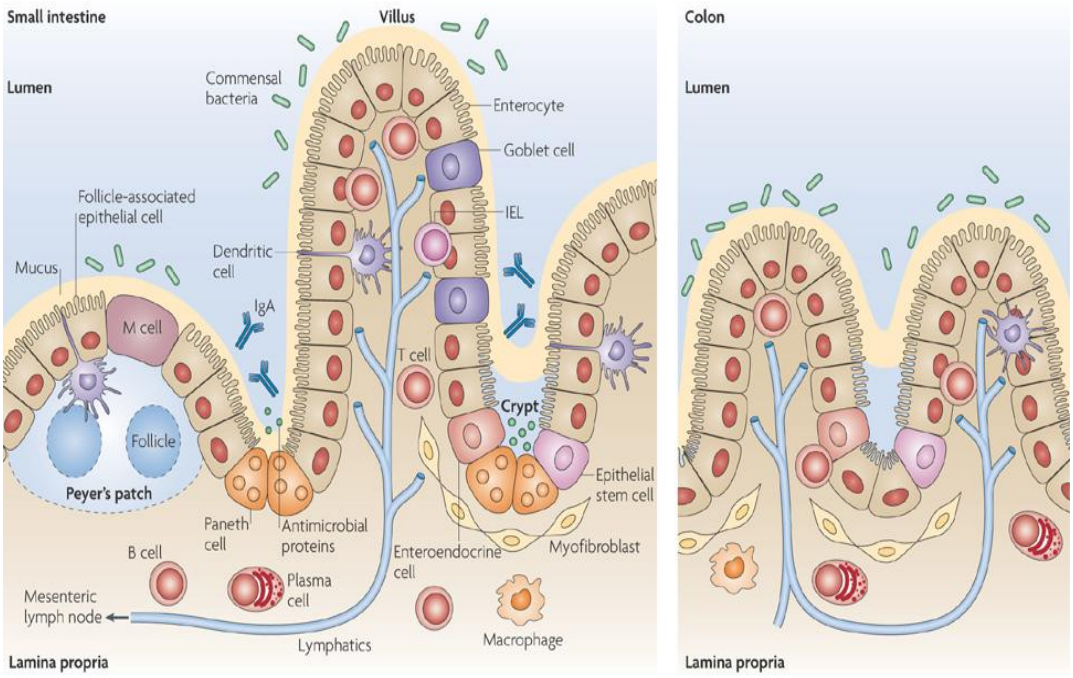

Figure 1.2 Anatomy of the intestinal immune system. A single layer of intestinal epithelial cells (IECs) provides a physical barrier that separates the trillions of commensal bacteria in the intestinal lumen from the underlying lamina propria. The IECs lining the lumen are bathed in nutrients, commensal bacteria, IgA and goblet cell-produced mucus. Epithelial stem cells proliferate and give rise to daughter cells with the potential to proliferate. These IECs then differentiate into villous or colonic enterocytes, which absorb nutrients (small intestine) and water (colon). In addition to differentiated enterocytes and goblet cells, progenitor IECs differentiate into both enteroendocrine cells, which secrete enteric hormones, and Paneth cells at the base of the small intestinal crypts. Beneath the IECs, the lamina propria is made up of stromal cells (myofibroblasts), B cells (especially IgA-producing plasma cells), T cells, macrophages and dendritic cells. Certain subsets of T cells and dendritic cells localize between the IECs. The small intestine has regions of specialized epithelium termed follicle-associated epitheliumand microfold (M) cells that overlie the Peyer's patches and sample the intestinal lumen. IEL, intraepithelial lymphocyte. Figure copied with permission from Abreu (2010)

A number of cells mediate the killing of microbes that occasionally breach the epithelial barrier. Firstly, innate immune cells such as tissue resident macrophage and migratory neutrophils quickly respond to the initial threat of infection.

Macrophages in the intestinal lamina propria are effective in phagocytosis and killing of microbes (Smythies et al., 2005; Smith et al., 2005). However, they are reported to lack innate response receptors including the receptors for LPS (CD14) and also do not produce proinflammatory cytokines, including IL-1, IL-6, IL-10, IL-12, TGF-beta, and TNF-alpha in response to an array of inflammatory stimuli (Smythies et al., 2005). This ensures that macrophages do not continuously respond to harmless bacteria at mucosal sites and acquire profound inflammatory anergy. Neutrophils, the most abundant leukocytes in circulating blood, also play a role in reducing the density of microbes at mucosal surfaces. To respond to an infection, they are rapidly released and migrate from the bone marrow with the contribution of inflammatory signals such as IL-6 and G-CSF and chemokine CXCL8 (IL-8).

Antigens taken up from the lumen by absorptive epithelial cells and specialized epithelial cells (microfold, or ,'M', cells) in mucosal inductive sites can be shuttled to, or directly captured by, 'professional' antigen-presenting cells APCs; including DCs, B lymphocytes, and macrophages) (Bilsborough and Viney, 2004). These APCs then migrate to inductive sites such as Peyer's patches, tonsils, and regional lymph nodes and present antigens to conventional $CD4^+$ and $CD8^+$ $\alpha\beta$T cells, all located in the inductive sites. Certain antigens may also be processed and presented directly by epithelial cells to neighboring intraepithelial T cells, including T cells with limited repertoire diversity ($\gamma\delta$T cells and NKT cells). Interestingly, the majority of intraepithelial lymphocytes and lamina propria lymphocytes, mainly T cells, possess a memory phenotype, suggesting that they

have been retained within the epithelial compartment as a result of previous antigen encounter although they are normally in a resting state to maintain tolerance to the presence of antigen-rich environment (Williams et al., 2012).

Both B and T cells are primed by antigen presenting cells at inductive sites, resulting in activation and differentiation of B and T cells. Then they migrate out of inductive sites, enter the circulation and then seed selected effector sites, mainly the mucosa of origin. Plasma B cells respond by synthesizing antibody, especially IgA, while T cells respond by secreting pro-inflammatory cytokines or instigating cytotoxic effector function. The anatomic affinity of such cells seems to be largely determined by site-specific integrins (homing receptors) on their surface and complementary mucosal tissue-specific receptors (addressins) on vascular endothelial cells. For example, α4β7 integrins expressed on T and B lymphocytes in the lamina propria bind to mucosal addressin cell adhesion molecule (MAdCAM1) which is expressed on the blood vessel wall of the gut. αEβ7 integrins predominant on intraepithelial lymphocytes bind to E-cadherin that is induced by epithelial cells (Williams et al., 2012).

Although the activation of immune cells is essential to prevent infection and eliminating pathogens, this inflammation is strictly controlled by regulating mechanisms so as not to generate excessive immunpathology and maintain tissue homeostasis.

1.8 Functional gene grouping involved in innate immune response to bacteria

The immune system can be divided into the innate and adaptive systems. Both systems are complementary and highly interrelated in the host defence system. While the innate immune system establishes immediate defensive effects on a broad range of pathogens, the adaptive immune system induces a delayed antigen-specific immune response. It is well known that the innate immune system not only induces immediate active defence responses, but also plays important roles in initiating and instructing the adaptive immune response (Janeway and Medzhitov, 2002). Limiting infections to a minimum in the early stage is very critical to limit the spread of foreign pathogen and to recovery from infections.

The recognition of foreign bacteria is the first and critical step in immune response. The recognition of innate immunity relies heavily on the limited number of receptors encoded by germ-line. These receptors evolved to recognize conserved structures or chemicals expressed by foreign pathogens, but not by the host. The molecular characteristics of these microbial components are named as pathogen-associated molecular patterns (PAMPs), and the host receptors that recognize these patterns are called pattern recognition receptors (PRRs). PRRs consist of several families such as Toll-like receptors (TLRs), Nod-like receptors (NLRs), RIG-I-like receptors (RLRs), C-type lectin receptors (CLRs), and DNA-sensing molecules (Kumar et al., 2009). Among them, the majority of TLR and NLR members play a pivotal role in recognition of pathogenic bacteria and their components. The PAMPs-PRRs binding initiates complex signaling pathways that activate various transcription factors, such as NF-κB and interferon regulatory

factors (IRFs), for the induction of antimicrobial responses via the expression of various antimicrobial peptides, proinflammatory cytokines, chemokines, and type I interferons (IFNs) (Trinchieri and Sher, 2007; Kawai and Akira, 2010). These responses initiate innate immunity and subsequently facilitate the eradication of the pathogen.

1.8.1 Toll-like receptor (TLR) signaling

1.8.1.1 Receptors & cofactors

Toll-like receptors (TLRs) are groups of important membrane PRRs specifically recognizing different PAMPs on viruses, bacteria, fungi, and protozoa. To date, 10 and 12 members of the TLR family have been identified in humans and mice, respectively. Of the TLRs, TLR 1, 2, 4, 5, 6, 9 are primarily dedicated to the recognition of various bacteria-derived molecules. Peptidoglycan (PGN) is sensed by TLR2 (Schwandner et al., 1999). In addition to PGN, TLR2 senses several other PAMPs, such as lipoteichoic acid (LTA), diacyl lipopeptides, and triacyl lipopeptides, when TLR2 forms a heterodimer with TLR1 or TLR6. For examples, TLR2/6 heterodimer responds to LTA and diacyl lipopeptide, whereas TLR1/2 heterodimer senses triacyl lipopeptides (Krutzik et al., 2003; Takeuchi et al., 2000; Takeuchi et al., 2002). Flagellin protein expressed by flagellated bacteria is recognized by TLR5. Un-methylated CpG DNA motif serves as a PAMP for TLR9, which is located on the endosomal membrane.

Lipopolysaccharide is a major cell wall component of Gram-negative bacteria and is primarily sensed by TLR4 (Poltorak et al., 1998). Studies indicate that recognition of LPS is also dependent on non-TLR carrier proteins and co-

receptors (Gioannini et al., 2004). Lipopolysaccharide molecules are amphiphilic and present in aggregate when they are released from Gram-negative bacteria. Studies have demonstrated that LPS-binding protein (LBP) dislocates endotoxin aggregates and catalyzes the movement of LPS monomers to membrane-associated CD14 expressed on phagocytic cells or soluble CD14 secreted into serum (Wright et al., 1990; Hailman et al., 1994). CD14 then transfers LPS to MD2, which is a molecule associated with TLR4 and is required for LPS recognition by TLR4 (Schromm et al., 2001).

Toll-like receptors are expressed in different immune cells like antigen presenting cells including DCs, macrophages, and B-cells as well as in non-immune cells like fibroblasts and epithelial cells. In different experimental systems, TLRs have been identified in most cell types, expressed either constitutively or in an inducible manner in the course of infection. Patterns of TLR expression in different cell types and anatomical tissue locations as well as mechanisms regulating TLR gene expression in response to inflammatory mediators are of great relevance and potential profound biological significance.

Binding between ligands and TLRs can induce signal transduction pathways and activate transcription factors within the host cells. The first step of TLR signaling requires the interaction between adaptor proteins and TLRs. The common adaptor molecules are MyD88 (myeloid differentiation primary-response protein 88), TRIF (TIR-domain-containing adaptor protein inducing IFN-beta, also known as TICAM-1), Mal (MyD88-adaptor-like protein, also known as TIR-domain-containing adaptor protein or TIRAP), TRAM (TRIF-related adaptor molecule)

(O'Neill et al., 2003). To induce different signaling cascades, different TLRs may recruit different adaptor molecules. TLR can be divided into the following main pathways: MyD88-dependent pathways, which drive the induction of inflammatory cytokines, and TRIF-dependent (Myd88-independent) pathways, which are responsible for the induction of type I interferon as well as inflammatory cytokines (Akira and Takeuchi, 2006).

1.8.1.2 MYD88-Dependent signaling

MyD88-dependent signaling is activated downstream of all TLRs except TLR3. MyD88 associates with the TIR domain of TLRs. Upon stimulation, MyD88 recruits IL-1 receptor-associated kinases (IRAKs) to TLRs through interaction of the death domains of both molecules. IRAKs are phosphorylated and then associate with tumor necrosis factor receptor-associated factor 6 (TRAF6), leading to the activation of NF-κB and mitogen-activated protein kinases (MAPKs) signaling pathways, and finally inducing the activation of transcription factors NF-κB and AP-1 (Akira and Takeuchi, 2006). Transcription factor IRF-5 was shown to associate with MyD88 and could be activated through unknown mechanism (Takaoka et al., 2005). These transcription factors induce the secretion of pro-infammatory cytokines.

MyD88 is essential for the inflammatory responses mediated by TLRs. MyD88 knockout mice showed no responses to the TLR4 ligand LPS regarding macrophage production of inflammatory mediators, B cell proliferation, or endotoxin shock (Kawai et al., 1999). IRAK family including IRAK1, IRAK2, IRAK3 (IRAK-M), and IRAK4 is also important in Myd88-depedent signaling

pathways. IRAK4 is activated initially and has an essential role in the activation of NF-κB and MAPK downstream of MyD88 (Akira and Takeuchi, 2006). IRAK-4 knockout mice showed severe impairment in the response to microbial components that stimulate TLR2, TLR3, TLR4, and TLR9 (Suzuki et al., 2002). IRAK1 and IRAK2 are activated sequentially, and activation of both kinases is required for robust activation of NF-κB and MAPK (Kawagoe et al., 2008). In contrast, IRAK-M has been indicated to play a negative inhibitory role in the TLR signaling pathway (Kobayashi et al., 2002).

1.8.1.3 TICAM1 (TRIF)-dependent (MYD88-independent) signaling

Studies have evidenced the existence of a MyD88-independent pathway. Kawai et al. (1999) reported that LPS-stimulated MyD88 knockout cells showed activation of NF-κB and JNK in response to TLR4 ligands, although these cells did not produce any inflammatory cytokines. Data also indicated that TLR4-induced IFN-β on murine macrophage was MyD88-independent (Toshchakov et al., 2002). Extensive molecular studies led to the identification of TRIF as the adaptor responsible for signaling in the MyD88-independent pathway (Yamamoto et al., 2003). The TRIF pathway can be exclusively induced by TLR3 and TLR4. TRIF-deficient mice were defective in both TLR3- and TLR4-mediated expression of IFN-β and activation of IRF-3 (Yamamoto et al., 2003).

During analysis of the MyD88-independent pathway, another TIR domain-containing adaptor, TIR domain-containing adaptor protein (TIRAP)/MyD88-adaptor-like (Mal) was also identified. An initial study reported that TIRAP/Mal specifically interacts with TLR4, and is involved in the TLR4-mediated MyD88-

independent signaling pathway (Horng et al., 2001). However, a study on TIRAP/Mal deficient macrophage in response to the TLR4 ligand showed similar results with those of MyD88 knockout macrophages, which included impaired inflammatory cytokine production and delayed activation of JNK and NF-κB (Horng et al., 2002). TLR4 ligand-induced activation of IRF-3 and expression of IFN-inducible genes were normally observed in TIRAP/Mal knockout macrophages, or even in mice lacking both MyD88 and TIRAP/Mal (Horng et al., 2002; Yamamoto et al., 2002). Therefore, TIRAP/Mal has a crucial role in the MyD88-dependent pathway, but not in the MyD88-independent pathway, via TLR4.

1.8.2. NOD-like receptor (NLR) signaling

NLRs can recognize microbial products as well as other intracellular danger signals, thereby initiating host defense pathways through the activation of the NF-kB response and caspases (Mariathasan et al., 2004). The two best characterized members of the NLR family are NOD1 and NOD2. Whereas NOD1 recognizes diaminopimelic acid produced primarily by Gram-negative bacteria, NOD2 is activated by muramyl dipeptide (MDP), a component of both Gram-positive and -negative bacteria. Activation of NOD proteins induces oligomerization and recruitment of downstream signaling molecules and transcriptional up-regulation of inflammatory genes.

Whereas NOD1 and NOD2 stimulation results primarily in activation of proinflammatory gene expression, other NLR proteins are involved in activation of caspases. Pathogens can be recognized by those NLRs in the cytosol, which

assemble a protein complex named inflammasome to activate caspase-1. Activated caspase-1 induces cellular apoptosis and promotes secretion of IL-1β and IL-18.

Inflammasome is composed of the adaptor ASC (associated speck-like protein containing a CARD), pro-caspase-1, and an NLR family member such as Ipaf (Ice protease-activating factor), NALP (NACHT LRR protein) 1, or NALP3. The best known is NALP3 (also called cryopyrin, NLRP3), which senses exogenous and host ligands such as bacterial peptidoglycan, ATP or uric acid (Petrilli et al., 2007; Mariathasan et al., 2006; Gasse et al., 2009). IPAF can directly activate caspase-1 in response to bacterial flagellin (Franchi et al., 2008).

1.8.3 Downstream signal transduction

The net result of TLR engagement of a relevant PAMP is the triggering of downstream signaling pathways, ultimately resulting in the generation of an antimicrobial proinflammatory response.

1.8.3.1 NF-kB pathway

There are five members of the mammalian *NF-κB* family, RelA, RelB, c-Rel, NF-κB1, and NF-κB2 (Hayden and Ghosh, 2004). They act as primary transcription factor found in all cell types and are involved in cellular responses to stimuli such as cytokines and stress. Normally they exist in unstimulated cells as homo- or hetero-dimers bound to a protein complex called inhibitor of kappa B (IκB). IκB inactivates NF-κB by masking the nuclear localization signals (NLS). Activation of NF-κB occurs via degradation of IκB, a process that is initiated by its

phosphorylation by IkappaB kinase (IKK). Phosphorylation also results in IkappaB ubiquitination and targeting to the proteasome. NF-kappaB can now enter the nucleus and regulate gene expression.

1.8.3.2 ERK pathway

Extracellular-signal-regulated kinases (ERKs) belong to the larger family of mitogen-activated protein (MAP) kinases that also includes ERK5, the c-JunNH2-terminal kinases (JNKs), and the p38 MAP kinases (Ramos, 2008). The extracellular signal regulated protein kinase (ERK) pathway involves sequential phosphorylation events that act in a cascade thus amplifying and enabling the transduction of signals from the cell membrane to the nucleus. ERK1/ERK2 (also called MAPK 3 and MAPK 1, respectively) are two distinct isoforms of ERK. MAP kinases are activated in response to many different signals such as growth factor receptors like epidermal growth factor (EGF) and cell adhesion receptors like integrins and this activation is predominantly found in plasma membrane (Ramos, 2008). In general, ligand binding of these receptors leads to GTP loading and activation of the small GTPase Ras, which recruits Raf into the complex on the membrane where Raf becomes activated (Jelinek et al., 1996). Raf subsequently phosphorylates two serine residues on sMAP/ERK kinase (MEK1/2, also known as MAP2K1 and MAP2K2), which in turn phosphorylates and thereby activates ERK (Her et al., 1993). Active ERK is then released from MEK and can dimerize and translocate into the nucleus (Khokhlatchev et al., 1998). In the nucleus ERK may phosphorylate many substrates including transcription factors. ERK1/2 can phosphorylate over 100 possible substrates with diverse

functions, thus activation of ERK can affect a broad array of cellular functions including proliferation, survival, apoptosis, motility, transcription, metabolism and differentiation (Ramos, 2008).

Therefore, MAPK/ERK pathway is one of the prime targets of bacteria like *H. pylori* by which it can induce uncontrolled proliferation and thus stimulate the induction of cell scattering and anti-apoptosis thus evading immune responses and creating a niche to survive. LPS has been shown to activate members of the mitogen-activated protein kinase (MAPK) family, including ERK1/2, c-Jun amino terminal kinases (JNKs), and p38 (van der Bruggen et al., 1999; Lucas and Flynn, 1999; Hambleton et al., 1996; Casey et al., 1994; Guha et al., 2001). Inhibition of MAPK kinase (MEK) in monocytes by a specific inhibitor U0126 reduced LPS induction of several inflammatory cytokines, including interleukin-1, interleukin-8, and TNF-α (Scherle et al., 1998).

1.8.3.3 p38/JNK pathway

Unlike NF-kB pathway in intestinal epithelial cells, which control intestinal immune homeostasis, a member of the mitogen-activated protein kinase (MAPK) family, p38α, in IECs is critical for chemokine expression, subsequent immune cell recruitment into the intestinal mucosa, and clearance of the infected pathogen (Kang et al., 2010). In fact, mice with p38α deletion in IECs suffer from a sustained bacterial burden after inoculation with *Citrobacter rodentium* (Kang et al., 2010). These animals are normal in epithelial integrity and immune cell function, but fail to recruit CD4+ T cells into colonic mucosal lesions. The expression of chemokines in IECs is impaired, which appears to be responsible

for the impaired T cell recruitment. p38 MAPK, also mediates the oxidative damage and reduced duodenal contractions caused by LPS. When p38 MAPK was inhibited by inhibitor SB203580, the increase in COX-2 expression and decrease in duodenal contractility was also inhibited, confirming that COX-2 expression is triggered by the p38 MAPK pathway (Gonzalo et al., 2010). Other types of MAPK such as the c-Jun NH2 terminal kinase (JNK) also have similar effects. JNK expression is increased in LPS-treated rabbits (Gonzalo et al., 2011).

1.8.4 Apoptosis

Apoptosis plays an important role in the immune system. Within this body system, apoptosis is used to regulate the function of immune system cells as well as to eliminate infected cells. Bacterial toxins are known as inducers of apoptosis in immune cells or other cell types. For examples, LPS induces apoptosis in macrophages through the production of TNF-α and nitric oxide (NO). LPS-treated monocytes/macrophages can also induce apoptosis of endothelial cells (EC) (Kirchner et al., 2004).

However, it has been shown that some genes are induced to inhibit apoptosis triggered by aopototic stimuli. *Birc3* (baculoviral IAP repeat-containing 3) or c-IAP2 (cell inhibitor of apoptosis protein 2) has a critical role in the maintenance of a normal innate immune inflammatory response. *Birc3* is induced by NF-kappaB (nuclear factor kappaB) when cells need to respond quickly to different apoptotic triggers. It is likely suppress apoptosis by facilitating the ubiquitination and turnover of active effector caspases in cells (Choi et al., 2009). In mice, LPS has been indicated to potentially upregulate *cIAP2* in macrophage and *cIAP2*$^{-/-}$

macrophage cells are highly susceptible to apoptosis in an LPS-induced proinflammatory environment (Conte et al., 2006).

Caspase recruitment domain 6 (CARD6) plays an important regulatory role in cell apoptosis. It is a protein that activates NF-kappaB signaling evoked by RIP1, RIP2, Bcl-10 and MEKK. The physiological function of CARD6 remains obscure. Despite the structural relatedness of CARD6 to the IFNiGTPases, which plays important roles in cell-autonomous immune responses against various pathogens, Dufner et al. (2008) failed to demonstrate a function for CARD6 in responses against the pathogens.

CARD9 is a caspase recruitment domain-containing signaling protein that is highly expressed in DCs and in macrophages and mainly found in lymphoid tissues and correlated with gastrointestinal lymphoid malignancy (Nakamura et al., 2005). Studies have indicated CARD9 as a central regulator of innate immunity. In fact, CARD9 has been suggested as a key adaptor in innate immune signaling by dectin-1-dependent NF-κB activation in response to fungal infection (Gross et al., 2006). CARD9 is also required for innate immune responses against intracellular pathogens by regulating Nod2-mediated activation of p38 and Jnk (Hsu et al., 2007). Using CARD9-deficient mice in the study, Hsu et al. (2007) showed that CARD9-deficient macrophages had defects in activation of the kinases p38 and Jnk and CARD9-deficient mice failed to clear infection after challenge with *Listeria monocytogenes.* In addition, CARD9 is found associated with BCL10 which function as an activator of apoptosis and NF-κB signaling (Bertin et al., 2000).

Apoptosis is affected through the activation of a cascade of intracellular proteases, collectively known as caspases. Caspase-1 (Casp1) was the first such protein identified on the basis of its sequence homology to the proapoptotic *Caenorhabditis elegans* gene product, ced-3. Casp1 (apoptosis-related cysteine peptidase) is also known as a cysteine protease responsible for the processing and releasing of IL-1β and IL-18, which are two cytokines critical for the induction of inflammation and the active Casp1 also cause rapid macrophage cell death called pyroptosis (Broz et al., 2010). Caspase-1-deficient mice have a defect in the maturation of pro-IL-1β and pro-IL-18 and are resistant to LPS-induced endotoxic shock. Siegmund et al. (2001) also reported that inhibition of Casp1 represents an anti-inflammatory strategy for intestinal inflammation. Caspase-8 is mainly known for its function as an initiator of the death-receptor-induced extrinsic pathway of apoptosis.

1.8.5 Antimicrobial Peptides

Antimicrobial peptides are important components of host defences against microbial invasions. Antimicrobial peptides are largely secreted into the lumen by specialized epithelial cells. Paneth cells, which are located in the base of the crypts, secrete α-defensins in the lumen where they are thought to have direct antimicrobial action that can limit bacterial load and prevent the invasion of pathogenic bacteria. Bactericidal permeability-increasing protein (BPI) is an antimicrobial peptide belonging to the lipid transfer/LPS-binding protein family. It plays important roles in defending against Gram-negative bacteria in the innate immune system. BPI binds LPS via their N-terminal domains and possesses

microbicidal and LPS-neutralizing activity (Iovine et al., 1997). Meanwhile, the C-terminal domain of BPI promotes bacterial attachment to neutrophils and monocytes, leading to phagocytosis (Xu et al., 2005). BPI has been shown to be expressed on mucosal epithelial cells as well as in neutrophil and eosinophil granules (Palmer et al., 2011; Levy et al., 2003).

Cathelicidins (CAMP) represent another major group of antimicrobial peptides found in mammals. Although several members of the family have been identified in mammalian species, to date only one homologue has been found in mice and humans. The human cathelicidin (hCAP18) is synthesized as a precursor 18 kD protein containing the bioactive peptide LL-37 at its C-terminus (Gudmundsson et al., 1996). In the GI tract, LL-37 gene and protein expression was found to be constitutively expressed in surface gastric epithelium and in epithelia of the fundic glands (Hase et al., 2003). The C-terminal antimicrobial peptide from a variety of cathelicidins has been found to be active against a broad range of microbes with a wide overlap in specificity. Several members of the cathelicidin family including LL-37 have the additional property of directly binding and neutralizing LPS. LL-37 has been shown to protect against endotoxic shock in a murine model of septicaemia (Bals et al., 1999).

Secretory leukocyte protease inhibitor (SLPI; also known as antileukoprotease, ALP) was identified as human protease inhibitor that controls excessive proteolysis by neutrophil serine proteases (Wiesner and Vilcinskas, 2010). SLPI was first isolated from bronchial secretions and was later identified to be also produced by many mucosal surfaces, keratinocytes, neutrophils and macrophages.

In addition to their serine protease inhibitory activity, SLPI possesses antimicrobial activity. SLPI is active against *E. coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Staphylococcus epidermidis.* The antimicrobial activity clearly does not depend on inhibition of serine proteases. Instead, disruption of the membranes of target organisms is observed, most likely depending of the cationic nature of these small proteins.

1.9. Conclusion

In conclusion tremendous knowledge has been accumulated during the last few decades about prion proteins and related diseases; however, the precise mechanism(s) of disease and how the disease is initiated still remains elusive. The exact pathway by which prions enter the host and breach the immune system is not known either. The main hypothesis is that exogenous prions enter the host through the food chain and the gastrointestinal tract. How prions are initially acquired from the gut lumen is not known. There have been suggestions that co-factors might play a role; however, these potential cofactors are under investigation. Soon after experimental intragastric or oral exposure with scrapie prions have been found in Peyer's patches, gut associated lymphoid tissues, and ganglia of the enteric nervous system. How the prions make it to these tissues is not clear. Most probably they are transported by the immune cells, especially DCs. The processes involved in the movement of the infectious agent from the gut to the CNS are incompletely understood. The precise functions of prion protein also are not known. Besides several functions inside the cell, there have been suggestions that prion proteins might play a role as antibacterial compounds.

Therefore it is important to continue to study the potential mechanism(s) of entry and the functions of prion proteins in various animal models.

1.10 Research hypotheses

We hypothesized that mouse (mo)PrP will permeate through the colon tissues of rats and mice. In addition, it is hypothesized that LPS from *E. coli* 0111:B4 would affect permeability of moPrP through the rat and mice colon tissues. We also hypothesized that detoxification of LPS by alkaline phosphatase will not affect the transport of moPrP through mice colon tissue. In addition, genes related to antibacterial responses in the colon would be affected by moPrP in both rats and mice and by combination of moPrP with LPS or detoxified (D)-LPS. Finally, it is hypothesized that LPS would be able to permeate through the intact colon tissue of rats and mice. The reasons for the aforementioned hypotheses were that: 1) previously it was shown that PrP^{Sc} is able to go through the colon tissue of cattle and sheep, 2) LPS is able to permeate through rumen and colon tissues of cattle; 3) LPS and moPrP interact with each other resulting in conversion of moPrP from alpha to a beta-rich isoform and able to create fibrils resistant to digestion by protease K.

1.11 Overall objectives

Therefore the objectives of this thesis research are: 1) to evaluate whether there is interspecies translocation of PrP through the colon tissue (mouse recombinant PrP will be used to evaluate whether it will permeate through the rat colon tissue); 2) to investigate whether LPS or detoxified (D)-LPS from *Escherichia coli* 0111:B4

influences permeability of moPrP through the colon tissue of rats and mice; 3) to assess whether moPrP affects antibacterial gene expression profile in the colon tissue of rats and mice; 4) to test whether combinations of moPrP with LPS or D-LPS are able to affect genes related to antibacterial responses; 5) to examine whether presence of LPS or D-LPS on the mucosal or serosal side of the colon tissue will affect permeability of moPrP and gene expression profiles in the colon tissue of rats or mice.

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CHAPTER 2: Recombinant mouse prion protein did not permeate through rat colonic tissue but affected expression of genes related to host immune responses under an Ussing chamber system

2.1 INTRODUCTION

Transmissible spongiform encephalopathies (TSEs), also known as prion diseases, are a group of progressive conditions that affect the brain and the nervous system of various animals including humans. Although it is known that the scrapie prion protein (PrP^{Sc}) is responsible for the development of TSE, the process by which PrP^{Sc} is transported through the gastrointestinal (GI) tract is still not clear.

While PrP^{Sc} enters the organism via the oral route, first it has to cross the gut-epithelial barriers. Less data are available about scrapie uptake and its transportation through the animal gastrointestinal tract and it has been suggested that Peyer's patches and M cells are the most probable sites for the intestinal uptake of the prions (Beekes and McBride, 2000; Miyazawa et al., 2010).

In order to assign a function to the PrP^{C} it has been suggested that PrP^{C} is required for self-renewal of hematopoietic cells (Zhang et al., 2006), activation of T-cells (Zomosa-Signoret et al., 2008), and metal transporting (Pauly and Harris, 1998). An increase in the PrP^{C} expression has been demonstrated during bacterial infections (Konturek et al., 2005) and inflammation like during skin diseases (Pammer et al., 1998). Antibacterial properties have been attributed to prion protein, killing both Gram-negative and Gram-positive bacteria and fungi (Pasupuleti et al., 2009). In addition, requirement of cellular prion protein for

intestinal barrier functions in patient with inflammatory bowel disease has been also reported by Petit et al. (2012).

Previous studies have reported interspecies transmission of prion protein (Bruce et al., 1994; Lasmezas et al., 1997; Jeffrey et al., 2001). *In vitro* research with the Ussing chamber system showed that Syrian hamster scrapie prion protein ($ShaPrP^{Sc}$) is able to translocate through bovine and ovine colons with bovine colon having much higher permeability for macromolecules of 4 kDa and above compared to the ovine one (McKie et al., 1999).

Recently, we demonstrated that *Escherichia coli* (*E.coli*) LPS interacts with the normal PrP^{C} and converts it into a β-rich isoform, resistant to proteinase K (Ametaj et al., 2010). Lipopolysaccharide bound to recombinant Syrian hamster prion protein (ShaPrP) under physiological conditions (saline solution and at 37 °C) was able to convert the protein into a β-rich oligomer that could form amyloid-like fibrils.

We hypothesised that recombinant mouse prion protein (moPrP) will permeate through rat colon mucosa and that addition of LPS from *E. coli* 0111:B4 might influence its transport through the rat colonic tissue. We also hypothesised that moPrP alone or in combination with LPS will affect the expression of genes related to rat antibacterial responses. Therefore the objectives of this investigation were to evaluate, under a Ussing chamber system, whether: 1) moPrP will translocate through the rat intact colon, 2) LPS will affect the transport of moPrP through the rat colonic tissue, and 3) moPrP alone or in combination with LPS will affects various genes related to antibacterial response in the rat colon.

2.2 MATERIALS AND METHODS

2.2.1 Animals and Experimental Design

Eight male rats JCR:LA-*cp*, age 105 ± 8.4 d and body weight 337 ± 18.3 g were used. All rats were fed the same diet before euthanization. The diet composition is shown in Table 2.1. All mice were healthy and did not show clinical signs of disease. All experimental procedures were approved by the University of Alberta Animal Care and Use Committee for Health Sciences and animals were cared for in accordance with the guidelines of the Canadian Council on Animal Care (1993).

Following euthanasia mice colons were removed (9-12 cm), and washed free of feces with Krebs buffer (Table 2.2). Then, the colon tissues were immersed into a Krebs buffer solution, and mounted into a thermostated (38.5 ˚C) Ussing chamber system (six ports in parallel per chamber), (EasyMount Diffusion Chambers, Physiologic Instruments, Harvard Apparatus Canada, St. Laurent, QC) within 40 min from the time of collection. The colon tissues (approximately 1.5 cm^2) were mounted in different Ussing chambers exposing an area of 0.3 cm^2. After mounting to the Ussing chamber, colon tissues were allowed to equilibrate in Krebs buffer (pH 7.4 on both sides of the chamber) for 15 min. Then, 100 µL of 11 mmol of glucose was added and electrical measurements were recorded for 10 min to evaluate tissue validity. After that, the Krebs buffer was replaced with new Krebs buffer with 2 different pHs (i.e., 5.5 and 7.4) in order to simulate acidic conditions (pH = 5.5) on the mucosal side and alkaline conditions (pH = 7.4) on the serosal side of the Ussing chambers. Tissues were left to equilibrate in the new buffer for 15 min before starting the experimental period of 40 min. The total

Ussing chamber procedure lasted 80 min. Ussing chambers in these studies were gassed continuously with 95% O_2 and 5% CO_2. The electrophysiological responses and ionic fluxes were measured using a VCC MC6 multichannel voltage/current clamp and A&A software (data not shown).

Four different treatments were applied to the Ussing diffusion chambers as follows. The control (CTR) group was treated with 70 µL of pyrogen-free H_2O on the mucosal side of the Ussing chamber. Treatment 1 contained 10 µg/mL of LPS on the mucosal side of the Ussing chamber (**LPS M**). Treatment 2 consisted of 10 µg/mL of recombinant moPrP (90-232) on the mucosal side of the Ussing chamber (**PrP M**). In treatment 3 both moPrP and LPS were added on the mucosal side of the Ussing chamber (**PrP + LPS M**) containing 10 µg/mL of LPS and 10 µg/mL of PrP.

Fluid samples were collected on both mucosal and serosal sides of the chambers at 40 min after the initiation of the treatments. All colon tissues were collected at the end of the experimental procedures and stored at -86 °C until analyses.

2.2.2 Measurement of Lipopolysaccharide

Ussing chamber fluid samples from both mucosal and serosal sides were taken for determination of LPS. Determination of LPS was done indirectly by measuring concentrations of fatty acids contained in the lipid A part according to Emmanuel et al. (2007). Briefly, 100 µL of the sample was freeze-dried. The freeze-dried samples were methylated by adding 1,000 µL of methanolic HCl 3N (Supelco,

Bellefonte, PA). The samples were then vortexed before placing them in a water bath at 50 °C and shaken every 5 min for 30 min. Methylated fatty acids were extracted by adding 50 µL of doubly distilled H_2O and 3 mL of hexane and shaking vigorously for 20 s. The top hexane portion was then removed using a disposable Pasteur pipette (Fisher Scientific, Fair Lawn, NJ) and dried under liquid nitrogen. The dried fatty acids were again dissolved in 150 µL of hexane containing internal standard (2 mg C17 in 1,000 3L of hexane) and injected into 100 mm × 0.25 mm with 0.2 µm film thickness capillary column (Supelco, Bellefonte, PA) in a Varian 3400 gas chromatography equipped with Varian 8100 autosampler (Varian, Canada, Inc. Mississauga, Ontario, Canada). Hydrogen was used as the carrier gas (18 psi head pressure). Injector temperature was programmed from 50 °C to 230 °C at 150 °C/min with a run time of 86 min. Detector temperature was set at 230 °C, and peak area integration for fatty acids were made using Galaxy software (Varian Inc., Walnut Creek, CA).

2.2.3 Western Blot Analyses

Concentration of total protein (TP) was determined in all samples collected using NanoDrop 8000 (Thermo Fisher Scientific, Mississauga, ON, Canada) and the sensitivity of Western blot was determined to be 5 µg. Twenty µg of total protein was used to detect the presence of moPrP on the mucosal and serosal sides of the Ussing chamber samples. Fluid samples were precipitated with methanol overnight at -20 °C. After that samples were mixed (1:1) on ice with non-reducing tris-glycine SDS sample buffer (Invitrogen, Burlington, ON, Canada) and heated at 99 °C for 10 min. Denatured samples (15 µL) were loaded onto 12% NuPage

bis-tris polyacrylamide mini gels (Invitrogen, Burlington, ON, Canada) and subjected to electrophoresis in NuPage MESS SDS running buffer in the XCell SureLock^{TM} (Invitrogen, Burlington, ON, Canada). Ten µL of SeeBlue Plus 2 Pre-stained standard protein (Invitrogen, Burlington, ON, Canada) were included in each gel. Proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Millipore Co., Billerica, MA) for 2 h at 60 mA during 1h. Western blot for the moPrP were carried out using Mab antiprion protein Sha31 (SPI-Bio Bertin, Burlington, ON, Canada). The first antibody was diluted 1:30,000 in TBS Tween 0.5% and only 5 mL was used to incubate the membrane at 4 °C temperature overnight with gentle shaking. After washing the membrane with 1X TBS the membrane was incubated with the second antibody, goat anti-mouse HRP conjugate (Biorad, Mississauga, ON, Canada). The second antibody was diluted 1:10,000 in 1X TBS 0.1% Tween containing 5% non-fat dried milk (Carnation, Markham, ON, Canada) and 15 mL was used to incubate the membrane at room temperature during 45 min with gentle shaking. Blots were developed by the enzyme chemiluminescence (ECL) method according to the manufacturer's instructions (Amersham, Oakvillae, ON, Canada).

2.2.4 RNA Extraction and Real-Time PCR Analyses

Total RNA was extracted from tissues using RNeasy Mini Kit (Qiagen, Mississauga, ON, Canada). The concentration and purity of the RNA was determined using NanoDrop 8000 spectrophotometer (Thermo Fisher Scientific, Mississauga, ON, Canada). To eliminate the possible amplification of contaminating genomic DNA, DNAse treatment was carried out and total RNA (1

μg) was reverse-transcribed to cDNA using the RT^2 First Strand Kit (Qiagen, Mississauga, ON, Canada) according to the manufacturer's instructions. The expression profiling of 84 key innate immune response genes in colon tissue was determined by qPCR in a StepOnePlus ABI Prism platform (Applied Biosystems, Burlington, ON, Canada) using the Rat Antibacterial Response PCR Array Kit (Qiagen, Mississauga, ON, Canada). The 84 genes are involved in signaling pathways such as toll-like receptor (TLR) signaling, nod-like receptor (NLR) signaling, other bacterial pattern recognition receptors (PRRs), downstream signal transduction, apoptosis**,** inflammatory response, cytokines & chemokines and antimicrobial peptides. The kit contained primers of 84 genes and 12 controls (5 housekeeping genes including Actin beta (*Actb*), Beta-2 microglobulin (*B2m*), Hypoxanthine phosphoribosyltransferase 1 (*Hprt1*), Lactate dehydrogenase A (*Ldha*), Ribosomal protein, large, P1 (*Rplp1*) as well as genomic DNA contamination–, reverse transcription–, and positive PCR–controls). The qPCR was carried out in a total of 25 μL PCR mixture, containing RT^2 SYBR Green Rox^{TM} qPCR Master Mix (Qiagen, Mississauga, ON, Canada) and cDNA, according to the manufacture recommendation. The amplification conditions were an initial step of 10 min at 95 °C, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The specificity of the amplification products was determined using a melting curve. qPCR data were normalized to housekeeping genes and quantified using the delta-delta comparative threshold (Ct) method, using an analysis tool from Qiagen. Normalized qPCR data were transformed from fold-

change and presented in n fold-regulation relative to CTR group according to the manufacture recommendation.

2.2.5 Statistical Analyses

Gene expression statistical analyses were carried out according to the manufacture recommendations. The *t*-test was used to determine whether the differences observed between the treatments on gene expression were statistically significant ($P \leq 0.05$). The relative differences in gene expression between the different treatments were defined as the relative quantities after normalization. Lipopolysaccharide data were analyzed with the Mixed procedure of SAS (SAS Institute Inc., 2002). The colon tissue was the experimental unit for all analysis. The animal was used as a random effect in the model. Differences between the treatment effects and means were classified by pairwise comparisons, and unless otherwise noted, differences were considered significant at $P \leq 0.05$.

2.3 RESULTS

2.3.1 Measurement of Lipopolysaccharide

Results of gas chromatography analyses indicated that 1 mg/mL of LPS from *E. coli* 0111:B4 provided by Sigma-Aldrich Canada Co. (Oakville, ON) contained 589 µg/mL of 3-hydroxy-myristic acid (3-OH-$C_{14:0}$) as the main fatty acid in the LPS molecule. The other fatty acids contained in the LPS were those of lauric acid ($C_{12:0}$ at 0.32 µg/mL), myristic acid ($C_{14:0}$ at 0.50 µg/mL), palmitic acid ($C_{16:0}$

at 0.14 µg/mL), and stearic acid ($C_{18:0}$ at 0.22 µg/mL). Results also showed that concentrations of all the fatty acids measured on the mucosal and serosal sides of the Ussing chamber samples were not different among the different treatments (Table 2.3).

2.3.2 Measurement of moPrP

Western blot analyses showed presence of moPrP on the mucosal side of the Ussing chamber samples in PrP M and PrP + LPS M. No moPrP was detected in the CTR chambers (CTR M or CTR S). Additionally, moPrP was not detected on the serosal side of the Ussing chamber samples (PrP S, PrP + LPS S, Figure 2.1). The size of the moPrP was 17 kDa. Data also showed that presence of LPS on the mucosal side of the chamber had no effect on the transport of moPrP through the rat colon mucosa.

2.3.3 Gene Expression Profiles in the Colon Tissue

Results of gene expression profiles for the colon tissue samples are shown in Table 2.4. A total of 9 genes were significantly affected by the treatments. From all genes analyzed, one gene was related to TLR signaling (*Map3k7ip1*); *Nod2* related to NOD-like receptor signaling (NLR), *Bcl10* gene was related to downstream signal transduction; *Il12a*, *Ripk1* and *Ripk2* were related to apoptosis; and three other genes were related to cell inflammatory responses (*Il6*, *Cxcl1*, and *Cxcl3*).

Data showed that when LPS alone was present on the mucosal side of the Ussing chamber *Ripk1*, *Ripk2,* and *Nod2* genes were down-regulated. In addition, when moPrP alone was added on the mucosal side of the chamber, *Il6* was up-regulated. Meanwhile when both LPS and moPrP were added on the mucosal side of the Ussing chamber, *Nod2*, *Il12a*, *Il6*, *Cxcl1*, *Cxcl3* expression were down-regulated, whereas *Map3k7ip1* and *Bcl10* gene expressions were up-regulated.

2.4 DISCUSSION

In this study we hypothesised that moPrP alone or in combination with LPS will affect expression of genes related to host antibacterial and immune responses in the rat colon under an Ussing chamber system. Indeed various genes were specifically down- or up-regulated in the presence of moPrP alone or in combination with LPS.

The most important finding of this investigation was that when moPrP was added on the mucosal side of the rat colon *Il6* was significantly up-regulated compared to the CTR group. Traditionally IL-6 has been considered as an activator of acute phase response (Heinrich *et al*., 1990) and a stimulatory factor for lymphocytes (Jones, 2005). Recent investigations have documented several IL-6 activities that are important for resolving innate immunity and initiating acquired immune responses. Interleukin-6 has been identified as one of the main factors in the network of regulators that govern the switching from the innate to adaptive immunity (Jones, 2005). Resolving an acute inflammatory episode involves

recruitment of neutrophils to the site of inflammation, followed by their clearance and replacement by a more sustainable population of mononuclear cells (Topley et al., 1996). This pattern of infiltration relies on IL-6 trans-signaling. Interestingly, the mRNA expression levels of pro-inflammatory cytokines IL-6, IL-1, and TNF-α were elevated in the ascending colon of Parkinson disease patients with respect to controls, and they correlated negatively with disease duration (Devos et al., 2013).

Addition of LPS alone on the mucosal side down-regulated expression of *Nod2*, *Ripk1*, *Ripk2*. Nod proteins are intracellular receptors for muramyl dipeptide (MDP) moieties from bacterial peptidoglycan. Interestingly, recent research demonstrated that the absence of *Nod2* (Nod2−/− mice) had a substantial protective effect in systemic LPS challenge experiments in mice (Pauleau and Murray, 2003). *Ripk1* and *Ripk2* belong to a class of kinases that function in cell survival and cell death mechanisms (Inohara *et al*., 1998; Chin *et al*., 2002). *Ripk1* is a mediator of tumor necrosis factor (TNF) receptor-1-induced cell activation or death, and *Ripk2*, is a key mediator of both innate immune signaling and adaptive immunity (Kobayashi *et al*., 2002). Upon ligand recognition, *Nod2* undergoes conformational changes and self-oligomerization. This is followed by the recruitment and activation of *Ripk2*, which is essential for the activation of NF-κB and MAPKs (Inohara *et al*., 2000; Park *et al*., 2007). Thus the results suggest that NF-κB activation of proinflammatory cytokines production were suppressed during treatment with LPS, under our experimental conditions. It has been reported that overproduction of cytokines via stimulation of monocytes and

macrophages with LPS and other TLR ligands is harmful to the host and can lead to septic shock and death (Dobrovolskaia and Vogel, 2002; Danner et al., 1991). As a form of protection against these deleterious effects, LPS exposure induces a transient state of tolerance or non-responsiveness to subsequent LPS challenge, thus avoiding cytokine-induced immunopathology (Dobrovolskaia and Vogel, 2002). Therefore, the experimental time and LPS concentration used in our experiment were not able to induce the LPS early response or activate the innate immune signaling; however, it might have induced a state of tolerance to LPS challenge.

Another finding of this study was that addition of both moPrP and LPS on the mucosal side of the chambers up-regulated genes related to TLR signaling (*Map3k7ip1*) and downstream signal transduction (*Bcl10)*. *Map3k7ip1* is a kinase, which forms a complex with *Map3k7ip2* and *Map3k7* and regulates the transcription factor NF-κB and production of proinflammatory cytokines IL-1 and TNF-alpha, which direct the adaptive immune response (Wang et al., 2001). *Bcl10* is increasingly recognized as an important mediator of LPS signaling events (Liu et al., 2004). Upregulation of *Bcl10* gene in response to presence of moPrP and LPS in our experiment is in agreement with other reports indicating that the product of this gene is a mediator of LPS activation of NF-κB and proinflammatory cytokine production in normal human intestinal epithelial cells (Bhattacharyya et al., 2007).

On the other hand, presence of both moPrP and LPS on the mucosal side of the chamber down-regulated expression of *Nod2*, *Il12a*, *Il6*, *Cxcl1*, *Cxcl3*. *Cxcl1* is

expressed by colon epithelial cells (Yang et al., 1997) and is regarded as a proinflammatory, chemotactic molecule that affects leukocyte migration (Baggiolini, 1998) and stimulates cell proliferation (Cochran et al., 1983). It also functions in wound repair and inflammation, attracts neutrophils, and promotes angiogenesis (Hogan et al., 1994; Rennekampff et al., 1997). *Ccl3* (macrophage inflammatory protein (MIP-1α) is a potent neutrophil chemoattractant *in vivo*. Its function is necessary to translate an innate-immune response into an acquired immune response (Luster, 2002) inducing the directional migration of targeted populations of leukocytes during periods of inflammation. *Nod2* is important in recognition of intracellular bacterial products. Interleukin-12 promotes Th1 cell differentiation and cell-mediated immunity. Liu et al. (2001) showed that anti-IL-12 treatment abrogates mucosal inflammation and decreases leukocyte infiltration (CD4 cells and macrophages), and down-regulates proinflammatory cytokines IFN-gamma and IL-2. Therefore, down-regulation of the aforementioned genes suggests that combination of moPrP and LPS on the mucosal side suppressed genes related to activation of innate immunity to control the inflammatory process.

PrP^{Sc} has been found in the enteric nervous system of orally challenged rodent models of TSE, suggesting that this might be the route of entry via the intestinal mucosa (Beekes and McBride, 2000). It has also been reported that increased intestinal permeability associated with infection or inflammation (McKay and Baird, 1999) may facilitate oral TSE infection and that administration of endotoxin leads to an increase in colonic and jejunal vascular permeability in rat

and mice (Boughton-Smith *et al.*, 1993; Han *et al*., 2004). We hypothesized that moPrP will permeate through rat colon under an Ussing chamber system and that LPS from *E. coli* 0111:B4 might facilitate the transport of moPrP. Our hypothesis was based on previous research conducted with bovine and ovine colon tissues indicating that $ShaPrP^{Sc}$ is able to permeate through the bovine and ovine colons (McKie et al., 1999). Results of our study showed that moPrP did not go through the intact colonic tissue of rats. These results are different with results of McKie et al. (1999) who showed permeation of $ShaPrP^{Sc}$ through bovine and ovine colon mucosal tissue. The reason for the discrepancy of the results might be related to different ways of preparation of the colon tissues. While McKie et al. (1999) removed the mucosa from the underlying circular and longitudinal smooth muscle layers we used an intact colon tissue in our experiment.

Data indicated that LPS did not go through the intact colon of rats. Previously we showed that LPS was able to translocate through the mucosal layer of cattle colon (Emmanuel et al., 2007). The reason for the discrepancy might be that in Emmauel's study we removed the muscular layer of the colon tissue. It is assumed that even if small amounts of LPS permeated the mucosal layer of the colon, it might have been endocytosed by the intestinal epithelial cells of the colon within the 40 min time period that our experiment lasted. On the other hand, Tomita et al. (2004) showed that the permeability of FITC-LPS in both the mucosal to serosal and serosal to mucosal directions in the rat colon epithelia was inhibited by unlabeled free LPS present in the Ussing chamber.

Lipopolysaccharide molecules form large aggregates and are endocytosed into the epithelial cells through the CD14 receptor and TLR-4 (Tomita et al., 2004).

2.5 CONCLUSIONS

moPrP alone did not translocate from mucosal to serosal side of the intact rat colon. The presence of LPS did not facilitate the transport of moPrP through the intact colonic tissue of rats. When LPS was added alone on the mucosal side of the Ussing chamber it suppressed three genes related to Nod-like receptor signaling (*Nod2*, *Ripk1*, and *Ripk2*). moPrP in the rat colon induced expression of *Il6* gene suggesting a potential role in switching from innate to adaptive immune responses. Lipopolysaccharide in combination with moPrP up-regulated expression of genes related to TLR and downstream signal transduction (*Map3k7ip1* and *Bcl10*, respectively) and down-regulated several genes related to inflammatory response (*IL-12a*, *Il6*, *Cxcl1*, and *Cxcl3*) in the rat colon. Further research is warranted to understand the functions of PrP^{C} on the intestinal tract and interspecies transmissibility of PrP^{C} transportation in the GI tract.

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Table 2.1 Composition of the diet used for rats

Nutrient	Amount
Protein, %	24.6
Fat (ether extract), %	4.8
Fat (acid hydrolysis), %	5.5
Total Saturated Fatty Acids,%	1.22
Total Monosaturated Fatty Acid,%	1.20
Fiber (Crude), %	4.1
Nitrogen-Free Extract (by difference), %	50.1
Total Digestive Nutrients, %	76.2
Gross Energy, kcal/gm	4.14
Physiological fuel Value, kcal/gm	3.42
Metabolizable Energy, kcal/gm	3.02
Ash,%	6.2

Table 2.2 Composition of Krebs buffer solution

Composition	Concentration (mM)	Amount (g)
NaCl	117	6.83748
KCl	4.8	0.35784
$CaCl_2$	2.5	0.367525
$MgCl_2$	1.2	0.243972
$NaHCO_3$	25	2.10025
NaH_2PO_4	1.2	0.143952
Glucose	11	1.98176

Table 2.3 Concentrations of fatty acids on the mucosal and serosal side of the Ussing chamber measured by gas chromatography

	Fatty acid				
Treatment	C12	C14	3OH-C14	C16	C18
	μg/mL				
Control[1]					
Mucosa	0.24	0.22	1,238	1.17	1.15
Serosa	0.11	0.24	1,137	1.45	1.78
LPS M[2]					
Mucosa	0.18	0.16	1,089	0.54	0.51
Serosa	0.13	0.12	978	0.80	0.93
PrP M+LPS M[3]					
Mucosa	0.11	0.11	1,111	0.39	0.40
Serosa	0.22	0.24	1,088	0.81	0.99
Pooled SEM	0.048	0.085	119.86	0.384	0.463
P-values	0.4238	0.7657	0.7328	0.2172	0.1895

[a,b]Means in the same column (within effect) with different superscripts differ significantly ($P \leq 0.05$).
[1]Pyrogen-free water was added on the musosal side of the chamber at 70 μL.
[2]LPS was added on the musosal side of the chamber at 10 μg/mL.
[3] LPS (10 μg/mL) and moPrP (10 μg/mL) were added on the mucosal side of the chamber.

Table 2.4 Antibacterial gene expression in rat colon according to the treatment. Results of gene expression are shown as fold-regulation relative to CTR[1] group

Genes	Description	LPS M[2]	PrP M[3]	LPS M+PrP M[4]
	Toll- like Receptor Signaling			
Map3k7ip1	Mitogen-activated protein kinase kinase kinase 7 interacting protein 1	-1.21±0.99	-1.1±0.57	1.57±0.58*
	NOD-like Receptor Signaling			
Nod2	Nucleotide-binding oligomerization domain containing 2	-2.46±0.61*	-1.38±0.57	-2.04±0.61*
	Downstream signal transduction			
Bcl10	B-cell lymphoma/leukemia 10	1.04±0.57	1.15±0.57	1.57±0.57*
	Apoptosis			
Il12a	Interleukin 12a	-1.54±0.56	-1.78±0.58	-2.03±0.02**
Ripk1	Receptor (TNFRSF)-interacting serine-threonine kinase 1	-1.93±0.59**	-1.1±0.58	-1.26±0.57
Ripk2	Receptor (TNFRSF)-interacting serine-threonine kinase 2	-1.89±0.01*	-1.73±0.57	-1.28±0.01
	Inflammatory response			
Il6	Interleukin 6	-1.2±1.00	2.90±0.58**	-5.15±2.14*
Cxcl1	C-X-C motif chemokine 1	1.04±0.66	1.83±0.0	-2.54±0.17*
Cxcl3	C-X-C motif chemokine 3 Precursor	1.04±0.5	1.82±0.58	-2.02±0.01*

Within a row, means with subscript were different relative to CTR group *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$.
[1]Pyrogen-free water was added on the musosal side of the chamber at 70 µL.
[2]LPS was added on the musosal side of the chamber at 10 µg/mL.
[3]moPrP was added on the mucosal side of the chamber at 10 µg/mL.
[4] LPS (10 µg/mL) and moPrP (10 µg/mL) were added on the mucosal side of the chamber.

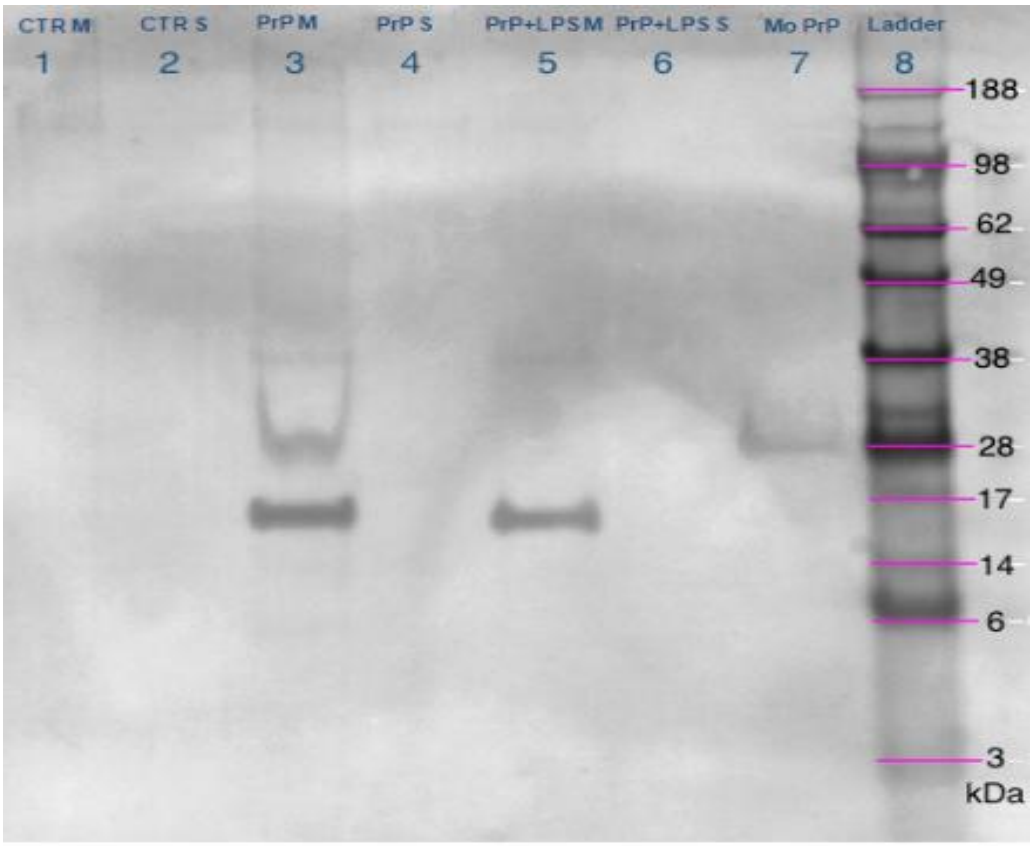

Figure 2.1 Detection of moPrP in mucosal and serosal side of the Ussing diffusion chambers in rat colon by Western blot
Lane 1: Control mucosal side, **Lane 2:** Control serosal side, **Lane 3:** moPrP (90-232) mucosal side, **Lane 4**: moPrP serosal side, **Lane 5:** moPrP + LPS mucosal side, **Lane 6:** moPrP + LPS serosal side, **Lane 7:** Positive CTR moPrP (29-232), **Lane 8:** Ladder

CHAPTER 3: Recombinant mouse prion protein alone or in combination with lipopolysaccharide enhanced expression of genes related to host antimicrobial responses in the colon of mice

3.1 INTRODUCTION

Transmissible spongiform encephalopathies (TSEs), also known as prion diseases, are a group of progressive conditions that affect the brain and the nervous system of many animals including humans. Although it is known that the pathological prion protein (PrP^{Sc}) is responsible for the development of TSE, the mechanism by which PrP^{Sc} enters the host is still unclear.

Oral infection is the major route of prion transmission for many prionoses including bovine spongiform encephalopathy (BSE), chronic wasting disease (CWD) (Belay *et al*., 2004), transmissible mink encephalopathy, scrapie (Andreoletti et al., 2000) and Creutzfeldt- Jacob disease (CJD) (Bruce et al., 1997).

While PrP^{Sc} enters the organism via the oral route, fewer data are available about its uptake and transportation through the animal gastrointestinal (GI) tract. It has been suggested that Peyer's patches are the most probable site for the intestinal uptake of the prion (Beekes and McBride, 2000).

The colon is considered to be relatively impermeable to macromolecules but this permeation greatly increases during stress and inflammatory states in all parts of the intestine, including the colon. Increased intestinal permeability associated with infection or inflammation (McKay and Baird, 1999) may facilitate oral TSE

infection. In a previous study it was reported that $ShaPrP^{Sc}$ translocated from the mucosal to serosal side of bovine and ovine colonic tissues (McKie et al., 1999). Cattle and sheep colonic tissues were permeable to $ShaPrP^{Sc}$ *in vitro* under Ussing chamber system. Bovine colonic tissue showed greater permeability to $ShaPrP^{Sc}$ compared with that of sheep. The authors suggested that greater bovine permeability to $ShaPrP^{Sc}$ may permit entry of much greater amounts of the ingested scrapie into the systemic circulation and thereby seed the transformation of PrP^{C} to PrP^{Sc} within the bovine reticuloendothelial system and brain. These authors concluded that scrapie might be transmitted *in vivo* more easily across the low resistance bovine colonic barrier than in other species.

Different studies have been conducted in order to assign a function to the PrP^{C}. It has been suggested that PrP^{C} is required for self-renewal of hematopoietic cells (Zhang *et al*., 2006), activation of T-cells (Zomosa-Signoret et al., 2008), or as a metal transporter (Pauly and Harris, 1998). An increase in PrP^{C} expression has been demonstrated during bacterial infection (Konturek *et al*., 2005) and inflammation in skin diseases (Pammer *et al*., 1998). Antibacterial properties have been attributed to prion protein, killing both Gram-negative and -positive bacteria and fungi. This antibacterial activity has been suggested to be mediated by the N-terminus of PrP^{C} (Pasupuleti *et al*., 2009).

Microarray studies have reported that alterations in gene expression take place during prion diseases. These studies have identify changes in expression of a great number of genes related to proteolysis, protease inhibition, cell growth, apoptosis, signal transduction, cell adhesion, and immune response (Xiang *et al*., 2004;

Sorensen *et al*., 2008; Filali *et al*., 2011). Those data suggest a possible role of PrP^C in inducing host innate and adaptive immunity.

In a previous study we demonstrated that E. *coli* LPS interacts with the ShaPrP and converts it into a β-rich isoform that could form amyloid-like fibrils and resistant to proteinase K, under physiological conditions (saline solution and at 37 °C) (Ametaj *et al.,* 2010). We hypothesized that recombinant mouse prion protein (moPrP) alone or in combination with LPS from *Escherichia coli* 0111:B4 will be able to permeate through mice intact colon and that during simulated endotoxemia LPS might facilitate translocation of moPrP from mucosal to the serosal side of mice colon. We also hypothesized that expression of genes related to host antibacterial responses will be induced in presence of moPrP alone or in combination with LPS. Therefore the objectives of this study were to test whether moPrP alone will permeate through the intact colonic tissue of mice and whether LPS will facilitate its transport under an Ussing diffusion chamber model. Additionally, expression of 84 genes related to antibacterial responses will be evaluated to assess the potential role of PrP^C in colonic immune responses.

3.2 MATERIALS AND METHODS

3.2.1 Animals and Experimental Design

To test our hypotheses two experiments were conducted. A total of 16 male mice of the FVB/N strain were used to collect colon samples. They were euthanized at age of 67 ± 7 days. All mice were fed the same diet before euthanization. The diet composition is shown in Table 3.1. Mice were all healthy with no clinical signs of

disease until killing before the experiment. All experimental procedures were approved by the University of Alberta Animal Care and Use Committee for Health Sciences and animals were cared for in accordance with the guidelines of the Canadian Council on Animal Care (1993).

Following euthanasia, mice colon was removed (9-12 cm), and washed free of feces with Krebs buffer (composition of Krebs buffer is shown in Table 2.2). Then, the colons were immersed into a Krebs buffer solution and mounted into a thermostated (37 ˚C) Ussing chamber system (six ports in parallel per chamber), EasyMount Diffusion Chambers (Harvard Apparatus, Montreal, QC, Canada) within 40 min from the time of collection. The colon tissues (approximately 1.5 cm^2) were mounted in different Ussing chambers exposing an area of 0.3 cm^2. Krebs buffer solution (7 mL) with an optimal pH (i.e. 7.4) for the rodent's colon tissue was added on the mucosal and serosal sides of the Ussing chamber. The chambers in these studies were gassed continuously with 95% O_2 and 5% CO_2, and temperature kept at 37 ºC. Colon tissues were allowed equilibrating for 15 min. The electrophysiological responses and ionic fluxes were measured using a VCC MC6 multichannel voltage/current clamp and A&A software (data not shown; Harvard Apparatus, Montreal, QC, Canada). The whole Krebs solution was collected on both sides of the chamber 40 min after the initiation of the experiment. All colon tissues were collected at the end of the experimental procedures and stored at -86 °C until analyses.

In the first experiment the mucosal sides of the chambers in the control group (CTR) were supplemented with 700 µL of pyrogen-free H_2O. Treatment 1

consisted in adding 700 µL of a solution containing 1 mg/mL of LPS from *Escherichia coli* O111:B4 strain on the mucosal side of the chamber (LPS M). Mucosal chambers in the treatment 2 were supplemented with 700 µL of a solution containing 1 mg/mL of recombinant mouse (mo)PrP (PrP M; 29-232). In treatment 3, 700 µL of a solution containing both LPS and moPrP at 1 mg/mL each on the mucosal side of the chambers (PrP M + LPS M) was added.

In the second experiment animal's diet and Ussing chamber conditions were the same as those in experiment 1. The mucosal sides of the chambers in the control group (CTR) were supplemented with 700 µL of pyrogen-free H_2O. Treatment 1 consisted in adding 700 µL of a solution containing 1 mg/mL of LPS from *E. coli* O111:B4 strain on the serosal side (LPS S) of the chamber. Mucosal chambers in the treatment 2 were supplemented with 700 µL of a solution containing 1 mg/mL of moPrP (PrP M; 29-232). In treatment 3, in order to simulate endotoxemia, 700 µL of a solution containing LPS at 1 mg/mL was added on the serosal side of the diffusion chambers and 700 µL of a solution containing moPrP at 1 mg/mL was added on the mucosal side (PrP M + LPS S).

3.2.2 Determination of LPS (see details in Chapter 2)

3.2.3 Western Blot (see details in Chapter 2)

3.2.4 RNA Extraction and Real-Time PCR

Total RNA was extracted from tissues using RNeasy Mini Kit (Qiagen, Mississauga, ON, Canada). The concentration and purity of the RNA was determined using NanoDrop 8000 spectrophotometer (Thermo Fisher Scientific,

Mississauga, ON, Canada). To eliminate the possible amplification of contaminating genomic DNA, DNAse treatment was carried out and total RNA (1 μg) was reverse-transcribed to cDNA using the RT^2 First Strand Kit (Qiagen, Mississauga, ON, Canada) according to the manufacturer's instructions. The expression profiling of 84 key innate immune response genes in colon tissue was determined by qPCR in a StepOnePlus ABI Prism platform (Applied Biosystems, Burlington, ON, Canada) using the Mice Antibacterial Response PCR Array Kit (Qiagen, Mississauga, ON, Canada). The 84 genes are involved in signaling pathways such as toll-like receptor (TLR) signaling, nod-like receptor (NLR) signaling, other bacterial pattern recognition receptors (PRRs), downstream signal transduction, apoptosis**,** inflammatory response, cytokines & chemokines and antimicrobial peptides. The kit contained primers of 84 genes and 12 controls (5 housekeeping genes including Actin beta (*Actb*), Beta-2 microglobulin (*B2m*), Glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*), Glucuronidase, beta (*Gusb*), and Heat shock protein 90 alpha (cytosolic), class B member 1 (*Hsp90ab1*) as well as genomic DNA contamination–, reverse transcription–, and positive PCR–controls). The qPCR was carried out in a total of 25 μL PCR mixture, containing RT^2 SYBR Green Rox^{TM} qPCR Master Mix (Qiagen, Mississauga, ON, Canada) and cDNA, according to the manufacture recommendation. The amplification conditions were an initial step of 10 min at 95 °C, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The specificity of the amplification products was determined using a melting curve. qPCR data were normalized to housekeeping genes and quantified using the delta-delta

comparative threshold (Ct) method, using an analysis tool from Qiagen. Normalized qPCR data were transformed from fold-change and presented in n fold-regulation relative to CTR group according to the manufacture recommendation.

3.2.5 Statistical Analyses (see details in Chapter 2)

3.3 RESULTS

3.3.1 Measurement of Lipopolysaccharide

Presence of LPS was determined indirectly by analyzes of fatty acids with Gas Chromatography. Data regarding concentrations of fatty acids on the mucosal and serosal sides of the Ussing chamber for both experiments are shown in Table 3.2 and 3.3. Results showed no significant differences among the treatments.

3.3.2 Measurement of moPrP

In both experiments the Western Blot results showed presence of moPrP only on the mucosal side, in all treatments, but not on the serosal side in mice colonic samples (Figure 3.1). Presence of LPS either on the mucosal or the serosal side of the chamber did not influence the transport of moPrP through the intact colonic tissue of mice.

3.3.3 Gene Expression Profiling

The results of gene expression profiles are shown in Table 3.4. Within the 40 minutes of the experiment presence of LPS on the mucosal side up-regulated the expression of 4 genes related to TLR signaling (*Tlr6, Irf5; P*≤ 0.05 and *P*≤ 0.01,

respectively) and inflammatory response (*Nlrp3* and *Slc11a1*; $P \leq 0.001$ and $P \leq 0.05$, respectively), respectively.

Additionally, when LPS was on the serosal side of the diffusion chamber it affected the expression of 9 genes (Table 3.4). TLR signaling genes *Tlr9, Irf5,* and *Ripk1* were up-regulated ($P \leq 0.05$). NOD-like receptor signaling *Nod2* expression was down-regulated ($P \leq 0.05$), whereas *Sugt1* gene was up-regulated ($P \leq 0.001$). *Card9* gene related to apoptosis was down-regulated ($P \leq 0.05$) and *Nlrp3* and *Tollip* related to immune response were both up-regulated ($P \leq 0.05$ and $P \leq 0.001$, respectively) including the chemokine expression gene *Ccl3* ($P \leq 0.05$). The inflammatory response gene *Nlrp3* expression was also up-regulated ($P \leq 0.05$) in presence of LPS on the serosal side.

When moPrP was added on mucosal side of the chamber (PrP M), only 6 genes were significantly affected by the treatment (Table 3.4). Four of them related to NOD like receptor signaling were up-regulated including *Irf5, Map3k7, Act1,* and *Sugt1* ($P \leq 0.05$, $P \leq 0.001$, $P \leq 0.01$, $P \leq 0.01$, respectively). *Casp1* related to apoptosis was down-regulated ($P \leq 0.05$) and antimicrobial peptide *Slpi* expression was up-regulated > than 10-fold ($P \leq 0.05$).

When moPrP and LPS were added on the mucosal side of the chamber (PrP M + LPS M) 11 genes were affected by the treatment, 10 of them were up-regulated and only *Card9* was down-regulated (Table 3.4). Toll-like receptor signaling genes *Tlr1, Map3k7,* and *Act1* were up-regulated ($P \leq 0.001$) and so did downstream signal transduction genes *Mapk14* and *Mapk8* ($P \leq 0.001$).

Additionally, 4 genes related to apoptosis *Card6, Irak1, Nfkbia*, and *Jun* were up-regulated ($P \leq 0.05$, $P \leq 0.001$, $P \leq 0.05$, and $P \leq 0.01$, respectively) while *Card9* remained down-regulated ($P \leq 0.001$). Inflammatory response genes *Tollip* and *Rac1* were also up-regulated ($P \leq 0.001$).

Interestingly, PrP M + LPS S treatment affected the highest number of genes (Table 3.4). Twenty one genes were up-regulated and only *Casp1* related to apoptosis remained down- regulated. Toll like receptor signaling genes, (*Tlr1, Tlr6, Irf5, Map3K7, Ripk1, Traf6),* NOD-like receptor signaling (*Nod1, Sugt1, Xiap*), downstream signal transduction (*Mapk14*), apoptosis (*Casp8, Irak1, Tnfrsf1a, Jun*), inflammatory response (*Myd88, Rela, Tirap, Tollip*), cytokine (*Il18),* and antibacterial peptides (*Camp, Slpi*) were up-regulated (see Table 3.4 for *P* values).

3.4 DISCUSSION

We hypothesized that moPrP alone or in combination with LPS from *E. coli* 0111:B4 will permeate through the intact mice colon. We also hypothesized that in case of simulated endotoxemia LPS might facilitate translocation of moPrP through the intact mice colon. In our first experiment moPrP alone or in combination with LPS was added on the mucosal side of the mice colon and in the second experiment endotoxemia was simulated by adding LPS on the serosal side of the diffusion chamber. In both experiments we could not detect presence of moPrP on the serosal sides of the chambers showing that there was no transport

of moPrP through the intact colonic tissue of mice. One reason for not detecting the moPrP on the serosal side might be the short time of the experiment (40 minutes). In support of this line of thinking Takakura et al. (2011) investigated the sequence of events during the invasion of orally administered PrPs through the intestinal mucosa and the spread into lymphoid tissues and the peripheral nervous system. They reported that orally administered PrPs was incorporated by intestinal epithelial cells in the follicle-associated epithelium and villi within 1 hour and PrP-positive cells accumulated in the subfollicle region of Peyer's patches a few hours thereafter. Another reason might be that PrP^{C} does not permeate through the colonic tissue but it is endocytosed by epithelial cells or taken by immune cells through lymphatic pathways to lymphoid tissues. Our data differ from those of McKie et al. (1999) who showed permeation of ShaPrPSc through colon tissues of cattle and sheep; however, it should be noted that the later investigators separated the mucosal layer of the colon tissue from the muscle layers.

Results of our experiments also showed that LPS from *E. coli* 0111:B4 did not go through the intact colonic tissue of mice. These results confirm our previous finding in rats that LPS is not transported from the mucosal to the serosal side of the intact colonic tissue of rats. In a previous experiment we reported that LPS translocates through cattle colon and rumen tissues (Emmanuel et al., 2007). However, it should be pointed out that in that experiment the mucosal layer was separated from the muscle circular and longitudinal layers underneath.

Microarray studies have reported various alterations in gene expression during prion diseases. Some of these studies have identified alteration in expression of a great number of genes related to proteolysis, protease inhibition, cell growth, apoptosis, signal transduction, cell adhesion, and immune response (Xiang et al., 2004; Sorensen et al., 2008; Filali et al., 2011). We also hypothesized that expression of genes related to host antibacterial immune responses will be induced by moPrP alone or in combination with LPS.

Results of our two experiments showed that when LPS was present on the mucosal side of the diffusion chamber *Tlr6* and inflamasome gene *Nlrp3* were up-regulated. Interestingly MyD88-dependent signaling pathway gene *Irf5* was also up-regulated. IRF5 has been shown to be associated with MyD88 and is activated through unknown mechanisms to activate pro-inflammatory cytokine gene transcription (Takaoka et al., 2005). TRIF-dependent pathway gene expression was not affected by presence of LPS on the mucosal side. Rathiinam et al. (2012) identified a TRIF pathway that licenses *Nlrp3* inflammasome activation by all Gram-negative bacteria. In our study we found up-regulation of *Nlrp3* gene expression but not TRIF-dependent pathway gene expression, probably indicating a *Nlrp3* TRIF-independent pathway activation. Moreover the up-regulation of *Slc11a1*, which plays a role in sequestration of Fe^{2+} suggests that the colon cells are responding to LPS by removing Fe^{2+} from the environment. Indeed, antimicrobial actions of *Slc11a1* are attributable to modulation of macrophage immune function and cellular iron metabolism; the latter affecting the availability of the essential nutrient Fe^{2+} for bacteria (Fritsche et al., 2012).

Additionally when LPS was added on the serosal side, it activated 5 pathways, a total of 9 genes, related to TLR signaling, NOD-like receptor signaling, inflammatory response and up-regulation of chemokine *Ccl3* and *Tlr9* gene. It has been previously described that *Tlr4* is an important sensor of LPS (Poltorak et al., 1998). Regulation of *Tlr2* and *Tlr4* expression by LPS was considered to be one of the mechanisms to control the overall responses of immune cells to bacteria. Interestingly, in our experiment, we could not find differences in gene regulation of *Tlr4* in the LPS treatments. We found that *Tlr9* was up-regulated when LPS was on the serosal side of the diffusion chamber. An et al. (2002) showed that in macrophages stimulated with LPS, *Tlr9* gene expression was up-regulated within 1 h and reached peak levels at about 3 h. Impaired lung bacterial clearance in mice with defective *Tlr4* or deletion of *Tlr9* was reported (Bhan et al., 2007) suggesting that *Tlr4* and *Tlr9* are required for optimal bacterial clearance.

We also found that when LPS was on the serosal side it induced a much broader immune response in mice colon including gene up-regulation of *MyD88* and TRIF-dependent pathway gene (*Ripk1*) suggesting that both pathways might be activated as well as the up-regulation of endosome gene (*Nlrp3*). *Nlrp3* plays an important role in activation of inflammasome and the release of the proinflammatory cytokine *IL-1* (Petrilli et al., 2007). *Nlrp3*$^{-/-}$ mice show impaired leukocyte and lymphocyte migration and decreased *Il1β*production (Miller et al., 2007). Moreover, *Ccl3* expression, a chemokine related to acquired immune response, was also up-regulated by LPS on the serosal side of mice colon. *Ccl3* (macrophage inflammatory protein MIP-1α) is a potent neutrophil

chemoattractant *in vivo*. Its function is necessary to translate an innate-immune response into an acquired immune response (Luster et al., 2002) inducing the directional migration of targeted populations of leukocytes during periods of inflammation. It has been previously reported that *Ccl3* is up-regulated by LPS in the brain endothelial cells (Chui and Dorovini- Zis, 2010) and in skeletal muscles during endotoxemia (Demoule et al., 2009). Indeed our results are similar with those obtained from previous authors. Increases in *Ccl3* expression are associated with the progression of tissue injury and inflammation during enteritis (Morteau et al., 2002), allergic inflammation (Das et al., 1999), sepsis (Standiford et al., 1995), and lung injury (Smith et al., 1994). Overall presence of LPS on the serosal side activated genes related to inflammation and recruitment of immune cells to the site of inflammation.

In the PrP M treatment, 5 genes related to TLR-signaling (*Irf5*, *Map3k7*, and *Act1*), NOD-like receptor (*Sugt1*) and antimicrobial peptides (*Slpi*) were up-regulated, while pro-inflammatory caspase gene (*Casp1*) was down-regulated compared to the CTR group. MyD88-dependent pathway, *Irf5* gene expression was induced. *Map3k7* is a component of a protein kinase signal transduction cascade that stimulates NF-κB activation and the *Mapk14* (p38) MAPK pathway. It participates in TLR and IL-1 signaling pathway and plays an essential role in innate and adaptive immune signaling cascades (Ninomiya-Tsuji et al., 1999; Wang et al., 2001). Recent studies have defined an essential role of *Map3k7* in T cell receptor (TCR) and B cell receptor expression and in the survival and development of mature B- and T-cells (Liu et al., 2006; Sato et al., 2005, 2006;

Schuman et al., 2009; Tang et al., 2008; Wan et al., 2006). Moreover, Qian et al. (2004) reported that *Act1* plays an important role in the homeostasis of B cells. Zomosa-Signoret et al. (2008) suggested that PrP^{C} may be involved in T-cell activation but its role in T-cell function is not yet determined. Here we report for the first time that moPrP up-regulated *Map3k7* and *Act1* expression in mice colonic tissue. It is speculated that moPrP might be involved in the survival and development of mature B- and T-cells through up-regulation of *Map3k7* and *Act1* gene expression. The gene related to antimicrobial peptide (*Slpi*) also was up-regulated suggesting an antibacterial activity of moPrP. *Slpi* protects tissues against the destructive action of neutrophil elastase at the site of inflammation. Antibacterial properties have been attributed to *Slpi* by suppressing bacterial growth and killing of both Gram-negative (*E. coli*) and -positive (*S. aureus*) bacteria (Hiemstra et al., 1996). Nakamura et al. (2003) suggested that endogenous *Slpi* has protective functions during septic shock in mice. In our experiment *Slpi* showed the highest levels of expression in presence of moPrP. Interestingly, comparative transcriptomic analysis between FVB/N and FVB/N Prnp knockout mice at early embryonic stages revealed that *Slpi* was highly down-regulated in FVB/N Prnp knockout mice embryo (Khalifé et al., 2011), indicating that PrP^{C} has a role in regulation of *Slpi* gene expression. Antibacterial properties have been attributed to prion protein, killing both Gram-negative and -positive bacteria and fungi (Pasupuleti et al., 2009). Down-regulation of pro-inflammatory caspase gene (*Casp1*), part of inflamasome, that affects the release of *Il1β* and *Il18*, might suggest that moPrP diminished pro-inflammatory

processes. In the same line with the aforementioned studies, our results support the idea that moPrP has antibacterial activity and a protective role, inducing expression of antimicrobial peptide *Slpi* and down-regulation of *Casp1* gene expression.

The combination of both moPrP and LPS on the mucosal (PrP M + LPS M) side up-regulated *Tlr1* expression. In addition, *Map3k7*, *Act1*, and *Rac1,* which are involve in survival and development of mature B- and T-cell signaling (Liu et al., 2006; Sato et al., 2005, 2006; Schuman et al., 2009; Tang et al., 2008; Wan et al., 2006), also were up-regulated. Moreover, down-stream signal transduction genes *Mapk14, Mapk8,* and *Jun* were up-regulated. Activation of the mitogen-activated protein kinase (MAPK) cascade after TLR stimulation enables innate immune cells to rapidly activate cytokine gene expression (Hammer et al., 2006). The activation of those proteins leads to AP1 and NF-kB activation. This might suggest that the combination of moPrP with LPS on the mucosal side was involved in stimulation of pro-inflammatory cytokines through up-regulation of *Mapk14, Mapk8,* and *Jun*. Epithelial cells of the gut express Tlr1 and activation of *Tlr1* is important because it affects recruitment of dendritic cells (DC, antigen presenting cells) to the site of infection (Kamdar et al., 2013). Moreover, genes related to apoptosis *Card6, Irak1, Nfkbia,* and *Jun* were up-regulated. It has been described that cellular prion protein is involved in apoptotic processes in the neuronal cells (O'Donovan et al., 2001; Solforosi et al., 2004). When added alone on the mucosal side of mice colon, moPrP did not have an affect on any of the genes related to apoptosis, but in combination with LPS it affected genes related

to apoptosis. It has been shown that a relatively severe chronic stimulation with LPS exacerbates neuronal death and motor neuron axon degeneration during chronic neurodegeneration (Nguyen et al., 2004; Cunningham et al., 2005). Our results suggest that the combination of moPrP and LPS on the mucosal side of mice colon enhances apoptotic processes potentially to shed the LPS-overloaded epithelial cells.

Combination of moPrP M + LPS S produced a broader gene response in the mice colon. A total of 22 genes and seven signaling pathways were affected by the treatment. TLR signaling MyD88-dependent pathway (*Irak1*, *Traf6*, and *Irf5*) and TRIF-dependent pathway (*Ripk1*) genes were up-regulated. In addition, genes related to NOD-like receptor signaling (*Nod1*, *Sugt1*, and *Xiap*) that recognize pathogen-associated molecular patterns in the intracellular compartment were up-regulated. NOD-like receptor signaling is important because it induces innate immune responses through cytosolic recognition of bacterial molecules (Park et al., 2007). More specifically, up-regulation of *Nod1* is related to recognition of peptidoglycan-related molecules containing the amino acid meso-diaminopimelic acid that are produced by most Gram-negative and specific Gram-positive bacteria (Chamaillard et al., 2003). Once activated, *Nod1* induces gene transcription through NF-κB and MAPK signaling pathways (Park et al., 2007). In this treatment also *Map3k7* and *Act1* were up-regulated. Moreover death-inducing signaling complex (DISC) mediated by genes *Tnfrsf1a* and *Casp8*, and *Jun* were up-regulated indicating that when moPrP was on the mucosal side and LPS on the serosal side apoptotic signaling took place. *Casp8* has been demonstrated to play

a key role in mediating Fas-induced extrinsic apoptotic pathway (Boldin et al., 1996 Fernandes-Alnemri et al., 1996; Muzio et al., 1996). Indeed, activated *Casp8* has been observed in postmortem brain tissue in Parkinson disease-related neurodegeneration (Hartmann et al., 2001). Furthermore, recent data have indicated a role of caspase-mediated apoptosis of intestinal epithelial cells in the pathogenesis of inflammatory bowel diseases such as Crohn's disease and ulcerative colitis and in the gut (Nenci et al., 2007; Sanders, 2005; Gunther et al., 2011). Apoptosis is a vital mechanism in multicellular organisms to shed cells during development, tissue homeostasis, and immune system function (Jacobson et al., 1997). Therefore it is suggested that when moPrP was added on the mucosal side of the colon tissue and LPS on the serosal side death-inducing signaling complex genes *Tnfrsf1a* and *Casp8* were up-regulated probably leading to apoptosis in order to eliminate the infected cells. Interestingly *Casp1* expression remained down-regulated and *Il18* was up-regulated showing that *Il18* can also be intracellularly activated by caspase-1-independent mechanism. In addition, antibacterial peptides expression *Camp* and *Slpi* were up-regulated, which indicates that moPrP induces antibacterial activities possibly through up-regulation of *Camp* and *Slpi* gene. In agreement with Hyeon et al. (2012) it is postulated that PrP^{C} may not be limited to prion diseases, but might play a role in induction of apoptosis and in activation of antibacterial responses during LPS toxicity or disease pathogenesis.

3.5 CONCLUSIONS

moPrP alone or in combination with the LPS did not result in the transport of moPrP protein through the intact colonic tissue of mice. Under simulated endotoxemia, LPS did not facilitate translocation of moPrP from mucosal to serosal side of intact mice colon. Expression of genes related to host antibacterial immune response were affected in presence of moPrP alone or in combination with LPS. To our knowledge this is the first report that moPrP specifically induced the expression of *Map3k7* and *Act1* which suggest moPrP might be involve in survival and development of mature B- and T-cells. moPrP induced expression of antimicrobial peptide *Slpi* and down-regulated *Casp1* gene expression suggesting an antibacterial role and anti-inflammatory role for prion protein. moPrP contributed to host defence against endoxemia through enhancing gene expression of antibacterial peptides. moPrP in combination with LPS up-regulated *Tlr1*, *Mapk14*, *Irak1,* and *Jun* gene expression. When moPrP was added on the mucosal side of the colon tissue and LPS on the serosal side, simulating endotoxemia, antibacterial responses and death-inducing signaling complex genes were up-regulated.

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Table 3.1 Composition of diet fed to mice

Nutrient	Amount
Protein, %	20
Fat (ether extract), %	5
Fat (acid hydrolysis), %	5.6
Total Saturated Fatty Acids,%	0.93
Total Monosaturated Fatty Acid,%	0.99
Fiber (Crude), %	4.7
Nitrogen-Free Extract (by difference), %	52.9
Total Digestive Nutrients, %	76.2
Gross Energy, kcal/gm	4.07
Physiological fuel Value, kcal/gm	3.41
Metabolizable Energy, kcal/gm	3.07
Ash,%	6.1

Table 3.2 Concentrations of fatty acids on the mucosal and serosal sides of the Ussing chamber (experiment 1)

	Fatty acid				
Treatment	C12	C14	3OH-C14	C16	C18
	μg/mL				
Control[1]					
Mucosa	2.12	2.14	109	2.07	2.29
Serosa	5.71	0.00	165	0.14	0.17
LPS M[2]					
Mucosa	2.56	0.13	160	0.22	0.24
Serosa	2.35	0.00	126	0.18	0.18
PrP M+LPS M[3]					
Mucosa	2.35	0.8	133	1.81	2.42
Serosa	2.80	3.18	230	4.36	5.95
Pooled SEM	2.23	0.96	56	1.83	2.60
P-values	0.24	0.45	0.52	0.50	0.54

[a,b]Means in the same column within effect with different superscripts differ significantly ($P \leq 0.05$).

[1]Pyrogen-free water was added on the musosal side of the chamber: 700 μL.

[2]LPS was added on the musosal side of the chamber at 100 μg/mL.

[3]LPS (100 μg/mL) and moPrP (100 μg/mL) were added on the mucosal side of the chamber.

Table 3.3 Concentrations of fatty acids on the mucosal and serosal sides of the Ussing chamber (experiment 2)

	Fatty acid				
Treatment	C12	C14	3OH-C14	C16	C18
	μg/mL				
Control[1]					
Mucosa	0.35	1.67	428	8.27[a]	13.50[a]
Serosa	0.54	1.82	400	9.48[a]	14.82[a]
LPS S[2]					
Mucosa	0.33	1.26	280	5.47[ab]	8.41[ab]
Serosa	0.29	1.10	338	6.35[ab]	9.53[ab]
PrP M+LPS S[3]					
Mucosa	0.32	0.94	391	3.65[b]	2.87[b]
Serosa	0.18	1.42	293	1.27[b]	1.55[b]
Pooled SEM	0.14	0.65	111	2.79	4.50
P-values	0.6642	0.9008	0.9534	0.0386	0.0481

[a,b]Means in the same column within effect with different superscripts differ significantly ($P \leq 0.05$).

[1]Pyrogen-free water was added on the musosal side of the chamber: 700 μL.

[2]LPS was added on the serosal side of the chamber at 100 μg/mL.

[3]LPS (100 μg/mL) was added on the serosal side of the chamber and moPrP (100 μg/mL) was added on the mucosal side of the chamber.

Table 3.4 Antibacterial gene expression in mice colon according to the treatment. Results of gene expression are shown as fold-regulation relative to CTR group

Gene[A]	Description[A]	LPS M	LPS S	PrP M	PrP M+ LPS M	PrP M+LPS S
Toll- like Receptor Signaling						
Tlr1	Toll-like receptor 1	1.20±2.31	1.32±0.68	-1.21±0.57	1.44±0.00***	1.32±0.58*
Tlr6	Toll-like receptor 6	2.97±0.37*	1.66±1.55	3.34±1.73	3.64±0.99	4.21±0.57*
Tlr9	Toll-like receptor 9	7.84±2.51	2.13±0.59*	3.39±1.05	1.49±0.58	3.42±0.68
Irf5	Interferon regulatory factor 5	3.75±1.14**	2.11±0.57*	4.19±1.17*	2.31±1.53	3.31±0.00*
Map3k7	Mitogen-activated protein kinase kinase kinase 7	-1.05±2.89	1.32±0.58	1.66±0.57***	1.44±0.01***	1.65±0.01**
Ripk1	Receptor (TNFRSF)-interacting serine-threonine kinase 1	1.20±1.53	1.33±0.58*	1.04±0.57	1.14±0.58	1.32±0.58*
Traf6	Tnf receptor-associated factor 6	-1.31±1.74	1.32±0.58	1.05±0.57	1.15±0.57	1.32±0.57*
Act1	Thymoma viral proto-oncogene 1	-1.05±0.58	1.31±0.57	1.66±0.58**	1.44±0.01***	1.66±0.01
NOD-like Receptor Signaling						
Nod1	Nucleotide-binding oligomerization domain containing 1	-1.05±1.00	1.67±0.58	1.68±1.00	1.45±0.57	3.32±0.57***
Nod2	Nucleotide-binding oligomerization domain containing 2	-1.66±1.52	-1.91±0.01*	1.32±0.58	-1.10±1.01	-1.92±1.00
Sugt1	SGT1, suppressor of G2 allele of SKP1 (S. cerevisiae)	1.20±2.31	1.67±0.0***	1.66±0.58**	1.15±0.58	2.09±0.58*
Xiap	X-linked inhibitor of apoptosis	-1.04±2.07	-1.20±0.01	1.32±0.00	1.15±0.58	1.33±0.58**
Downstream signal transduction						
Mapk14	Mitogen-activated protein kinase 14	1.19±2.32	1.04±0.58	1.05±0.57	1.44±0.01***	1.32±0.58**
Mapk8	Mitogen-activated protein kinase 8	1.20±2.31	1.32±0.57	1.32±1.00	1.44±0.01***	1.66±0.02
Apoptosis						
Card 6	Caspase recruitment domain family, member 6	1.92±2.0	1.32±0.58	2.08±1.16	2.26±0.58*	2.54±0.53
Card9	Caspase recruitment domain family, member 9	1.18±0.64	-1.22±0.07*	-1.20±0.57	-1.38±0.00***	1.05±0.56
Casp1	Caspase 1	1.20±0.57	1.05±0.57	-1.52±0.00*	-1.39±1.00	-1.52±0.58**
Casp8	Caspase 8	1.20±2.3	1.29±0.57	1.32±0.00	-1.10±0.58	0.31±0.58*
Irak1	Interleukin-1 receptor-associated kinase 1	1.20±2.30	1.32±0.57	1.32±0.99	1.44±0.00***	1.32±0.58**
Tnfrsf1a	Tumor necrosis factor receptor superfamily, member 1a	-1.04±2.88	1.33±0.58	1.06±0.57	1.16±0.57	1.33±0.57**
Nfkbia	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha	1.91±3.05	1.32±0.57	1.33±1.00	2.29±0.57*	1.32±0.57
Jun	Jun oncogene	-1.31±2.89	1.32±0.00	1.32±1.15	2.30±0.00**	2.09±0.57*

Inflammatory response						
Myd88	Myeloid differentiation primary response gene 88	-1.33±1.75	-1.21±0.99	1.31±0.99	1.44±0.99	1.32±0.58*
Nlrp3	NLR family, pyrin domain containing 3	6.03±2.08***	4.16±0.57*	4.17±2.01	2.85±0.59	2.07±0.57
Rela	V-rel reticuloendotheliosis viral oncogene homolog A (avian)	1.50±2.3	1.66±0.57	1.33±0.58	1.44±1.15	1.65±0.57*
Tirap	Toll-interleukin 1 receptor (TIR) domain-containing adaptor protein	-1.05±2.08	1.32±0.57	1.05±0.58	1.14±0.57	1.32±0.57*
Tollip	Toll interacting protein	1.20±2.32	1.66±0.01***	1.05±0.58	1.45±0.01***	1.32±0.58*
Slc11a1	Solute carrier family 11 (proton-coupled divalent metal ion transporters), member 1	1.48±0.78*	1.69±1	1.67±1.53	1.46±1.00	1.67±0.00
Rac1	RAS-related C3 botulinum substrate 1	1.20±2.30	1.32±0.59	1.05±0.57	1.45±0.01***	1.32±0.58
Cytokines and Chemokines						
Ccl3	Chemokine (C-C motif) ligand 3	3.81±2.00	3.31±0.57*	1.02±1.57	1.44±0.57	1.02±1.05
Il18	Interleukin 18	1.51±3.2	1.31±0.58	1.66±1.00	2.28±1.56	1.65±0.58*
Antimicrobial peptides						
Camp	Cathelicidin antimicrobial peptide	1.15±0.04	1.29±0.06	1.33±0.57	-1.10±1.1	2.12±0.59*
Slpi	Secretory leukocyte peptidase inhibitor	3.83±1.3	5.26±1.21	10.55±3.21*	7.30±4.36	42.24±0.58***

Within a row, means with subscript were different relative to CTR group *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$.

[A]Gene symbol and description. **CTR**: Pyrogen-free water was added on the musosal side of the chamber (700 µL). **LPS M**: Lipopolysaccharide (LPS) was added on the mucosal side of the Ussing chamber at 100 µg/mL; **LPS S**: LPS was added on the serosal side of the Ussing chamber at 100 µg/mL; **PrP M**: moPrP (29-232) was added on the mucosal side of Ussing chamber at 100 µg/mL; **PrP M + LPS M**: moPrP (100 µg/mL) and LPS (100 µg/mL) were added on the mucosal side **PrP M + LPS S** LPS (100 µg/mL) was added on the serosal side of the chambers and moPrP (100 µg/mL) was added at the mucosal side.

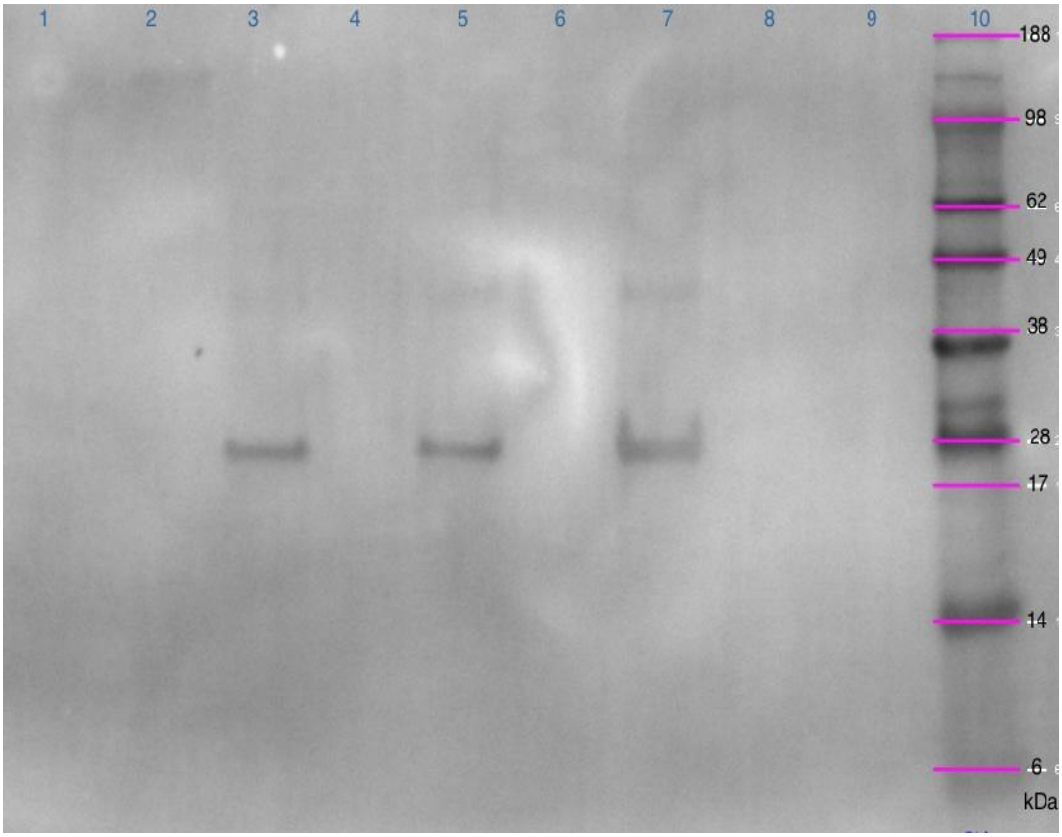

Figure 3.1 Detection of moPrP on mucosal and serosal sides of the Ussing diffusion chambers in mice colon by Western blot.
Lane 1: Control mucosal side; **Lane 2:** Control serosal side; **Lane 3:** moPrP (29-232) M mucosal side; **Lane 4**: moPrP M serosal side, **Lane 5:** moPrP M + LPS M mucosal side, **Lane 6:** moPrP M + LPS M serosal side; **Lane 7:** moPrP + LPS S mucosal side; **Lane 8:** moPrP M + LPS S serosal side; **Lane 9:** Negative control; **Lane 10:** Ladder.

CHAPTER 4: Effects of detoxified lipopolysaccharide on mouse recombinant prion protein translocation through the mice colon and antibacterial gene expression

4.1 INTRODUCTION

Prion diseases or transmissible spongiform encephalopathies (TSEs) are fatal neroudegenerative disorders associated with the conformational conversion of GPI-linked cellular prion protein (PrP^{C}) into a β-sheet rich isoform known as PrP^{Sc}. Prion infections can be induced by oral challenge and are presumably acquired naturally by consumption of prion-contaminated food as shown for kuru, Creutzfeldt- Jacob disease (CJD) (Bruce et al., 1994), bovine spongiform encephalophathy (Foster et al., 2001), scrapie (Andreoletti et al., 2000), and chronic wasting disease (CWD) (Belay et al., 2004). Previous studies have shown that after oral exposure to pathogenic prions, PrP^{Sc} accumulation is found in the gut lymphoid tissues or in the enteric nervous system prior to its presence in the central nervous system (Andreoletti et al., 2000; Beekes and McBride, 2000). Therefore, the uptake and translocation of the infectious agent from the gastrointestinal (GI) tract prior to its spread through the different systems and tissues of the host is considered to be a critical step in transmission during natural prion infections.

After digestion, the gastrointestinal mucosa possesses limited protection against prion infections. An *in vitro* study using Ussing chamber system by McKie et al. (1999) indicated that prions permeate through the colonic mucosal layer via a

paracellular route. Prions were also found to cross the mucosal barrier *in vitro* via M cell, a unique epithelial cell, specialized for antigen sampling for mucosal associated lymphoid tissue (Okamoto et al., 2003). Translocation of prion also occurs through other cell types at epithelial level including intestinal epithelial cells (i.e., enterocytes) and migratory dendritic cells (Morel et al., 2005; Huang et al., 2002). Enterocytes can transcytose fragments of PrP^{Sc} complexed with ferritin or internalize them via laminin receptor binding and endocytosis (Morel et al., 2005; Mishra et al., 2004). Migratory bone marrow derived DC can also tranport PrP^{Sc} directly from the intestinal lumen (Huang et al., 2002).

However, it is unknown whether there are cofactors in the gastrointestinal tract which might facilitate permeation of prion protein through the intestinal mucosa. Inflammation and bacterial infection have been shown to influence prion susceptibility. The permeability of all parts of the intestine, including colon, which is considered to be relatively impermeable to macromolecules, greatly enhance during stress and inflammatory states (Berin et al., 1997; Malin et al., 1996). In addition, an increase in prion protein expression in the mucous membrane of digestive tract has been also demonstrated during bacterial infection (Konturek et al., 2005) and inflammation (Pammer et al., 1998). Recently it was shown that *Helicobacter pylori* infection induces up-regulation of PrP^{C} expression in gastric mucosa (Konturek et al., 2005). Another investigation showed that *Salmonella typhimurium* increases the susceptibility to prion diseases (Sigurdson et al., 2009). Nevertheless, the mechanism by which this occurs still remains unclear.

Could lipopolisaccharide (LPS) at the mucosal entry site affect prion translocation? Lipopolysaccharide, a cell wall component of Gram-negative bacteria, also known as endotoxin, may play a major role in development of several diseases including sepsis, atherosclerosis, inflammatory bowel disease, and rheumatoid arthritis. (Doi et al., 2009; Stoll et al., 2004; Gardiner et al., 1995; Yoshino et al., 2000). The GI tract is a large reservoir of LPS since it is exposed to a high density and diversity of Gram-negative bacteria. Lipopolysaccharide can increase the mucosal permeability and make the gastrointestinal tract become "leaky" by altering the expression of gut tight junction proteins. Emmanuel et al. (2007) reported that LPS was able to penetrate the mucosa and translocate through bovine rumen and colon *in vitro* under Ussing chamber system. Ametaj et al. (2010) indicated that LPS was able to bind moPrP and to catalytically induce a β-rich conformation of moPrP, which is resistant to proteinase K. Since LPS interacts with moPrP it might be possible that this might affect the transport of moPrP through the colon tissue. We hypothesized that detoxifying LPS by removing the phosphate groups may have an effect on the activity of LPS and its interaction with prion and possibly its transport through the GI tract. Alkaline phosphatase (AP), which is abundantly expressed in the GI tract where antigens, like LPS, enter to the host have been proved to have a role in the natural defence system against LPS insults (Bates et al., 2007). Alkaline phosphatase is able to remove a single phosphate group from the lipid A moiety, thus reducing the toxicity of LPS (Koyama et al., 2002).

Here we investigate whether detoxified (D)-LPS affects translocation of moPrP through mice colon and whether moPrP or the combination of moPrP with D-LPS alter the host immune responses. We used an Ussing diffusion chamber model to study the permeability of the mice colon and an antibacterial response PCR array to determine the effects of moPrP and D-LPS on gene expression profile in the colonic tissue of mice.

4.2 MATERIALS AND METHODS

4.2.1 Animals

Eight healthy male mice of the FVB/N strain at age 114 ± 5 d old were used to collect colon samples. All mice were fed the same diet before euthanization (Table 3.1). All experimental procedures were approved by the University of Alberta Animal Care and Use Committee for Health Sciences and animals were cared for in accordance with the guidelines of the Canadian Council on Animal Care (1993).

4.2.2 Detoxified LPS preparation

LPS was detoxified using alkaline phosphatase (ALP) to dephospharylate lipid A moiety of LPS (Beumer et al., 2003). To generate detoxified LPS (D-LPS), 1 µg LPS/ml was incubated with 1 EU ALP/ml (SIGMA-ALDRICH, Oakville, ON, Canada) for 3h at 37°C (Fiechter, 2007).

4.2.3 Ussing Chamber Study

After mice were euthanized, colon tissue was immediately excised, gently flushed with cold Krebs buffer to remove the luminal contents, opened along mesenteric border, and not stripped of external muscle layers. From each mouse, 6 pieces of the colon segment (1.5 cm^2) were mounted between the mucosal and serosal reservoirs of easymount Ussing diffusion chambers (Physiologic Instruments INC., Harvard Apparatus Canada, St. Laurent, QC) with 0.3 cm^2 exposed area. This entire procedure was done within 40 minutes, and during the experiment tissues were bathed on mucosal and serosal sides with 7 mL of Krebs buffer (pH 7.4) oxygenated continuously with 95% O2/5% CO2 at the temperature of 37 $^{\circ}C$. The Krebs buffer solution included 11 mmol/L glucose as an energy source and contained (in mmol/L) 117 NaCl, 1.2 NaH_2PO_4, 1.2 $MgCl_2$, 2.5 $CaCl_2$, 4.8 KCl, and 25 $NaHCO_3$ (Table 2.2)

The tissues were allowed to equilibrate for 15 min before the electrical variables were evaluated during the 40 min of the experiment. Treatments were added as follows: 1) control group (CTR), 700 µl pyrogen-free H_2O added on the mucosal side of the first chamber; 2) treatment 1, 700 µL of a solution containing 1mg/mL of LPS from *Escherichia coli* O111: B4 added on the mucosal side (LPS-M); 3) treatment 2, 700 µL of a solution containing detoxified (D)-LPS added on the serosal side (D-LPS-S); 4) treatment 3, 700 µl of a solution containing 1 mg/mL moPrP (29-232) added on the mucosal side (PrP-M); 5) treatment 4, 700 µL of a solution containing D-LPS and moPrP added on the mucosal side (D-LPS/PrP-M) at 1 mg/mL each; 6) treatment 5, 700 µL of a solution containing moPrP added on

the mucosal side and 700 µL of a solution containing D-LPS added on the serosal side (PrP-M/D-LPS-S), at 1 mg/mL each. Two pairs of electrodes in each chamber were used to monitor the transmural potential difference (PD, in mV) and to inject a current that maintained zero PD (short-circuit current [Isc] expressed as mA/cm2). The electrophysiological responses and ionic fluxes were measured using a VCC MC6 multichannel voltage/current clamp and A&A software (data not shown). Samples (350 µL) were collected on both sides of the chamber at 40 min after initiation of the treatment. At the end of the experiment, colon tissues were collected for gene expression analyses.

4.2.4 Detection of moPrP (see details in Chapter 2)

4.2.5 Extraction of total RNA and Real-Time PCR (see details in Chapter 3)

4.2.6 Statistical Analyses

Gene expression statistical analyses were carried out according to the manufacture recommendations. The *t*- test was used to determine whether the differences observed between the treatments on gene expression were statistically significant ($P \leq 0.05$). The relative differences in gene expression between the different treatments were defined as the relative quantities after normalization. The colon tissue was the experimental unit for all analysis. The animal was used as a random effect in the model. Differences between the treatment effects and means were classified by pairwise comparisons, and unless otherwise noted, differences were considered significant at $P \leq 0.05$.

4.3 RESULTS

4.3.1 moPrP Detection by Western Blot Analyses

Samples from the eight treatment groups were analyzed for the moPrP abundance in both mucosal and serosal sides of the Ussing chamber system (Figure 4.1). In lanes 1 and 2, there were no bands observed confirming that the experiment was free from contamination. Samples from mucosal side containing moPrP and serosal side without moPrP were loaded in lanes 3 and 4 respectively. We observed a strong band only in lane 3 but not in lane 4. In lanes 5 and 6, samples from the mucosal side containing moPrP and the serosal side detoxified LPS were respectively loaded and prion protein was detected only on the mucosal side but not on the serosal side. Finally, we loaded samples from the mucosal side that contain detoxified LPS and moPrP in lane 7 and serosal side containg buffer alone in lane 8. There were no moPrP bands detected in both lanes 7 and 8.

4.3.2 Gene Expression in the Colon

The expression profiling of 84 key innate immune response genes in mice colon tissue was performed using Mouse Antibacterial Response RT² Profiler PCR Array. The 84 genes are involved in signaling pathways such as toll-like receptor (TLR) signaling, nod-like receptor (NLR) signaling, other bacterial pattern recognition receptors (PRRs), downstream signal transduction, apoptosis**,** inflammatory response, cytokines & chemokines and antimicrobial peptides. The

differentially expressed genes functional grouping, fold regulation and *P* values in all the treatment groups are shown in Table 4.1.

Fifteen genes were differentially regulated in mice colon tissue that contains moPrP on the mucosal side as compared to the control colon tissue that had pyrogen free water. Among these, 4 were down-regulated and the remaining eleven were up-regulated. *Slpi* showed the maximum up-regulation (9.19-fold change) followed by *Tlr4* (7.32-fold change). A relatively lower magnitude of fold regulation was observed for the down-regulated genes where *Il12a* showed the maximum with -2.75-fold change. These differentially expressed genes can be categorized into different functional groupings such as toll-like receptor signaling (*Tlr4, Irak3, Tirap & Lbp*), nod-like receptor (NLR) signaling (*Nlrpa, Nlrp3, Nod2, Casp1, Sugt1 & Tnf*), apoptosis (*Casp1, Il12a, Tnf &tnfrsf1*), inflammatory response (*Lbp, Nlrp3, Tirap, Tlr4 &Tnf*), cytokines and chemokines (*Il12a and Il18*) and antimicrobial peptides (*Slpi*).

The mRNA expression of *Nlrp1a* and *Slc11a1* were significantly ($P \leq 0.05$) higher in the treatment groups when LPS was added on the mucosal side of mice colon tissue with 1.61 and 1.54 fold change respectively. However, when D-LPS alone was added on the serosal side, there were no differentially expressed genes at $P \leq 0.05$. Three genes such as *Nlrp1a* ($P = 0.05$), *Ripk2* ($P = 0.07$) and *Sugt1* ($P = 0.05$) showed tendencies as their *P* values range between 0.05 and 0.1. These three genes: *Nlrp1a*, *Ripk1* and *Sugt1* also showed < 2 fold change increase with 1.4, 1.89 and 1.4 respectively.

In mice colon tissue that contain prion protein on the mucosal side and detoxified LPS on the serosal side 11 genes were differentially expressed. Among these, 8 genes were up-regulated (*Slpi, Tlr4, Cd14, Irak1, Irak3, Jun, Ltf, Sugt1*) and three genes (*Dmbt1, Irf7* and *Mapk3*) were down-regulated. *Slpi* (30.58 fold change) showed the maximum fold change up-regulation followed by *Tlr4* (4.83) and *Cd14* (3.06) whereas *Dmbt1* (-5.28) and *Irf7* (-5.26) showed higher magnitude of down-regulation. The pathways potentially affected by the differentially expressed genes include toll-like receptor signaling (*Cd14, Tlr4, Irak1* and *Irf7*), downstream signal transduction (*Jun, Mapk3*), apoptosis (*Cd14, Irak1and Jun)*, inflammatory response (*Cd14* and *Tlr4*) and antimicrobial peptides (*Slpi*).

Furthermore, when the combination of moPrP and D-LPS added to the mucosal side only 8 genes were differentially regulated. Among these, 6 genes (*Slpi*, *Tlr4*, *Cxcl1*, *Irak3*, *Jun*, and *Tnf*) were up-regulated, while *Irf7* and *Mapk3* were down-regulated.

Interestingly, when moPrP alone (mucosal side) or combined with D-LPS either in the mucosal or serosal side, *Slpi, Tlr4* and *Irak3* were consistently up-regulated. The magnitude of up-regulation for *Slpi* and *Tlr4* was exceptionally higher in all the three comparison groups as compared to the other differentially regulated genes. Interferon regulator factor 7 (Irf7) was down-regulated in the treatment groups that involve moPrP in the mucosal side and D-LPS either in the mucosal or serosal side.

4.4 DISCUSSION

Transmissible spongiform encephalopathies (TSEs) are considered to be acquired orally and transmissibility involves bypassing the epithelial barrier (Kaatz et al., 2012) where prions are thought to gain access to the central nervous system via physical interaction with peripheral nerve fibers of interic nervous system (Beekes and McBride, 2007). As the mechanism by which these orally introduced prions crosses the gut epithelium is not well established, we tested the hypothesis that translocation of *in vitro* synthesized mouse recombinant prion protein might be modulated by detoxified LPS (D-LPS) D-LPS in an Ussing chamber model. Our results indicate neither the presence of D-LPS on the serosal side nor the presence of moPrP alone on the mucosal side showed evidence of moPrP translocation from the mucosal to serosal side of the intact colonic tissue of mice. This result is not in agreement with the observation made by (McKie et al., 1999) that showed permeability of labeled, inactivated prion protein across bovine and ovine colon. This discrepancy might be explained by the difference in molecular structure of the protein, where we used the mouse recombinant prion protein (moPrP) as opposed to the PrP^{sc} that is proteinase K resistant and also to the methodology of tissue preparation which included removal of muscle layer versus intact tissue that we used in our experiment.

The *in vitro* experiment to evaluate whether or not LPS, D-LPS and moPrP induce the expression of genes related to anti-microbial response in mice colon was studied under an Ussing chamber model. When LPS was added to the

mucosal side of mice colon tissue very few genes were differentially expressed. This result is in agreement with previous studies that intestinal epithelial cells limit dysregulated LPS signaling by down-regulating the expression of *Md*-2 and *Tlr4* while keeping the remainder of the intracellular LPS signaling pathway functionally intact (Abreu et al., 2001). However, more genes were differently expressed by the treatment of LPS alone on the mucosal side of the chamber as compared to the data in Chapter 3. This might be explained by the difference in the age of mice used in two experiments. Tateda et al. (1996) indicated that aged mice are more sensitive to the lethal toxicities of LPS and LPS-induced cytokine production than young mice. On the other hand, when LPS was detoxified by alkaline phosphatase and added on the serosal side of the mouse colon, there was no gene that was differentially expressed. Besides intestinal epithelial cells hyporesponsiveness to LPS, there is experimental evidence that D-LPS lacking the phosphate group in the lipid A functional moiety is unable to induce gene expression. The two phosphate groups of LPS in the lipid A part are of crucial importance for its biological activity (Brandenburg et al., 1993). Together with the acyl chains, phosphate groups are responsible for the binding of LPS to myeloid differentiation-2-protein (MD-2) initiating MD-2/Tlr4 signaling complex (Gangloff and Gay, 2004) and determining the release of inflammatory mediators. This appears consistent with our results where we observed LPS being able to initiate the expression of few genes. On the contrary, there was no significant gene expression regulation ability of D-LPS was observed. This explains the fact

that the phosphate group of the LPS is of crucial importance in triggering the expression of inflammatory response genes (Brandenburg et al., 1993).

When prion protein was added on the mucosal side and D-LPS on the serosal side a different set of genes were differentially expressed where *Slpi*, *Tlr4*, and *Cd14* were the most expressed. Research in mice has shown correlation of *Tlr4* mRNA expression and sensitivity to LPS both *in vivo* and *in vitro* where Tg mice possessing the highest copy number of *Tlr4* respond to LPS to the most (Bihl et al., 2003) giving survival advantage during early infection. Besides, over-expression of human CD14 in transgenic mice renders mice hypersensitive to LPS and increases their susceptibility to endotoxin shock (Ferrero et al., 1993). It has been also well documented that *Slpi* exerts an anti-inflammatory effect on the immune system by inhibiting *TNF-α* production in macrophages (Jin et al., 1997) interfering with breakdown of IkappaBbeta (Ward and Lentsch, 2002), competitively binding to the NF-κB consensus-binding site and inhibiting NF-κB activation in macrophages and nuetrophils (Taggart et al., 2005). Besides, *Slpi* is reported to have antibacterial (Fahey and Wira, 2002) and antifungal (Jin et al., 1997) activities suggesting another function of cellular PrP^{C} to induce antimicrobial compound production. On the other hand, the expression of Irf7 and Dmbt1 were decreased suggesting the potential compromise of type I interferon (*Ifn*) signaling network (Le Bon and Tough, 2002; Paun and Pitha, 2007) as *Irf-7* is the master regulator of type-I interferon-dependent immune responses (Honda et al., 2005). Similarly, the expression of *Dmbt1* was decreased suggesting that moPrP may not activate NF-κB via *Dmbt1* (Rosenstiel et al., 2007).

When moPrP alone was added on the mucosal side, *Slpi* and *Tlr4* showed increased mRNA expression (9.19 and 7.34 fold change) followed by *Irak3, Tnf, Nlrp3* and *Nod2* indicating the role of moPrP in immune activation. Tumor necrosis factor-α is a potent proinflammatory cytokine which plays a central role in inflammation and immunity (Baud and Karin, 2001). Rosenstiel et al. (2003) showed that *Tnf-α* up regulates *Nod2* protein and mRNA in epithelial cell lines and primary IECS. This up-regulation is dependent on NF-κB activation and binding of NF-κB to two different sites of the *Nod2* promoter. Moreover, upon activation, *Nlrp3* forms inflammasome complex with Asc and controls activation of *Caspase 1* that in turn cleaves pro-IL-1β and pro-IL-18 into the biologically active forms (Dinarello, 2009). Since endogenous *Nlrp3* expression in immune cells is insufficient to permit inflammasome activation, increased expression of *Nlrp3* to a functional level is quite important (Bauernfeind et al., 2009; Franchi et al., 2009). Thus, the up-regulation of *Nlrp3* in this experiment explains *Nlrp3* inflammasome activation in order to mediate inflammatory response. It is also notable that Interleukin-1 receptor-associated kinase-3 (*Irak3*) expression was increased in this comparison group indicating its role in limiting inflammatory response as *Irak3* is an important negative regulator of TLR-mediated cell signaling (Kobayashi et al., 2002) by preventing dissociation of Irak1 and Irak4 from *MyD88* and formation of *Irak – Tnf* receptor-associated factor-6 (TRAF6) complexes (van 't Veer et al., 2007). We also observed that having the moPrP in contact with mucosal side of colon tissue increased the expression of *Tnf-α* and decreased expression of *Il12a* and *Il18* indicating its anti-inflammatory effect.

Chikano et al., (2000) showed that administration of *Il-12* and *Il-18* to BALB/C induced intestinal inflammation.

In Prnp-knockout embryos at E7.5 the expression of *Slpi* was observed to be down regulated (Khalife et al., 2011), moreover, it has been shown that Slpi production in epithelial cells can be induced by *Tnf-α* (Sallenave et al., 1994). Moreover, Slpi protects the local tissue against the detrimental consequences of inflammation inhibiting the proteases, such as cathepsin G, elastase, and trypsin from neutrophils, chymotrypsin and trypsin from pancreatic acinar cells; and chymase and tryptase from mast cells (Jin et al., 1997; Gipson et al., 1999; He et al., 2003). Besides, *Slpi* is reported to have antibacterial (Fahey and Wira, 2002) and antifungal (Jin et al., 1997) activities suggesting another function of cellular PrP^C to induce antimicrobial compound production.

In addition, increased level of NF-κB and neural sensitivity to oxidative stress in cultured cells isolated from prion protein knockout mice has been reported (Brown et al., 2002). In another study, the normal prion protein (PrP^C) has been shown as a potential actor of proliferation and differentiation of epithelial cells through the interaction with c-Src, desmosome-and cytoskeleton-associated proteins indicating its involvement in the homeostasis of epithelia (Morel et al., 2008). Recently, it has been reported that Aβ binds to PrP^C with high affinity (Chen et al., 2010) and its N-terminal fragment bind to amyloid β oligomers and inhibits their neurotoxicity *in vivo* (Fluharty et al., 2013). These lines of evidences are in agreement with our results that showed the potential role of prion protein (moPrP) as part of the immune system.

The epithelial cells lining the gastrointestinal membranes separate the sterile internal melieu of the body from the contaminated external environment. Studies have shown that *Slpi* is constitutively expressed in epithelial cell lines and primary cultures (Abbinante-Nissen et al., 1995; Maruyama et al., 1994) and the expression can be increased by proinflammatory stimuli such as *Tnf-α* and *Il-1β* (Sallenave et al., 1994; Maruyama et al., 1994). On the other hand, the intestinal epithelial cells (IEC) express low level of *Tlr4* and *MD*-2 and are LPS unresponsive as the response to LPS is mediated by its interaction with Tlr4 in conjunction with *Md*-2 and *Cd14* and transduced via the Il-1 receptor signaling complex to activate NF-κB activation and proinflammatory cytokine expression (Jiang et al., 2000; Zhang et al., 1999), Our result showed consistently increased expression of *Slpi* and *Tlr4* mRNA in treatment groups that involve moPrP indicating moPrP mediated induction of *Tlr4* that could lead to the activation of inflammatory response. It is well established that inflammatory cytokines such as *Tnf –α* and *Il-1β* increase the expression of *Slpi* from the epithelial cells with diverse functions such as anti-microbial, anti-inflamatory, anti-protease.

4.5 CONCLUSIONS

In conclusion, *in vitro* synthesized moPrP did not cross the colon epithelial barrier in an Ussing chamber model; neither D-LPS had an effect on its translocation. However, moPrP markedly increased the expression of *Slpi* and *Tlr4* in mice colon tissue suggesting the role of prion protein as modulator of cellular immunity. The increased expression of *Tlr4* and *Cd14* could lead to the activation

of NF-κB signaling pathway and expression of inflammatory cytokines. Besides mediating the inflammatory response, the cytokines increase the expression of *Slpi* from the intestinal epithelial cells. As it is evidenced by multiple experimental findings, the expression of *Slpi* is regulated in intestinal epithelial cells and its increased expression correlates with anti-bacterial, anti-inflammatory and anti-protease functions. This study provides evidence that prion protein (moPrP) might mediate the activation of cellular immunity in the mice colon by modulating the expression of genes that mediate inflammatory response.

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Table 4.1 Antibacterial gene expression in mice colon according to the treatment. Results of gene expression are shown as fold-regulation relative to CTR[1] group.

Gene	Description	LPS M[2]	D-LPS S[3]	PrP M[4]	PrP M+D-LPS S[5]	PrP M+D-LPS M[6]
	Toll- like Receptor Signaling					
Cd14	CD14 antigen	1.56±0.45	1.01±0.62	1.83±0.56	3.06±1.0**	2.21±0.58
Tlr4	Toll-like receptor 4	1.64±0.99	3.81±1.12	7.32±0.01***	4.83±1.15*	5.56±0.58**
Irak3	Interleukin-1 receptor-associated kinase 3	2.31±0.87	2.10±0.75	3.65±0.01***	1.90±0.59*	3.50±0.59*
Irf7	Interferon regulatory factor 7	-1.09±1.25	-1.46±0.74	-1.74±0.58	-5.26±1.53**	-3.64±0.01**
Tirap	Toll-interleukin 1 receptor (TIR) domain-containing adaptor protein	1.29±1.52	1.35±0.71	1.83±0.01*	1.51±1.14	1.74±0.56
Lbp	Lipopolysaccharide binding protein	-1.02±1.39	1.59±0.06	1.83±0.01*	-1.04±1.00	2.21±0.99
	NOD-like Receptor Signaling					
Nlrp1a	NLR family, pyrin domain containing 1A	1.61±1.42*	1.40±0.24*	1.45±0.07**	1.91±0.62	2.19±0.61
Nod2	Nucleotide-binding oligomerization domain containing 2	1.15±0.82	1.54±0.52	1.83±0.01*	-1.66±1.53	1.75±0.57
Sugt1	SGT1, suppressor of G2 allele of SKP1 (S. cerevisiae)	1.33±1.05*	1.40±0.31*	2.30±0.58*	1.52±0.99**	1.38±0.57
	Other Bacterial Pattern Recognition Receptors					
Dmbt1	Deleted in malignant brain tumors 1	1.11±1.14	1.08±0.24	1.15±0.01	-5.28±1.52**	-1.45±1.00
Zbp1	Z-DNA binding protein 1	1.29±0.97	1.56±0.57	1.15±0.57	-1.31±01.00*	-1.14±0.00
	Downstream signal transduction					
Chuk	Conserved helix-loop-helix ubiquitous kinase	-1.13±1.66	-1.50±1.25	-2.18±0.56*	-2.08±0.57	-2.28±0.57
Mapk3	Mitogen-activated protein kinase 3	1.25±1.50	-1.29±0.57	-22.46±0.01	-2.63±1.53*	-2.29±0.58*
Map2k3	Mitogen-activated protein kinase kinase 3	1.28±0.96	-1.18±1.27	-1.37±0.01*	-1.04±0.58	-1.14±0.01
	Apoptosis					
Casp1	Caspase 1	1.42±1.19	1.03±0.66	-1.74±0.58*	-1.66±0.57	-1.82±0.56
Irak1	Interleukin-1 receptor-associated kinase 1	1.35±1.10*	1.13±0.82	1.15±0.58	1.51±0.59**	1.38±0.57
Jun	Jun oncogene	1.41±1.15	1.11±0.79	1.83±0.57	1.51±1.01**	2.18±0.58**
Nfkb1	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1, p105	1.42±1.16**	1.03±0.62	-1.37±0.02*	-1.04±1.15	-1.13±0.01
Tnfrsf1a	Tumor necrosis factor receptor superfamily, member 1a	1.42±1.19**	1.13±0.81	1.45±0.01**	-1.32±1.00*	1.10±0.57
	Inflammatory response					
Nlrp3	NLR family, pyrin domain containing 3	2.81±0.74*	1.36±0.82	2.30±0.01**	2.43±1.00	4.43±1.16
Rela	V-rel reticuloendotheliosis viral oncogene homolog A (avian)	1.36±1.10*	1.03±0.65	1.15±0.58	-1.05±1.15	1.09±0.58

Slc11a1	Solute carrier family 11 (proton-coupled divalent metal ion transporters), member 1	1.54±1.40*	1.82±0.51	2.29±0.59	1.91±1.16	1.76±1.00
Tirap	Toll-interleukin 1 receptor (TIR) domain-containing adaptor protein	1.29±1.52	1.35±0.71	1.83±0.01*	1.51±1.14	1.74±0.56
Tnf	Tumor necrosis factor	1.69±1.18	2.05±1.28	2.30±0.58*	-1.31±1.00*	2.78±0.58*
	Cytokines and Chemokines					
Cxcl1	Chemokine (C-X-C motif) ligand 1	2.35±0.78	-1.07±1.97	2.88±1.73	1.91±0.57	10.98±0.58*
Il12a	Interleukin 12A	1.41±1.16	-1.29±1.14	-2.75±0.00***	-1.31±1.00	-1.17±1.76
Il18	Interleukin 18	-1.14±1.32	-1.64±1.15	-2.18±0.58*	-1.65±1.00	-1.82±0.57
	Antimicrobial peptides					
Slpi	Secretory leukocyte peptidase inhibitor	1.05±1.54	1.48±1.28	9.19±0.58*	30.58±2.08*	35.16±0.57***

Within a row, means with subscript were different relative to CTR group *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$.

[1]Pyrogen-free water was added on the musosal side of the chamber at 700 µL.

[2]LPS was added on the mucosal side of the chamber at 100 µg/mL.

[3]D-LPS was added on the serosal side of the chamber at 100 µg/mL.

[4]moPrP was added on the mucosal side of the chamber at 100 µg/mL.

[5]D-LPS (100 µg/mL) was added on the serosal side of the chamber and Prion (100 µg/mL) was added on the mucosal side of the chamber.

[6]D-LPS (100 µg/mL) was added on the mucosal side of the chamber and Prion (100 µg/mL) was added on the mucosal side of the chamber.

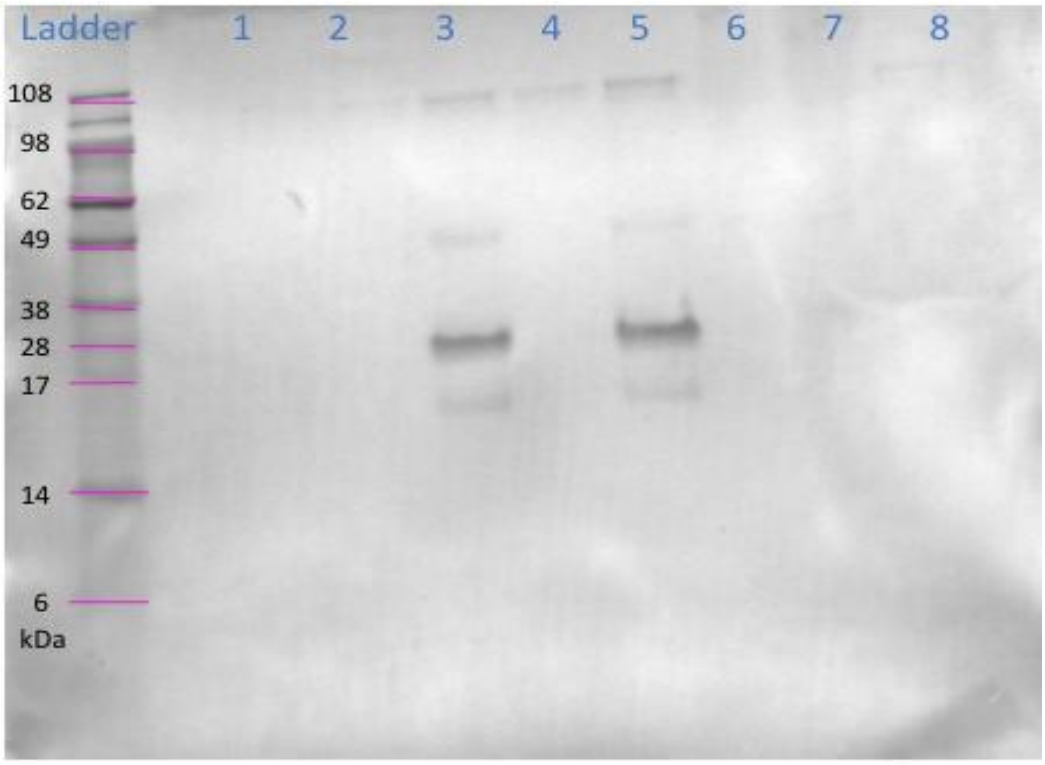

Figure 4.1 Detection of moPrP on mucosal and serosal sides of the Ussing diffusion chambers in mice colon by Western blot.
Lane 1: Control mucosal side; **Lane 2:** Control serosal side; **Lane 3:** moPrP (29-232) M mucosal side; **Lane 4**: moPrP M serosal side, **Lane 5:** moPrP M + D-LPS S mucosal side, **Lane 6:** moPrP M + D-LPS S serosal side; **Lane 7:** moPrP M + D-LPS M mucosal side; **Lane 8:** moPrP M + D-LPS M serosal side

CHAPTER 5: General Discussion

The physiologic functions of the normal cellular PrP^C remain poorly understood despite the well-characterized role of the misfolded molecule in the pathogenesis of neurodegenerative diseases. This thesis research achieved characterization of selected immune and antibacterial gene expression in the colon tissue of rats and mice under an Ussing chamber model and treatments with moPrP or LPS alone or combinations of moPrP with LPS or D-LPS. Several genes related to immune and antibacterial responses were affected by moPrP alone or in combination with LPS or D-LPS. This investigation showed that one of the unrecognized functions of moPrP in the colon tissue might be to activate antibacterial immune responses of the host.

Another issue tackled by this investigation was whether moPrP would go through the intact colon tissue of rats and mice. Understanding the route of entry of prion protein into the host is important to develop strategies for its prevention. It has been assumed that the oral route is the common route of infection. This research indicated that moPrP does not permeate through the intact colon tissue of rats or mice. This does not imply that moPrP does not enter into the host intestinal epithelial cells. Other investigators have already indicated that intestinal epithelial cells are able to endocytose prion protein (Morel et al., 2005). However, what happens to PrP after endocytosis to the intestinal epithelail cells it is not well characterized and might be related to binding to DCs and transportation through lymphatic system to the local lymph nodes (Natale et al., 2011). Transportation of

moPrP from the mucosal to the serosal side of the diffusion chamber was not proven by the present investigation. In addition, LPS or D-LPS did not facilitate the transport of moPrP through the intact colon tissue of rats and mice.

Stimulation of expression of a greater number of genes by combination of moPrP with LPS implies that moPrP and LPS might be internalized in the intestinal epithelial cells of the colon and serve as a danger signal to the host to activate antibacterial responses.

Another interesting observation was that detoxification of LPS by alkaline phosphatase showed that LPS loses its functions when phosphate groups are removed, as indicated by the complete lack of stimulation of gene expression.

5.1 Ussing chamber model and potential future experiments

Experiments conducted with the intact colon tissue of rats and mice showed that moPrP did not permeate from the mucosal to the serosal side or vice versa through the intact colon tissue and suggested that transportation of PrP after passing the epithelial layer might involve immune cells. It should be noted that experiments conducted from other authors have demonstrated that PrP^{Sc} is able to pass through the epithelial cell layers of cattle and sheep (McKie et al., 1999); however, the methodology used by those authors was different from ours; they removed the muscle layers from the colon tissue. Another difference was that they used PrP^{Sc}, whereas we used a recombinant moPrP. The protease resistant PrP^{Sc} can easily enter the epithelial cells (i.e., enterocytes, M cells or DCs) (Martin et

al., 2011). Results also suggested that transportation of moPrP through the gastrointestinal tract involves mechanisms that require tracking of movement inside the cell and organelles of the epithelial cells. For example, laminin receptor on enterocytes has been demonstrated to bind and internalize PrP^{Sc} inside the epithelial cells (Da Costa Dias et al., 2011). However, tracking the transport of PrP inside the epithelial cells was beyond the scope of this investigation.

Our results also showed that LPS was not transported from the mucosal to the serosal side of the intact colon tissue of rats or mice. In a previous experiment with cattle where muscle layers of the colon and rumen tissues were removed, we demonstrated that LPS was able to go through the intestinal epithelial layers of rumen and colon tissues (Emmanuel et al., 2007). It should be noted that rat and mice colon tissues are very delicate and it is very difficult to remove the muscle layers without damaging the epithelial layer itself. However, it will be interesting to note that in future potential experiments radio labeling of the molecules and tracking of their transport through the colon tissue would be suggested.

An issue that was encountered during the experiment was that samples from the control group had also substantial amounts of typical LPS fatty acids present in similar concentrations with the treatment groups. The reason for that might be that there are numerous bacteria that are attached to the colon tissue. It is impossible to remove those bacteria from the colon tissue, and during the Ussing chamber experiments it is possible that they were released into the solution. Processing of samples with chemicals for extraction of fatty acids potentially killed those bacteria and released bacterial LPS in the solution. Therefore, it is important that

in future experiments to filter the solutions for bacteria and then run the samples for LPS content.

Data also showed that LPS or D-LPS did not facilitate the transport of moPrP through rat or colon tissue. The reason we hypothesized that LPS might facilitate the permeation of moPrP through intestines was that we showed that LPS interacts with moPrP and converts it into a beta-rich isoform (PrPbeta) (Ametaj et al., 2010). Since PrP^{Sc} can easily enter intestinal epithelial cells we also hypothesized that PrPbeta might enter the intestinal epithelial cells and permeate the colon tissue. Expression of many genes, when LPS was combined with moPrP, suggests that there was internalization of moPrP; however, the protein did not go from the mucosal to the serosal side of the diffusion chamber.

5.2 Antibacterial gene expression profiling

Experiments conducted indicated that LPS and moPrP were able to activate various genes in the colon tissue of rats and mice. Regarding LPS, results showed that not very many genes were affected by its presence on the mucosal side; however, the number of genes affected was doubled when LPS was on the serosal side. The reason for that might be that there are no receptors for LPS on the apical side of the epithelial cells. Indeed Hornef et al. (2003) indicated intracellular recognition of LPS by TLR-4 in the intestinal epithelial cells. The same authors demonstrated that LPS receptors are located in the Golgi apparatus of intestinal crypt epithelial cells and not on the apical side of epithelial cells. Therefore,

recognition of LPS needs internalization into the Golgi apparatus of the intestinal cells. Based on minimal gene expression by LPS our data suggest that colon epithelial cells internalized minimal amounts of LPS. The reason for this might be that the type of LPS that we used was not recognized as a danger signal by the colon. However, when LPS was added on the serosal side of the diffusion chamber the number of genes increased. This agrees with the reported observations that the receptors for LPS are located on the basement membrane of the epithelial cells rather than on the apical side (Abreu et al., 2003).

Regarding gene expression by moPrP, a greater number of genes were affected when compared to LPS on the mucosal side. The fact that moPrP activated various genes related to antibacterial responses and those of innate and adaptive immunity suggests that moPrP is internalized and that one of the main functions of moPrP in the murine GI tract is to activate host immune responses. Normally prion protein is not free in the intestinal lumen. However, presence of free PrP might serve as a signal for the host that there are dangers of bacterial infections. Previously it has been shown that PrP is able to kill both Gram-negative and -positive bacteria (Pasupuleti et al., 2009). Therefore, the release of PrP in the gut lumen might be a mechanism of the host to kill pathogenic bacteria. Recently other authors demonstrated that PrP^C participates in the regulation of the response of microglia to *Mycobacterium bovis* infection through the upregulation of pro-inflammatory cytokines and the modulation of apoptosis by interference with the intrinsic apoptotic pathway (Ding et al., 2013).

Combination of moPrP with LPS or D-LPS affected more antibacterial genes than compared to moPrP or LPS alone. It is interesting to note that one of the most expressed genes by moPrP was *Slpi*, an antimicrobial peptide. This suggests that one of the functions of moPrP is to activate host genes related to antimicrobial activity. Moreover, when moPrP was combined with LPS or D-LPS on mucosal or serosal sides the same gene (i.e., *Slpi*) was up-regulated 3- and 4-fold more than when moPrP was added alone. This suggests that combination of moPrP with LPS or D-LPS is a stronger signal to the host for presence of bacteria. Previously we indicated that LPS binds to moPrP and converts it into a beta-rich isoform and that LPS and moPrP form large aggregates together (Ametaj et al., 2010). It is possible that these aggregates are endocytosed by epithelial cells and serve as a signal for presence of LPS. That might be the reason why *Tlr4* gene was activated only when moPrP and LPS were added together and not when LPS or moPrP were added alone.

Data also indicated that the colon did not respond strongly to free LPS. There are abundant amounts of free LPS in the lumen of the GI tract as a result of continuous death of Gram-negative bacteria. It seems like it is not in the host interest to respond to free LPS unless it becomes a danger to the host. However, presence of PrP in the gut lumen, which is supposedly secreted by the host when pathogenic bacteria are present, and its combination with LPS might be a danger signal for presence of harmful bacteria. This line of thinking is supported by reports of increased PrP expression and secretion during bacterial infections of various mucosal layers including the stomach during *Helicobacter pylori*

infection (Konturek et al., 2005) and during infections of the mammary gland (Ligios et al., 2011). Moreover, Martin et al. (2011) demonstrated that endogenous prion protein attenuated experimentally induced colitis suggesting that PrP^{C} has a cytoprotective and anti-inflammatory function within the murine colon.

Cross-species experiment, using moPrP in rats, showed that moPrP was able to induce expression of only one gene (i.e., *Il6*). This suggests that moPrP was internalized; however, it was not able to induce a larger number of genes. The dose of moPrP used also might have affected the results, because a 10-fold lower dose (10 µg/mL) was used with rat's experiment versus mice's experiments (100 µg/mL). Combination of moPrP with LPS was able to affect more genes in the rat colon, confirming that cross-species recognition of PrP is possible and that LPS and PrP are a stronger signal to the host. Other authors have proved cross-species oral infectivity with PrP^{Sc} from various species (Da Costa Dias et al., 2011).

Another interesting finding was that LPS or D-LPS were not able to activate *Tlr4*. The latter is known to be a specific receptor for LPS. This again suggests that this receptor is located inside the epithelial cells and not on the apical side of the cells. Indeed Abreu et al. (2003) showed that intestinal epithelium suppresses TLR-4 and MD-2 expression and is LPS unresponsive. The same authors demonstrated that LPS sensing on the intestinal epithelial cells occurs on the basolateral membrane and not on the apical side.

Detoxification of LPS by alkaline phosphatase inactivated LPS and this type of LPS was not able to affect expression of colon genes related to immunity or antibacterial response. Results suggest that deactivation of LPS by alkaline phosphatase might be a method for neutralizing the harmful effects of LPS in the GI tract.

5.3 Conclusions

Overall, results of this thesis research are promising and suggesting that one of the potential functions of moPrP might be its involvement in host immune responses and more specifically in activation of antibacterial responses in the gut. This opens new avenues in studying the antibacterial role of moPrP in the gut lumen. This also suggests that moPrP might be a danger signal for the host against pathogenic bacteria. The lack of transport of moPrP through the colon tissue suggests that the mechanism of transport of moPrP through the gut tissue involves more intricate transport mechanisms rather than simple diffusion. Although LPS did not facilitate permeation of moPrP through the colon tissue it is interesting that addition of LPS with moPrP in the diffusion chamber activated the highest number of genes related to immune and antibacterial responses in the colon when compared to moPrP or LPS alone. Interaction of moPrP with LPS might serve as a stronger signal to the host for presence of bacteria and mounting of an immune response. Detoxification of LPS neutralized LPS to the point that no host gene responses were obtained suggesting that detoxification of LPS might be a beneficial process to protect the host from harmful effects of LPS.

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