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IDENTIFICATION AND CHARACTERIZATION OF THE "GUT VASCULAR BARRIER"

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LIST OF ABBREVIATIONS

AID	Activation Induced cytidine Deaminase
AJ	Adherent Junction
ALT	Alanine aminotransferases
BBB	Blood Brain Barrier
CD	Cluster of differentiation
cDNA	Copy-Deoxyribonucleic acid
CFU	Colony Forming Unit
CLDN	Claudin
CNS	Central Nervous System
DAPI	4',6-diamidin-2-fenilindolo
DC	Dendritic Cell
DNA	Deoxyribonucleic Acid
DTH	Delayed Type Hypersensitivity
DTT	Dithiothreitol
EC	Endothelial Cell
EDTA	Ethylenediaminetetraacetic acid
EGC	Enteric Glial Cell
FAE	Follicle Associated Epithelium
FITC	Fluorescein isothiocyanate
GALT	Gut-Associated Lymphoid Tissue
GFAP	Glial Fibrillary Acidic Protein
GOF	Gain-of-function
GSK3β	Glycogen Synthase Kinase 3 β
HBSS	Hank's Balanced Salt Solution
Hh	Hedgehog
IBD	Inflammatory Bowel Disease
IF	Immunofluorescence
Ig	Immunoglobulin
ILF	Isolated Lymphoid Follicles
JAM	Junctional Adhesion Molecule
KO	Knock-out
LAM	Leukocyte Adhesion Molecule
LP	Lamina Propria

LPS	Lipopolysaccharide
LYVE-1	Lymphatic Vessel Endothelial Hyaluronan Receptor
MAMP	Microbe-Associated Molecular Patterns
mLN	Mesenteric lymph node
Muc	Mucin
MyD88	Myeloid Differentiation primary response 88
NOD	Nucleotide-binding Oligomerization Domain
NVU	Neurovascular Unit
P-gp	P-glycoprotein
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PE	R-Phycoerythrin
PFA	Paraformaldehyde
PGE2	Prostaglandin E2
PLVAP	Plasmalemma Vesicle-Associated Protein
PPAR-γ	Proliferator-Activated Receptor-γ
PPs	Peyer's Patches
PRR	Pattern recognition receptor
RA	Retinoic Acid
REG	Regenerating islet-derived protein
RNA	Ribonucleic Acid
SHh	Sonic Hedgehog
SI	Small Intestine
SPI	Salmonella Pathogenicity Island
TCR	T Cell Receptor
TGF-β	Transforming Grow Factor-β
TJ	Tight Junction
TLR	Toll-like Receptor
TSLP	Thymic Stromal Lympopoietin
TTSS	Type III Secretion System
VE	Vascular endothelial
WT	Wild type
ZO	Zonula occludens
α-SMA	α-Smooth Muscle Actin

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ABSTRACT

In order to protect the body from a wide range of harmful environmental agents, the intestine has developed a number of barrier mechanisms to limit the entry of potential hazards. These include the physical barrier formed by the epithelial layer and the intestinal immune system that is important to induce either tolerance against food antigens and intestinal flora or inflammatory responses against dangerous microorganisms.

It has been demonstrated that tolerance against commensal bacteria is strictly compartmentalized, in the sense that the systemic immune system is completely unprimed by these bacteria. It was demonstrated that the mLNs function as a “firewall” confining induction of tolerance to the mucosa while the systemic immune system remains ignorant to these bacteria.

However, in these studies how the bacterial flora is excluded from the entrance in the bloodstream via the intestinal blood vessels has not been analyzed.

Here, we describe a new barrier that we called the GVB (gut vascular barrier) that plays a fundamental role in controlling the spreading of molecules and bacteria to systemic sites. We found that intestinal endothelial cells (ECs) express the main components of TJs (occludin, JAM-A, Cldn-12, ZO-1 and cingulin) and AJs (VE-cadherin and junctional β-catenin), indicating the presence of a barrier that excludes bacteria from passing through the paracellular route. In addition, we observed the existence of a “gut vascular unit” (GVU) whereby ECs were associated with enteric glial cells and pericytes, whose role in the establishment of the endothelial barrier phenotype remains to be analyzed.

Moreover, we show that GVB integrity could be modified by *Salmonella typhimurium* infection. Indeed, upon infection ECs up-regulated the expression of Plvap, that has been previously used as a marker of immature/damaged vascular barrier in the brain, and up-regulated caveolin-1, the major component of caveolae. These changes correlated with a higher permeability of the endothelium to small molecules and to bacteria.

One way by which *S. typhimurium* could modify the barrier properties of the intestinal blood vessels could be through the negative regulation of the Wnt/β-catenin signaling pathway. Indeed, we found that the activation of β-catenin was reduced upon *Salmonella* infection *in vitro*. Consistently, we found that *Salmonella* was incapable to modify ECs permeability and to spread systemically in mice where β-catenin was constitutively activated by genetic means only in vascular ECs. Furthermore, it appeared that the TTSS encoded by Salmonella pathogenicity island-2 was involved in the regulation of Wnt/β-catenin signaling pathway in ECs.

Finally, preliminary results show that the microbiota could induce GVB maturation and maintenance. However, the mechanisms involved in these processes as well as the bacterial species responsible for this process have not been investigated yet.

INTRODUCTION

In order to protect itself from a wide range of harmful environmental agents, the body has developed a number of barrier mechanisms to limit the entry of potential hazards.

The organ mostly challenged by foreign material is the intestine. It has to constantly deal with innocuous food antigens and with an enormous number of commensal microbes that reaches 10^{14} bacteria in the human intestine, with close to 1000 distinct species, without considering archea, fungi, and viruses (Eberl, 2010). In the lower intestine, these organisms have evolved together with the host establishing a mutualistic relationship. Indeed, the microbes benefit from a constant nutrient supply while the host benefits from microbial degradation of plant polysaccharides and other dietary substances, of xenobiotics and they provide a barrier against potential pathogens (Hooper et al., 2012). Therefore, the union between the host and his microflora can be seen as a new functional entity termed a “superorganism” (Eberl, 2010). Despite the symbiotic relationship, the presence of the microbiota poses immense health challenges. For this reason, the intestine has adapted different strategies to limit opportunistic invasions by the resident microbiota and to maintain symbiotic host-microorganism relationship avoiding pathologies such as bacteremia and chronic inflammation (Hooper and Macpherson, 2010). Two main methods are used for these purposes: the structural compartmentalization that minimizes bacterial–epithelial cell contact and the presence of specialized immune cells responsible in handling intestinal bacteria and food antigens in a tolerant way (Fig. 1.1).

1.1 Physical Barrier

1.1.1 The epithelial barrier

The intestinal epithelium is a permeable barrier essential in preventing the uncontrolled passage into the host connective tissue of food or bacteria, and is involved in regulating the fluid absorption and secretion. Intercellular tight junctions (TJ) and adherens junctions (AJ), located near the apical surface of the intestinal epithelial cells, prevent paracellular traffic, whereas the brush border on the apical side avoids microbial attachment and invasion (Brown et al., 2013). Apart from the columnar absorptive epithelium, several specialized cells are involved in the creation and maintenance of the intestinal homeostasis. One of them is the M cell, located in the epithelium lining the secondary lymphoid organs such as Peyer's patches (PP), colonic patches and isolated lymphoid follicles. These cells are characterized by irregular microvilli on the apical membrane, consistent with their function to transport antigens, and a by pocket-like microfold structure on the basal side that contains T and B lymphocytes, macrophages and dendritic cells (DC) (Goto and Kiyono, 2012). Additional support for the maintenance of the barrier function is given by other specialized epithelial cells such as goblet cells, important for the formation of the mucus layer, and Paneth cells, that secrete antimicrobial peptides (Fig. 1.1), both of which will be described in detail in the next sections.

In the last decade, it has become evident that the epithelial layer is not merely a physical barrier but it takes part in immunological processes discriminating between harmless and potentially harmful stimuli. Indeed, epithelial cells express a wide range of pattern recognition receptors (PRR), including Toll-like receptors (TLR) as well as nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) that recognize bacterial microbe-associated molecular patterns (MAMP) shared by pathogens and the gut microbiota. The key mechanism to discriminate invasive pathogens, which can cross the epithelial monolayer, from the commensal microbiota, and to prevent responses to the

abundant bacterial components in the gut lumen preserving the ability to mount responses against pathogens is the compartmentalization of PRRs to the basolateral membrane or into the cytosol (Artis, 2008; Iliev et al., 2007; Rakoff-Nahoum et al., 2004). For example, TLR5, which recognizes bacterial flagellin, is expressed exclusively on the basolateral membrane allowing intestinal epithelial cells to induce an inflammatory response only against invading bacteria (Gewirtz et al., 2001).

Additionally, commensal bacteria can actively modulate inflammatory responses modulating NF-κB signaling pathway in the epithelial cells. Indeed, it has been shown that apical TLR9 stimulation by commensal bacteria induces the ubiquitination of IκB that accumulates in the cytoplasm preventing NF-κB activation, whereas basolateral TLR9 signals results in the activation of NF-κB transcriptional activity (Lee et al., 2006). Non-pathogenic bacteria can reduce NF-κB activation also by inducing the expression of peroxisome proliferator-activated receptor gamma (PPAR-γ), which favors the export of RelA NF-κB subunit out of the nucleus reducing its transcriptional activity (Kelly et al., 2004).

Intestinal epithelial cells contribute to maintenance of the intestinal hypo-responsiveness to harmless stimuli also interacting with the underlying antigen presenting cells and conditioning them through the secretion of cytokines (Iliev et al., 2007). Such conditioning renders DCs “tolerogenic”, i.e. unable to induce inflammatory responses and capable to induce tolerogenic T cell responses (Iliev et al., 2009a; Iliev et al., 2009b) (Rimoldi et al., 2005). In contrast, in presence of inflammatory signals, the intestinal epithelium releases inflammatory cytokines and chemokines that recruit non-educated DCs, competent in inducing inflammatory responses (Iliev et al., 2007).

Numbers of factors, known to be produced by epithelial cells, are involved in the DC conditioning. Among those, transforming growth factor-β (TGF-β), retinoic acid (RA), prostaglandin (PG) E2 and thymic stromal lympopoietin (TSLP) have been shown to play a major role in the induction of tolerance (Iliev et al., 2007).

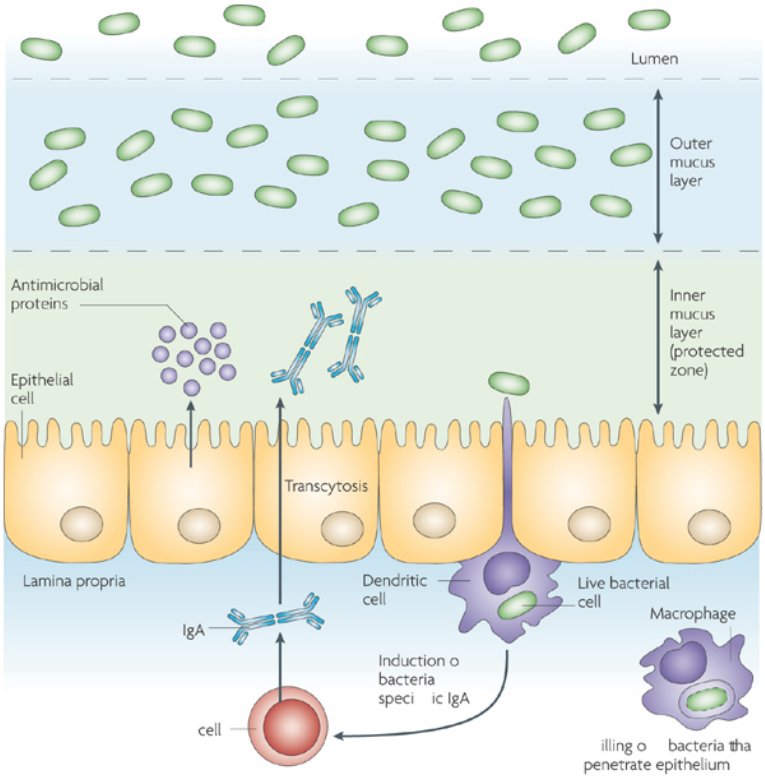

Figure 1.1: Physical and immunologic barriers for intestinal microbiota containment (Hooper, 2009). Goblet cells secrete mucins that assemble into a mucus layer. Bacteria are abundant in the outer mucus layer, whereas the inner layer remains sterile. Together with the mucus layer, antimicrobial proteins further help to avoid direct contact between bacteria and epithelial cells. LP-DCs can actively sample bacteria from the lumen extending their dendrites between the epithelial cells. Migrating into the Peyer's patches and mesenteric lymph nodes, DCs loaded with bacteria induce B cells to differentiate into IgA+ plasma cells. Recirculating plasma cells produce IgA that are then transcytosed across the epithelium where they limit bacterial penetration into the host tissues.

1.1.2 Mucus Layer

The first layer of defense between the gut and the external environment is the mucus layer. Specialized epithelial cells, namely goblet cells, secrete mucin glycoproteins that form a thick gel-like mucus layer that in the colon has been estimated to be around 50 µm in mice and 100µm in rat (Johansson et al., 2011). It is composed of two different layers:

the outer lose and easily removable mucus and the inner epithelium-adherent layer. Staining the intestine with 16S ribosomal RNA probes has revealed that unlike the inner mucus layer that is completely sterile, commensals colonize the outer layer (Johansson et al., 2008) that avoids their wash out and allows their growth providing glycans as nutrient source. Studies on mucus composition have established that it contains carbohydrates, immunoglobulins, cellular proteins, as well as lipids and electrolytes but the major component of both layers is mucin (Muc)2. Muc2 is a large glycoprotein characterized by a protein core heavily O-glycosylated with numerous carbohydrate chains that are important for Muc2 function (Johansson et al., 2008). Different studies in Muc2 deficient mice that lack mucus layer have demonstrated that Muc2 mucin plays an important role in the maintenance of intestinal physiology. Indeed, it has been shown that Muc2–/– mice develop spontaneous colitis by 7 weeks of age (Van der Sluis et al., 2006) as well as colon cancer after 6–12 months (Velcich et al., 2002). Furthermore Muc2–/– mice are more susceptible to infection by enteric pathogens such as *Salmonella typhimurium* (Zarepour et al., 2013) or *Citrobacter rodentium* (Bergstrom et al., 2010). This is due to the fact that the lack of Muc2 and therefore deficiency of both mucus layers facilitates direct contact between pathogens and epithelium as well as the contact with commensal microbes, that in this way are able to translocate across the epithelium and into the lamina propria (Bergstrom et al., 2010).

Recently, *Shan et al.* have demonstrated that the mucus acts not only as a physical barrier between gut tissue and intestinal bacteria but it also influences the function of antigen presenting cells, in particular DCs, and epithelial cells contributing in establishing tolerance toward food and microbiota-derived antigens. Indeed, on one side, DCs conditioned with glycosylated Muc2 show reduced expression of pro-inflammatory cytokines such as interleukin (IL)-12 counteracted by an increased production of anti-inflammatory cytokines (IL-10) when stimulated with lipopolysaccharide (LPS). This results in the generation of regulatory T (T_{reg}) cells. On the other side, upon Muc2

stimulation intestinal epithelial cells produce molecules that support DC regulatory function, such as TGF-β, TSLP and IL10 (Shan et al., 2013).

1.1.3 Antimicrobial peptides

The second way by which the bacteria-epithelial cell contact is limited is the secretion of antimicrobial peptides that are particularly important in the small intestine since it lacks a continuous mucus layer (Brown et al., 2013). Several antimicrobial peptides have been identified such as defensins and C-type lectins that are able to kill bacteria via enzymatic digestion of bacterial cell wall or inner membrane, and lipocalins that are able to inhibit bacterial growth by interfering with the acquisition of essential metals like iron (Brown et al., 2013). The expression of these proteins is regulated by different mechanisms. Indeed some defensins, for example some α-defensins that are produced by neutrophils and Paneth cells located at the base of the small intestinal crypts, are expressed constitutively without any bacterial stimulation (Putsep et al., 2000). However, other antimicrobial peptides are produced upon the ligation of PRRs, such as TLRs or NOD receptors. For example, it has been shown that some C-type lectins, such as regenerating islet-derived protein (REG)-3β and REG-3γ are expressed in the small intestine upon TLR engagement and myeloid differentiation primary response 88 (MyD88) signaling pathway activation (Vaishnava et al., 2008) similarly to some α-defensins that are expressed upon NOD2 recognition of bacterial muramyl dipeptide (Kobayashi et al., 2005).

The functional importance of antimicrobial proteins was demonstrated using gain-of-function and loss-of-function animal models. Indeed, it has been demonstrated that mice lacking Paneth cells have an increased penetration of commensal bacteria and pathogens such as *Salmonella*. Indeed without antimicrobial peptides, *S. typhimurium* is able to overcome the epithelial barrier and spread systemically (Vaishnava et al., 2008). The resistance to this pathogen could be restored in transgenic mouse models by inducing the production of the human defensin-5 in the mouse gut (Salzman et al., 2003).

The importance of antimicrobial peptides for the maintenance of the intestinal homeostasis has been supported also by studies on human pathologies. Indeed, reduced levels of α-defensins were detected in samples from Crohn's disease patients (Wehkamp et al., 2005) suggesting a role for enteric α-defensins in the pathogenesis of inflammatory bowel diseases.

1.2 Immune Barrier

1.2.1 IgA

A further line of defense to uncontrolled bacterial translocation through the host epithelial cell layer involves the secretion of immunoglobulin (Ig)A.

IgAs specific for the intestinal microflora are produced upon stimulation of B cells by DCs that have sampled luminal bacteria in the PPs (Macpherson and Uhr, 2004) or directly from the gut lumen (Rescigno et al., 2001). Together with DCs, also epithelial cells can directly induce class switch recombination from IgM to IgA by producing B cell activating factor belonging to the TNF family (BAFF) and the proliferation-inducing ligand (APRIL) upon TLR stimulation. BAFF and APRIL recognized by their receptor on B cells induces the activation of NFκB pathway that up-regulates the expression of the activation-induced cytidine deaminase (AID), the enzyme required for the class switch recombination (Macpherson et al., 2012).

Induced IgAs in the intestinal lymphoid follicles undergo recirculation from the mucosa through the lymph into the thoracic duct to home back via the blood stream in the intestine as IgA-producing plasma cells. The homing back to the site of induction is possible because retinoic acid (RA) produced by intestinal DCs up-regulates the α4β7 integrin and the CCR9 and CCR10 chemokine receptors on B cells that bind respectively to the chemokines CCL25 and CCL28 expressed in the intestinal mucosa (Mora et al., 2006).

Once in the intestine the IgA dimers are transported through the epithelium in the intestinal lumen via the polymeric Ig receptor present at the basolateral membrane of the epithelial cells. The “secretory” IgAs are then released in the lumen by the proteolysis of the extracellular domain of the polymeric Ig receptor (Macpherson et al., 2012).

The induction of secretory IgA in the gut is specifically induced by the microflora since germ-free mice have reduced numbers of mucosal IgA-producing cells (Slack et al., 2012) but it is independent on the presence of germinal centers and T cells. Indeed, Tumor necrosis factor (TNF) receptor–I deficient mice that have rudimentary PPs and no B cell follicles and T cells deficient mice (T cell receptor (TCR) β-/- δ-/-) show similar levels of IgA-producing plasma cells specific for the microbiota compared to normal C57Bl/6 mice (Macpherson et al., 2000). This indicates that a large portion of mucosal IgAs are produced in a “primitive” fashion, independently from T-cell-mediated immunity. However some reports have demonstrated also a T-cell dependent component of IgA induction in which Foxp3+ regulatory T cells are involved (Cong et al., 2009).

Different mechanisms of action of IgA have been suggested. Peterson *et al.* have found that germ-free immunodeficient Rag1-/- mice administered with *Bacteroides thetaiotaomicron* show a more robust innate immune response compared to wild-type (WT) mice and to $Rag1^{-/-}$ mice implanted with *B. thetaiotaomicron* specific IgA-producing hybridoma cells (Peterson et al., 2007). This finding indicates that IgA can act reducing intestinal pro-inflammatory signaling. Moreover, IgA may also have a role in controlling the composition of the microbiota. Indeed, mice lacking AID enzyme show an overgrowth of predominantly of the segmented filamentous bacteria (SFB) (Suzuki et al., 2004), which have been associated to an increase of Th17 inflammatory responses (Ivanov et al., 2009) (Gaboriau-Routhiau et al., 2009). Beyond “buffering” the mucosal responses, IgAs could act directly on commensal colonization of the intestine by influencing bacterial growth rate or survival (Slack et al., 2012).

1.2.2 Mucosal Dendritic cells

The intestinal immune system, generally referred to as GALT (gut-associated lymphoid tissue) is usually divided into effector sites, which consist of lymphocytes scattered throughout the intestinal epithelium and lamina propria (LP), and organized tissues, like PPs, mesenteric lymph nodes (mLNs) and the smaller isolated lymphoid follicles (ILFs), responsible for the induction of immune responses (Fig. 1.2). PPs and ILFs are located in the small intestine, whereas isolated clusters of lymphoid cells similar to ILFs are found in the colon (Mowat, 2003).

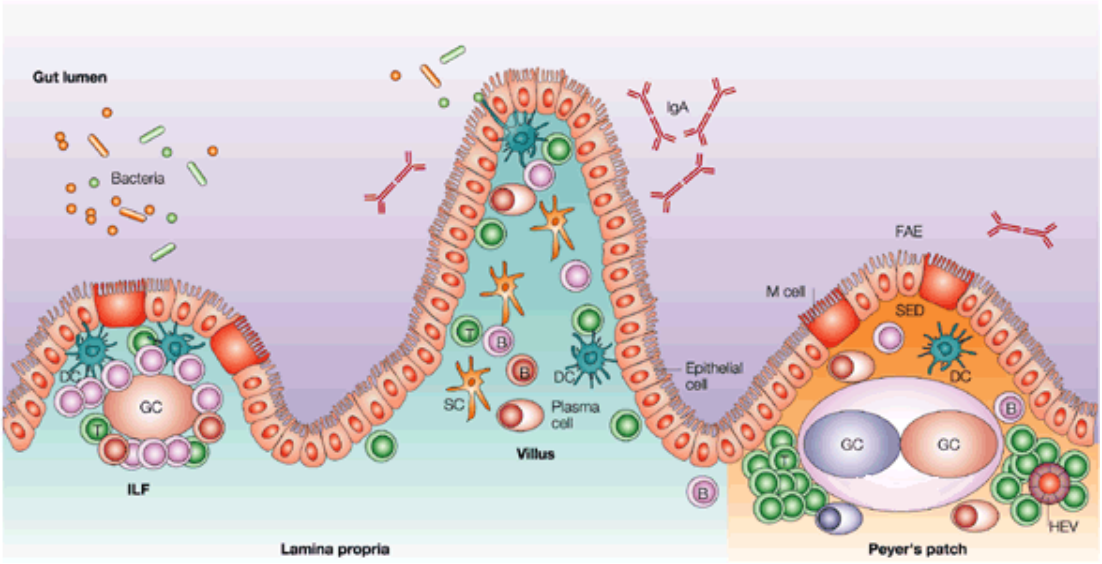

Figure 1.2: Schematic representation of GALT. Organized lymphoid structures - Peyer's patches and isolated lymphoid follicles - and effector sites - epithelium and lamina propria are depicted (Fagarasan and Honjo, 2003).

Different cell types compose the intestinal immune barrier in the intestine, but between them an essential role in maintaining the immune homeostasis is played by DCs.

In the PPs different populations of DCs were identified. On the basis of their cell-surface receptor expression they were classified into $CD11c^{hi}CD11b^{+}CD8\alpha^{-}$ (myeloid) DCs localized in the sub-epithelial dome, $CD11c^{hi}CD11b^{-}CD8\alpha^{+}$ (lymphoid) DCs present in the interfollicular regions and $CD11c^{hi}CD11b^{-}CD8\alpha^{-}$ DCs located at both sites (Iwasaki and Kelsall, 2001). Important functional distinctions also exist between these subpopulations. Indeed the $CD11b^{+}$ subset is endowed with the ability to induce IL-10-producing T cells and prime Th2 cells. On the other hand, $CD8\alpha^{+}$ and $CD11b^{-}$ $CD8\alpha^{-}$ PP DCs drive the differentiation of Th1 cells (Iwasaki and Kelsall, 2001). DCs from PPs can also be described in terms of their expression of the chemokine receptors CX3CR1 and CCR6. $CX3CR1^{+}$ DCs are associated with the follicle-associated epithelium (FAE) during the steady state, whereas $CCR6^{+}$ DCs are recruited from the dome region to the FAE during infection (Salazar-Gonzalez et al., 2006).

A large number of mononuclear cells, including macrophages and DCs, reside also in the intestinal LP. In the last decade, there was a growing interest on the different subpopulations of these mononuclear phagocytes and on their role in regulating mucosal innate and adaptive immune responses in both the steady-state and inflammatory settings.

Within the small intestine lamina propria, two developmentally and functionally non-overlapping populations of mononuclear phagocytes have been classified depending on the expression of CX3CR1 (the fractalkine receptor) and CD103 (αE integrin) (Fig. 1.3)(Schulz et al., 2009; Varol et al., 2009).

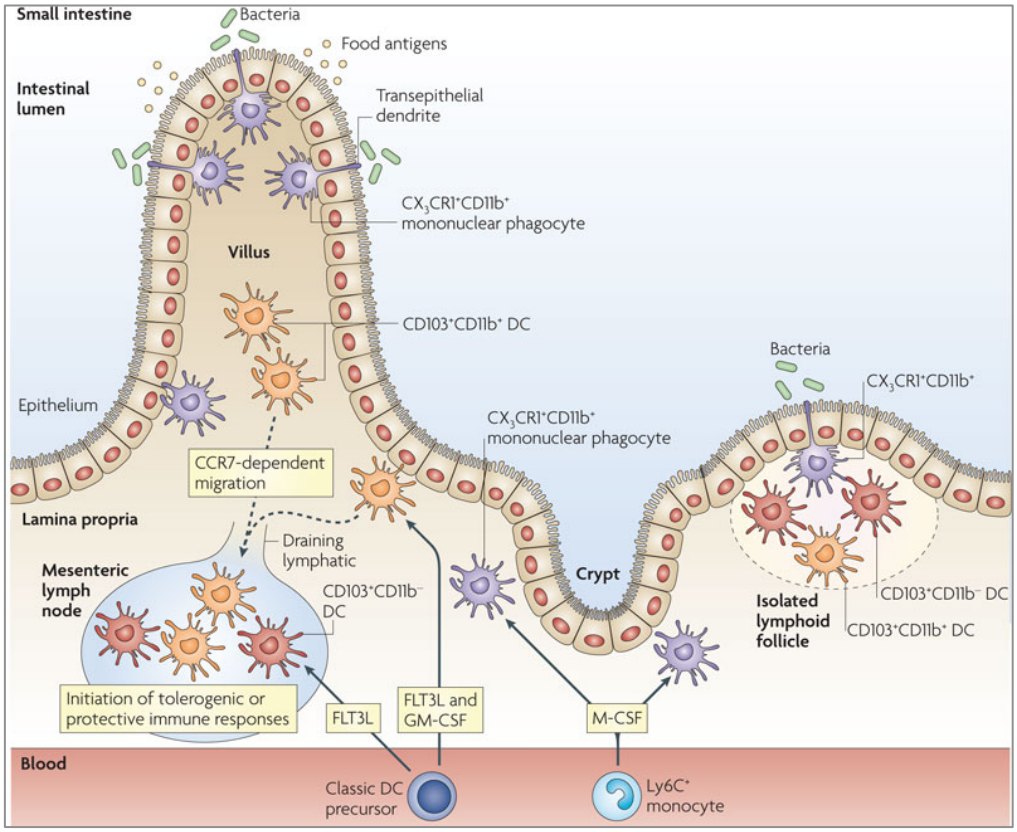

Figure 1.3: Mononuclear phagocytes in the small intestinal lamina propria (Varol et al., 2010). Classical DC precursors give rise to $CD103^+$ lamina propria DCs that can be subdivided into a $CD11b^-$ and a $CD11b^+$ population. Upon antigen stimulation, $CD11b^+CD103^+$ DCs migrate in a CCR7-dependent manner to the mLN. $Ly6C^+$ monocytes differentiate locally into CX_3CR1^+ mononuclear phagocytes endowed with the capacity to penetrate epithelium by extending trans-epithelial dendrites and sample luminal antigens. CX_3CR1^+ LP mononuclear phagocytes are not able to migrate to lymph nodes.

$CD103^+$ LP cells represent *bona fide* DCs. They develop from non-monocytic precursor cells (Varol et al., 2009) and they expand in response to fms-like tyrosine kinase 3 ligand (Flt3L) and granulocyte macrophage colony stimulating factor (GM-CSF) (Bogunovic et al., 2009; Schulz et al., 2009). They were found to express CCR7, allowing them to migrate to the mLN (Bogunovic et al., 2009; Jaensson et al., 2008; Schulz et al., 2009) where they induce, at steady state, the differentiation of naive $CD4^+$ T cells into $FoxP3^+$ regulatory T cells, through a mechanism dependent on RA and TGF-β (Coombes et al., 2007; Sun et al., 2007). Furthermore they induce the expression of the gut-homing

molecules CCR9 and α4β7 on responding T cells (Johansson-Lindbom et al., 2005). Under inflammatory conditions the tolerogenic features of the $CD103^+$ DCs are lost. Indeed, in a model of T-cell induced colitis, $CD103^+$ DCs sorted from mLNs have reduced ability to induce $FoxP3^+$ regulatory T cells but they polarize T cells toward a Th1 phenotype (Laffont et al., 2010).

$CX3CR1^+$ cells are now defined as macrophages, which derive from $Ly6C^{high}$ blood monocytes (Varol et al., 2009) and are dependent on macrophage colony stimulating factor (M-CSF) for their development (Schulz et al., 2009). This population has been identified as the one capable to sample the intestinal lumen extending protrusion through the epithelial cells (Niess et al., 2005) without disrupting the epithelial integrity (Rescigno et al., 2001). Moreover, TLR ligands and microbes such as *Salmonella* have been shown to increase the luminal sampling of $CX3CR1^+$ cells (Chieppa et al., 2006), which has been demonstrated to be dependent on MyD88 signaling pathway (Arques et al., 2009). Recently, it has been found that LP $CX3CR1^+$ cells can acquire antigens also from the blood stream that are then cross-presented to $CD8^+$ T cells which then express IL-10, IL-13 and IL-9 and could migrate into the intraepithelial compartment (Chang et al., 2013).

Although they are loaded efficiently with luminal antigens, it has been shown that $CX3CR1^+$ cells cannot migrate from LP to mLNs indeed they do not up-regulate CCR7 even in presence of LPS (Schulz et al., 2009). Moreover, they poorly prime naive T cells, but preferentially support Th1/Th17 cell differentiation (Niess and Adler, 2010), which is favoured by commensal-derived factors, such as ATP (Atarashi et al., 2008). However, CX3CR1 has been shown to be import to oral tolerance establishment. For instance, CX3CR1-deficient mice showed impaired local T_{reg} cell expansion and abrogated establishment of oral tolerance due to a reduced production of IL-10 by CX3CR1-expressing macrophages (Hadis et al., 2011).

In inflammatory conditions such as during a DSS-induced colitis model, DC-depleted mice reconstituted with $Ly6C^{high}$ monocyte with a resulting imbalance of the $CD11b^+$ $CX3CR1^+$ versus the $CD11b^-$ $CD103^{high}$ LP subsets, show severe signs of colitis indicating that

Ly6C^{hi} monocyte-derived CD11b^{+} CX3CR1^{+} have a pro-inflammatory phenotype (Varol et al., 2009).

Recent studies on intestinal dendritic cells and macrophages have added further insight on the populations present in the LP and their functions. Indeed, *Cerovic et al*. have found that not all the CX3CR1^{+} cells can be classified as macrophages. Indeed, a migratory population of CD103^{-} CX3CR1^{int} has been found in the lymph which has characteristics comparable to the ones of the "classical" CD103^{+} DC subset as they respond to Flt3 and present efficiently the antigen to CD4 and CD8 T cells, although they induce Th1 and Th17 cell polarization (Cerovic et al., 2013). *Diehl at al.* have investigated the possibility that also the CX3CR1^{high} population is able to migrate into the mLNs in a CCR7-dependent manner upon antibiotic-induced dysbiosis (Diehl et al., 2013).

On the other hand, using 2-photon microscopy, CD103^{+} cells have been found localized in the intestinal epithelium and they were found able to extend dendrites toward the lumen to take up bacteria but not soluble antigens (Farache et al., 2013).

1.3 Oral tolerance vs Systemic ignorance

The intestine is exposed continuously to a vast amount of foreign antigens that it has to discriminate. Indeed, it must distinguish between pathogens and harmless antigens, such as food proteins and commensal bacteria. For the first ones strong immune responses are required. By contrast, active immunity against harmless antigens would be detrimental, for instance hypersensitivity responses against dietary antigens or the microbiota can lead to inflammatory disorders such as coeliac disease and Crohn's disease, respectively (Mowat, 2003). Therefore, the usual response to gut antigens is the induction of what is called oral tolerance. However, an important difference exists between the oral tolerance to gut bacteria and to food antigens: whereas the tolerance to food proteins affects both local and systemic immune responses, tolerance to gut microbes is only local while the

systemic immune system is left ignorant to these bacteria (Macpherson et al., 2000; Macpherson and Smith, 2006; Pabst and Mowat, 2012). Therefore in the next paragraphs, I will use the term oral tolerance to indicate the tolerance to food antigens and systemic ignorance referring to the (local) tolerance to the microbiota.

1.3.1 Antigen uptake

Physical barriers, described in the previous paragraphs, prevent the access of harmful bacteria into the underlying tissue. However, in the gut there are different ways in which antigens can reach and be taken up by LP antigen presenting cells (Fig. 1.4). One entry port for antigens is formed by M cells, which are present within the epithelium overlaying the Peyer's patches and lymphoid follicles. These cells actively transport molecules from the lumen into the underlying dome region (Pabst and Mowat, 2012). It is not yet clear whether the M-cell mediated antigen uptake is important for oral tolerance induction. Indeed, some reports shows that the lack of PP, by administration of lymphotoxin-β receptor antibody during gestation, reduced oral tolerance (Fujihashi et al., 2001), while others demonstrate that normal oral tolerance can be inducted in the absence of PPs (Spahn et al., 2001; Spahn et al., 2002). Moreover, M cells have been identified also in the intestinal villous epithelium, which are, similarly to PPs-M cells, capable to transport antigens and to induce antigen-specific immune responses in a PP-independent manner (Jang et al., 2004).

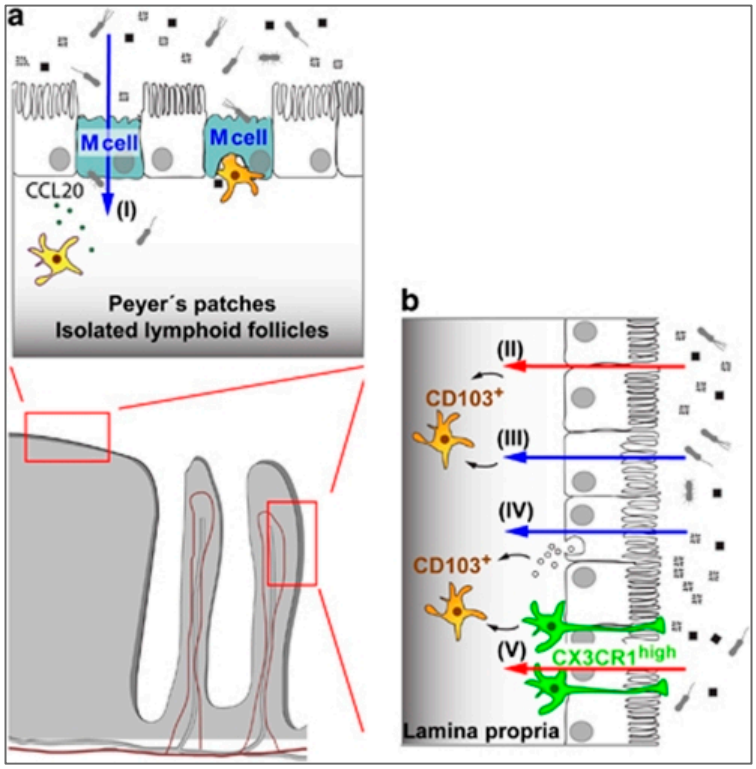

Figure 1.4: Mechanisms of antigen uptake in the gut (Pabst and Mowat, 2012). (I) Transcellular transport of particulate antigens via M cells; (II) Paracellular passage of soluble antigens; (III) Transcellular transport of antigens across epithelial cells; (IV) Exosome-dependent transport of antigens via MHC-II^{+} enterocytes; (V) CX3CR1^{high} macrophages sample luminal antigens extending dendrites across the epithelial layer. The antigens that reach the LP are taken up by the migratory CD103^{+} DCs.

Other routes by which antigens can reach LP have also been described. For instance, it has been reported that antigens can be transported by neonatal Fc receptor for IgG from the gut lumen into the intestinal lamina propria (Kelsall and Rescigno, 2004). Antigens with low molecular weight can diffuse into the LP though the tight junctions between epithelial cells while high molecular weight molecules can be transported through the epithelium by transcytosis. Luminal material can be transported into the intestinal tissue also within exosomes derived from major histocompatibility complex class II (MHC-II) expressing epithelial cells (Pabst and Mowat, 2012). Furthermore, it has been demonstrated that CX3CR1-expressing mononuclear phagocytes can directly sample antigens from the lumen extending dendrites crossing tight junctions between epithelial

cells without compromising the integrity of the epithelial barrier (Rescigno et al., 2001) (Niess et al., 2005). This process of antigen uptake has been shown to be mediated by MyD88-dependent signaling evoked by the interaction of epithelial TLRs with microbial products (Chieppa et al., 2006). Recently, it has been demonstrated that also the $CD103^+$ DCs, patrolling the epithelial layer, can extend dendrites toward the lumen and sample particulate antigens (Farache et al., 2013). Additionally, at steady state goblet cells deliver soluble antigens with low molecular weight from the intestinal lumen to underlying $CD103^+$ cells (McDole et al., 2012).

1.3.2 Oral tolerance establishment

It is known from many years that animals fed with a protein have reduced subsequent response to a systemic challenge with the same antigen (Macpherson and Smith, 2006). These classical experiments of delayed type hypersensitivity (DTH) responses are used to evaluate the establishment of oral tolerance to food proteins.

Although the principal characteristic of oral tolerance is the systemic T cell hyporesponsiveness, it is well known that the main site of oral tolerance induction is the mLN. Indeed, it has been shown that mice lacking mLNs but not the one lacking PPs, cannot induce systemic tolerance to food antigens (Spahn et al., 2002; Worbs et al., 2006). However, different observations suggested that oral tolerance could be induced outside the GALT. For instance, the intraperitoneal injection of serum from tolerized mice induces tolerance in the recipient animal (Macpherson and Smith, 2006). Furthermore, antigen delivery via the portal vein induces a response similarly to oral delivered antigens (Goubier et al., 2008). The induction of oral tolerance is possible outside the GALT because orally administered antigens can disseminate systemically and reach the liver via the portal circulation (Li et al., 2004; Pabst and Mowat, 2012).

Different specialized antigen presenting cells are involved in oral tolerance induction such as liver sinusoidal endothelial cells, Kupffer cells and plasmacytoid DCs favor oral

tolerance induction (Thomson and Knolle, 2010). The importance of liver as “extra-intestinal” place where oral tolerance is established is demonstrated also by the finding that portacaval shunting prevents the establishment of oral tolerance (Callery et al., 1989).

1.3.3 Systemic immune ignorance

Contrary to food proteins, intestinal microbiota is highly immunogenic indeed very low numbers of these bacteria given intravenously induce strong immune responses (Macpherson et al., 2000), indicating that in the case of commensal bacteria tolerance is established locally in the GALT while the systemic immune system remains ignorant. This setup is of primary importance because it avoids the tolerization of the systemic immune system, which is then able to respond efficiently to systemic sepsis from a commensal or a related bacterium (Macpherson and Smith, 2006).

This compartmentalization is preserved by mLNs that work as a system of containment, or “firewall” (Hooper and Macpherson, 2010). Indeed, bacteria that have penetrated epithelial barrier or that have been sampled by intestinal DCs to induce mucosal immunity are carried to the mLNs where they remain confined since bacteria loaded-DCs cannot reach the thoracic duct and, hence, the systemic circulation (Fig. 1.5) (Macpherson and Smith, 2006). The fundamental role of mLNs as firewall is demonstrated by the fact that after oral administration of *Enterobacter cloacae* (an aerobic component of the microbiota of SPF mice in some colonies), bacteria were recovered only from mLNs and not from spleen and liver that remain sterile (Macpherson and Uhr, 2004). Moreover, C57/BL6 mice in specific pathogen-free (SPF) conditions showed no serum IgG or IgA specific for *E. cloacae* but only specific secretory IgA in the intestinal washings (Macpherson et al., 2000). By contrast, removing mLNs before infecting mice via oral gavage resulted also in spleen colonization (Macpherson and Uhr, 2004) demonstrating that adaptive systemic ignorance is lost.

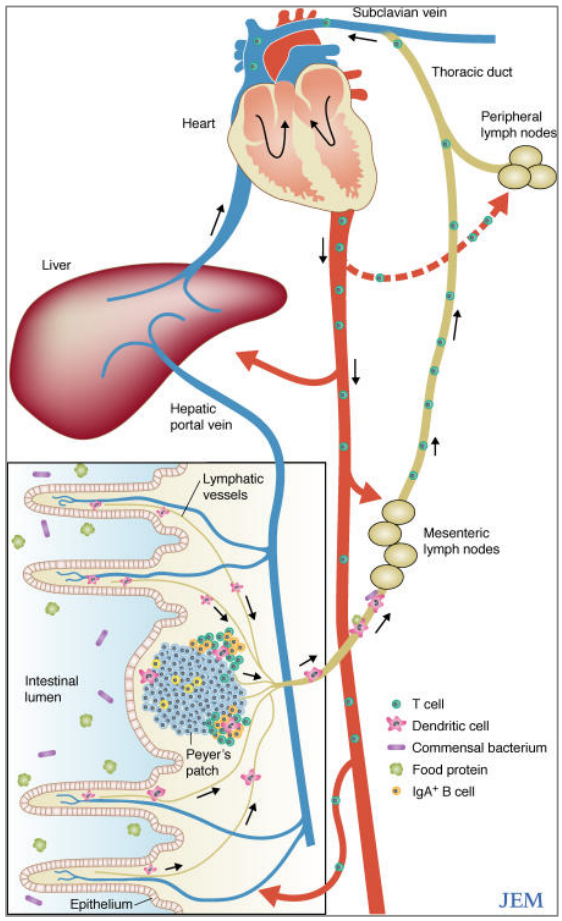

Figure 1.5: Anatomy of immune responses to antigens of intestinal origin (Macpherson and Smith, 2006). Food proteins can induce both local and systemic tolerance, reaching the mLNs via the lymph or the liver via the hepatic portal vein. On the contrary, commensal bacteria carried by DCs to the mLNs induce only local immune tolerance while the systemic immune system remains unprimed. This is possible since the mLNs act as "firewall" that block further penetration of bacteria in the thoracic duct.

Voedisch *et al.* have also demonstrated that mLNs block the access to systemic tissue to *Salmonella* functioning as a barrier preventing lethal systemic infection (Voedisch et al., 2009).

Together with the fundamental role of mLNs in preventing bacteria systemic dissemination, we envisage also the presence of a barrier at the endothelial cell level that blocks the penetration of bacteria into the blood stream via the blood capillaries located just beneath the epithelium (see Results section).

1.4 Blood Brain Barrier

In 1904 Paul Ehrlich discovered that dyes injected into the vascular system were taken up by all organs except the brain and the spinal cord (Engelhardt, 2003). However, the term "blood brain barrier" (BBB) was coined later by Lewandowsky that demonstrated that neurotoxic agents were effective only when directly injected into the brain but not when injected into the vascular system (Engelhardt, 2003). Thanks to the use of electron-microscopy, we have now deeper knowledge on which cell types form the BBB and how they modulate its development and maintenance.

1.4.1 Structure and function of BBB

The BBB is a selective barrier critical for the protection of CNS from toxins and from fluctuations in blood composition and, on the other hand, it is fundamental for the delivery of nutrients to the brain (Liebner et al., 2011).

The BBB is formed by endothelial cells (ECs) that, with their structural properties, tightly restrict the trafficking of ions, molecules and cells between the blood and the brain. These properties include: specialized TJs and AJs between adjacent ECs that limit the paracellular movement of ions and molecules; the expression of transporters required for the movement of nutrients, like water-soluble amino acids and glucose, into the brain and for transporting out potentially toxic substances; lower rates of transcytosis compared to peripheral endothelium and the lack of fenestrations. Moreover, ECs limit extravasation of immune cells from the blood into the brain reducing the expression of leukocyte adhesion molecules (LAMs) (Abbott et al., 2006; Siegenthaler et al., 2013).

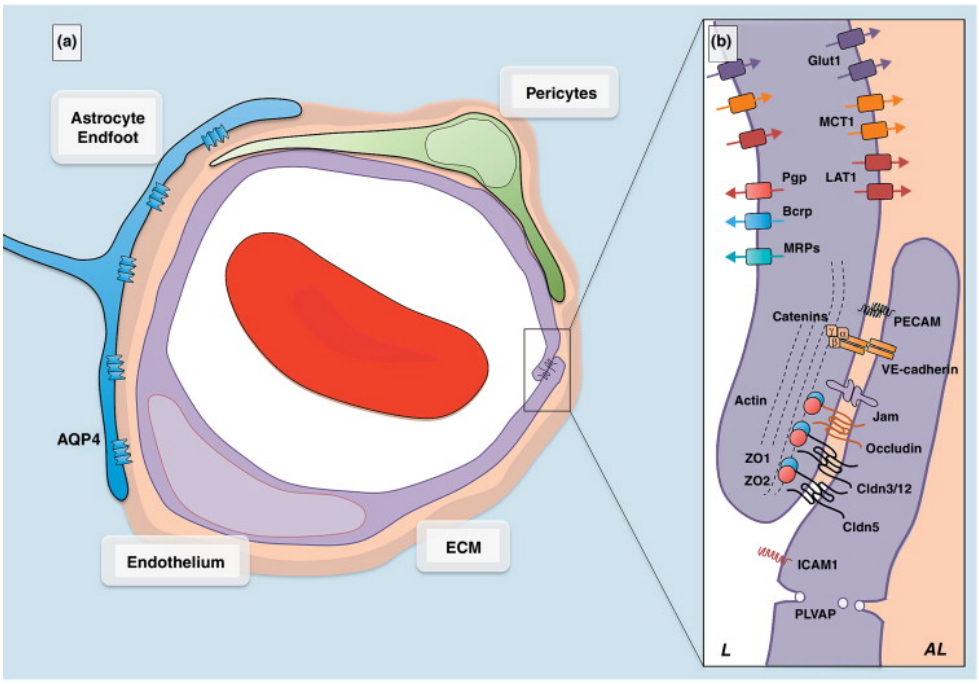

Figure 1.6: Schematic representation of the BBB (Siegenthaler et al., 2013). (a) Cellular components of the BBB. Capillaries (purple) in the CNS are covered by a pericyte (green), which are embedded in the vascular extracellular matrix (orange). Astrocytes (blue), extending cellular processes, contact the blood vessels. Together these cells form the Neuro-Vascular unit. (b) Barrier components of the brain ECs. TJs and AJs create a tight paracellular barrier and polarize the cells creating distinct luminal and abluminal membrane compartments, each of which is characterized by the expression of different transporters. The ECs are also characterized by low rates of transcytosis, mediated by low levels of Plvap protein. In addition ECs express low levels of leukocyte adhesion molecules, including ICAM1.

The junctional complexes between endothelial cells include AJs and TJs, which are both important for the maintenance of cell-cell adhesion and for the activation of signaling pathways that control many EC functions (Paolinelli et al., 2011).

In AJs, which are important for initiating cell-to-cell contacts, transmembrane cadherin proteins, in particular the endothelial specific Vascular Endothelial (VE)-cadherin, span the intercellular cleft and are linked through the cytoplasmic domain to the scaffolding proteins p120-catenin and β-catenin or plakoglobin, which, in turn, through the binding to α-catenin, promote the anchorage to the actin cytoskeleton (Dejana, 1996; Giannotta et al., 2013). Although the extracellular domain of VE-cadherin is necessary for clustering, the

intracellular association to the actin cytoskeleton is necessary to provide strength and cohesion to the junction (Navarro et al., 1995). Studies carried out using ECs with a null mutation of VE-cadherin (Vittet et al., 1997) or using an anti-VE-cadherin blocking monoclonal antibody (Corada et al., 1999) have highlighted the importance of VE-cadherin for the maintenance of the vascular integrity.

TJs are particularly abundant and complex in the BBB where there is the need to strictly control paracellular permeability to polar solutes. They are formed by transmembrane proteins such as occludin (Furuse et al., 1993), claudins (Furuse et al., 1998), and junctional adhesion molecules (JAM) (Martin-Padura et al., 1998) linked to a number of cytoplasmic scaffolding and regulatory proteins such as Zonula occludens (ZO)-1, ZO-2, ZO-3 and cingulin (Abbott et al., 2010). These junctions in the brain capillaries significantly block the paracellular transport also of small ions such as Na+ and Cl-, so that the transendothelial electrical resistance (TEER), which is typically 2–20 ohm x cm^2 in peripheral capillaries, can be more than 1,000 ohm x cm^2 in brain endothelium (Abbott et al., 2006).

Occludin was the first integral membrane protein to be found associated to TJ (Furuse et al., 1993). This protein was shown to be dispensable for TJ formation since occludin-deficient mice are viable and develop normal TJs in most tissues (Saitou et al., 2000). By contrast, proteins belonging to claudin protein family were found to be necessary for the formation of TJ strands (Morita et al., 1999). The claudin gene family includes more than 20 isoforms with high homology with occludin and are differently expressed among different tissues (Engelhardt, 2003). Peculiar of the brain endothelium are claudin (Cldn)-5, Cldn-12 (Nitta et al., 2003) and Cldn-3 (Liebner et al., 2008) that are believed to be responsible for the establishment of endothelial barrier function. Indeed, it has been demonstrated that in experimental allergic encephalomyelitis and in glioblastoma multiforme the selective loss of Cldn-3, together with the loss of Cldn-1 and Cldn-5, is associated with a loss of BBB integrity (Wolburg et al., 2003) (Liebner et al., 2000). Moreover, it has been shown that the increased Cldn-3 expression by Wnt/β-catenin

pathway activation in ECs is associated to an enhanced barrier formation (Liebner et al., 2008). Furthermore, claudin-5 deficient mice have a severely compromised and leaky BBB and die shortly after birth (Nitta et al., 2003). Therefore it appears that disappearance of either Cldn-3 or Cldn-5 from the tight junctional complexes can result in a compromised BBB.

Together with occludin and claudins also JAM-A, B and C are expressed by brain endothelial cells and are involved in the formation and the maintenance of the tight junctions and of BBB integrity (Wyss et al., 2012; Yeung et al., 2008).

The effectiveness of the TJs is regulated via the intracellular scaffold proteins ZO-1, ZO-2 and ZO-3 which link the junctional molecules claudin and occludin via cingulin to the cytoskeleton and to several cytoplasmic signaling molecules that are involved in controlling the assembly and disassembly of TJ (Abbott et al., 2006). For instance, it has been shown that ZO-1 is a substrate of protein kinase C, which is crucial for the formation and regulation of TJs (Stuart and Nigam, 1995).

In brain capillaries TJs, together with AJs, beyond the “gate” function, are also required for the maintenance of cell polarity, i.e. a correct differential distribution of enzymes and carriers between luminal and abluminal compartments (Paolinelli et al., 2011). The brain endothelial transporters that supply the brain with nutrients include the Glut-1 glucose carrier, Lat-1 amino acid carrier and transporters for nucleosides. Other transporters include the luminal P-glycoprotein (P-gp) and multidrug resistance-related proteins (MRP) that require ATP or the Na+ gradient created by the abluminal Na+,K+-ATPase to move compounds against a concentration gradient (Abbott et al., 2006).

However, brain ECs in culture do not have all these features characteristic of the BBB-phenotype indicating that these properties are induced by interactions with other cell types, namely pericytes, astrocytes and microglia, that together are termed as neurovascular unit (NVU) (Liebner et al., 2011).

1.4.2 Barriergenesis induction and regulation

Stewart and Wiley with transplantation experiments have demonstrated for the first time that vessels derived from the coelomic cavity of chick embryos transplanted in the embryonic quail brain acquired BBB characteristic while brain vessels implanted in mesodermal tissue lack barrier characteristics (Stewart and Wiley, 1981). These observations are the direct evidence that the ECs are not “committed” to have barrier characteristics but these are induced by the neural tissue during embryogenesis.

The earliest BBB marker to be expressed is the glucose transporter Glut-1 (at rodent embryonic day 12) (Daneman et al., 2010). It is initially expressed on both luminal and abluminal sides of the ECs while later in development, due to an increased demand for glucose, it has a higher localization at the abluminal membrane (Engelhardt, 2003). Although some TJ proteins such as cldn-5, occludin and ZO-1 are also present in blood vessels at the same embryonic stage (Daneman et al., 2010) the barrier functionality at this stage is incomplete since BBB becomes impermeable to horseradish peroxidase only at E13 in chick and at E15 in rats (Siegenthaler et al., 2013). At these early stages of development the BBB leakage could be attributed to the fact that brain blood vessels have still leaky properties such as high levels of trancytosis – identified by the expression of plasmalemma vesicle associated protein (Plvap) – and the expression of LAMs, in particular of ICAM-1 that promote leukocyte infiltration (Daneman et al., 2010). During the late gestation and the postnatal stages (in rodents) there is a gradual maturation of the BBB with an increased density and complexity of TJs (Kniesel et al., 1996) and the loss of the expression of ICAM-1 and Plvap (Daneman et al., 2010).

The cell types that together form the NVU have been shown to play an important role during all these phases of the BBB development.

One of the main players are the pericytes, a cell population that has been found at high density in the brain and closely associated to the nascent vessels at the early stages of development (Daneman et al., 2010). *In vitro* BBB model obtained co-culturing mouse

brain ECs with primary rat brain pericytes have suggested that pericytes are able to regulate BBB properties (Dohgu et al., 2005). Recently it has been demonstrated that brain pericytes are required for the stabilization of newly formed vessels and for development and maintenance of the BBB. Indeed, mice that lack the platelet derived growth factor B (PDGFBB) signaling that is required for pericytes recruitment in the brain show ECs hyperplasia, increased vessel diameter and permeability (Daneman et al., 2010). Moreover, the lack of pericytes coverage during brain development have been associated to an increased expression of “leaky” vascular barrier, such as Plvap and LAMs while “tight” barrier features, i.e. TJ proteins and transporters, remained unchanged (Armulik et al., 2010; Daneman et al., 2010). These *in vivo* studies also suggested that pericytes are necessary for the establishment of astrocytes-vessel contact, which are important for the maintenance of BBB properties during the postnatal period (Armulik et al., 2010).

Indeed, in the postnatal period (that corresponds to the second half of gestation in humans) astrocytes contact the brain endothelium via cellular processes or end-feet and start to influence the endothelial barrier properties (Daneman et al., 2010). One features of the astrocytes is the polarity of the cellular membrane at the end-feet structure. Indeed, some proteins, such as the water channel Aquaporin-4 (Aqp4), are expressed at the abluminal surface of the astrocyte processes. However the connection between the expression of Aqp4 and the maintenance of the BBB is not yet known (Liebner et al., 2011).

In vitro studies, using astrocyte-conditioned medium or the direct contact between astrocytes and ECs (Dehouck et al., 1990; Rubin et al., 1991), together with *in vivo* experiments in which astrocytes were transplanted into non-neural tissue (Janzer and Raff, 1987) have demonstrated their role in barrier maturation.

Recent studies have described different ways by which astrocytes are able to promote BBB integrity. One of these is the activation of the Hedgehog (Hh) pathway. Indeed Alvarez *et al*. have demonstrated that both human and mouse astrocytes secrete Sonic

Hh (Shh) and that brain ECs express high levels of the receptor Patched-1 and of the downstream protein Smoothened suggesting that this pathway is used by these two cellular populations to communicate (Alvarez et al., 2011). The inactivation of Hh pathway in ECs leads to a decrease in the expression of TJ proteins, to higher vascular permeability and to increased leukocyte extravasation, all markers of BBB disruption (Alvarez et al., 2011).

Additionally, the binding of the astrocyte-derived retinoic acid to its receptor RARβ on human ECs has been implicated in inducing barrier properties. Indeed, RA increases the expression of VE-cadherin, ZO-1 together with Glut-1 and P-gp transporters (Mizee et al., 2013).

Although all this evidence shows the important role of astrocytes and pericytes in enhancing BBB development, embryonic neural progenitors have been suggested to play an important role in inducing BBB properties in the initial phase of development. Indeed, even in the absence of pericytes, in an embryonic phase in which astrocytes are not yet generated, brain ECs express different BBB-associated proteins (Daneman et al., 2010).

The central role for canonical Wnt ligands as neural progenitor signal for BBB development has emerged from different animal models that target the Wnt ligands or directly the β-catenin (Daneman et al., 2009; Liebner et al., 2008; Stenman et al., 2008).

Wnt ligands are a family of 19 secreted glycoproteins able bind to Frizzled (Fzd) and low-density lipoprotein receptor-related protein (LRP) receptors. In the canonical Wnt signaling, receptor ligation leads to the inactivation of a destruction complex composed by glycogen syntase kinase-3β (GSK-3β), the scaffolding protein axin and adenomatosis polyposis coli (APC) avoiding the phosphorylation of β-catenin and therefore its proteosome-mediated degradation. If β-catenin is not phosphorylated, it translocates to the nucleus where it regulates the transcription of target genes by binding to T-cell factor/ lymphoid enhancing factor (Tcf/LEF) transcription factors (Paolinelli et al., 2011). Moreover, Wnt/β-catenin signaling pathway activation can be regulated also by secreted

antagonists, such as Dickkopf homologs (Dkk1–4) and secreted frizzled-related protein families (sFRPs, 1–5) (MacDonald et al., 2009) (Fig. 1.7).

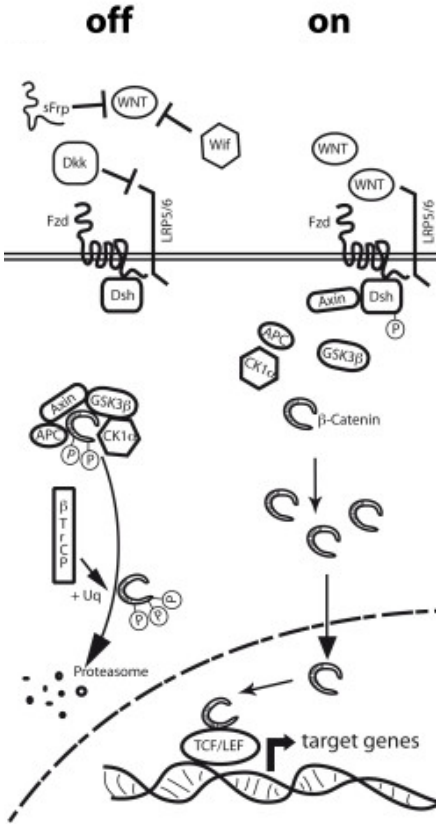

Figure 1.7. Scheme of Canonical Wnt/β-catenin signaling pathway (Liebner and Plate, 2010). When no Wnt ligands are present or are inhibited by WIF, sFRPs and Dkk. cytosolic β-catenin is targeted to proteolytic degradation through phosphorylation by the APC-Axin-GSK3β destruction complex. In the "on-state" stimulation of Fzd receptors and their co-receptors Lrp5/6 by Wnt ligands, leads the accumulation of β-catenin in the cytoplasm, which is then able to enter into the nucleus activating target gene transcription through association with Tcf/LEF transcription factors.

Using β-catenin-activated expression of nuclear β-galactosidase reporter mice, it was demonstrated that Wnt/β-catenin signaling pathway is activated in the vessels penetrating into the embryonic brain till E17.5 when its activation decreases (Liebner et al., 2008). The Wnt signaling follows the same kinetics of brain angiogenesis and BBB development supporting the idea that Wnt ligands are involved in brain vascularization

and induction of the BBB properties. Indeed, Liebner, *et al*. have found that the Wnt/β-catenin pathway activation by Wnt3a induces the up-regulation of Cldn-3 and correspondingly a decrease of the Plvap "leaky" protein (Liebner et al., 2008). Moreover, other studies have demonstrated that also Wnt7a-mediated activation of Wnt/β-catenin pathway induces the up-regulation of BBB markers including Glut-1 and other transporters such as slc7a1 and slc7a5 (Daneman et al., 2009; Stenman et al., 2008).

The Wnt signaling pathway is responsible not only for barrier maturation during embryogenesis but also for the BBB maintenance in the adult. Indeed, the inactivation of the pathway in postnatal mouse brain ECs leads to the down-regulation of the cldn3 and the up-regulation of Plvap (Liebner et al., 2008).

ECs lose their barrier properties also under pathological conditions such as ischemia, brain tumors or Alzheimer's disease. In these conditions BBB microvessels up-regulate Plvap (Carson-Walter et al., 2005; Sparks et al., 2000) that is therefore associated to immature or disrupted BBB.

Plvap protein has been localized to caveolae and trans-endothelial channels of fenestrated capillaries (Herrnberger et al., 2012b; Stan et al., 2012). Studies using Plvap-deficient mice have demonstrated the importance of Plvap also in the regulation of the permeability of fenestrated endothelial (not the brain since it has a continuous ECs layer). Indeed, mixed background Plvap -/- mice show loss of plasma proteins, tissue edema and dyslipidemia that finally results in multiple organ disfunction (Herrnberger et al., 2012b; Stan et al., 2012).

1.5 *Salmonella typhimurium* infection

Salmonella enterica serovars are Gram-negative facultative intracellular bacteria that through food and water can cause local gastroenteritis or systemic disease called typhoid fever, depending on the serovar. Indeed, in humans infections by *Salmonella enterica*

serovar Typhi (hereafter referred to as *S. typhi*) causes typhoid fever while *Salmonella enterica* serovar Typhimurium (referred to as *S. typhimurium*) induces only locally restricted infection. By contrast, mice are susceptible to oral infection with *S. typhimurium,* but not *S.typhi*, resembling the human systemic disease (Voedisch et al., 2009). Therefore *S. typhimurium* oral infection in mice is widely used as model of human systemic infection. The resistance of mice to *S.typhi* was firstly associated to an inhibitory effect of the host intestinal microbiota on the growth of the inoculated *Salmonella*. However, germfree mice were equally resistant to *S.typhi* systemic infection indicating that the microbiota does not influence the ability of different *Salmonella* serovars to spread systemically (Collins and Carter, 1978). Interestingly, Collins *at al.* have also demonstrated that the ability of *S.typhi* to reach distal organs such as spleen and lung is not obtained even in the absence of phagocytic cells that are necessary for bacterial clearance (Collins and Carter, 1978) indicating that other mechanisms are involved in the discrimination between local and systemic disease caused by different *Salmonella* strains.

1.5.1 Mechanisms of invasion

The preferential site of entry for *S. typhimurium* are the M cells present in the PPs (Jones et al., 1994; Pascopella et al., 1995). M cells continuously sample the gut lumen and transport particulate antigens including live bacteria into the LP where they are taken up by immune cells (Pabst and Mowat, 2012). One way by which *Salmonella* is transported in the PPs is through the caveolae (Lim et al., 2010).

Caveolae are formed by lipid rafts rich in sphingolipids and cholesterol that oligomerize in the presence of caveolin-1 and caveolin-2 to form 60-80 nm diameter pits (Parton and Simons, 2007). They have been implicated in endocytosis and transcytosis, cholesterol trafficking, signaling and they can be used as entry portals by different pathogens such as *E coli* (Sukumaran et al., 2002) and *Salmonella* (Hoeke et al., 2013; Lim et al., 2010). In particular, it has been shown that *Salmonella* can down-regulate caveolin-2 expression by

increasing the expression of miR-29a both *in vivo* and *in vitro* (Hoeke et al., 2013). Experiments using epithelial cell lines have suggested that the reduced expression of caveolin-2 causes defects in epithelial cell renewal, which can favour pathogen invasion through the damaged epithelium (Hoeke et al., 2013). By contrast, Lim *et al*. have shown that *Salmonella* induces the up-regulation of caveolin-1 in PPs isolated from old mice favouring its trancytosis (Lim et al., 2010).

Together with this passive transport, *Salmonella* is also able to induce rearrangements of the M cells cytoskeleton resulting in loss of integrity of the PP epithelium that allows rapid spreading to the organs before an immune response can be initiated (Jones et al., 1994; Pascopella et al., 1995). Moreover, it has been shown that *Salmonella* could transverse the gut epithelium, a process dependent on type III secretion system (TTSS)-1 for epithelial cell invasion and on TTSS-2 for trafficking to the basolateral side (Muller et al., 2012).

In addition to the passage through the M cells, *Salmonella* can invade the intestinal LP via an active sampling mechanism mediated by mononuclear phagocytes expressing CX3CR1 that have been described in the section 'Mucosal dendritic cells'.

Salmonella, like other intracellular bacterial pathogens have the ability to multiply inside vacuoles in the infected host cells. These vacuoles first acquire markers of the early endosomes, such as the early endosomal antigen 1 (EEA1), which are then replaced by markers of the late-endosomal system including Rab7, the lysosomal glycoproteins LAMP-1, LAMP-2 and LAMP-3/LIMP-1 and the vacuolar ATPase responsible for phagosome acidification. However these vacuoles never acquire the mannose-6-phosphate receptor (M6PR), which delivers lysosomal hydrolases to the endosomal system. Therefore *S. typhimurium* actively manipulates host cell factors to create an intracellular compartment, distinct from a classical phagosome permissive for bacterial growth (Bakowski et al., 2008).

Two distinct TTSS encoded on *Salmonella* pathogenicity islands (SPIs) 1 and 2 are involved in invasion and survival in the host cells (Fig. 1.8).

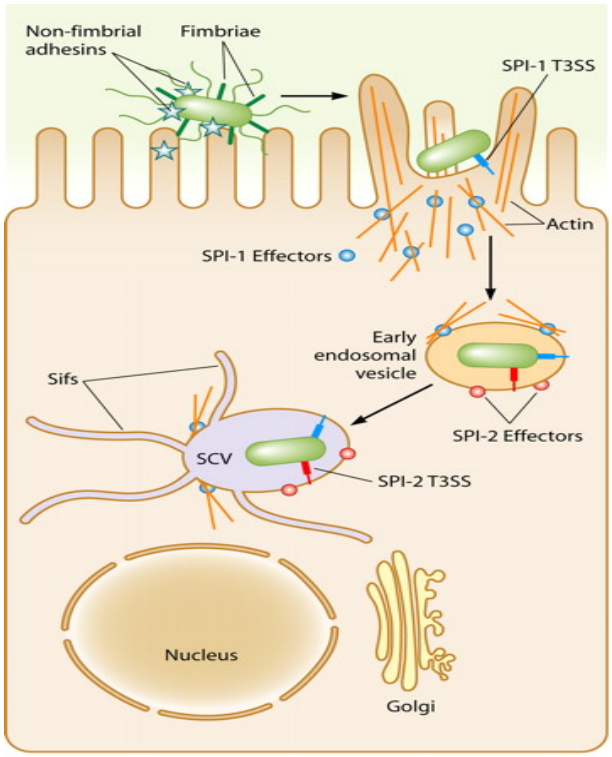

Figure 1.8. SPI-1 and SPI-2 TTSS in *Salmonella* infection. (modified from (Gilbreath et al., 2011)) After adhesion to the epithelium through fimbrial and nonfimbrial adhesins, *Salmonella* spp are translocated across the epithelial cell using SPI-1 T3SS effectors. SPI-2 effectors are then required for intracellular survival and for the maturation of the early endosome vesicles to the specialized vacuole, termed as *Salmonella*-containing vacuole (SCV). The SCV localizes near the Golgi apparatus that serves as an intracellular replicative niche.

TTSSs are needle-like protein complexes that translocate bacterial virulence proteins (effectors) from the bacterial cytoplasm directly into the host-cell cytoplasm. These translocated effectors are able to alter host-cell functions such as signal transduction, cytoskeletal architecture, membrane trafficking, and cytokine gene expression (Galan, 2001).

Genes of the SPI-1 TTSS are activated upon contact with epithelial cells and are required for translocating effectors across the host cell plasma membrane (Galan, 2001). Some of these effector proteins, such as SopB, SopE, SopE2 and SipA are able to disrupt the

epithelial cell barrier by altering the localization of TJ proteins such as ZO-1 and occludin (Boyle et al., 2006). Such disruption may lead also to the exposure of TLR localized on the basal membrane of the epithelial cells to bacterial ligands inducing indirectly the activation of inflammatory response (Galan, 2001).

TTSS-1 mutant strains administered by the oral route show an attenuated phenotype, demonstrating that SPI-1 encoded genes are necessary for the efficient entry of *Salmonella* into the host tissue (Galan and Curtiss, 1989). However TTSS-1 deficient strains are still able to reach the intestinal LP by a mechanism that is independent from the *Salmonella* intrinsic capability to invade the intestinal epithelium. The way by which also TTSS-1 mutant *Salmonella* penetrate into the intestine is through DCs, indeed depletion of DCs during the early phases of infection strongly reduces the bacterial spreading (Hapfelmeier et al., 2008). In line with this finding, it was shown that TTSS-1 is necessary for the colonization of PP, liver and spleen but not of mLNs, which indicates that the colonization of mLNs is mediated, by DCs sampling and migration to the draining lymph node (Martinoli et al., 2007; Voedisch et al., 2009).

SPI-1 TTSS effector proteins have also been implicated in the stimulation of polymorphonuclear leukocytes (PMN) recruitment (SipA protein) (Lee et al., 2000) and in the induction of fluid accumulation and diarrhea (SopB, SopD, and SopA) (Wood et al., 2000). However, TTSS-1 secreted protein SipB has been shown to be responsible for the induction of apoptosis in macrophages via caspase-1 activation (Hersh et al., 1999).

The SPI-2 encoded TTSS has been associated to the ability of *Salmonella* to survive in host cells and to spread systemically (Ochman et al., 1996). Indeed, *S. typhimurium* mutated strains in TTSS-2 genes cannot establish systemic infection upon intra-peritoneal injection (Shea et al., 1996). *In vitro* studies have shown that expression of SPI-2 is induced inside the macrophages in the acidic phagosomal environment, which induces the activation of SPI-2-encoded regulatory system SsrA/B (Cirillo et al., 1998; Deiwick et al., 1999). Interestingly, Cirillo *et al.* have found that in contrast to SPI-1 deficient strains that are not able to colonize PPs but are still able to reach mLNs, SPI-2 mutants invade

PPs but are not found in the mLNs, liver and spleen indicating that SPI-2 is required to avoid *Salmonella* clearance by macrophages (Cirillo et al., 1998). Recently, it has been demonstrated by 2-photon microscopy that TTSS-2 is also involved in the invasion of the epithelium. Indeed, mutants for TTSS-2 reach the LP through the epithelial layer less efficiently compared to the WT strain (Muller et al., 2012).

AIM OF THE STUDY

The intestine is continuously challenged by a vast amount of foreign antigens including a large community of commensal bacteria, collectively called the microbiota. Although the microbiota has established a symbiotic relationship with the host, it poses immense health challenges. For this reason, the intestine has adapted different strategies to limit opportunistic invasions by the resident microbiota avoiding pathologies such as bacteremia and chronic inflammation. In the past years, a lot of effort was made at understanding how the mucosal tolerance and the systemic ignorance is maintained, focusing in particular on the role of the epithelial cells and of the intestinal immune system.

In this thesis, we investigated whether the intestinal endothelial cells, beyond the physical barrier and the intestinal immune system, form another layer of protection avoiding indiscriminate trafficking of molecules and bacteria from the gut into the blood stream.

So we proceeded through these steps using as model the blood-brain barrier whose features have been extensively studied:

- Identification of the “intestinal vascular unit” (counterpart of the neurovascular unit found in the brain) and analysis of the intestinal endothelial cell phenotype, focusing on the composition of the TJs and AJs;
- Identification of markers to define the intestinal endothelial barrier integrity, starting from the analysis of the expression of Plvap, Cldn-3 and caveolin-1 since the up-regulation of Plvap and caveolin-1 together with the down-regulation of Cldn-3 was associated to damaged BBB. To induce endothelial damage, we infected mice with *Salmonella typhimurium*;
- Functional studies of the gut vascular barrier in which vascular permeability to molecules with different molecular mass was measured;

- Study of the mechanisms behind intestinal endothelial barrier modifications. We focused on Wnt/β-catenin signaling pathway since in the brain the activation of this signaling pathway leads to enhanced endothelial barrier properties. Moreover, we investigated if and how *Salmonella* could interfere with Wnt/β-catenin signaling pathway activation in endothelial cells to favour its systemic spreading.

MATERIALS AND METHODS

3.1 Mice

8-10 weeks old WT C57BL/6J mice were purchased from Harlan Laboratories.

For some experiments, β-catenin$^{lox(ex3)/lox(ex3)}$ mice (Harada et al., 1999) (from E. Dejana, FIRC Institute of Molecular Oncology, Italy) were used. In these mice the exon3 of the β-catenin gene (*Catnb*) that contains the serine/threonine residues phosphorylated by the GSK3β kinase, was flanked by two *LoxP* sequences. These mice were crossed with *Cdh5*(PAC)-CreERT2 mice (Monvoisin et al., 2006) (from E. Dejana), where upon tamoxifen treatment the Cre recombinase is expressed in VE-cadherin positive endothelial cells. To induce recombination *Cdh5*(PAC)-CreERT2/β-catenin$^{lox(ex3)/lox(ex3)}$ mice were fed with tamoxifen-enriched food (TAM 400, Harlan) for two weeks. Upon treatment, the β-catenin exon3 is excised resulting in a gain-of-function (GOF) for the β-catenin in an endothelial specific manner.

Cdh5(PAC)-CreERT2 /β-catenin$^{lox(ex3)/lox(ex3)}$ mice were screened for the presence of Cre enzyme by PCR using primers CreA 5'-CCA AAA TTT GCC TGC ATT ACC GGT CGA TGC-3' and CreB 5'-ATC CAG GTT ACG GAT ATA GT-3'. The cycling protocol used is the following:

Step	Temp. (°C)	Time (s)	
1	95	300	
2	95	60	for 40 cycles
3	58	30	
4	70	45	
5	72	600	

Table. 3.1: *Cdh5*(PAC)-CreERT2 /β-catenin$^{lox(ex3)/lox(ex3)}$ genotyping cycling protocol.

To ensure that the exon 3 was efficiently excised in the endothelial cells of the intestine, small pieces of intestinal tissue were lysed, genomic DNA was extracted and tested for

the presence of the exon 3-deleted β-catenin allele. The PCR amplification protocol is the following:

Step	Temp. (°C)	Time (s)	
1	95	300	
2	95	60	
3	65	30	for 40 cycles
4	72	120	
6	72	900	

Table. 3.2: Cycling protocol used to analyze cre-mediated recombination in mouse intestinal tissues.

Primers pair used to verify the cre-mediated recombination is: Del-Fw 5'-GCT GCG TGGG ACA ATG GCT AC-3' and Del-Rv 5'-TGA GCC CTA GTC ATT GCA TAC-3'. If the cre recombinase is present, the exon 3 is excised from the β-catenin gene in endothelial cells, and therefore two amplification bands are expected after genomic PCR: the lower one which is the deletion band and the upper one that corresponds to the wild-type allele present in non-ECs present in the preparation (Fig. 3.1).

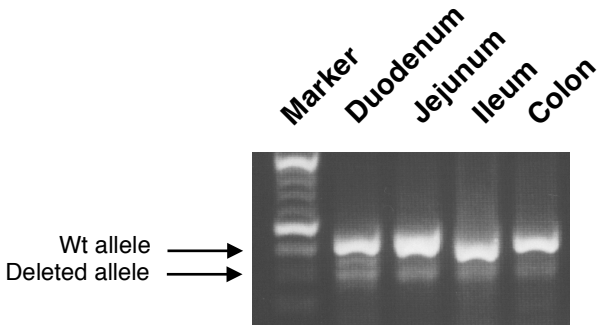

Figure 3.1: Cre mediated deletion of β-catenin exon 3 *in vivo.* Genomic PCR of duodenum, jejunum, ileum and colon from Cdh5(PAC)-CreERT2/β-catenin$^{lox(ex3)/lox(ex3)}$ mice. The lower band corresponds to the deleted allele and its weaker intensity is due to the high amount of non-ECs present in the lysate in which the recombination event does not occur.

For *in vitro* experiments, MyD88 KO mice, and TLR4 KO mice, obtained by Dr S Akira (Osaka University, Japan), were used.

Mice were bred and maintained at IFOM-IEO Campus animal facility under specific pathogen-free conditions. All experiments were performed in accordance with the guidelines established in the Principles of Laboratory Animal Care (directive 86 /609 /EEC).

3.2 Bacteria

S. typhimurium strains on SL1344 background were used for *in vivo* and *in vitro* experiments. For the *in vivo* experiments, an *aroA* mutant of *Salmonella*, auxotrophic for aromatic amino acids was used. It is characterized by an attenuated ability to replicate *in vivo*. For the *in vitro* experiments the strains used were: WT invasive strain (FB62); a *S. typhimurium* strain defective for the survival into the phagosome (BA83, SPI-2 KO); a noninvasive strain (BA34, SPI-1 KO) or into an endotoxin mutant (FB61, msbB). Moreover in some experiments WT DH5α *E. coli* strain or a modified strain expressing invasin from *Yersinia enterocolitica* was used. Bacterial strains were grown at 37° C in Luria broth supplemented with the appropriate antibiotics.

3.3 Lung endothelial cells isolation

For each isolation, three to four mice were used. Mouse lungs were removed from the thoracic cavity and placed in Hank's Balanced Salt Solution (HBSS) 1X. Lungs were cut into small pieces and then incubated with collagenase A (1,5 mg/mL; Roche Diagnostic) and DNase I (8 U/mL; Roche Diagnostic) for 75 min at 37°C under agitation. After the incubation, cell suspension was filtered through a 70-µm cell strainer and then through a 40-µm filter and cell suspension was centrifuged for 5 min at 1200 rpm. The red blood cells were lysed with a hypotonic lysis buffer, washed and the cell pellet was subjected to the CD45 positive selection using CD45 Microbeads kit (Miltenyi Biotec). CD31 positive

cells were then purified from the CD45 negative population using the CD31 MACS beads (Miltenyi Biotec). After selection, the $CD45^{-}CD31^{+}$ cells were washed with complete MCDB131 medium (GIBCO), then resuspended in complete MCDB131 containing 100 µg/mL heparin (Sigma-Aldrich) and 50 µg/mL EC growth supplement (homemade from calf brain) and plated into a gelatin-coated T-75 tissue culture flask.

3.4 *In vitro* infection

$7*10^5$ lung ECs were seeded in 6 well plates coated with gelatin. When they reached confluence (after 2 days), cells were infected with a ratio cell:bacteria 1:10 for 90 min. Cells were then washed and medium was replaced with complete MCDB131 containing 100 µg/mL gentamycin. After 150 min ECs were lysed for the subsequent RNA analysis.

3.5 Mice infection

C57BL/6J, β-catenin$^{lox(ex3)/lox(ex3)}$ or *Cdh5*(PAC)-CreERT2 /β-catenin$^{lox(ex3)/lox(ex3)}$ mice were infected with 10^9 *S.typhimurium* ΔaroA via oral gavage and after 2h, 6h, 24h and 48h blood was collected from heart and serum was tested for the presence of alanine aminotransferases (ALT, Sentinel Diagnostic) following manufacturer protocol. PPs, mLNs, spleen and liver were harvested and incubated 1h at 37°C with gentamycin to kill external bacteria. Organs were then digested with 1mg/ml collagenase D (Roche) for 30 minutes at 37°C and cells were plated on terrific broth agar plates together with sodium deoxycholate 1%, necessary to lyse the cells.

3.6 Immunofluorescence

Duodenum, jejunum and ileum of untreated or infected mice were fixed o/n in paraformaldehyde (PFA), L-Lysine pH 7.4 and NaIO4 (periodate-lysine-paraformaldehyde (PLP) fixative buffer). Then they were washed, dehydrated with 20% sucrose for at least 4 hours and included in OCT. 10 µm cryosections were rehydrated, blocked with 0.1M Tris-HCl pH 7.4, 2% fetal bovine serum (FBS), 0.3% Triton X-100 before staining them with Plvap, Cldn-3, Cldn-5, Cldn-12, ZO-1, JAM-A, Occludin, VE-cadherin, GFAP, CD31, CD34, cingulin antibodies. Primary antibodies were incubated o/n at 4°C. Slices were then incubated with the appropriate fluorofore-conjugated secondary antibody. Before imaging, nuclei were counterstained with 4',6-diamidin-2-fenilindolo (DAPI). Confocal microscopy was performed on a Leica TCS SP5 laser confocal scanner mounted on a Leica DMI 6000B inverted microscope equipped with motorized stage. Violet (405nm laser diode), blue (488nm argon laser), yellow (561nm laser diode) and red (633nm laser diode) laser lines that have been used for excitation. All images were acquired with a HCX PL APO 40X (NA 1.25) oil immersion objective. Software used for all acquisitions was Leica LAS AF and ImageJ or Imaris (Bitplane) for images analysis.

3.7 Permeability assay

C57/BL6, β-catenin$^{lox(ex3)/lox(ex3)}$ and VE-Cadherin-CreER^{T2}/β-catenin$^{lox(ex3)/lox(ex3)}$ mice were orally infected with 10^9 *S.typhimurium* ΔaroA. Immediately after infection or after 1h, 4h or 24h were anesthetized with 2, 2, 2-Tribromoethanol and intestinal loops were exteriorized and ligated in different parts of the small intestine. 2mg of FITC-Dextran 20 KDa (Sigma-Aldrich) were injected in the loop. After 1h blood samples were taken from the heart and the presence of the fluorofore was measured at fluorimeter ($\lambda_{Exitation}$: 485 nm, $\lambda_{Emission}$: 530nm).

3.8 RNA isolation and quantitation of gene expression by real-time PCR

Total RNA was purified from cells using RNeasy Kits (QIAGEN). cDNA synthesis was performed using SuperScript III reverse transcriptase (Invitrogen) and random hexamers. Real-time PCR reactions were carried out using the SYBR Green PCR kit on the Applied Biosystems 7500 Fast Real-Time PCR System. Expression levels for each sample were normalized to the expression levels of *Rpl32*. Results were quantified using the $2^{-\Delta\Delta Ct}$ method.

Gene	Forward (5'-3')	Reverse (5'-3')
Rlp32	AAGCGAAACTGGCGGAAAC	TAACCGATGTTGGGCATCAG
Axin2	TGACCGACGATTCCATGTC	GTTCCACAGGCGTCATCTC
Plvap	TACGCGACGTGAGATGGAG	GATGATAGCGGCGATGAAG

Table. 3.3: Primer Table

3.9 2-Photon intravital microscopy

Before starting the intravital experiments mice were starved o/n to limit the presence of feces into the intestine, which could interfere with the acquisition of the images. Mice were left untreated or infected for 4 hours with 10^9 *S.typhimurium* ΔaroA before anesthetizing them with 3-4% inhaled isoflurane. Animals were kept under anesthesia for all the experiment, for maximum 2h and then sacrificed. For surgery, a 1-cm long incision was made on the skin to expose the peritoneal wall. An additional small incision was made on the peritoneum to expose the abdominal cavity. A 3- to 4-cm loop from the ileum was externalized and the mucosal surface was exposed by making a 1-cm cut longitudinally through the gut wall (Fig. 3.2). To avoid the loss of blood from broken capillaries, a cauterizer was used to cut the intestine. The intestinal content was removed washing with PBS, without disrupting the mucosal layer, and the outer layer of the intestine was fixed to

a glass slide with surgical glue. The opened intestine was covered with a glass coverslip and the space between the two glass slides was filled with PBS. Once the surgery is completed, the animal was placed on a heating surface set at 37°C and 2mg 4KDa or 0.5mg 70KDa-FITC Dextran (Sigma-Aldrich) were injected into the tail vein. Intravital imaging was performed on a Leica TCS SP5 laser confocal scanner mounted on a Leica DM 6000CFS upright microscope, equipped with a Chameleon-XR (Coherent) Ti:Sapphire laser source directly coupled to the scanning head of the microscope using an infrared port. A Leica HCX APO L20X (NA 1.0) water immersion objective was employed for the analysis. For time-lapse image acquisition the stacks (with a z-step of 3 μm) over a depth of about 70 μm were acquired every 30 s and the two-photon excitation of FITC dye was performed at λ = 900 nm with a 512x512 pixel size and a pixel dwell time of 2.8 msec.

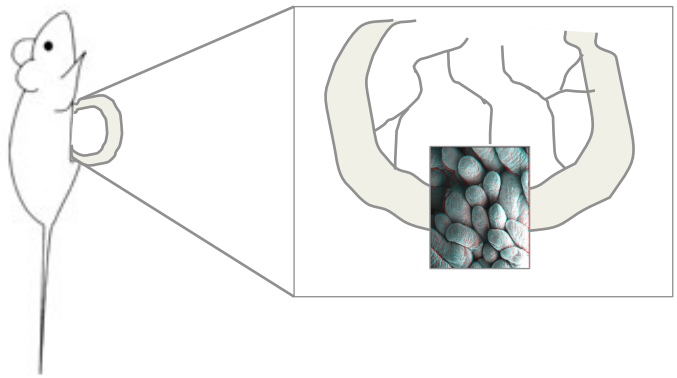

Figure 3.2: Surgery scheme. An incision was made on the skin and then on the peritoneum to expose the abdominal cavity. A 3- to 4-cm loop from the distal ileum was exposed. A small cut was done using a cauterizer along the intestinal wall to expose the mucosal layer.

3.10 Analysis of intravital imaging of vascular permeability

For image analysis at select time-points, an area of interest (5×5 μm) was chosen inside and outside, considering as outside the region immediately adjacent to the blood vessel. The fluorescence intensity was determined in the areas of interest for both the FITC-dextrans. For each time-point, the fluorescence intensity was measured for 10 areas

inside and 10 areas outside the blood vessels. To quantify the permeability of the intestinal blood vessels, the ratio between the outside and the inside fluorescence intensity and percent fluorescence was calculated by normalizing on the fluorescence ratio at the start of the acquisition.

3.11 Antibiotic treatment protocol

Animals were administered with ampicillin (1 g/L), vancomycin (500 mg/L), neomycin sulfate (1 g/L), and metronidazole (1 g/L; all antibiotics from MP Biomedicals) *ad libitum* in drinking water for four weeks (Rakoff-Nahoum et al., 2004) before infecting them with 10^9 *S.typhimurium* ΔaroA. Since it is known that mice generally refrain from drinking the antibiotic cocktail for the bad taste of metronidazole, we frequently check the mice to be sure of their welfare. Moreover the depletion of intestinal microbiota was confirmed plating the feces at the end of the treatment.

3.12 Statistics

Statistical differences were evaluated using GraphPad Prism software. Values were compared using either a student t-test for single variable, 2-way ANOVA for two variables or non-parametric 2-tailed Mann-Whitney tests depending on the distribution of the data. In all the cases, the statistical test used is indicated in each figure legend.

Results were represented as Mean ± SEM. *$p<0.05$, **$p<0.01$, ***$p<0.001$.

RESULTS

4.1 "Gut Vascular Unit" identification and characterization

4.1.1 Cellular composition of GVU and intestinal endothelial cell phenotype characterization

The blood brain barrier is a specialized structure formed by the brain microvascular endothelium that strictly controls the exchanges between blood and brain parenchyma protecting the central nervous system from the constantly changing milieu of the blood stream (Paolinelli et al., 2011). Also in the intestine there is the necessity to confine the foreign potentially dangerous material present in the gut lumen outside from blood stream to avoid systemic dissemination. It has been demonstrated that commensal bacteria that reach the lymphatics do not penetrate into the thoracic duct and therefore they do not reach the systemic circulation since they remain confined into the mLNs (Hooper and Macpherson, 2010; Macpherson et al., 2012). However, what are the mechanisms by which bacteria are excluded from systemic dissemination through the blood vessels are still unknown.

We hypothesized that also in the intestine there was a control of the endothelial permeability and we wondered whether there was a structure similar to the one described for the BBB. We used the knowledge on the BBB characteristics as model to characterize the intestinal vascular barrier and the intestinal endothelial cells phenotype.

In the BBB, ECs display peculiar features such as the lack of fenestration, low pinocytic activity, the presence of elaborated junctional complexes that includes TJ and AJ proteins and the polarized expression of selective transporters (Daneman and Rescigno, 2009; Paolinelli et al., 2011). The brain ECs were found associated with pericytes, embedded by

the basal lamina, neurons, microglia and astrocytes endfeet that altogether constitute the neurovascular unit (NVU), essential for CNS homeostasis (Cardoso et al., 2010).

In accordance with previous studies that have demonstrated that there is an extensive network of glial cells within the LP, with processes that reach the epithelial cell layer (Bush et al., 1998; Neunlist et al., 2007; Neunlist et al., 2008) and blood capillaries (Hanani and Reichenbach, 1994), we found that in the mouse gut, enteric glial cells, identified with the expression of the intermediate filament GFAP, are abundant in the intestinal LP and they extend their projections to the villi tips (Fig. 4.1). Moreover, similar to astrocytes in the brain, GFAP-positive glia forms several endfeet-like structures surrounding blood vessels particularly when they are located underneath the epithelial basal membrane (Fig. 4.1). Furthermore, ECs are associated with pericytes, stained with α-smooth muscle actin (α-SMA; Fig. 4.1).

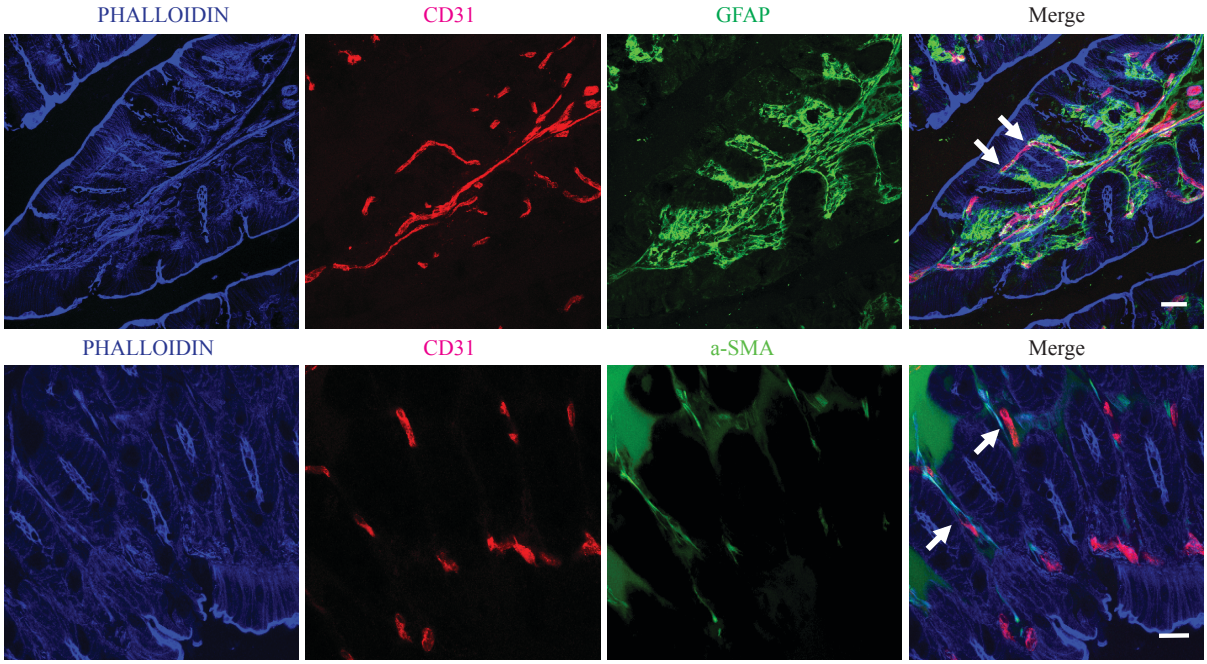

Figure 4.1: Intestinal Vascular Unit characterization. Confocal images showing blood vessels (red, CD31/PECAM-1), enteric glial cells (green, GFAP) in the upper panel or pericytes (green, α-SMA) in the lower panel in cryosections from intestines of C57/BL6 mice. Actin filaments were stained with phalloidin (blue). Scale bars: in the upper panels 30 μm; in the lower panels 20 μm.

One peculiar feature of BBB capillaries is the presence of elaborated junctional complexes that includes TJ and AJ proteins. As shown in Figure 4.2A-F, similar to the cerebral endothelium, intestinal ECs express the main components of TJs, such as occludin (Fig. 4.2A), the transmembrane junctional adhesion molecule–A (JAM-A, Fig 4.2C), claudin-5 (Fig. 4.2E) and low levels of claudin-12 (Fig. 4.2F), as well as the cytoplasmic accessory proteins ZO-1 (Fig. 4.2B) and cingulin (Fig. 4.2D) involved in the connection of integral TJs to the actin cytoskeleton. Moreover, as shown in Figure 4.2B-C-D-F, also the intestinal epithelium expresses components of TJs.

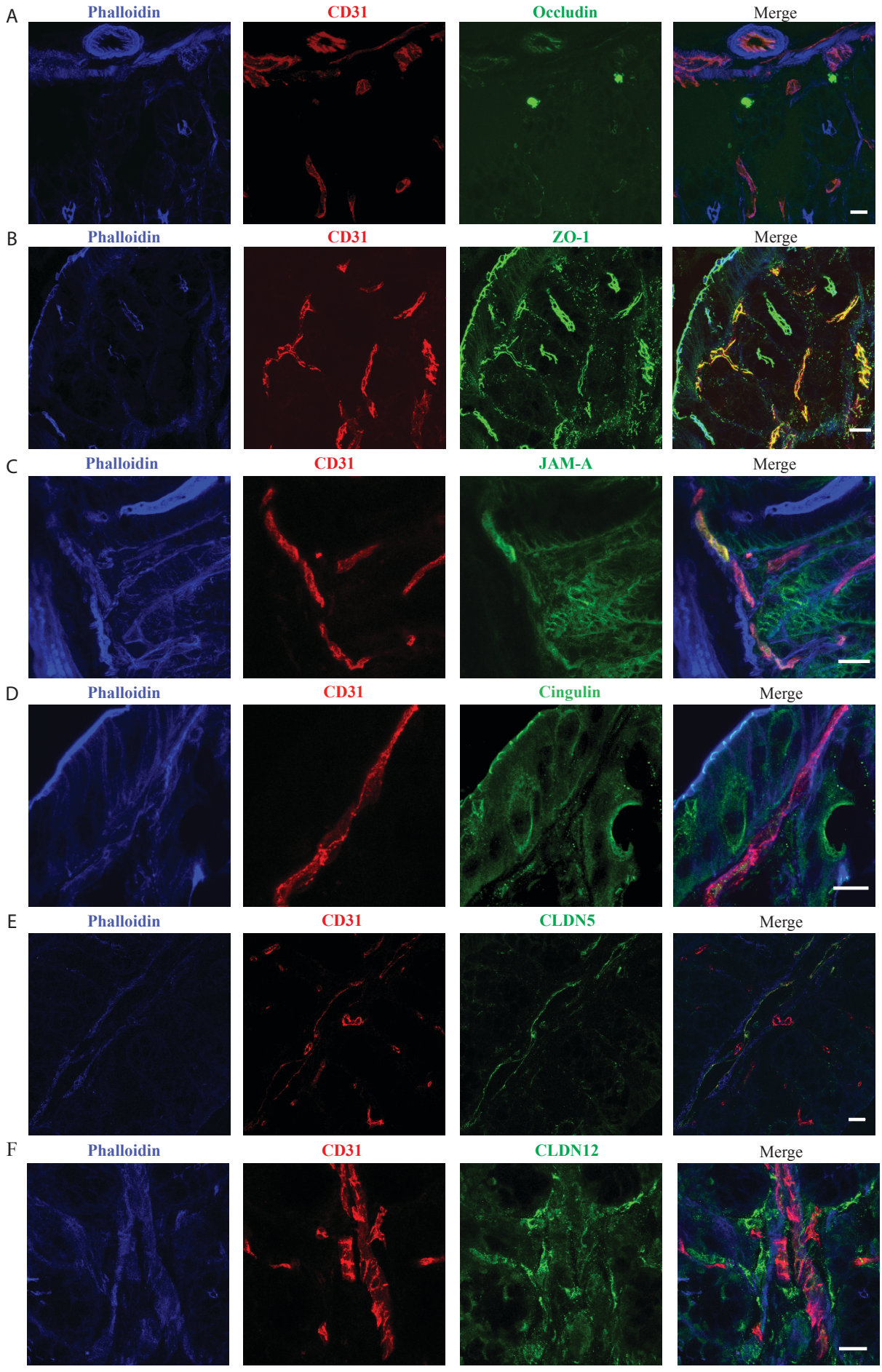
A
Phalloidin
CD31
Occludin
Merge
B
Phalloidin
CD31
ZO-1
Merge
C
Phalloidin
CD31
JAM-A
Merge
D
Phalloidin
CD31
Cingulin
Merge
E
Phalloidin
CD31
CLDN5
Merge
F
Phalloidin
CD31
CLDN12
Merge

Figure 4.2: Intestinal endothelial cells express TJ proteins. Confocal images showing the localization of TJ proteins in blood vessels (CD31/PECAM-1, red). In green (A) Occludin, (B) ZO-1, (C) JAM-A, (D) cingulin, (E) claudin-5, (F) claudin-12. Every intestinal section was stained also with phalloidin (blue). Scale bars: (A, D) 10 μm, (B, C, E, F) 20 μm.

As shown in Figure 4.2E, claudin-5 seems to be expressed by a subset of CD31 positive vessels. To assess whether claudin-5 is differentially expressed by blood vessels and lymphatics, we stained the former with CD34 and the latter with LYVE-1 antibodies and Cldn-5 localization was evaluated. In Figure 4.3 the immunostaining demonstrates that Cldn-5 is expressed by LYVE-1 lymphatic endothelium but not by CD34 blood vessels.

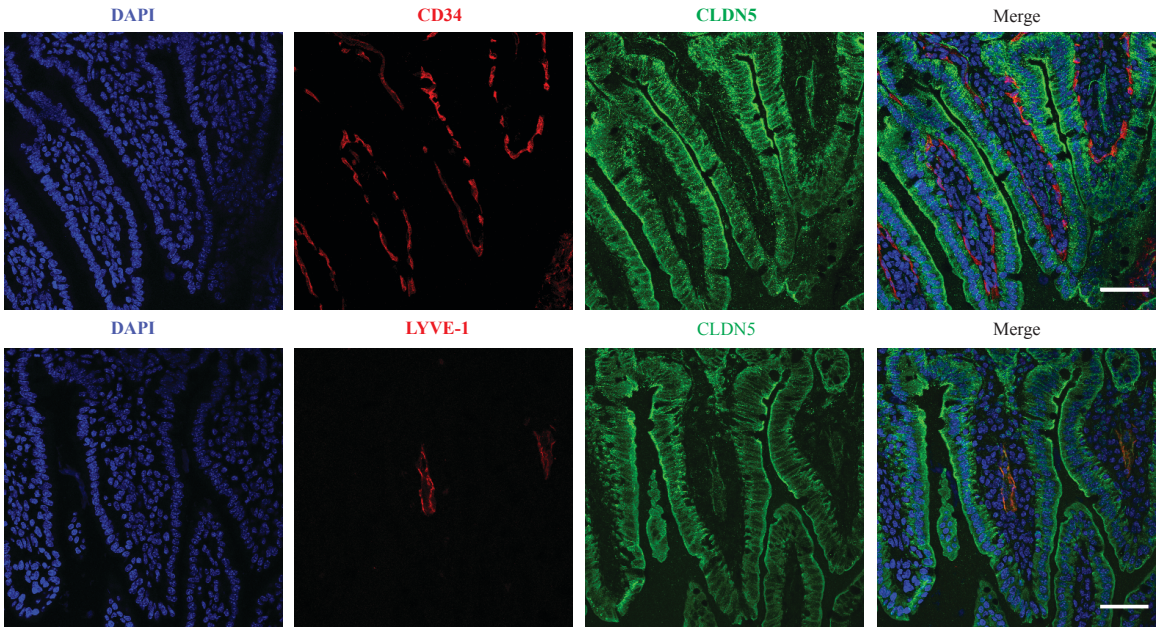

Figure 4.3: Lymphatics but not blood vessels express claudin-5. Confocal images showing the localization of claudin-5. In the upper panels, intestinal sections were stained for Cldn-5 (green) and CD34 (red) to mark blood vessels while in the lower panels, sections were labeled with Cldn-5 (green) and LYVE-1 (red) which is specifically expressed by lymphatic vessels. Every intestinal section was stained also with DAPI (blue) that marks cell nuclei. Scale bars: 50 μm.

Essential for the formation of the TJs are the AJs since they hold ECs together. In the gut, endothelial cells express the transmembrane VE-cadherin (Fig. 4.4) that forms AJs by homophilic interaction. As shown in Figure 4.4, intestinal epithelial cells as well as endothelium express also β-catenin in the intercellular junctions.

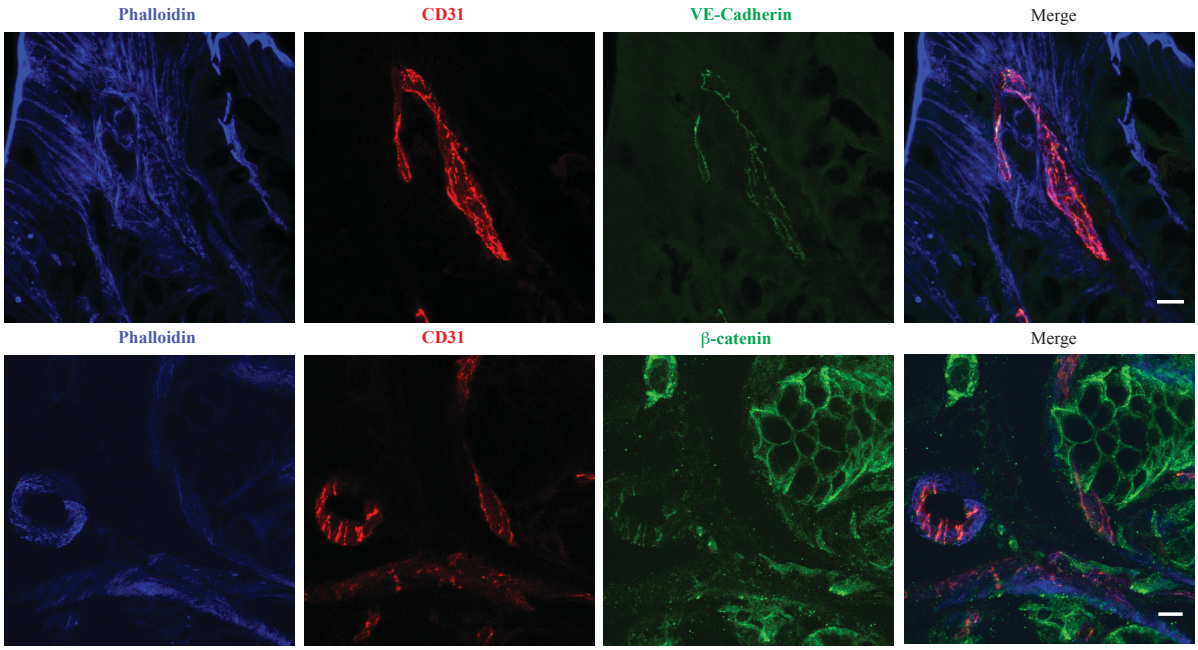

Figure 4.4: Adherens Junction proteins expression in intestinal endothelial cells. Confocal images showing the expression of AJ proteins on blood vessels (CD31/PECAM-1, red). In green VE-cadherin (upper panel) or β-catenin (lower panel). Every intestinal section was stained also with phalloidin (blue). Scale bars: 10 μm.

Taken together these results demonstrate that in the intestine we can define a "gut vascular unit" (GVU) composed by pericytes, enteric glia that extends cellular processes around intestinal vessels and endothelial cells.

Moreover, we found that intestinal endothelial cells express a peculiar set of TJs that probably allows these cells to restrict the trafficking of molecules within the paracellular space. However, the expression of transporters involved in the movement of substances between the intestinal LP and the blood together with the expression on ECs of leukocyte

adhesion molecules necessary for movement of immune cells from the blood into LP remain to be evaluated to have a complete picture of the intestinal endothelium properties.

4.1.2 *S. typhimurium* modifies barrier properties of the intestinal endothelium

In order to clearly define whether there is a vascular barrier in the intestine we analyzed the expression of Plasmalemma Vesicle Associated Protein-32 (Plvap/ MECA-32/ PV1) since in the brain it has been demonstrated that Plvap expression negatively correlates with the differentiation of the vasculature to form the BBB (Liebner et al., 2008). Indeed, when the endothelium differentiates to form the BBB at approximately E17, concomitantly to the activation of Wnt/β-catenin signaling pathway, MECA-32 antigen expression is downregulated (Hallmann et al., 1995) leading to increased barrier properties of the endothelium (Liebner et al., 2008). By contrast, BBB microvessels up-regulate Plvap under pathological conditions such as brain tumors or stroke (Carson-Walter et al., 2005). Analyzing Plvap expression in the small intestine by immunofluorescence, we found that at steady state all the 3 parts of the small intestine, namely duodenum, jejunum and ileum, do not express Plvap (Fig. 4.5). However, when mice were orally infected with *Salmonella typhimurium*, a pathogen capable to spread systemically in the mouse, we found that at early phases of infection Plvap was slightly up-regulated in $CD34^+$ blood vessels of the jejunum and ileum and after 6h it was highly up-regulated in the same parts of the small intestine (Fig. 4.5). By contrast in the duodenum after infection Plvap is expressed at low levels only after 6h post-infection. Following the kinetics of Plvap expression, we found that at 24h after infection, the protein tends to return to basal levels in all the tracts of the intestine (Fig. 4.5).

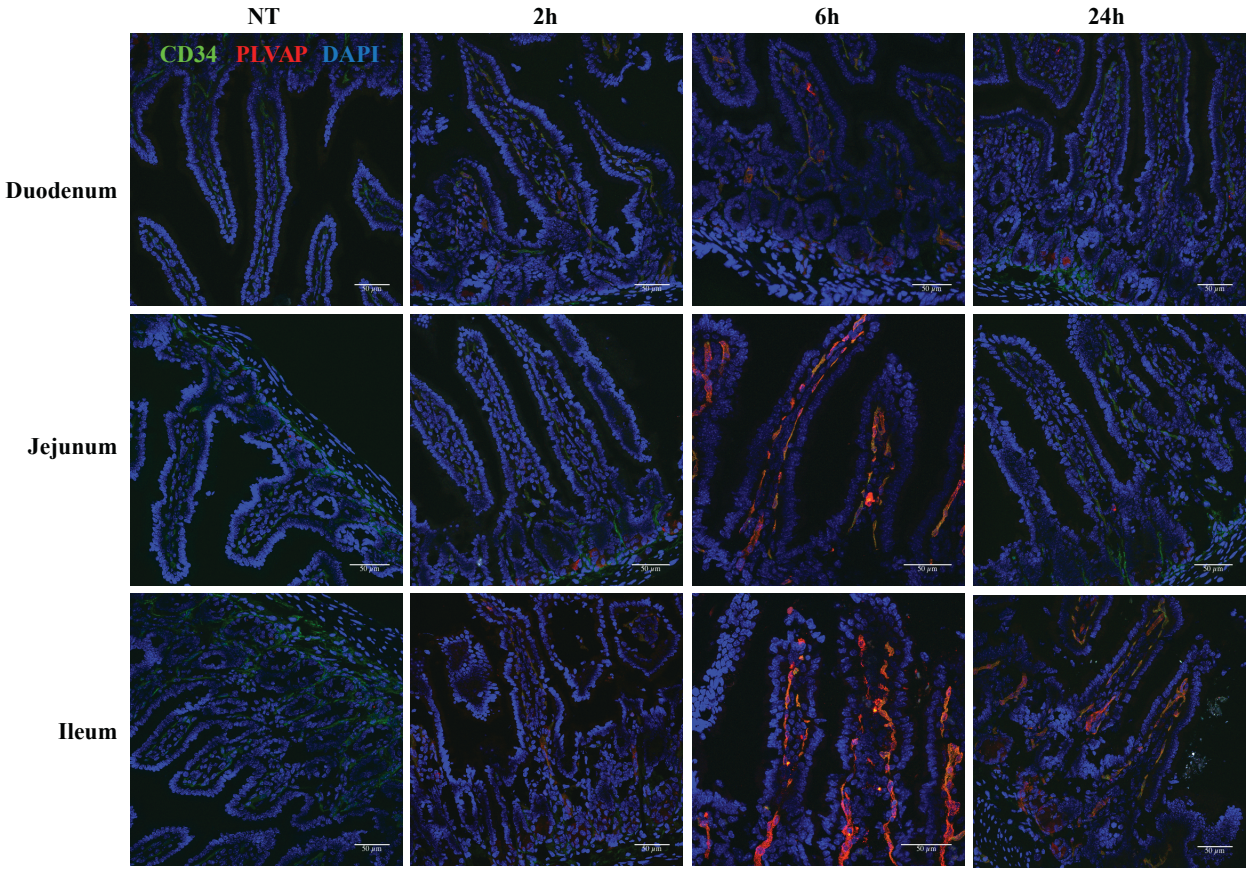

Figure 4.5: Plvap expression on intestinal blood vessels. Confocal images showing blood vessels (green, CD34), Plvap (red) and cell nuclei (blue) in cryosections from different SI tracts of C57/BL6 mice at different time points after oral gavage with 10^9 *S.typhimurium* ΔaroA. Bars: 50μm. Images are representative of three independent experiments.

4.1.3 *Salmonella* spreads systemically after oral infection

To address whether the upregulation of Plvap correlates with reduced barrier properties of the intestinal endothelium and therefore with a systemic spreading of *S. typhimurium,* we challenged C57/BL6J mice intragastrically with 10^9 *S. typhimurium* ΔaroA. We used this auxotrophic mutant with low replicative capabilities *in vivo* because it allows us to evaluate the spreading of the bacteria reducing the effect of the bacterial replication on colony forming unit (CFU) counts.

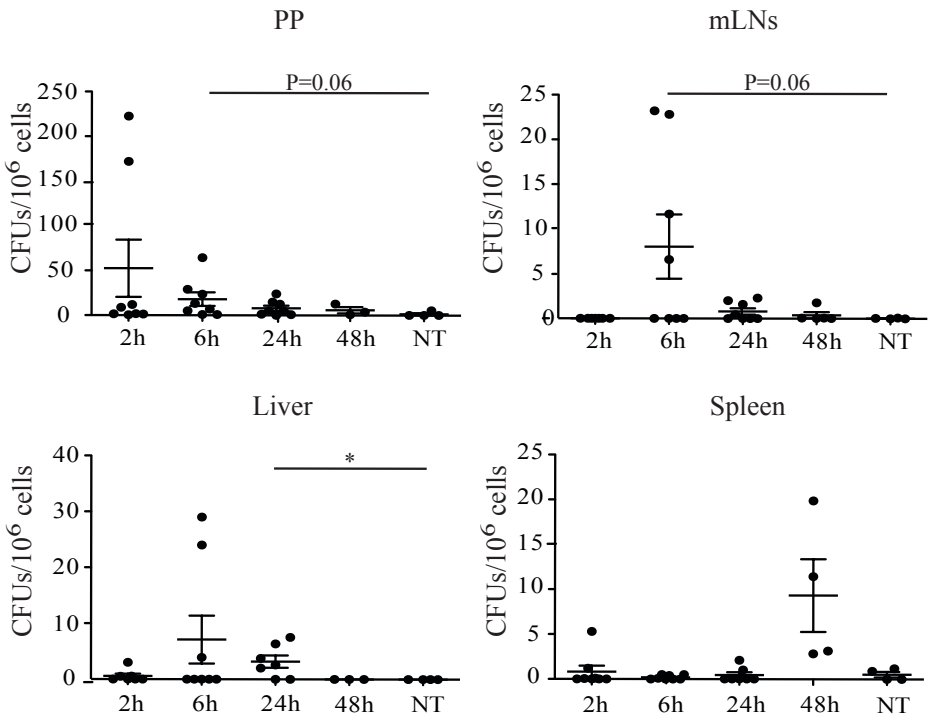

Figure 4.6: *S. typhimurium* spreading after oral administration. Mice were orogastrically inoculated with 10^9 *S.typhimurium* ΔaroA bacteria. After 2h, 6h, 24h and 48h CFUs in PPs, mLNs, spleen and liver were determined. Each data point represents an individual mouse. Error bars represent SEM. Results are pooled from 3 independent experiments. Student unpaired t-test with Welch's correction for unequal variances was used to evaluate statistical significance. *P<0.05.

We found that during the early phases of infection the first organs to be involved are the PPs and later mLNs and liver and finally the spleen are colonized (Fig. 4.6). Moreover, between 6h and 24h when there is the highest number of CFU in the liver, also alanine transaminases (ALT), indicators of liver damage, are high in the serum (Fig. 4.7).

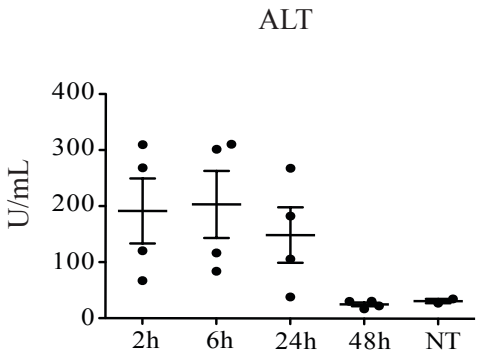

Figure 4.7: Alanine transaminases after *S. typhimurium* infection. Blood from mice infected with 10^9 *S. typhimurium* ΔAroA was collected and serum was tested for the presence of alanine transaminases (ALT), as indicators of liver damage. Each circle represent the result obtained from one single mice. Error bars represent SEM. Data are representative of 3 independent experiments with similar results.

4.1.4 *S.typhimurium* infection modulates caveolae formation in intestinal vessels

Different studies have demonstrated that Plvap is necessary for the formation of endothelial diaphragms in the fenestrae and caveolae both *in vitro* (Ioannidou et al., 2006; Stan, 2004) and *in vivo* (Herrnberger et al., 2012a; Herrnberger et al., 2012b; Stan et al., 2012) where the presence of diaphragms in organs with fenestrated vessels like the intestine are important in maintaining the endothelial barrier function. Indeed, Plvap deficient mice, that have impaired formation of diaphragms and consequently a leaky fenestrated endothelium, showed a selective loss of plasma proteins, edema, dyslipidemia and lethal protein-losing enteropathy (Stan et al., 2012). In the context of the blood-brain barrier, caveolin-1, the major component of the caveolae, has been associated to BBB breakdown, since in the early phase after barrier damage it is upregulated in the vessel of the lesion (Nag et al., 2007). Moreover it has been demonstrated that caveolae are involved in the entry of virus and bacteria such as *E.coli* K1 through brain microvascular endothelial cells (Sukumaran et al., 2002) and *S. typhimurium*, which has been

demonstrated to be transported into the intestinal M cells through caveolin-1 expressing caveolae (Lim et al., 2010).

Based on these published data, we wondered whether also in intestinal endothelial cells caveolin-1, the caveolae membrane scaffolding protein, is expressed and if its expression is modulated during infection along with Plvap. Therefore we analyzed by immunofluorescence the expression of caveolin-1 in the intestine of untreated mice and mice orally infected with 10^9 *S. typhimurium* bacteria after different time points.

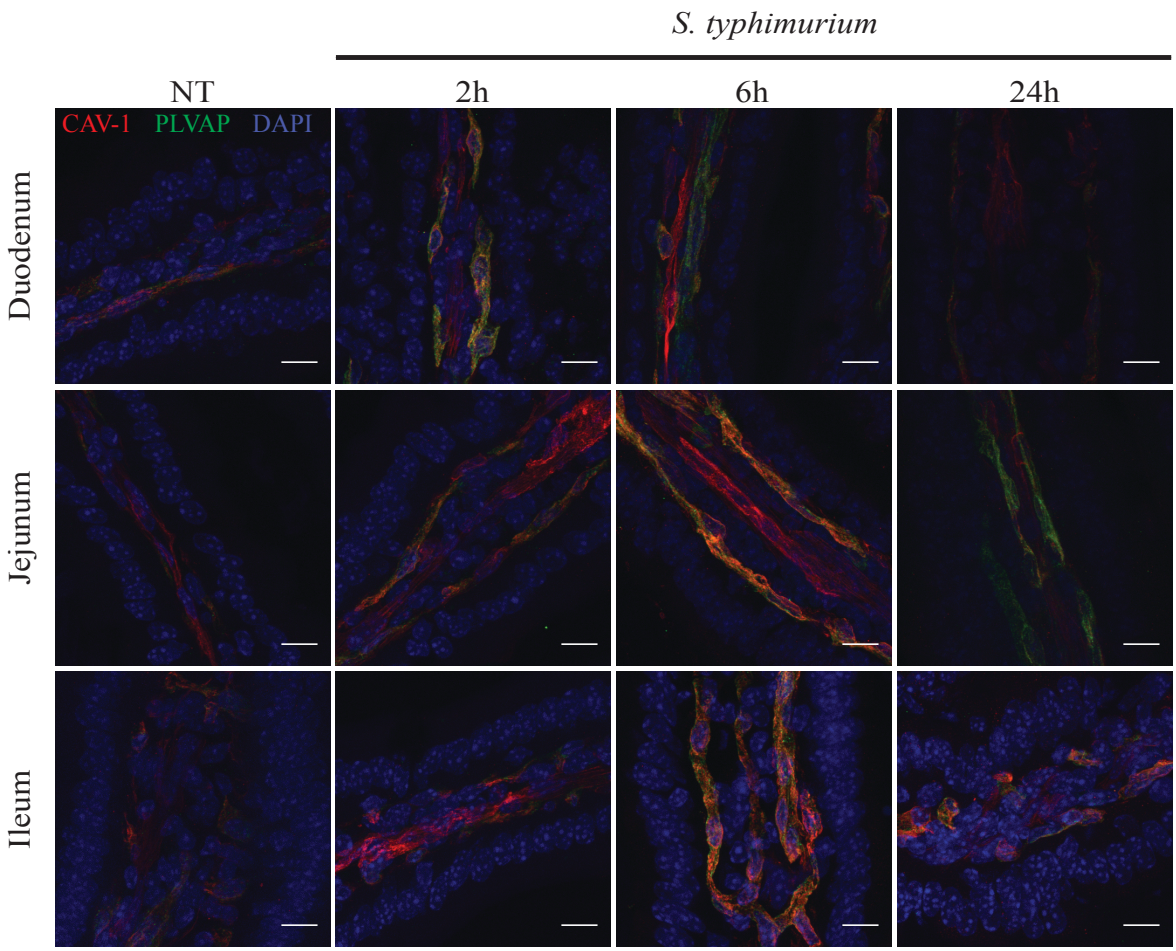

Figure 4.8: Caveolin-1 is expressed in lymphatic and blood endothelial cells in the intestine and it is up-regulated upon infection. Confocal images showing Plvap expression (stained in green), caveolin-1 (CAV-1 in red) and nuclei were stained with DAPI (blue) in cryosections from different SI tracts of C57/BL6 mice at different time points after oral gavage with 10^9 *S.typhimurium* ΔaroA. Bars: 10 μm. Images are representative of three independent experiments.

We found that caveolin-1 was expressed in all the three part of the small intestine in both blood vessels, that after infection could be identified by the expression of Plvap, and in lymphatic vessels, that can be recognized also based on their position in the center of the villi. As shown in Fig. 4.8, at steady state caveolin-1 is expressed at low levels in all the SI, while after infection we found that it is up-regulated. In detail, in the duodenum *Salmonella* infection induces an higher expression of cav-1 first in the blood vessels and then in the lymphatics, and its expression is restored at low levels after 24 hours p.i. On the contrary, in the jejunum and ileum, we found a higher expression of caveolin-1 both in the blood and lymphatic vessels at 2h and 6h p.i. In the ileum the presence of caveolae remains high also after 24 hours after infection.

Together, these results suggest that *Salmonella* infection is able to modulate the formation of caveolae in intestinal blood and lymphatic vessels and to change the Plvap expression on blood vessels modifying the endothelial barrier state.

4.1.5 Claudin-3 expression on intestinal epithelium after *S. typhimurium* infection

Together with the modulation of Plvap expression, it was demonstrated that in the brain Wnt/β-catenin signaling pathway is also able to up-regulate the expression of Claudin-3 (Cldn-3) resulting in barrier maturation (Liebner et al., 2008). Starting from these findings, we wondered whether also in intestinal vessels Cldn-3 is modulated after infecting mice with *S. typhimurium*. We found that Cldn-3 is not expressed by intestinal ECs but interestingly its expression is modulated by the infection in the epithelium confirming what has been recently demonstrated by Corr *et al.* (Corr et al., 2013). We showed that Cldn-3 is up-regulated after infection especially after 6 hours p.i. in the duodenum and jejunum while in the ileum it is expressed at steady state while it is down-regulated after infection (Fig. 4.9). Moreover, at 2 hours after infection we found a more basolateral localization of Cldn-3 compared to the other time points where it is localized at cell-cell junctions.

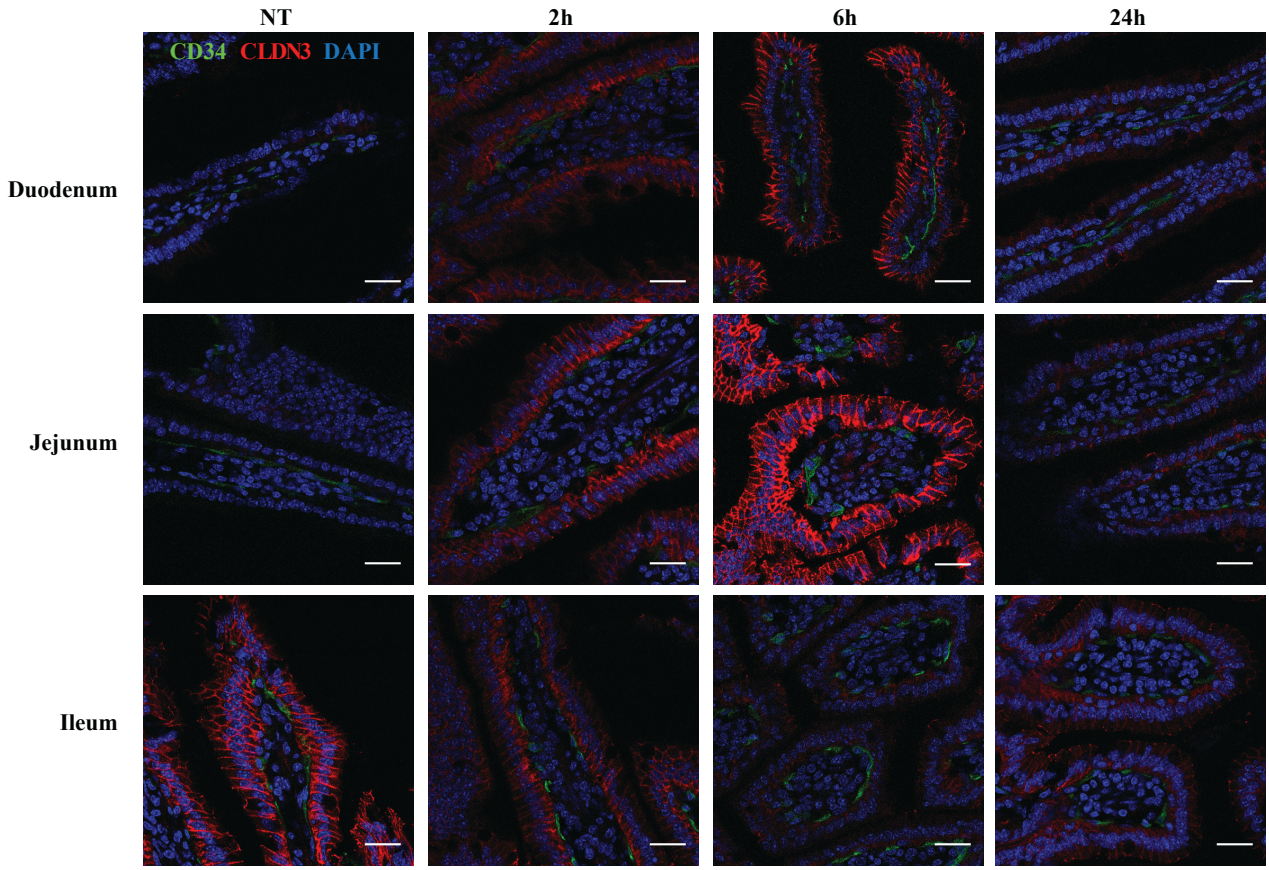

Figure 4.9: Claudin-3 expression on intestinal epithelial cells after *S. typhimurium* infection. Confocal microphotographs showing blood vessels (green, CD34), Cldn-3 (red) and DAPI (blue) in 10 μm thick cryosections from different tracts of the small intestine of C57/Bl6 mice at different time points after oral gavage with 10^9 *S.typhimurium* ΔAroA. Bars: 50 μm.

4.1.6 Permeability of the intestinal blood vessels is modified by *S. typhimurium* infection

To understand whether after *S. typhimurium* infection the vascular barrier function is compromised, we tested endothelial permeability injecting 2 mg of 20 KDa FITC-Dextran in intestinal loops exteriorized from C57/Bl6J mice orally infected with 10^9 *S.typhimurium* ΔAroA. At different time points after *Salmonella* infection blood was harvested and tested for the presence of the fluorophore. As controls, intestinal loops from mice not infected were injected with the fluorophore-conjugated dextran. We found an accumulation of

FITC-dextran in the blood serum between 4h and 24h after infection, when conjugated dextran is injected in the duodenum or jejunum, while if it is injected in the ileum it reaches the blood stream after 24h (Fig. 4.10).

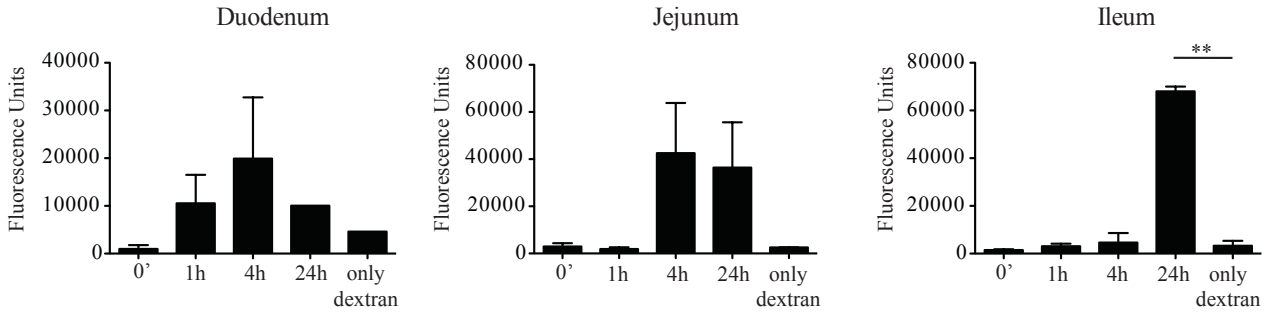

Figure 4.10: *S. typhimurium* infection modifies barrier properties of intestinal endothelium. C57/BL6 mice were orally infected with 10^9 *S.typhimurium* ΔAroA and immediately or after 1h, 4h or 24h were anesthetized and in different parts of the SI, intestinal loops were exteriorized and ligated. 2 mg of FITC-Dextran 20 KDa were injected in the loop. After 1h blood was harvested from the heart and tested for the presence of the fluorophore. As control, uninfected mice were injected in the duodenum, jejunum or ileum with FITC-Dextran and blood was collected after 1h. Serum fluorescence was measured by a fluorimeter and from each value the fluorescence of the serum collected from an untreated mice was subtracted to eliminate the basal level of fluorescence. Results represent mean±SEM. n=4, for each condition. Statistical significance was evaluated using Student unpaired t-test with Welch's correction for unequal variances. ** $p<0.01$.

To assess that the increased passage of dextran was not simply due to an increased epithelial permeability, we decided to assess the inside-out increased vessel permeability. We adapted the protocol described by Xu and colleagues (Xu et al., 2012) to the multi-photon confocal microscope present in our imaging facility to our purpose to analyze the modification of intestinal vessels barrier properties. Briefly, following anesthesia, a loop from the ileum of untreated mice or mice infected for 4 hours with 10^9 *S.typhimurium* ΔAroA was externalized and the mucosa was exposed by making an incision avoiding blood loss. Just before imaging, FITC-dextran with different molecular weights were injected into the tail vein and the extravasation of the dye was monitored via 2-photon

microscopy for at least 10 min every 30 s. As control dye, 4 KDa FITC-dextran was used. As shown in Fig. 4.11 in both untreated and *Salmonella* treated mice, the loss of intravascular fluorescence was very rapid and the visualization of lamina propria blood vessels morphology was lost immediately after fluorophore injection since 4 KDa dextran was not retained but it was accumulated in the intestinal lumen.

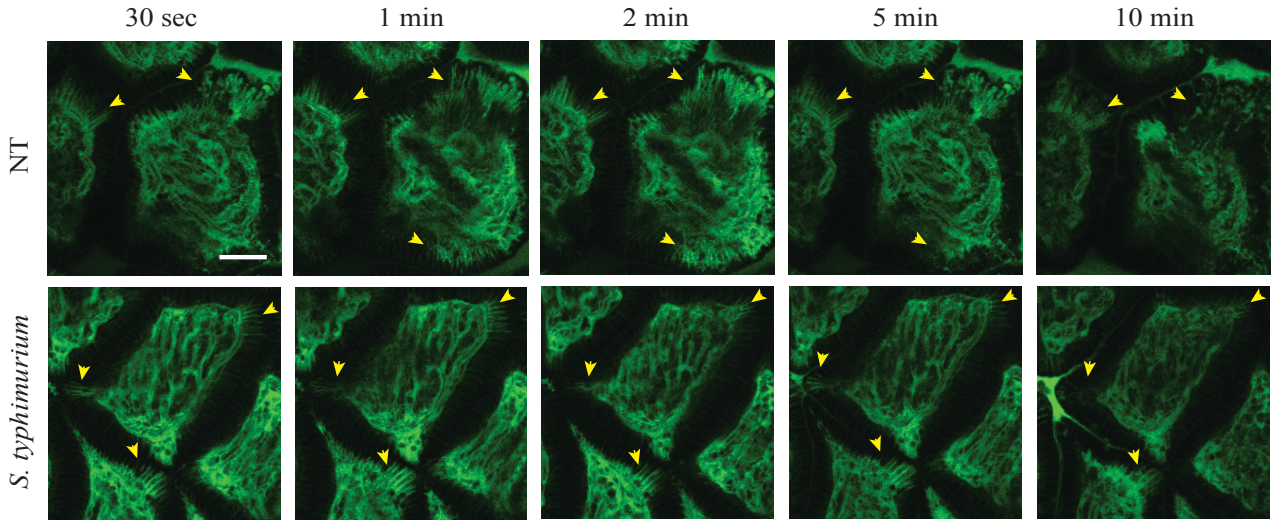

Figure 4.11: Intestinal vessels are highly permeable to 4 KDa FITC-Dextran. Intestinal blood vessels permeability to 4 KDa FITC-dextran was visualized by intravital 2-photon microscopy. 2 mg 4 KDa FITC-Dextran were injected i.v. and intestine was immediately imaged. Time-lapse images were acquired every 30s for 10 min. Yellow arrows indicate fluorophore extravasation through the gut epithelium into the intestinal lumen. Scale bar: 20 µm. $n = 3$.

On the contrary, if 70 KDa FITC-dextran was injected i.v. in untreated C57/BL6J mice, it remains almost completely within the vessel (Fig. 4.12, upper panels), while when mice were infected with *Salmonella* the fluorophore started to extravasate just after few seconds after starting the imaging (Fig. 4.12, lower panels). After infection, 70 KDa dextran was able to extravasate and also to pass the epithelium indicating that *Salmonella* is able to modify both epithelial cell permeability, as extensively demonstrated, but also vascular barrier permeability.

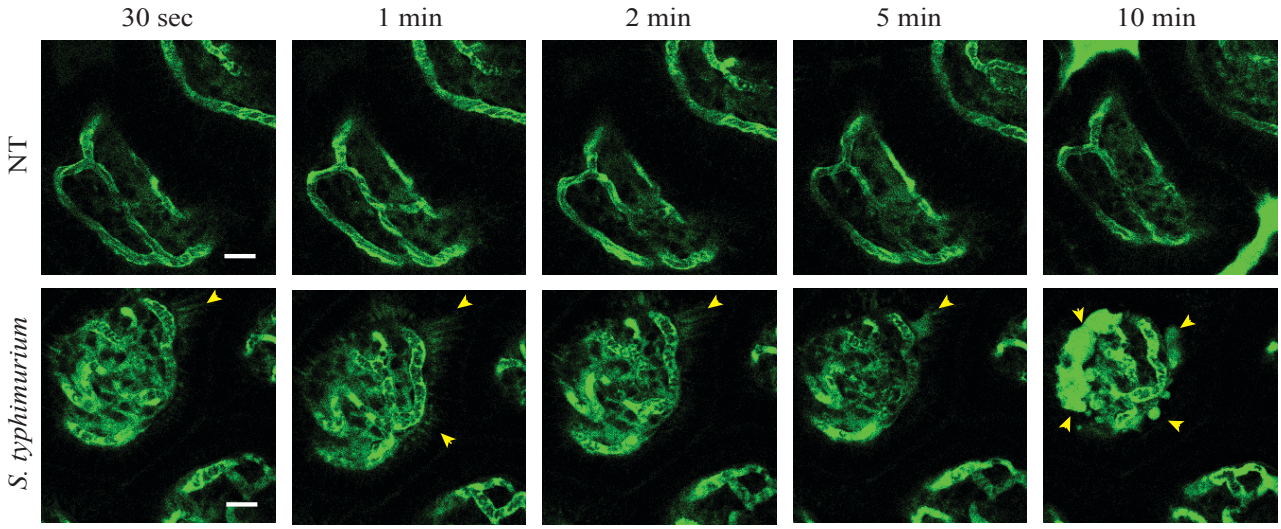

Figure 4.12: *Salmonella* infection increases the vascular permeability to 70 KDa dextran. Intestinal blood vessels permeability to 70 KDa FITC-dextran was visualized by intravital 2-photon microscopy. 0.5 mg 70 KDa FITC-Dextran were injected into the tail vein just before imaging in untreated or *Salmonella* infected mice (4h). Time-lapse images were acquired every 30s for 10 min. Yellow arrows indicate fluorophore exit from the vessels. Scale bar: 20 µm. *n* = 3.

A ratiometric analysis of extra- versus intravascular fluorescent dye was also conducted to quantify the difference between the permeability to the dextrans with different molecular weight. At 10 min after dye injection, the relative fluorescence of 70KDa dextran injected in *Salmonella* infected mice is about 5.5-fold higher than the fluorescence of the same dextran injected into untreated mice. Conversely, the permeability of intestinal blood vessels to 4KDa dextran is equivalent between infected and non-infected mice (Fig. 4.13).

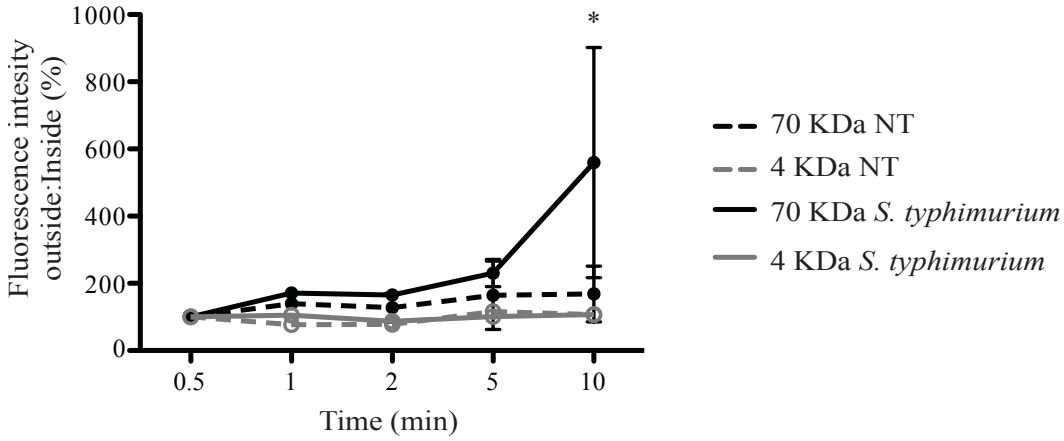

Figure 4.13: Ratiometric analysis of fluorescence intensity of extra- versus intravascular dextrans in untreated and *Salmonella*-infected mice. An area of 5×5 µm was chosen inside and outside the blood vessel. The fluorescence intensity was determined in the areas for both the FITC-dextrans in untreated and infected mice. For each time-point, the fluorescence intensity was measured for 10 areas inside and 10 areas outside the blood vessel and the ratio between the outside and the inside fluorescence intensity was calculated and normalized on the fluorescence ratio at time 0. The ratio was plotted in percentage over time. Results are represented as mean±SEM. Statistical significance was evaluated using 2-way ANOVA. * $p<0.05$.

4.2 Mechanisms behind the intestinal endothelial barrier modifications

4.2.1 Bacterial invasiveness is not involved in the changes of the intestinal endothelial barrier

We have shown that *S. typhimurium* is able to modify the endothelial barrier properties in the gut and to colonize distal organs, such as liver and spleen. Therefore, we wondered whether this could be explained only by the capacity of *Salmonella* to cross the epithelial barrier and hence to reach the endothelial barrier or by the fact that *Salmonella* is able to actively modulate a signaling cascade necessary for the modification of the intestinal endothelial barrier. To answer to the first question we infected mice with a non-pathogenic strain of *E. coli* or with an *E. coli* strain made capable to cross the epithelial barrier by the expression of the *Yersinia enterocolitica* Inv protein (*E. coli*pInv).

Yersinia invasin, a protein of the outer membrane, has been demonstrated to be involved in the entry of the enteropathogenic bacterium into non-phagocytic cells through the binding to β1 integrin. The binding to the integrin activates the focal adhesion kinase and cytoskeletal proteins that lead to the formation of pseudopods that engulf the bacteria into the host cell (Alrutz and Isberg, 1998).

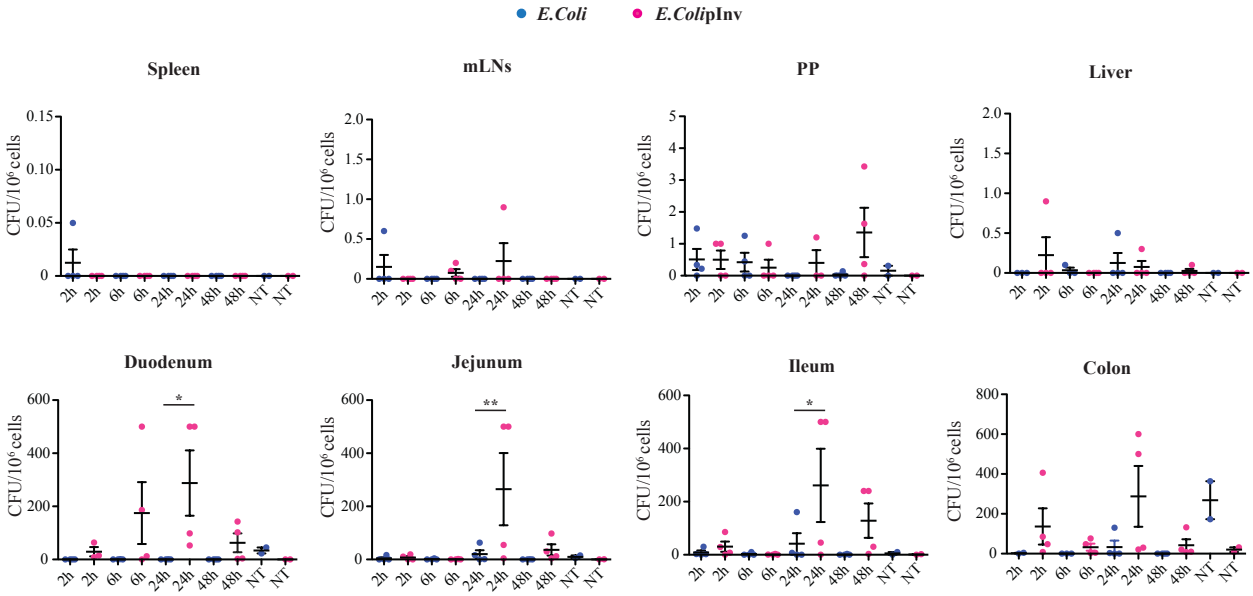

Figure 4.14: *E.coli* as well as the invasive strain *E.coli*pInv are not able to spread in the systemic circulation after oral administration. Mice were orogastrically inoculated with 10^9 *E.coli* (blue dots) or *E.coli*pInv (red dots). After 2h, 6h, 24h and 48h CFUs in PPs, mLNs, spleen, liver, small intestine, divided into duodenum, jejunum, ileum, and colon were determined. Each data point represents an individual mouse. Error bars represent SEM. To evaluate statistical significance between the groups, 2-way ANOVA was used. *P<0.05, **P<0.01.

Although we were able to find *E.coli* expressing the *Yersinia* invasin in the duodenum, jejunum, ileum and colon meaning that it was able to cross the epithelial layer and to reach the intestinal lamina propria (Fig .14, lower panels) and few bacteria were also found in the PPs and in the mLNs, we did not find *E.coli*pInv in the liver and spleen even after 48 hours p.i. (Fig 4.14, upper panels, red dots), meaning that, although it is invasive, *E.coli*pInv is not able to disseminate systemically. As control, mice were infected with a non-pathogenic *E.coli* strain, which was found neither in the intestine nor in the mLNs, liver and spleen (Fig 4.14, blue dots). These data suggest that the capacity of *Salmonella* to cross the intestinal endothelium and to modify its barrier characteristics is not simply due to the fact that it is able to cross the gut epithelium and reach the underlying vessels

but to an active process probably involving the activation/repression of signaling pathways and/or modification of cell-cell junctions.

4.2.2 *S. typhimurium* negatively regulates Wnt/β-catenin signaling pathway

The second hypothesis that could explain the capacity of *Salmonella* to disseminate systemically is its ability to actively modify the intestinal endothelial barrier by modulating a signaling pathway.

Canonical Wnt/β-catenin signalling pathway was demonstrated to be involved in vascular development in the embryo (Cattelino et al., 2003) and in particular in the formation of the blood-brain barrier. Indeed, at embryonic day 9.5 there is a marked activation of β-catenin in endothelial cells of brain capillaries which then decreases between days 15.5 and 17.5 (Liebner et al., 2008). In the brain, the activation of β-catenin correlates with the down-regulation of Plvap and maturation of the BBB. Moreover, it has been shown that Wnt/β-catenin signaling pathway is involved in *S. typhimurium* infection in intestinal epithelial cells (Duan et al., 2007; Liu et al., 2010; Sun et al., 2005; Zhang et al., 2012). Thus, we hypothesized that β-catenin activation may be responsible for the establishment also of the "gut vascular unit" and that it could be regulated by *S. typhimurium* also in endothelial cells. To support this hypothesis, we used an *in vitro* system in which primary lung endothelial cells, isolated from C57/BL6 mice, were infected with *S. typhimurium* and the expression of *Axin2* gene, one of the downstream targets of β-catenin, was analyzed by RT-PCR. We used primary lung endothelial cells for the *in vitro* experiments because of the lack of established mouse intestinal endothelial cell lines and we were not yet able to obtain a large number of primary murine intestinal endothelial cells and to propagate them in culture. We found that lung ECs infected with the *Salmonella* WT strain express less *Axin2* compared to the Wnt3a treated cells, that has been used as positive control and compared to the untreated cells (Fig. 4.15). Lung ECs were also infected with mutated *Salmonella* strains, specifically a SPI-2 deleted strain (BA83), a noninvasive SPI-1 mutant

strain (BA34) or an endotoxin mutant (FB61, msbB). Only when cells were infected with BA83 strain the down-regulation of *Axin2* was abrogated (Fig. 4.15) indicating that probably proteins encoded by T3SS pathogenicity islands 2 are involved in blocking the activation of the Wnt/β-catenin signaling pathway in the endothelium. Moreover, we infected ECs also with WT *E. coli* strain or an invasive *E.coli* strain (DH5αp*Inv*). Analyzing *Axin2* expression levels, only the invasive *E. coli* strain but not the non-pathogenic one is able to reduce the activation of β-catenin (Fig. 4.15).

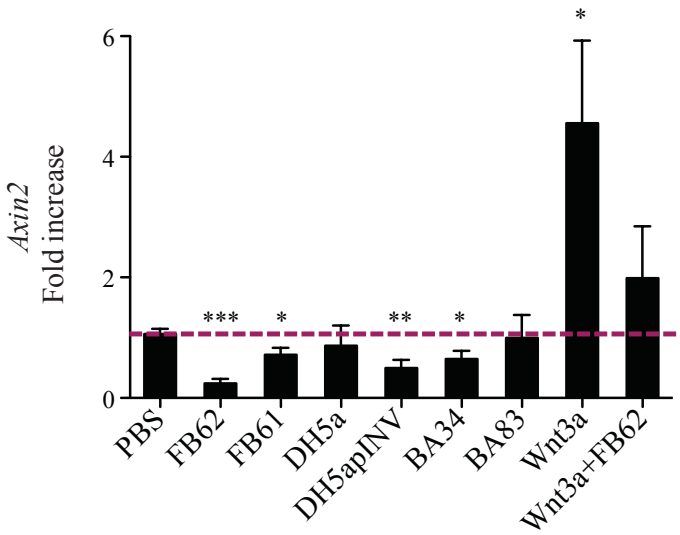

Figure 4.15: Wnt/β-catenin signaling pathway activation state after infection with different strains of *S. typhimurium* and *E. coli*. Primary lung endothelial cells were infected with WT *S.typhimurium* (FB62), a SPI-2 deleted strain (BA83), a noninvasive SPI-1 mutant strain (BA34), an endotoxin mutant (FB61, msbB) or with a WT DH5α *E. coli* strain or a modified strain expressing invasin from *Y. enterocolitica*. Cells were infected with a MOI 1:10 for 1h, washed and left with medium containing gentamycin for 90 min. Alternatively, cells were treated with 100 ng/ml of recombinant Wnt3a as positive control. Cells were then lysed and expression of *Axin2* was assessed by RT-PCR. Results are pooled from 3 independent experiments. Results represent mean±SEM. Statistical significance between untreated (PBS) and treated samples was evaluated using two-tailed Student's t-test. *p<0.05, **p<0.01, ***p<0.001.

In the *in vivo* experiment, shown in Figure 4.14, we found that *E.coli*pInv was not able to disseminate systemically and therefore we were expecting that, unlike *S. typhimurium,*

invasive *E.coli* should not be able to down-regulate *Axin2*. However, we unexpectedly observed that *E.coli*p*Inv* is able to down-regulate *Axin2* although to a lower extent than *S. typhimurium* (Fig. 4.15). These results suggest that *S. typhimurium* and an invasive strain of *E. coli*, at least *in vitro*, are able to affect the translocation of β-catenin to the nucleus where it binds lymphoid enhancer factor (Lef)/T cell factor (TCF) and modulates the transcription of target genes, such as *Axin2*. It remains to be established whether *Axin2* can be considered as the best marker of all of the target genes of Wnt signaling and whether the two bacteria control differently the Wnt pathway and hence a panel of target genes should be analyzed to have a complete picture. In addition, it would be interesting to know whether the difference in the downregulation of *Axin2* in response to *Salmonella* or *E. coli*pInv can account for the different behavior of the two bacteria *in vivo*. Moreover, it seems that TTSS SPI-2 is involved in the modulation of β-catenin activity. The possible implication *in vivo* could be that infection with *Salmonella* reduces the stability of β-catenin in the intestinal endothelium consequently inducing the up-regulation of Plvap and, in general, the destabilization of the barrier to favour its systemic spreading.

4.2.3 MyD88 signaling regulates β-catenin activation state in endothelial cells

Toll-like receptors (TLRs) act as primary sensors for microbial components whose engagement by the bacterial ligands culminates with the activation of the transcription factor nuclear factor-kappaB (NF-kappaB). The endothelial cells express a number of TLR, such as TLR2, 3, 4 and 6 in the brain endothelium (Nagyoszi et al., 2010) or TLR4 (Ogawa et al., 2003) and 5 (Maaser et al., 2004) in the intestinal endothelial cells. Once vascular endothelial cells are stimulated by LPS, they become activated and are characterized by an enhanced expression of cell adhesion molecules (CAM) with an increased leukocyte adhesion and tissue recruitment from the circulation (Ogawa et al., 2003).

In epithelial cells, *Salmonella* is known to activate upon TLR engagement both the pro-inflammatory NF-κB signaling pathway and the β-catenin signaling pathway. Moreover it has been demonstrated a direct physical interaction between the NF-κB p50 subunit and β-catenin upon infection (Duan et al., 2007; Sun et al., 2005).

Since we have shown that *S. typhimurium* infection induces the reduction of β-catenin activation (Fig 4.16) in endothelial cells we wondered whether this modulation is mediated by TLRs and by MyD88 through which most surface TLRs, such as TLR2 and TLR4, signal.

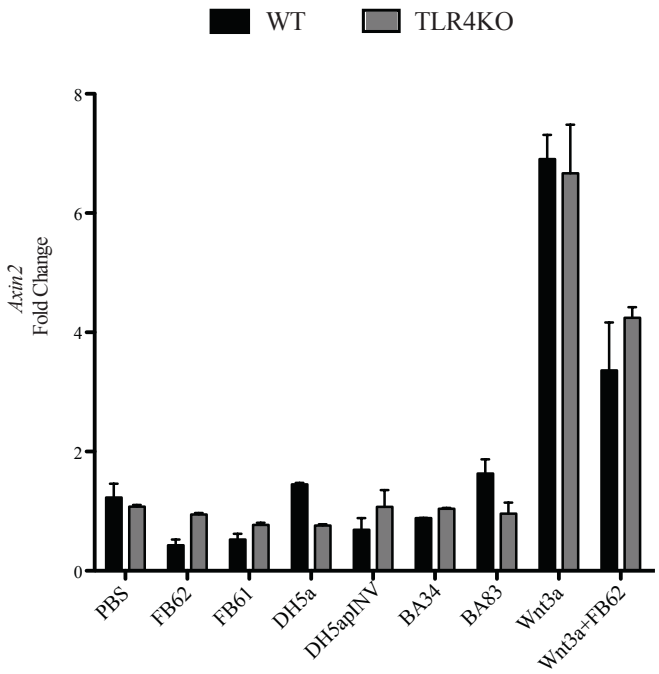

Figure 4.16: TLR4 engagement in endothelial cells is not required for Wnt/β-catenin signaling pathway. Primary lung endothelial cells from WT or TLR4-deficient mice were infected with WT *S.typhimurium* (FB62), a SPI-2 deleted strain (BA83), a noninvasive SPI-1 mutant strain (BA34), an endotoxin mutant (FB61, msbB) or with a non-pathogenic (DH5α) or invasive (DH5αpINV) *E. coli* strains. Cells were infected with a MOI 1:10 for 1h, washed and left with medium containing gentamycin for 90 min. As positive control, cells were treated with 100 ng/ml of recombinant Wnt3a. Cells were then lysed and expression of *Axin2* was assessed by RT-PCR. Results represent mean±SEM.

For this purpose, primary lung endothelial cells isolated from WT, TLR4 KO and MyD88 KO mice were infected with WT *Salmonella* or mutated strains for SPI-2, SPI-1 or endotoxin or with *E. coli* WT or invasive strains and *Axin2* expression was evaluated as indicator of β-catenin activation. As shown in Fig. 4.16, TLR4 ligation is not involved in the reduced activation of β-catenin that we observed with WT lung endothelial cells. However, the effect seemed to be mediated by the signal transduction protein MyD88 since the canonical Wnt/β-catenin signaling pathway is completely shut off in MyD88 deficient endothelial cells (Fig. 4.17). Interestingly, the absence of MyD88 in endothelial cells induces the reduction of β-catenin activation even in the absence of TLR ligands or in presence of the Wnt3a, known to activate the Wnt/β-catenin pathway and therefore the transcription of *Axin2* (Fig. 4.17).

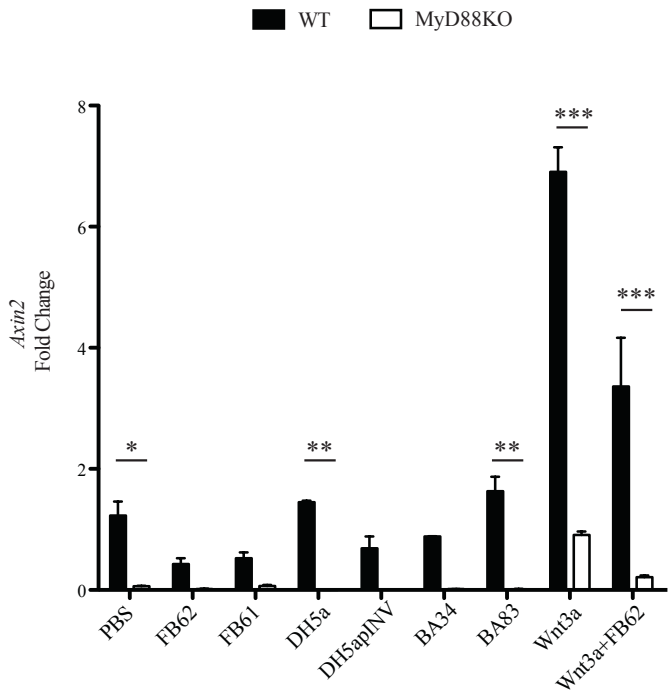

Figure 4.17: β-catenin activation after infection in endothelial cells is dependent on MyD88. Primary lung endothelial cells from WT or MyD88 KO mice were infected with WT *S.typhimurium* (FB62), a SPI-2 mutated strain (BA83), a noninvasive SPI-1 mutant strain (BA34), an endotoxin mutant (FB61) or with a WT DH5α *E. coli* strain or a modified strain expressing invasin from *Y. enterocolitica*. As control for the β-catenin activation state, cells were treated with 100 ng/ml of recombinant Wnt3a. ECs were then lysed and expression of *Axin2* was assessed by RT-PCR. Results represent mean±SEM. Statistical significance between WT and KO samples was evaluated using two-way ANOVA test. *p<0.05, **p<0.01, ***p<0.001.

This effect is confirmed also by analyzing the expression of *Plvap*, another gene inversely modulated by the activation of β-catenin in endothelial cells. Indeed, as shown in Fig. 4.18, MyD88 deficiency caused the up-regulation of *Plvap* gene independently from the presence of bacteria and Wnt ligands.

These preliminary results suggest an unknown interaction between β-catenin and MyD88 adaptor protein. An explanation for the interaction between MyD88 and β-catenin could be that MyD88 is important to maintain a basal level of active β-catenin that could be involved in the maintenance of endothelial barrier similarly to what happens for the intestinal epithelium in which Wnt/β-catenin signaling is important for the maintenance of intestinal homeostasis (Fevr et al., 2007). MyD88 is downstream of most TLRs and of IL-1/IL-18 signaling pathways (Warner and Nunez, 2013). Hence, basal activation of β-catenin may be the response of either IL-1 signaling or endogenous ligands of TLRs via MyD88.

Moreover during infection it has been shown that MyD88 plays an important role in controlling the systemic spreading of *C. rodentium* (Gibson et al., 2008) that could be obtained also by regulating Wnt/β-catenin pathway at endothelial barrier level.

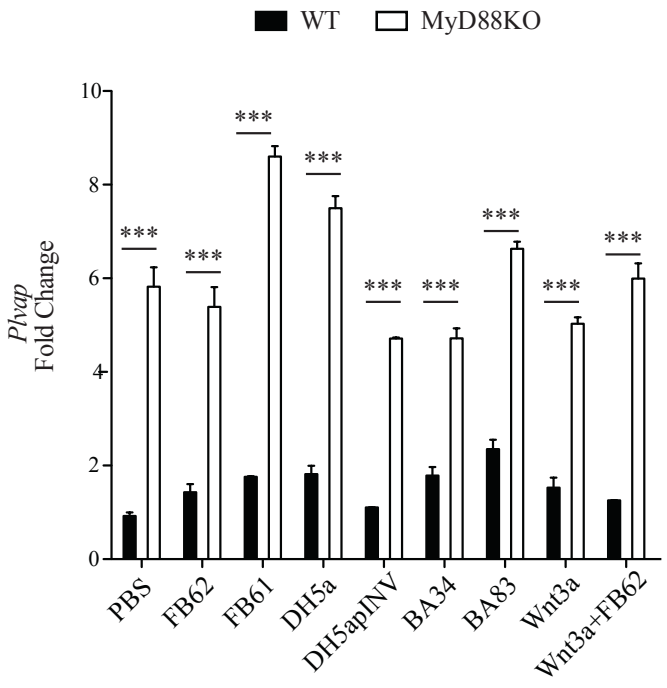

Figure 4.18: Plvap expression after infection of endothelial cells is dependent on MyD88. Primary lung endothelial cells from WT or MyD88 KO mice were infected with WT *S.typhimurium* (FB62), a SPI-2 (BA83), SPI-1 (BA34), or endotoxin (FB61) mutated strains or with DH5α *E. coli* or invasin-expressing strains. As control for the β-catenin activation state, cells were treated with 100 ng/ml of recombinant Wnt3a. ECs were then lysed and expression of *Plvap* was assessed by Real Time-PCR. Results represent mean±SEM. Statistical significance between WT and KO samples was evaluated using two-way ANOVA test. ***p<0.001.

4.2.4 Wnt/β-catenin signaling pathway controls *Salmonella* systemic spreading *in vivo*

To assess the role of β-catenin in the formation of the gut-vascular barrier *in vivo,* we used a mouse line in which β-catenin is active specifically in endothelial cells in an inducible way. In β-catenin$^{lox(ex3)/lox(ex3)}$ mice, the exon3 of the β-catenin gene (*Catnb*), that contains the serine/threonine residues phosphorylated by the GSK3β kinase, is flanked by two *LoxP* sites (Harada et al., 1999). These mice were crossed with *Cdh5*(PAC)-CreERT2 mice where Cre recombinase is inserted downstream of VE-cadherin (*Cdh5*) promoter (Monvoisin et al., 2006). Upon tamoxifen treatment the Cre recombinase is expressed in VE-cadherin positive endothelial cells and the β-catenin exon3 is excised resulting in a gain-of-function (GOF) mouse strain, i.e. where β-catenin becomes constitutively active. We decided to use this mouse line and not a mouse line in which β-catenin is activated in all the tissue because in the intestine β-catenin is known to be involved in intestinal epithelial cell proliferation and differentiation (Fevr et al., 2007; Pinto et al., 2003) and also because we wanted to assess the role of β-catenin activation only in endothelial cells as *Salmonella* affects β-catenin activation in epithelial cells (Liu et al., 2010; Zhang et al., 2012).

Hence, we infected intragastrically *Cdh5*(PAC)-CreERT2 X β-catenin$^{lox(ex3)/lox(ex3)}$ GOF mice with 10^9 *S. typhimurium* ΔaroA. Before starting the experiments, excision of exon3 was verified by PCR (see Materials and Methods section).

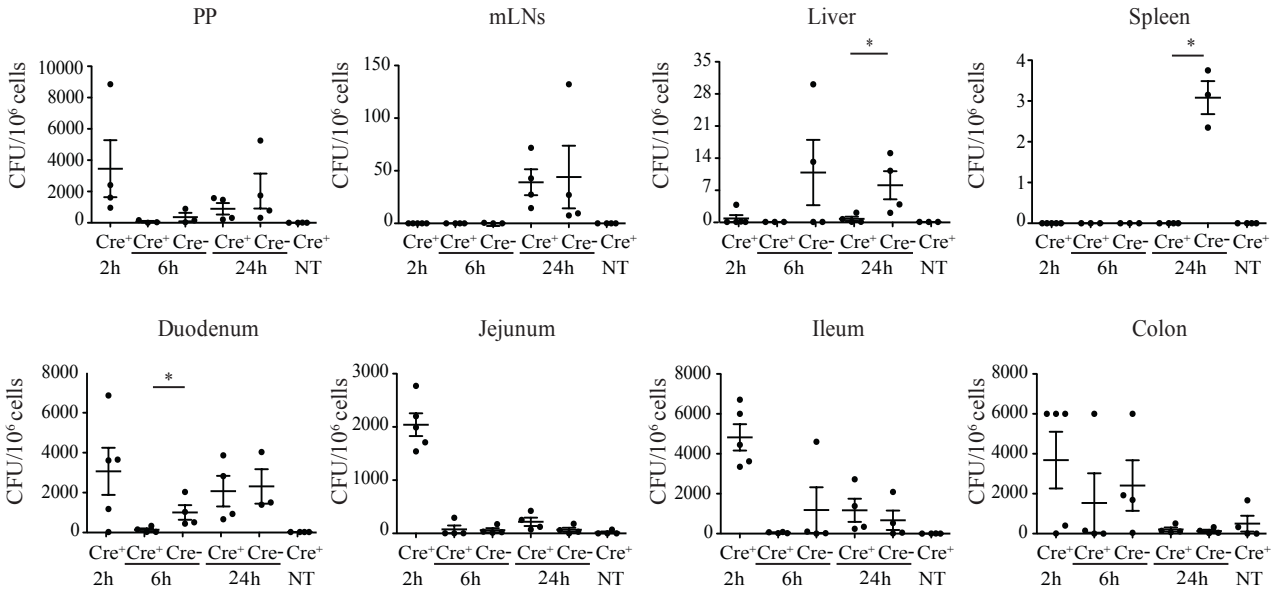

Figure 4.19: *S. typhimurium* systemic spreading is reduced in β-catenin gain-of-function mice. *Cdh5*(PAC)-CreERT2 X β-catenin$^{lox(ex3)/lox(ex3)}$ GOF mice or β-catenin$^{lox(ex3)/lox(ex3)}$ control mice were infected p.o. with 10^9 *S.typhimurium* ΔaroA. After 2h, 6h and 24h CFUs in PPs, mLNs, spleen, liver, small intestine and colon were determined. Each data point represents an individual mouse. Error bars represent SEM. Results are representative of 2 independent experiments with similar results. Student unpaired t-test with Welch's correction for unequal variances was used to determine statistical significance. *P<0.05.

If β-catenin is involved in modulating the endothelial barrier in the gut, we expect that when β-catenin is made constitutively active, the barrier integrity should be maintained and therefore *Salmonella* should not be able to spread systemically.

As expected, we found that *S. typhimurium* was able to invade and colonize different segments of the small intestine and colon of GOF mice and Cre-negative control mice in the same way (Fig. 4.19, lower panels), indicating that the constitutive activation of β-catenin in endothelium did not have indirect effects on intestinal epithelium permeability. However, between 6h and 24h when there was the highest number of CFU in the liver of control mice, we found fewer bacteria in the livers of GOF mice. The same result was obtained also for the spleen at 24h post infection (Fig. 4.19, upper panels). These data indicate that when β-catenin is activated in blood ECs, *Salmonella* is not able to modify

the endothelial barrier and spread systemically indeed it cannot reach either the liver or the spleen crossing the intestinal blood vessels but it can only reach the mLNs (Fig. 4.19, upper panels) by the lymphatics where probably it will remain confined (Voedisch et al., 2009).

4.2.5 Permeability of the intestinal blood vessels is modified by *S. typhimurium* infection

To assess the functionality of the intestinal barrier in *Cdh5*(PAC)-CreERT2 X β-catenin$^{lox(ex3)/lox(ex3)}$ GOF mice at steady state and after challenge with *S. typhimurium*, we tested endothelial permeability injecting 2 mg of 20 KDa FITC-conjugated dextran in intestinal loops exteriorized from Cre^{+} GOF mice and Cre^{-} control mice orally infected with 10^9 *S.typhimurium* ΔAroA.

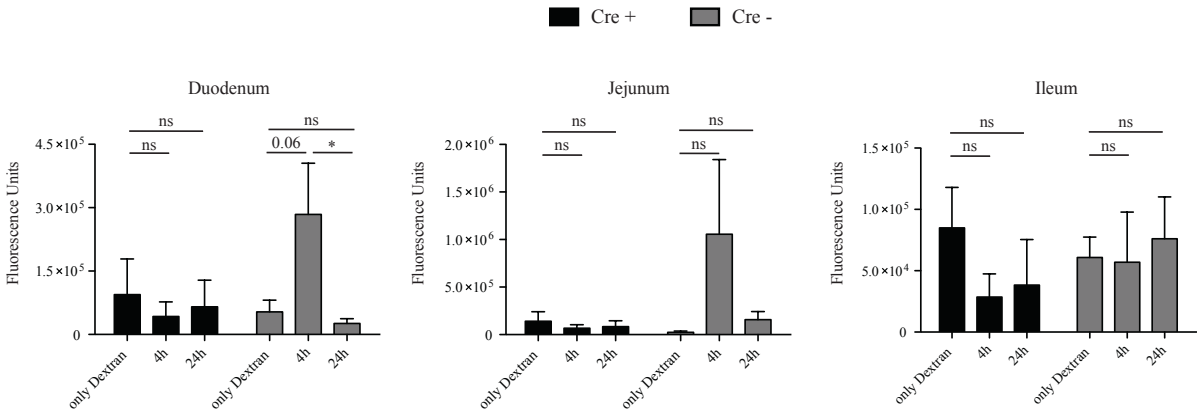

Figure 4.20: *S. typhimurium* do not modifies endothelial permeability of intestinal blood vessels of *Cdh5*(PAC)-CreERT2 X β-catenin$^{lox(ex3)/lox(ex3)}$ gain-of-function mice. *Cdh5*(PAC)-CreERT2 X β-catenin$^{lox(ex3)/lox(ex3)}$ GOF mice and cre-negative control mice were orally infected with 10^9 *S.typhimurium* ΔAroA and after 4h or 24h were anesthetized and intestinal loops were exteriorized and ligated. 2 mg of FITC-Dextran 20 KDa were injected in the loop. After 1h blood was harvested from the heart and tested for the presence of the fluorophore. As control uninfected mice were injected in the duodenum, jejunum or ileum with FITC-Dextran and blood was collected after 1h. Serum fluorescence was measured at fluorimeter and from each value the fluorescence of the serum collected from naive mice was subtracted to eliminate the basal

level of fluorescence. Results represent mean±SEM. Statistical significance was evaluated using Student unpaired t-test with Welch's correction for unequal variances. *p<0.05, ns: not significant.

Analyzing the fluorescence in the blood serum we found an accumulation of FITC-Dextran at 4h after infection, when conjugated dextran was injected in the duodenum or jejunum of cre recombinase negative control mice, as expected from the previous experiments (Fig. 4.10), but not in the β-catenin GOF mice where permeability to the 20 KDa dextran is not modified even after infection (Fig. 4.20). In contrast, when fluorophore-conjugated dextran was injected in ligated ileum in all time points analyzed there was only a slight difference between GOF and control mice (Fig. 4.20). Moreover, we noticed a difference between the results of the permeability experiments carried out with Cre- control mice and the WT C57/BL6J mice (purchased from Harlan) when FITC-dextran was injected in the ileum. This discrepancy could be explained taking into account the fact that the ileum is most colonized part of the small intestine by the intestinal microbiota and that it is highly possible that the composition of the intestinal flora is different between animals bred in our animal facility and mice purchased from an external company, indicating a possible role of the microbiota in the modulation of vascular barrier function in the gut. It is likely that a different composition of the microbiota may modify the epithelial (for example by modulating Cldn-3 expression) and/or endothelial barrier, thus influencing the segment of the intestine that is more prone to be invaded by *Salmonella*.

All together these results suggest that *Salmonella* influences β-catenin activation state in intestinal endothelial cells *in vivo* to facilitate its spreading to systemic districts.

4.3 Role of microbiota in GVU formation

4.3.1 Colonization of the intestine by the microbiota induces barrier maturation

The intestinal microbiota exerts a multiplicity of functions in our body such as nutrient absorption, xenobiotic metabolism, angiogenesis, maturation of the immune system and plays a key role in the intestinal barrier maturation (Hooper et al., 2001; Natividad and Verdu, 2013). Another uninvestigated role of the intestinal flora could be to induce the formation of the GVU during the postnatal period and to maintain vascular barrier integrity during adulthood.

To understand the role of gut microbiota in the establishment of vascular barrier we analyzed the presence of bacteria in the organs of C57/BL6 mice after weaning when the switch from lactation to solid food is known to change the bacterial composition of the offspring gut. We tested different time points after weaning but we found the highest difference between day 8 and day 10 post-weaning. We found that at day 8 some bacteria of the intestinal flora reached both the mLNs and the liver, while at day 10 we did not find any bacteria in both organs (Fig. 4.21).

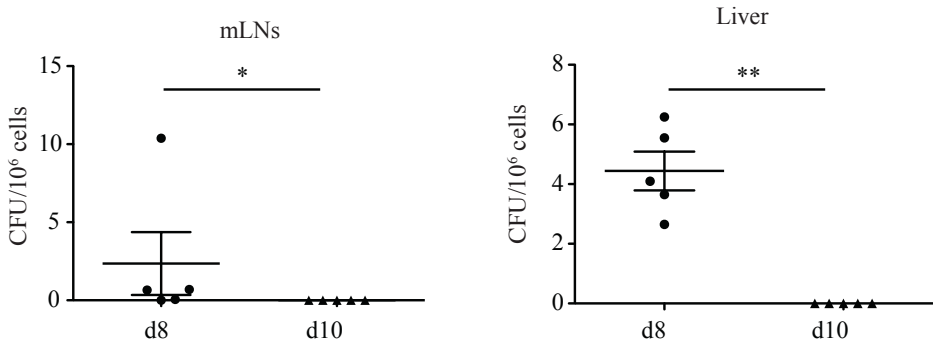

Figure 4.21: Intestinal vascular barrier became mature after weaning. C57Bl/6 mice were weaned and after 8 or 10 days bacterial number in the mLNs and liver were evaluated. Each data point represents an individual mouse. Error bars represent SEM. Mann-Whitney test was used to determine statistical significance. *P<0.05, *P<0.01.

These results suggest that in the early phases of life, the GVU can be modified by the diet (from breast milk to solid food) and/or by the gut microbiota that may be participating in the formation and maturation of the intestinal barrier. However, till now we cannot clearly dissect out the role of the microbiota on epithelial and on endothelial barrier maturation.

Another interesting aspect of the relationship between the intestinal microbiota of the offspring and pregnancy is the exchange of bacteria between the maternal gut and the newborn. Indeed, although it is commonly accepted that the intrauterine environment is sterile, it has been reported the presence of bacteria in the intrauterine environment that are probably involved in the prenatal colonization of the newborn. *Enterococcus, Streptococcus, Staphylococcus*, or *Propionibacterium* bacterial genera were found in the umbilical cord blood suggesting translocation of the mother's gut bacteria via the bloodstream (Jimenez et al., 2005; Matamoros et al., 2013). Moreover, another strong influence in the development of the newborn intestinal microbiota is breast milk. In fact, in breast milk bacterial strains from *Streptococcus* and *Staphylococcus* genera were isolated (Fernandez et al., 2013; Matamoros et al., 2013). How the maternal gut microbiota reaches the newborn via umbilical cord blood or via the milk through the lactating gland is unknown. In both cases, a role for dendritic cells has been hypothesized (Fernandez et al., 2013; Jimenez et al., 2005), but another possibility could be that during pregnancy the maternal gut-vascular barrier may be more permissive to the microbiota.

To test this hypothesis we harvested the intestine of female mice as soon as the offspring were weaned and we stained them for Plvap molecule that we have identified as marker of vascular barrier damage.

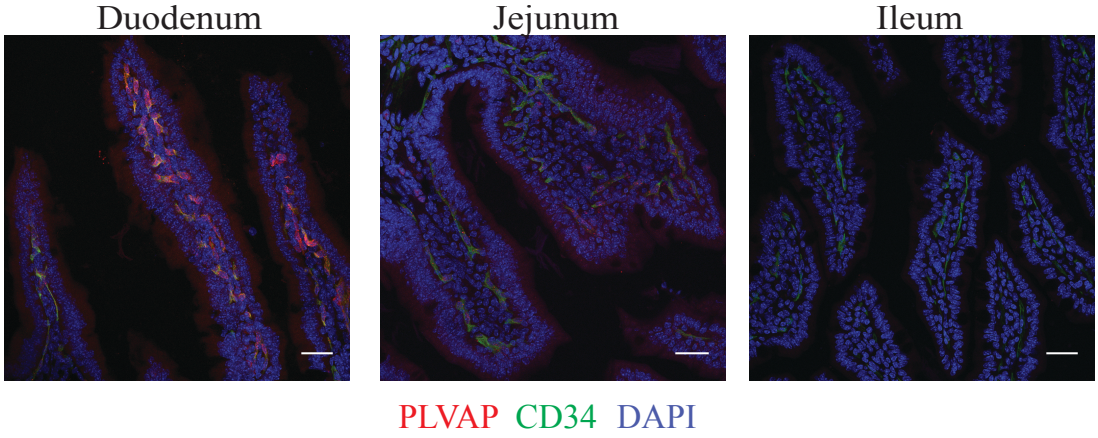

Figure 4.22: Plvap expression on intestinal blood vessels of lactating females. Confocal images showing blood vessels (green, CD34), Plvap (red) and DAPI (blue) in cryosections from duodenum, jejunum and ileum of C57/BL6 female mice at the end of lactation. Bars: 30μm.

Analyzing the small intestine of lactating females, we found that Plvap is expressed in the duodenum and at lower levels in the jejunum (Fig. 4.22), while in normal mice we have previously shown that at steady state Plvap is not express in any part of the small intestine. Although this is still only a preliminary result, it may indicate that during pregnancy the intestinal vascular barrier could be more permeable explaining why the microbiota is found in the systemic circulation or in the mammary gland.

4.3.2 Antibiotic treatment increases *S. typhimurium* systemic spreading capabilities

Another possible role of the microbiota is to maintain the GVU integrity in order to preserve the health of our body.

For this reason we treated mice with broad spectrum antibiotics (ampicillin, vancomycin, neomycin sulfate and metronidazole) for 4 weeks before challenging them with *S.typhimurium* ΔaroA.

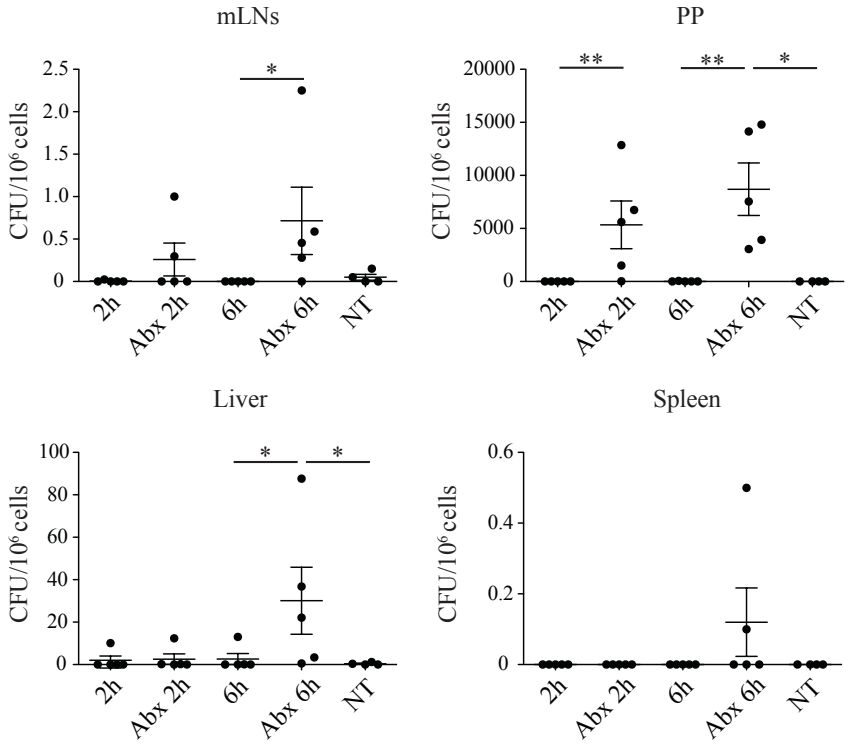

Figure 4.23: *S. typhimurium* spreading after antibiotic treatment. C57/BL6 mice received in the drinking water a mixture of antibiotics (Abx) to eliminate most of the endogenous microbiota (ampicillin, metronidazole, neomycin, vancomycin). After 4 weeks, mice were inoculated p.o. with 10^9 *S.typhimurium* ΔaroA bacteria. After 2h or 6h CFUs in PPs, mLNs, spleen and liver were determined. Each data point represents an individual mouse. Error bars represent SEM. Results are pooled from 2 independent experiments. Student unpaired t-test with Welch's correction for unequal variances *P<0.05, **P<0.01.

As shown in Fig. 4.23, antibiotic treatment resulted in an increase in the translocation of *Salmonella* to the liver and spleen. This result suggests that the reduced number of the microbiota present into the intestine after antibiotic treatment could bring the gut vascular barrier to an immature state which is more permissive for *Salmonella* entry. However, we cannot exclude that the increased translocation of bacteria after antibiotic treatment is also due to a higher ability of *Salmonella* to penetrate the epithelial barrier in the absence of competing microbiota.

DISCUSSION

In this work we have identified an intestinal vascular barrier that controls the systemic dissemination of food antigens and invasive bacteria that can be either accidentally ingested with the diet or be resident within the microbiota.

Drawing a parallel between the well-known BBB and the newly identified gut vascular barrier (GVB) many similarities can be found. For both of them, the body has evolved a set of mechanisms to avoid the indiscriminate movement of molecules, cells and bacteria from blood to the brain parenchyma in the BBB and from the subepithelial space to the blood in the case of the GVB. However, the BBB and GVB display different characteristics due to the fact that these two endothelia should fulfill distinct functions. For instance, the BBB should avoid the uncontrolled movement of any substances from the blood into the brain parenchyma to protect the CNS from the constantly changing milieu of the blood stream. For this reason, the brain endothelium is continuous, paracellular trafficking is avoided by TJs, transcytosis is limited and the controlled movement of substances is achieved by the polarized expression of transporters (Abbott et al., 2006; Siegenthaler et al., 2013). By contrast, the intestinal endothelium is not continuous and it should be permeable to nutrients, due to the absorptive function of the gut. However, it displays barrier characteristics to preserve the body from the spreading of intestinal microbiota and pathogens that could cross the epithelium and has size limit exclusion to large molecules.

Indeed molecules with low molecular weight can diffuse via the paracellular route while high molecular weight molecules can be transported by transcytosis. The paracellular trafficking of molecules with high molecular mass is avoided by the presence of TJs and AJs proteins at cell-to-cell junctions.

We found that, similar to the cerebral endothelium, intestinal ECs express the main components of TJs (occludin, JAM-A, Cldn-12, as well as the cytoplasmic proteins ZO-1 and cingulin) and AJs (VE-cadherin and junctional β-catenin). Moreover, in line with

previous studies that have demonstrated that there is an extensive network of glial cells within the LP with processes that reach the epithelial cell layer (Bush et al., 1998; Neunlist et al., 2007; Neunlist et al., 2008) and blood capillaries (Hanani and Reichenbach, 1994), we found that in the mouse gut, enteric glial cells (the equivalent of astrocytes in the brain) entail contacts with blood vessels, particularly when they are located in proximity to the epithelium. Different studies have highlighted the importance of enteric glial cells in the intestine. Indeed mice lacking $GFAP^+$ cells died for fulminant jejuno-ileitis characterized by massive destruction of the epithelial layer together with microvascular disturbances that result in bacterial spreading into the blood (Bush et al., 1998). Enteric glia was found to regulate mucosal barrier function secreting S-nitrosoglutathione (Savidge et al., 2007) which induces an increased expression of ZO-1 and occludin in the epithelium (Flamant et al., 2011). Whether gut glial cells may confer a barrier phenotype also to intestinal ECs, resembling the astrocytes in the brain, is still unknown. This possibility is however supported by the finding that transplantation of enteric glia into the damaged spinal cord accelerates the repair of vasculature at the site of injury and the induction of barrier properties (Jiang et al., 2005).

Gut ECs were found associated also to pericytes that together with enteric glial cells form what we call “gut vascular unit” (GVU). However the influence of these cells on the ECs barrier phenotype remains to be established.

We hypothesize that the role of the GVB is to exclude from the systemic circulation bacteria or other unwanted molecules that have been translocated from the gut lumen. To define clearly whether there is a functional vascular barrier we analyzed the expression of Plvap protein as barrier marker.

Different reports have demonstrated that in the BBB, Plvap is a marker of “leaky” vascular barriers (Armulik et al., 2010; Daneman et al., 2010) and Liebner *et al.* have found that Wnt/β-catenin signaling pathway activation is responsible for the down-regulation of Plvap expression on ECs during barrier maturation (Liebner et al., 2008).

Analyzing the expression of Plvap in the intestinal blood vessels we found that it is not expressed at steady state but it is up-regulated in the jejunum, ileum and to a smaller extent in the duodenum upon infection with *S. typhimurium* following the same kinetics of *Salmonella* spreading to the liver. This is in accordance with the observation that *in vitro Salmonella* is able to reduce β-catenin activation and therefore could induce Plvap up-regulation. Moreover, we found that TTSS encoded by pathogenicity islands 2 are involved in blocking the activation of the Wnt/β-catenin signaling pathway in the endothelium since TTSS-2 mutated strain fail to down-regulate *Axin2,* used as marker of Wnt/β-catenin signaling pathway activation.

Interestingly, Cirillo *et al.* have found that SPI-2 mutants are able invade PPs but were not found in the mLNs, liver and spleen and this was linked to the fact that SPI-2 is required to avoid *Salmonella* clearance by macrophages (Cirillo et al., 1998). In light of our results, the inability of TTSS-2 mutant to spread systemically could also be due to the fact that they are not able to pass the intestinal endothelium and reach the blood stream.

Moreover, we found that an *E.coli* strain expressing *Y. enterocolitica* invasin gene was able to reduce the activation of β-catenin although it was not able to disseminate systemically *in vivo*. These results suggests that other Wnt/β-catenin target genes should be analyzed to have a complete picture and to be sure that *Axin2* can be considered as the best marker of all of the target genes of Wnt signaling. Another possibility is that the difference between the behavior of *Salmonella* or *E. coli*pInv can be in the modulation of other signaling pathways.

The involvement of the Wnt/β-catenin signaling pathway in maintenance of the integrity of the GVB is demonstrated also by the experiments carried out with *Cdh5*(PAC)-CreERT2 X β-catenin$^{lox(ex3)/lox(ex3)}$ mice where, upon tamoxifen treatment, β-catenin becomes constitutively active in endothelial cells. We used this mouse line and not a mouse line in which β-catenin is activated in all the tissue because in the intestine β-catenin is known to be involved in intestinal epithelial cell proliferation and differentiation (Fevr et al., 2007; Pinto et al., 2003) and also because we wanted to assess the role of β-catenin activation

only in endothelial cells as *Salmonella* affects β-catenin activation in epithelial cells (Liu et al., 2010; Zhang et al., 2012). Using these mice we found that when β-catenin is activated in vascular ECs, *S. typhimurium* is not able to spread systemically indeed it cannot reach either the liver or the spleen crossing the intestinal blood vessels. However, *Salmonella* can still reach the mLNs by the lymph but does not recirculate through the thoracic duct into the blood circulation since mLNs work as "firewall" (Hooper and Macpherson, 2010) (Macpherson and Smith, 2006). Thus the mLN firewall can limit also pathogen spreading to systemic sites.

Together with the down-regulation of the "leaky" protein Plvap, the BBB acquires the expression of tight junction proteins during the establishment of endothelial barrier function. These proteins include Cldn-5 (Nitta et al., 2003) and Cldn-3 whose expression is increased by Wnt/β-catenin pathway activation in ECs (Liebner et al., 2008).

Analyzing the expression of these proteins in the intestine we found that Cldn-5 is only expressed in the lymphatics while Cldn-3 is not expressed in the ECs. However, we found that Cldn-3 is expressed by the epithelial layer and its expression and cell localization are modulated by *Salmonella* infection in line with what has been recently demonstrated by Corr *et al.* (Corr et al., 2013). The importance of Cldn-3 in the establishment of epithelial barrier function has been demonstrated also by the study on probiotics by Patel *et al.* Indeed, the treatment of neonatal mice that show an immature epithelial barrier, with *Lactobacillus rhamnosus* GG induces an increased expression of Cldn-3 in the epithelium and the maturation of barrier function (Patel et al., 2012). Interestingly, the authors pointed out that this probiotic strain has a protective function at low doses while the administration of a high dose of live probiotic induces death of the animals because of sepsis. This suggests that neonatal mice, beyond an immature epithelial barrier, could have also an immature vascular barrier still permeable to bacteria.

Patel and colleagues have also shown that the maturation of the intestinal epithelium is abrogated in MyD88-/- mice indicating that MyD88-dependent TLR signaling may play a role in inducing intestinal barrier function (Patel et al., 2012). We suspected that MyD88

could also be involved in the maintenance of the GVB. Indeed, we found that the absence of MyD88 in endothelial cells induced the reduction of β-catenin activation concomitantly with the up-regulation of *Plvap* gene even in the absence of exogenous TLR or Wnt ligands indicating that MyD88 is required in ECs for the maintenance of endothelial barrier properties (or at least for the inhibition of "leaky" proteins like Plvap), via a still unknown mechanism.

The importance of MyD88 in the control of the permeability of the gut vascular endothelium together with a role in the maintenance of the epithelial barrier integrity is suggested by different studies in which MyD88-deficient mice show higher susceptibility to *C. rodentium* infection compared to WT controls – the bacterium was clearly identified inside the blood vessels of KO mice – (Gibson et al., 2008) and higher susceptibility to DSS-induced colitis, although the pathology seems to be not directly associated to bacteremia but to a compromised expression of cytoprotective factors by MyD88-/- epithelium (Rakoff-Nahoum et al., 2004). Additionally, it was found that the lack of MyD88 adapter-like (Mal) protein on non-hematopoietic cells induces increased susceptibility to oral infection with *S. typhimurium*. Furthermore, Mal-deficient mice show perturbed barrier function with increased systemic spreading of *Salmonella* and increased blood permeability following infection (Corr et al., 2013).

During the early phase of BBB breakdown, blood vessels in the site of lesion express another marker of leaky endothelial barrier, namely caveolin-1 (Nag et al., 2007), which has been associated to caveolae. Interestingly, in organs with fenestrated vessels like the intestine, Plvap protein was found localized at stomatal diaphragms of fenestrae and caveolae and it was found to be important for the maintenance of the endothelial barrier function (Herrnberger et al., 2012b; Stan et al., 1999; Stan et al., 2012).

It has been demonstrated that caveolae are involved in the entry of viruses and bacteria such as *E.coli* K1 and *Salmonella* (Hoeke et al., 2013; Lim et al., 2010). In particular, it has been shown that *Salmonella* can down-regulate caveolin-2 expression by increasing the expression of miR-29a both *in vivo* and *in vitro* (Hoeke et al., 2013). In the small

intestine we found that caveolin-1 was expressed in blood vessels and in lymphatics and interestingly, we found that after infection it is up-regulated in both of them. The expression of caveolin-1, and therefore the increase in caveolae formation, could be another way by which *Salmonella* could spread in both the lymph and the blood.

Together with the increase of caveolin-1 and of Plvap we functionally tested the presence of endothelial barrier using fluorophore-conjugated dextrans. Two approaches have been used: an “outside-inside” method in which dextran was injected into ligated intestinal loops and the presence of dextran in the serum was measured and an “inside-out” approach in which dextrans with different molecular weight were injected i.v. and the extravasation of the dextrans was visualized using 2-photon microscopy.

Using both methods it was clear that *Salmonella* infection could reduce the functionality of the intestinal vascular barrier. In particular, using 2-photon microscopy we found that under steady state the GVB was permeable to 4KDa dextran but not to 70KDa dextran, indicating that the endothelial barrier in the intestine did not restricts the trafficking of small molecules – unlike the BBB in which the size exclusion is for molecules with molecular mass higher than 500 Da (Pardridge, 2005). However, the size exclusion limit of the GVB has to be identified.

Using the same experimental set up, we assessed also the functionality of the intestinal barrier in *Cdh5*(PAC)-CreERT2 X β-catenin$^{lox(ex3)/lox(ex3)}$ GOF mice at steady state and after challenge with *S. typhimurium*. As expected, we found that in mice with a constitutively active β-catenin, ECs permeability to the dextran is not modified even after infection.

Interestingly, we noticed a difference in the permeability to dextran of the ileal endothelium in Cre- control mice and WT C57/BL6J mice (purchased from Harlan). This discrepancy could be explained taking into account the fact that the composition of the intestinal flora is different between animals bred in our animal facility and mice purchased from an external company, indicating a possible role of the microbiota in the modulation of vascular barrier function in the gut. It is possible that components of the microbiota may modify both the endothelial barrier and the epithelial one. For instance, microbiota could

modulate Cldn-3 expression in the epithelium so that higher expression of Cldn-3 in the ileum could tighten the epithelial barrier avoiding the trafficking of molecules in the last part of the intestine. This modulation cannot be excluded since we have found a modulation of Cldn-3 expression by *S. typhimurium*. Indeed we showed that Cldn-3 is up-regulated in the duodenum and jejunum and down-regulated in the ileum during infection. The down-regulation of Cldn-3 in the ileum could induce an increased epithelial permeability in this intestinal tract and it could be also possible that this favours DCs to sample luminal antigens extending their dendrites through the epithelial layer, in accordance to what was found by Chieppa *et al.* who showed an increased number of dendrites in the ileum upon *Salmonella* infection (Chieppa et al., 2006) (Fig. 5.1).

Of note, although the results of "outside-inside" permeability assays could be influenced by the presence of another barrier – the epithelium – with the 2-photon experiments where an "inside-outside" method was used, we could analyze the effect of *Salmonella* on endothelial permeability excluding the effects on the epithelium.

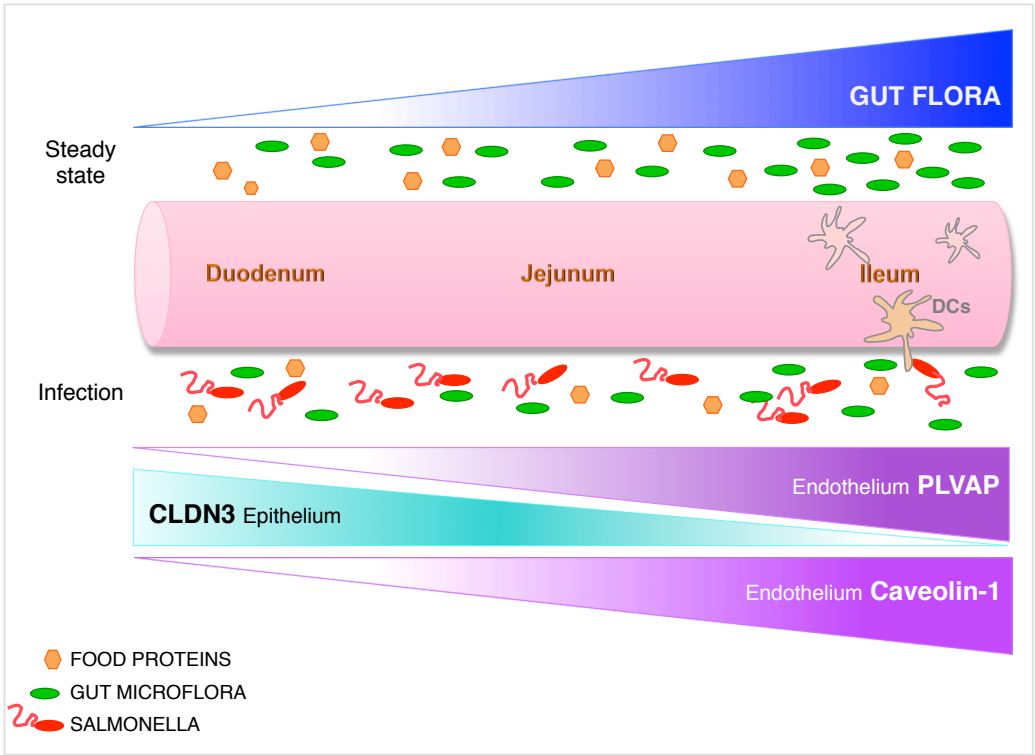

Figure 5.1 Scheme of the findings of this work. We have shown that at steady state Plvap is not expressed in the endothelial cells of the small intestine similarly to Caveolin-1, which is low express in the blood vessels. Upon infection both proteins were up-regulated mostly in blood vessels of the jejunum and ileum. Reducing the integrity of the endothelial barrier in these parts of the SI, *Salmonella* could favour its systemic spreading. Moreover, at steady state we found high expression of Cldn-3 in the epithelial layer of the ileum but not in the duodenum and jejunum. During *S. typhimurium* infection, Cldn-3 expression is up-regulated in the duodenum and jejunum and down-regulated in the ileum where the epithelial barrier could be less tight than the steady state. It could be possible that this could favour luminal antigens sampling by DCs extending their dendrites through the epithelial layer, in accordance to what was found by Chieppa *et al.* who showed an increased number of dendrites in the ileum upon *Salmonella* infection.

Since the intestinal microbiota was shown to play a key role in the intestinal barrier maturation (Hooper et al., 2001; Natividad and Verdu, 2013) we wondered whether it could induce also the formation of the GVB during the postnatal period and maintain vascular barrier integrity during adulthood. Preliminary data show that soon after weaning the epithelial and/or endothelial barrier are not yet formed since we found intestinal bacteria in the liver and mLNs. However, after 10 days post-weaning we did not find any bacteria in these organs indicating that the endothelial barrier maturation can be induced by the gut microbiota, although we cannot exclude the contribution of the diet (from breast milk to solid food). Moreover, till now we cannot clearly dissect out the role of the microbiota on epithelial and on endothelial barrier maturation.

On the other hand, the role of the microbiota in the maintenance of the GVB during adulthood was addressed treating mice with broad-spectrum antibiotics before challenging with *S.typhimurium*. We found that antibiotic treatment resulted in an increase in the translocation of *Salmonella* to the liver and spleen suggesting that the reduced number of the microbiota could modify the gut vascular barrier so that it is more permissive for *Salmonella* entry. However, we cannot exclude that the increased translocation of bacteria after antibiotic treatment is also due to a higher ability of *Salmonella* to penetrate the epithelial barrier in the absence of competing microbiota.

The importance of the intestinal endothelial barrier was also suggested by studies on human diseases. For instance, it was shown that one feature of IBD is the increased intestinal vascular permeability that leads to tissue edema and damage (Oshima et al., 2001). Interestingly, the alteration of the vascular permeability in IBD patients is not restricted to the intestinal vessels but affects also the vasculature of other organs such as the brain (Hathaway et al., 1999).

Furthermore, it has been found that in neonates, non-typhoidal *Salmonella* spp (included *S. typhimurium* serovar) that are usually able to cause only local infection, could cause bacteriemia and meningitis (van Sorge et al., 2011), suggesting that during infancy the gut endothelial barrier is still permeable and also non-typhoidal *Salmonella* spp are able to spread systemically and reach the brain. Another possibility is that in neonates the intestinal endothelium has not yet acquired some characteristics that can be involved in the discrimination between typhoidal and non-typhoidal *Salmonella enterica* serovars.

In conclusion, we have identified and characterized the gut vascular barrier and we have shown that it plays a fundamental role in controlling the spreading of molecules and bacteria to systemic sites. To have a complete picture of the features of this newly discovered barrier we will analyze the gene expression profile of mouse isolated intestinal ECs with a next-generation sequencing approach.

Moreover, we showed that *Salmonella typhimurium* infection could modify GVB integrity. One way by which *S. typhimurium* is able to modify the barrier properties of the intestinal blood vessels is through the negative regulation of the Wnt/β-catenin signaling pathway. *In vitro* we have identified the TTSS-2 as the possible negative regulator of the β-catenin signaling pathway, therefore in next future we will investigate the role of TTSS-2 *in vivo*.

Another interesting point that we would like to examine in depth is the role of the microbiota in the maturation and maintenance of the GVB. To this aim we will analyze the

barrier properties in the endothelium of newborns, before and after weaning and in mice treated with antibiotics.

Finally, we will evaluate whether also in the human intestine there is a GVU as in the mouse gut and if it will be the case we will use an organ culture method developed in our laboratory to infect human intestine with *Salmonella* to analyze the endothelial cells phenotype.

BIBLIOGRAPHY

Abbott, N.J., A.A. Patabendige, D.E. Dolman, S.R. Yusof, and D.J. Begley. 2010. Structure and function of the blood-brain barrier. *Neurobiol Dis* 37:13-25.

Abbott, N.J., L. Ronnback, and E. Hansson. 2006. Astrocyte-endothelial interactions at the blood-brain barrier. *Nat Rev Neurosci* 7:41-53.

Alrutz, M.A., and R.R. Isberg. 1998. Involvement of focal adhesion kinase in invasin-mediated uptake. *Proc Natl Acad Sci U S A* 95:13658-13663.

Alvarez, J.I., A. Dodelet-Devillers, H. Kebir, I. Ifergan, P.J. Fabre, S. Terouz, M. Sabbagh, K. Wosik, L. Bourbonniere, M. Bernard, J. van Horssen, H.E. de Vries, F. Charron, and A. Prat. 2011. The Hedgehog pathway promotes blood-brain barrier integrity and CNS immune quiescence. *Science* 334:1727-1731.

Armulik, A., G. Genove, M. Mae, M.H. Nisancioglu, E. Wallgard, C. Niaudet, L. He, J. Norlin, P. Lindblom, K. Strittmatter, B.R. Johansson, and C. Betsholtz. 2010. Pericytes regulate the blood-brain barrier. *Nature* 468:557-561.

Arques, J.L., I. Hautefort, K. Ivory, E. Bertelli, M. Regoli, S. Clare, J.C. Hinton, and C. Nicoletti. 2009. Salmonella induces flagellin- and MyD88-dependent migration of bacteria-capturing dendritic cells into the gut lumen. *Gastroenterology* 137:579-587, 587 e571-572.

Artis, D. 2008. Epithelial-cell recognition of commensal bacteria and maintenance of immune homeostasis in the gut. *Nat Rev Immunol* 8:411-420.

Atarashi, K., J. Nishimura, T. Shima, Y. Umesaki, M. Yamamoto, M. Onoue, H. Yagita, N. Ishii, R. Evans, K. Honda, and K. Takeda. 2008. ATP drives lamina propria T(H)17 cell differentiation. *Nature* 455:808-812.

Bakowski, M.A., V. Braun, and J.H. Brumell. 2008. Salmonella-containing vacuoles: directing traffic and nesting to grow. *Traffic* 9:2022-2031.

Bergstrom, K.S., V. Kissoon-Singh, D.L. Gibson, C. Ma, M. Montero, H.P. Sham, N. Ryz, T. Huang, A. Velcich, B.B. Finlay, K. Chadee, and B.A. Vallance. 2010. Muc2 protects against lethal infectious colitis by disassociating pathogenic and commensal bacteria from the colonic mucosa. *PLoS Pathog* 6:e1000902.

Bogunovic, M., F. Ginhoux, J. Helft, L. Shang, D. Hashimoto, M. Greter, K. Liu, C. Jakubzick, M.A. Ingersoll, M. Leboeuf, E.R. Stanley, M. Nussenzweig, S.A. Lira, G.J. Randolph, and M. Merad. 2009. Origin of the lamina propria dendritic cell network. *Immunity* 31:513-525.

Boyle, E.C., N.F. Brown, and B.B. Finlay. 2006. Salmonella enterica serovar Typhimurium effectors SopB, SopE, SopE2 and SipA disrupt tight junction structure and function. *Cell Microbiol* 8:1946-1957.

Brown, E.M., M. Sadarangani, and B.B. Finlay. 2013. The role of the immune system in governing host-microbe interactions in the intestine. *Nature immunology* 14:660-667.

Bush, T.G., T.C. Savidge, T.C. Freeman, H.J. Cox, E.A. Campbell, L. Mucke, M.H. Johnson, and M.V. Sofroniew. 1998. Fulminant jejuno-ileitis following ablation of enteric glia in adult transgenic mice. *Cell* 93:189-201.

Callery, M.P., T. Kamei, and M.W. Flye. 1989. The effect of portacaval shunt on delayed-hypersensitivity responses following antigen feeding. *The Journal of surgical research* 46:391-394.

Cardoso, F.L., D. Brites, and M.A. Brito. 2010. Looking at the blood-brain barrier: molecular anatomy and possible investigation approaches. *Brain Res Rev* 64:328-363.

Carson-Walter, E.B., J. Hampton, E. Shue, D.M. Geynisman, P.K. Pillai, R. Sathanoori, S.L. Madden, R.L. Hamilton, and K.A. Walter. 2005. Plasmalemmal vesicle associated protein-1 is a novel marker implicated in brain tumor angiogenesis. *Clin Cancer Res* 11:7643-7650.

Cattelino, A., S. Liebner, R. Gallini, A. Zanetti, G. Balconi, A. Corsi, P. Bianco, H. Wolburg, R. Moore, B. Oreda, R. Kemler, and E. Dejana. 2003. The conditional inactivation of the beta-catenin gene in endothelial cells causes a defective vascular pattern and increased vascular fragility. *J Cell Biol* 162:1111-1122.

Cerovic, V., S.A. Houston, C.L. Scott, A. Aumeunier, U. Yrlid, A.M. Mowat, and S.W. Milling. 2013. Intestinal CD103(-) dendritic cells migrate in lymph and prime effector T cells. *Mucosal Immunol* 6:104-113.

Chang, S.Y., J.H. Song, B. Guleng, C.A. Cotoner, S. Arihiro, Y. Zhao, H.S. Chiang, M. O'Keeffe, G. Liao, C.L. Karp, M.N. Kweon, A.H. Sharpe, A. Bhan, C. Terhorst, and H.C. Reinecker. 2013. Circulatory antigen processing by mucosal dendritic cells controls CD8(+) T cell activation. *Immunity* 38:153-165.

Chieppa, M., M. Rescigno, A.Y. Huang, and R.N. Germain. 2006. Dynamic imaging of dendritic cell extension into the small bowel lumen in response to epithelial cell TLR engagement. *The Journal of experimental medicine* 203:2841-2852.

Cirillo, D.M., R.H. Valdivia, D.M. Monack, and S. Falkow. 1998. Macrophage-dependent induction of the Salmonella pathogenicity island 2 type III secretion system and its role in intracellular survival. *Molecular microbiology* 30:175-188.

Collins, F.M., and P.B. Carter. 1978. Growth of salmonellae in orally infected germfree mice. *Infect Immun* 21:41-47.

Cong, Y., T. Feng, K. Fujihashi, T.R. Schoeb, and C.O. Elson. 2009. A dominant, coordinated T regulatory cell-IgA response to the intestinal microbiota. *Proc Natl Acad Sci U S A* 106:19256-19261.

Coombes, J.L., K.R. Siddiqui, C.V. Arancibia-Carcamo, J. Hall, C.M. Sun, Y. Belkaid, and F. Powrie. 2007. A functionally specialized population of mucosal CD103+ DCs induces Foxp3+ regulatory T cells via a TGF-beta and retinoic acid-dependent mechanism. *The Journal of experimental medicine* 204:1757-1764.

Corada, M., M. Mariotti, G. Thurston, K. Smith, R. Kunkel, M. Brockhaus, M.G. Lampugnani, I. Martin-Padura, A. Stoppacciaro, L. Ruco, D.M. McDonald, P.A. Ward, and E. Dejana. 1999. Vascular endothelial-cadherin is an important determinant of microvascular integrity in vivo. *Proc Natl Acad Sci U S A* 96:9815-9820.

Corr, S.C., E.M. Palsson-McDermott, I. Grishina, S.P. Barry, G. Aviello, N.J. Bernard, P.G. Casey, J.B. Ward, S.J. Keely, S. Dandekar, P.G. Fallon, and L.A. O'Neill. 2013. MyD88 adaptor-like (Mal) functions in the epithelial barrier and contributes to intestinal integrity via protein kinase C. *Mucosal Immunol*

Daneman, R., D. Agalliu, L. Zhou, F. Kuhnert, C.J. Kuo, and B.A. Barres. 2009. Wnt/beta-catenin signaling is required for CNS, but not non-CNS, angiogenesis. *Proc Natl Acad Sci U S A* 106:641-646.

Daneman, R., and M. Rescigno. 2009. The gut immune barrier and the blood-brain barrier: are they so different? *Immunity* 31:722-735.

Daneman, R., L. Zhou, A.A. Kebede, and B.A. Barres. 2010. Pericytes are required for blood-brain barrier integrity during embryogenesis. *Nature* 468:562-566.

Dehouck, M.P., S. Meresse, P. Delorme, J.C. Fruchart, and R. Cecchelli. 1990. An easier, reproducible, and mass-production method to study the blood-brain barrier in vitro. *Journal of neurochemistry* 54:1798-1801.

Deiwick, J., T. Nikolaus, S. Erdogan, and M. Hensel. 1999. Environmental regulation of Salmonella pathogenicity island 2 gene expression. *Molecular microbiology* 31:1759-1773.

Dejana, E. 1996. Endothelial adherens junctions: implications in the control of vascular permeability and angiogenesis. *The Journal of clinical investigation* 98:1949-1953.

Diehl, G.E., R.S. Longman, J.X. Zhang, B. Breart, C. Galan, A. Cuesta, S.R. Schwab, and D.R. Littman. 2013. Microbiota restricts trafficking of bacteria to mesenteric lymph nodes by CX(3)CR1(hi) cells. *Nature* 494:116-120.

Dohgu, S., F. Takata, A. Yamauchi, S. Nakagawa, T. Egawa, M. Naito, T. Tsuruo, Y. Sawada, M. Niwa, and Y. Kataoka. 2005. Brain pericytes contribute to the induction and up-regulation of blood-brain barrier functions through transforming growth factor-beta production. *Brain research* 1038:208-215.

Duan, Y., A.P. Liao, S. Kuppireddi, Z. Ye, M.J. Ciancio, and J. Sun. 2007. beta-Catenin activity negatively regulates bacteria-induced inflammation. *Lab Invest* 87:613-624.

Eberl, G. 2010. A new vision of immunity: homeostasis of the superorganism. *Mucosal Immunol* 3:450-460.

Engelhardt, B. 2003. Development of the blood-brain barrier. *Cell and tissue research* 314:119-129.

Fagarasan, S., and T. Honjo. 2003. Intestinal IgA synthesis: regulation of front-line body defences. *Nat Rev Immunol* 3:63-72.

Farache, J., I. Koren, I. Milo, I. Gurevich, K.W. Kim, E. Zigmond, G.C. Furtado, S.A. Lira, and G. Shakhar. 2013. Luminal bacteria recruit CD103+ dendritic cells into the intestinal epithelium to sample bacterial antigens for presentation. *Immunity* 38:581-595.

Fernandez, L., S. Langa, V. Martin, A. Maldonado, E. Jimenez, R. Martin, and J.M. Rodriguez. 2013. The human milk microbiota: origin and potential roles in health and disease. *Pharmacol Res* 69:1-10.

Fevr, T., S. Robine, D. Louvard, and J. Huelsken. 2007. Wnt/beta-catenin is essential for intestinal homeostasis and maintenance of intestinal stem cells. *Mol Cell Biol* 27:7551-7559.

Flamant, M., P. Aubert, M. Rolli-Derkinderen, A. Bourreille, M.R. Neunlist, M.M. Mahe, G. Meurette, B. Marteyn, T. Savidge, J.P. Galmiche, P.J. Sansonetti, and M. Neunlist. 2011. Enteric glia protect against Shigella flexneri invasion in intestinal epithelial cells: a role for S-nitrosoglutathione. *Gut* 60:473-484.

Fujihashi, K., T. Dohi, P.D. Rennert, M. Yamamoto, T. Koga, H. Kiyono, and J.R. McGhee. 2001. Peyer's patches are required for oral tolerance to proteins. *Proc Natl Acad Sci U S A* 98:3310-3315.

Furuse, M., K. Fujita, T. Hiiragi, K. Fujimoto, and S. Tsukita. 1998. Claudin-1 and -2: novel integral membrane proteins localizing at tight junctions with no sequence similarity to occludin. *J Cell Biol* 141:1539-1550.

Furuse, M., T. Hirase, M. Itoh, A. Nagafuchi, S. Yonemura, S. Tsukita, and S. Tsukita. 1993. Occludin: a novel integral membrane protein localizing at tight junctions. *J Cell Biol* 123:1777-1788.

Gaboriau-Routhiau, V., S. Rakotobe, E. Lecuyer, I. Mulder, A. Lan, C. Bridonneau, V. Rochet, A. Pisi, M. De Paepe, G. Brandi, G. Eberl, J. Snel, D. Kelly, and N. Cerf-Bensussan. 2009. The key role of segmented filamentous bacteria in the coordinated maturation of gut helper T cell responses. *Immunity* 31:677-689.

Galan, J.E. 2001. Salmonella interactions with host cells: type III secretion at work. *Annual review of cell and developmental biology* 17:53-86.

Galan, J.E., and R. Curtiss, 3rd. 1989. Cloning and molecular characterization of genes whose products allow Salmonella typhimurium to penetrate tissue culture cells. *Proc Natl Acad Sci U S A* 86:6383-6387.

Gewirtz, A.T., T.A. Navas, S. Lyons, P.J. Godowski, and J.L. Madara. 2001. Cutting edge: bacterial flagellin activates basolaterally expressed TLR5 to induce epithelial proinflammatory gene expression. *J Immunol* 167:1882-1885.

Giannotta, M., M. Trani, and E. Dejana. 2013. VE-cadherin and endothelial adherens junctions: active guardians of vascular integrity. *Dev Cell* 26:441-454.

Gibson, D.L., C. Ma, K.S. Bergstrom, J.T. Huang, C. Man, and B.A. Vallance. 2008. MyD88 signalling plays a critical role in host defence by controlling pathogen burden and promoting epithelial cell homeostasis during Citrobacter rodentium-induced colitis. *Cell Microbiol* 10:618-631.

Gilbreath, J.J., W.L. Cody, D.S. Merrell, and D.R. Hendrixson. 2011. Change is good: variations in common biological mechanisms in the epsilonproteobacterial genera Campylobacter and Helicobacter. *Microbiology and molecular biology reviews : MMBR* 75:84-132.

Goto, Y., and H. Kiyono. 2012. Epithelial barrier: an interface for the cross-communication between gut flora and immune system. *Immunological reviews* 245:147-163.

Goubier, A., B. Dubois, H. Gheit, G. Joubert, F. Villard-Truc, C. Asselin-Paturel, G. Trinchieri, and D. Kaiserlian. 2008. Plasmacytoid dendritic cells mediate oral tolerance. *Immunity* 29:464-475.

Hadis, U., B. Wahl, O. Schulz, M. Hardtke-Wolenski, A. Schippers, N. Wagner, W. Muller, T. Sparwasser, R. Forster, and O. Pabst. 2011. Intestinal tolerance requires gut homing and expansion of FoxP3+ regulatory T cells in the lamina propria. *Immunity* 34:237-246.

Hallmann, R., D.N. Mayer, E.L. Berg, R. Broermann, and E.C. Butcher. 1995. Novel mouse endothelial cell surface marker is suppressed during differentiation of the blood brain barrier. *Dev Dyn* 202:325-332.

Hanani, M., and A. Reichenbach. 1994. Morphology of horseradish peroxidase (HRP)-injected glial cells in the myenteric plexus of the guinea-pig. *Cell and tissue research* 278:153-160.

Hapfelmeier, S., A.J. Muller, B. Stecher, P. Kaiser, M. Barthel, K. Endt, M. Eberhard, R. Robbiani, C.A. Jacobi, M. Heikenwalder, C. Kirschning, S. Jung, T. Stallmach, M. Kremer, and W.D. Hardt. 2008. Microbe sampling by mucosal dendritic cells is a discrete, MyD88-independent step in DeltainvG S. Typhimurium colitis. *The Journal of experimental medicine* 205:437-450.

Harada, N., Y. Tamai, T. Ishikawa, B. Sauer, K. Takaku, M. Oshima, and M.M. Taketo. 1999. Intestinal polyposis in mice with a dominant stable mutation of the beta-catenin gene. *EMBO J* 18:5931-5942.

Hathaway, C.A., C.B. Appleyard, W.H. Percy, and J.L. Williams. 1999. Experimental colitis increases blood-brain barrier permeability in rabbits. *The American journal of physiology* 276:G1174-1180.

Herrnberger, L., K. Ebner, B. Junglas, and E.R. Tamm. 2012a. The role of plasmalemma vesicle-associated protein (PLVAP) in endothelial cells of Schlemm's canal and ocular capillaries. *Exp Eye Res* 105:27-33.

Herrnberger, L., R. Seitz, S. Kuespert, M.R. Bosl, R. Fuchshofer, and E.R. Tamm. 2012b. Lack of endothelial diaphragms in fenestrae and caveolae of mutant Plvap-deficient mice. *Histochem Cell Biol* 138:709-724.

Hersh, D., D.M. Monack, M.R. Smith, N. Ghori, S. Falkow, and A. Zychlinsky. 1999. The Salmonella invasin SipB induces macrophage apoptosis by binding to caspase-1. *Proc Natl Acad Sci U S A* 96:2396-2401.

Hoeke, L., J. Sharbati, K. Pawar, A. Keller, R. Einspanier, and S. Sharbati. 2013. Intestinal Salmonella typhimurium infection leads to miR-29a induced caveolin 2 regulation. *PLoS One* 8:e67300.

Hooper, L.V. 2009. Do symbiotic bacteria subvert host immunity? *Nature reviews. Microbiology* 7:367-374.

Hooper, L.V., D.R. Littman, and A.J. Macpherson. 2012. Interactions between the microbiota and the immune system. *Science* 336:1268-1273.

Hooper, L.V., and A.J. Macpherson. 2010. Immune adaptations that maintain homeostasis with the intestinal microbiota. *Nat Rev Immunol* 10:159-169.

Hooper, L.V., M.H. Wong, A. Thelin, L. Hansson, P.G. Falk, and J.I. Gordon. 2001. Molecular analysis of commensal host-microbial relationships in the intestine. *Science* 291:881-884.

Iliev, I.D., G. Matteoli, and M. Rescigno. 2007. The yin and yang of intestinal epithelial cells in controlling dendritic cell function. *The Journal of experimental medicine* 204:2253-2257.

Iliev, I.D., E. Mileti, G. Matteoli, M. Chieppa, and M. Rescigno. 2009a. Intestinal epithelial cells promote colitis-protective regulatory T-cell differentiation through dendritic cell conditioning. *Mucosal Immunol* 2:340-350.

Iliev, I.D., I. Spadoni, E. Mileti, G. Matteoli, A. Sonzogni, G.M. Sampietro, D. Foschi, F. Caprioli, G. Viale, and M. Rescigno. 2009b. Human intestinal epithelial cells promote the differentiation of tolerogenic dendritic cells. *Gut* 58:1481-1489.

Ioannidou, S., K. Deinhardt, J. Miotla, J. Bradley, E. Cheung, S. Samuelsson, Y.S. Ng, and D.T. Shima. 2006. An in vitro assay reveals a role for the diaphragm protein PV-1 in endothelial fenestra morphogenesis. *Proc Natl Acad Sci U S A* 103:16770-16775.

Ivanov, II, K. Atarashi, N. Manel, E.L. Brodie, T. Shima, U. Karaoz, D. Wei, K.C. Goldfarb, C.A. Santee, S.V. Lynch, T. Tanoue, A. Imaoka, K. Itoh, K. Takeda, Y. Umesaki, K. Honda, and D.R. Littman. 2009. Induction of intestinal Th17 cells by segmented filamentous bacteria. *Cell* 139:485-498.

Iwasaki, A., and B.L. Kelsall. 2001. Unique functions of CD11b+, CD8 alpha+, and double-negative Peyer's patch dendritic cells. *J Immunol* 166:4884-4890.

Jaensson, E., H. Uronen-Hansson, O. Pabst, B. Eksteen, J. Tian, J.L. Coombes, P.L. Berg, T. Davidsson, F. Powrie, B. Johansson-Lindbom, and W.W. Agace. 2008. Small intestinal CD103+ dendritic cells display unique functional properties that are conserved between mice and humans. *The Journal of experimental medicine* 205:2139-2149.

Jang, M.H., M.N. Kweon, K. Iwatani, M. Yamamoto, K. Terahara, C. Sasakawa, T. Suzuki, T. Nochi, Y. Yokota, P.D. Rennert, T. Hiroi, H. Tamagawa, H. Iijima, J. Kunisawa, Y. Yuki, and H. Kiyono. 2004. Intestinal villous M cells: an antigen entry site in the mucosal epithelium. *Proc Natl Acad Sci U S A* 101:6110-6115.

Janzer, R.C., and M.C. Raff. 1987. Astrocytes induce blood-brain barrier properties in endothelial cells. *Nature* 325:253-257.

Jiang, S., M.I. Khan, Y. Lu, E.S. Werstiuk, and M.P. Rathbone. 2005. Acceleration of blood-brain barrier formation after transplantation of enteric glia into spinal cords of rats. *Exp Brain Res* 162:56-62.

Jimenez, E., L. Fernandez, M.L. Marin, R. Martin, J.M. Odriozola, C. Nueno-Palop, A. Narbad, M. Olivares, J. Xaus, and J.M. Rodriguez. 2005. Isolation of commensal bacteria from umbilical cord blood of healthy neonates born by cesarean section. *Curr Microbiol* 51:270-274.

Johansson, M.E., J.M. Larsson, and G.C. Hansson. 2011. The two mucus layers of colon are organized by the MUC2 mucin, whereas the outer layer is a legislator of host-microbial interactions. *Proc Natl Acad Sci U S A* 108 Suppl 1:4659-4665.

Johansson, M.E., M. Phillipson, J. Petersson, A. Velcich, L. Holm, and G.C. Hansson. 2008. The inner of the two Muc2 mucin-dependent mucus layers in colon is devoid of bacteria. *Proc Natl Acad Sci U S A* 105:15064-15069.

Johansson-Lindbom, B., M. Svensson, O. Pabst, C. Palmqvist, G. Marquez, R. Forster, and W.W. Agace. 2005. Functional specialization of gut CD103+ dendritic cells in the regulation of tissue-selective T cell homing. *The Journal of experimental medicine* 202:1063-1073.

Jones, B.D., N. Ghori, and S. Falkow. 1994. Salmonella typhimurium initiates murine infection by penetrating and destroying the specialized epithelial M cells of the Peyer's patches. *The Journal of experimental medicine* 180:15-23.

Kelly, D., J.I. Campbell, T.P. King, G. Grant, E.A. Jansson, A.G. Coutts, S. Pettersson, and S. Conway. 2004. Commensal anaerobic gut bacteria attenuate inflammation by regulating nuclear-cytoplasmic shuttling of PPAR-gamma and RelA. *Nature immunology* 5:104-112.

Kelsall, B.L., and M. Rescigno. 2004. Mucosal dendritic cells in immunity and inflammation. *Nature immunology* 5:1091-1095.

Kniesel, U., W. Risau, and H. Wolburg. 1996. Development of blood-brain barrier tight junctions in the rat cortex. *Brain research. Developmental brain research* 96:229-240.

Kobayashi, K.S., M. Chamaillard, Y. Ogura, O. Henegariu, N. Inohara, G. Nunez, and R.A. Flavell. 2005. Nod2-dependent regulation of innate and adaptive immunity in the intestinal tract. *Science* 307:731-734.

Laffont, S., K.R. Siddiqui, and F. Powrie. 2010. Intestinal inflammation abrogates the tolerogenic properties of MLN CD103+ dendritic cells. *European journal of immunology* 40:1877-1883.

Lee, C.A., M. Silva, A.M. Siber, A.J. Kelly, E. Galyov, and B.A. McCormick. 2000. A secreted Salmonella protein induces a proinflammatory response in epithelial cells, which promotes neutrophil migration. *Proc Natl Acad Sci U S A* 97:12283-12288.

Lee, J., J.H. Mo, K. Katakura, I. Alkalay, A.N. Rucker, Y.T. Liu, H.K. Lee, C. Shen, G. Cojocaru, S. Shenouda, M. Kagnoff, L. Eckmann, Y. Ben-Neriah, and E. Raz. 2006. Maintenance of colonic homeostasis by distinctive apical TLR9 signalling in intestinal epithelial cells. *Nature cell biology* 8:1327-1336.

Li, W., S.T. Chou, C. Wang, C.S. Kuhr, and J.D. Perkins. 2004. Role of the liver in peripheral tolerance: induction through oral antigen feeding. *American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons* 4:1574-1582.

Liebner, S., M. Corada, T. Bangsow, J. Babbage, A. Taddei, C.J. Czupalla, M. Reis, A. Felici, H. Wolburg, M. Fruttiger, M.M. Taketo, H. von Melchner, K.H. Plate, H. Gerhardt, and E. Dejana. 2008. Wnt/beta-catenin signaling controls development of the blood-brain barrier. *J Cell Biol* 183:409-417.

Liebner, S., C.J. Czupalla, and H. Wolburg. 2011. Current concepts of blood-brain barrier development. *The International journal of developmental biology* 55:467-476.

Liebner, S., A. Fischmann, G. Rascher, F. Duffner, E.H. Grote, H. Kalbacher, and H. Wolburg. 2000. Claudin-1 and claudin-5 expression and tight junction morphology are altered in blood vessels of human glioblastoma multiforme. *Acta Neuropathol* 100:323-331.

Liebner, S., and K.H. Plate. 2010. Differentiation of the brain vasculature: the answer came blowing by the Wnt. *Journal of angiogenesis research* 2:1.

Lim, J.S., H.E. Choy, S.C. Park, J.M. Han, I.S. Jang, and K.A. Cho. 2010. Caveolae-mediated entry of Salmonella typhimurium into senescent nonphagocytotic host cells. *Aging Cell* 9:243-251.

Liu, X., R. Lu, S. Wu, and J. Sun. 2010. Salmonella regulation of intestinal stem cells through the Wnt/beta-catenin pathway. *FEBS Lett* 584:911-916.

Maaser, C., J. Heidemann, C. von Eiff, A. Lugering, T.W. Spahn, D.G. Binion, W. Domschke, N. Lugering, and T. Kucharzik. 2004. Human intestinal microvascular endothelial cells express Toll-like receptor 5: a binding partner for bacterial flagellin. *J Immunol* 172:5056-5062.

MacDonald, B.T., K. Tamai, and X. He. 2009. Wnt/beta-catenin signaling: components, mechanisms, and diseases. *Dev Cell* 17:9-26.

Macpherson, A.J., D. Gatto, E. Sainsbury, G.R. Harriman, H. Hengartner, and R.M. Zinkernagel. 2000. A primitive T cell-independent mechanism of intestinal mucosal IgA responses to commensal bacteria. *Science* 288:2222-2226.

Macpherson, A.J., M.B. Geuking, E. Slack, S. Hapfelmeier, and K.D. McCoy. 2012. The habitat, double life, citizenship, and forgetfulness of IgA. *Immunological reviews* 245:132-146.

Macpherson, A.J., and K. Smith. 2006. Mesenteric lymph nodes at the center of immune anatomy. *The Journal of experimental medicine* 203:497-500.

Macpherson, A.J., and T. Uhr. 2004. Induction of protective IgA by intestinal dendritic cells carrying commensal bacteria. *Science* 303:1662-1665.

Martin-Padura, I., S. Lostaglio, M. Schneemann, L. Williams, M. Romano, P. Fruscella, C. Panzeri, A. Stoppacciaro, L. Ruco, A. Villa, D. Simmons, and E. Dejana. 1998. Junctional adhesion molecule, a novel member of the immunoglobulin superfamily that distributes at intercellular junctions and modulates monocyte transmigration. *J Cell Biol* 142:117-127.

Martinoli, C., A. Chiavelli, and M. Rescigno. 2007. Entry route of Salmonella typhimurium directs the type of induced immune response. *Immunity* 27:975-984.

Matamoros, S., C. Gras-Leguen, F. Le Vacon, G. Potel, and M.F. de La Cochetiere. 2013. Development of intestinal microbiota in infants and its impact on health. *Trends Microbiol* 21:167-173.

McDole, J.R., L.W. Wheeler, K.G. McDonald, B. Wang, V. Konjufca, K.A. Knoop, R.D. Newberry, and M.J. Miller. 2012. Goblet cells deliver luminal antigen to CD103+ dendritic cells in the small intestine. *Nature* 483:345-349.

Mizee, M.R., D. Wooldrik, K.A. Lakeman, B. van het Hof, J.A. Drexhage, D. Geerts, M. Bugiani, E. Aronica, R.E. Mebius, A. Prat, H.E. de Vries, and A. Reijerkerk. 2013. Retinoic acid induces blood-brain barrier development. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 33:1660-1671.

Monvoisin, A., J.A. Alva, J.J. Hofmann, A.C. Zovein, T.F. Lane, and M.L. Iruela-Arispe. 2006. VE-cadherin-CreERT2 transgenic mouse: a model for inducible recombination in the endothelium. *Dev Dyn* 235:3413-3422.

Mora, J.R., M. Iwata, B. Eksteen, S.Y. Song, T. Junt, B. Senman, K.L. Otipoby, A. Yokota, H. Takeuchi, P. Ricciardi-Castagnoli, K. Rajewsky, D.H. Adams, and U.H. von Andrian. 2006. Generation of gut-homing IgA-secreting B cells by intestinal dendritic cells. *Science* 314:1157-1160.

Morita, K., H. Sasaki, M. Furuse, and S. Tsukita. 1999. Endothelial claudin: claudin-5/TMVCF constitutes tight junction strands in endothelial cells. *J Cell Biol* 147:185-194.

Mowat, A.M. 2003. Anatomical basis of tolerance and immunity to intestinal antigens. *Nat Rev Immunol* 3:331-341.

Muller, A.J., P. Kaiser, K.E. Dittmar, T.C. Weber, S. Haueter, K. Endt, P. Songhet, C. Zellweger, M. Kremer, H.J. Fehling, and W.D. Hardt. 2012. Salmonella gut invasion involves TTSS-2-dependent epithelial traversal, basolateral exit, and uptake by epithelium-sampling lamina propria phagocytes. *Cell host & microbe* 11:19-32.

Nag, S., R. Venugopalan, and D.J. Stewart. 2007. Increased caveolin-1 expression precedes decreased expression of occludin and claudin-5 during blood-brain barrier breakdown. *Acta Neuropathol* 114:459-469.

Nagyoszi, P., I. Wilhelm, A.E. Farkas, C. Fazakas, N.T. Dung, J. Hasko, and I.A. Krizbai. 2010. Expression and regulation of toll-like receptors in cerebral endothelial cells. *Neurochem Int* 57:556-564.

Natividad, J.M., and E.F. Verdu. 2013. Modulation of intestinal barrier by intestinal microbiota: pathological and therapeutic implications. *Pharmacol Res* 69:42-51.

Navarro, P., L. Caveda, F. Breviario, I. Mandoteanu, M.G. Lampugnani, and E. Dejana. 1995. Catenin-dependent and -independent functions of vascular endothelial cadherin. *J Biol Chem* 270:30965-30972.

Neunlist, M., P. Aubert, S. Bonnaud, L. Van Landeghem, E. Coron, T. Wedel, P. Naveilhan, A. Ruhl, B. Lardeux, T. Savidge, F. Paris, and J.P. Galmiche. 2007. Enteric glia inhibit intestinal epithelial cell proliferation partly through a TGF-beta1-dependent pathway. *Am J Physiol Gastrointest Liver Physiol* 292:G231-241.

Neunlist, M., L. Van Landeghem, A. Bourreille, and T. Savidge. 2008. Neuro-glial crosstalk in inflammatory bowel disease. *J Intern Med* 263:577-583.

Niess, J.H., and G. Adler. 2010. Enteric flora expands gut lamina propria CX3CR1+ dendritic cells supporting inflammatory immune responses under normal and inflammatory conditions. *J Immunol* 184:2026-2037.

Niess, J.H., S. Brand, X. Gu, L. Landsman, S. Jung, B.A. McCormick, J.M. Vyas, M. Boes, H.L. Ploegh, J.G. Fox, D.R. Littman, and H.C. Reinecker. 2005. CX3CR1-mediated dendritic cell access to the intestinal lumen and bacterial clearance. *Science* 307:254-258.

Nitta, T., M. Hata, S. Gotoh, Y. Seo, H. Sasaki, N. Hashimoto, M. Furuse, and S. Tsukita. 2003. Size-selective loosening of the blood-brain barrier in claudin-5-deficient mice. *J Cell Biol* 161:653-660.

Ochman, H., F.C. Soncini, F. Solomon, and E.A. Groisman. 1996. Identification of a pathogenicity island required for Salmonella survival in host cells. *Proc Natl Acad Sci U S A* 93:7800-7804.

Ogawa, H., P. Rafiee, J. Heidemann, P.J. Fisher, N.A. Johnson, M.F. Otterson, B. Kalyanaraman, K.A. Pritchard, Jr., and D.G. Binion. 2003. Mechanisms of endotoxin tolerance in human intestinal microvascular endothelial cells. *J Immunol* 170:5956-5964.

Oshima, T., F.S. Laroux, L.L. Coe, Z. Morise, S. Kawachi, P. Bauer, M.B. Grisham, R.D. Specian, P. Carter, S. Jennings, D.N. Granger, T. Joh, and J.S. Alexander. 2001. Interferon-gamma and interleukin-10 reciprocally regulate endothelial junction integrity and barrier function. *Microvascular research* 61:130-143.

Pabst, O., and A.M. Mowat. 2012. Oral tolerance to food protein. *Mucosal Immunol* 5:232-239.

Paolinelli, R., M. Corada, F. Orsenigo, and E. Dejana. 2011. The molecular basis of the blood brain barrier differentiation and maintenance. Is it still a mystery? *Pharmacological research : the official journal of the Italian Pharmacological Society* 63:165-171.

Pardridge, W.M. 2005. The blood-brain barrier: bottleneck in brain drug development. *NeuroRx : the journal of the American Society for Experimental NeuroTherapeutics* 2:3-14.

Parton, R.G., and K. Simons. 2007. The multiple faces of caveolae. *Nature reviews. Molecular cell biology* 8:185-194.

Pascopella, L., B. Raupach, N. Ghori, D. Monack, S. Falkow, and P.L. Small. 1995. Host restriction phenotypes of Salmonella typhi and Salmonella gallinarum. *Infect Immun* 63:4329-4335.

Patel, R.M., L.S. Myers, A.R. Kurundkar, A. Maheshwari, A. Nusrat, and P.W. Lin. 2012. Probiotic bacteria induce maturation of intestinal claudin 3 expression and barrier function. *Am J Pathol* 180:626-635.

Peterson, D.A., N.P. McNulty, J.L. Guruge, and J.I. Gordon. 2007. IgA response to symbiotic bacteria as a mediator of gut homeostasis. *Cell host & microbe* 2:328-339.

Pinto, D., A. Gregorieff, H. Begthel, and H. Clevers. 2003. Canonical Wnt signals are essential for homeostasis of the intestinal epithelium. *Genes & development* 17:1709-1713.

Putsep, K., L.G. Axelsson, A. Boman, T. Midtvedt, S. Normark, H.G. Boman, and M. Andersson. 2000. Germ-free and colonized mice generate the same products from enteric prodefensins. *J Biol Chem* 275:40478-40482.

Rakoff-Nahoum, S., J. Paglino, F. Eslami-Varzaneh, S. Edberg, and R. Medzhitov. 2004. Recognition of commensal microflora by toll-like receptors is required for intestinal homeostasis. *Cell* 118:229-241.

Rescigno, M., M. Urbano, B. Valzasina, M. Francolini, G. Rotta, R. Bonasio, F. Granucci, J.P. Kraehenbuhl, and P. Ricciardi-Castagnoli. 2001. Dendritic cells express tight junction proteins and penetrate gut epithelial monolayers to sample bacteria. *Nature immunology* 2:361-367.

Rimoldi, M., M. Chieppa, V. Salucci, F. Avogadri, A. Sonzogni, G.M. Sampietro, A. Nespoli, G. Viale, P. Allavena, and M. Rescigno. 2005. Intestinal immune homeostasis is regulated by the crosstalk between epithelial cells and dendritic cells. *Nature immunology* 6:507-514.

Rubin, L.L., D.E. Hall, S. Porter, K. Barbu, C. Cannon, H.C. Horner, M. Janatpour, C.W. Liaw, K. Manning, J. Morales, and et al. 1991. A cell culture model of the blood-brain barrier. *J Cell Biol* 115:1725-1735.

Saitou, M., M. Furuse, H. Sasaki, J.D. Schulzke, M. Fromm, H. Takano, T. Noda, and S. Tsukita. 2000. Complex phenotype of mice lacking occludin, a component of tight junction strands. *Molecular biology of the cell* 11:4131-4142.

Salazar-Gonzalez, R.M., J.H. Niess, D.J. Zammit, R. Ravindran, A. Srinivasan, J.R. Maxwell, T. Stoklasek, R. Yadav, I.R. Williams, X. Gu, B.A. McCormick, M.A. Pazos, A.T. Vella, L. Lefrancois, H.C. Reinecker, and S.J. McSorley. 2006. CCR6-mediated dendritic cell activation of pathogen-specific T cells in Peyer's patches. *Immunity* 24:623-632.

Salzman, N.H., D. Ghosh, K.M. Huttner, Y. Paterson, and C.L. Bevins. 2003. Protection against enteric salmonellosis in transgenic mice expressing a human intestinal defensin. *Nature* 422:522-526.

Savidge, T.C., P. Newman, C. Pothoulakis, A. Ruhl, M. Neunlist, A. Bourreille, R. Hurst, and M.V. Sofroniew. 2007. Enteric glia regulate intestinal barrier function and inflammation via release of S-nitrosoglutathione. *Gastroenterology* 132:1344-1358.

Schulz, O., E. Jaensson, E.K. Persson, X. Liu, T. Worbs, W.W. Agace, and O. Pabst. 2009. Intestinal CD103+, but not CX3CR1+, antigen sampling cells migrate in lymph and serve classical dendritic cell functions. *The Journal of experimental medicine* 206:3101-3114.

Shan, M., M. Gentile, J.R. Yeiser, A.C. Walland, V.U. Bornstein, K. Chen, B. He, L. Cassis, A. Bigas, M. Cols, L. Comerma, B. Huang, J.M. Blander, H. Xiong, L. Mayer, C. Berin, L.H. Augenlicht, A. Velcich, and A. Cerutti. 2013. Mucus Enhances Gut Homeostasis and Oral Tolerance by Delivering Immunoregulatory Signals. *Science*

Shea, J.E., M. Hensel, C. Gleeson, and D.W. Holden. 1996. Identification of a virulence locus encoding a second type III secretion system in Salmonella typhimurium. *Proc Natl Acad Sci U S A* 93:2593-2597.

Siegenthaler, J.A., F. Sohet, and R. Daneman. 2013. 'Sealing off the CNS': cellular and molecular regulation of blood-brain barriergenesis. *Current opinion in neurobiology*

Slack, E., M.L. Balmer, J.H. Fritz, and S. Hapfelmeier. 2012. Functional flexibility of intestinal IgA - broadening the fine line. *Frontiers in immunology* 3:100.

Spahn, T.W., A. Fontana, A.M. Faria, A.J. Slavin, H.P. Eugster, X. Zhang, P.A. Koni, N.H. Ruddle, R.A. Flavell, P.D. Rennert, and H.L. Weiner. 2001. Induction of oral tolerance to cellular immune responses in the absence of Peyer's patches. *European journal of immunology* 31:1278-1287.

Spahn, T.W., H.L. Weiner, P.D. Rennert, N. Lugering, A. Fontana, W. Domschke, and T. Kucharzik. 2002. Mesenteric lymph nodes are critical for the induction of high-dose oral tolerance in the absence of Peyer's patches. *European journal of immunology* 32:1109-1113.

Sparks, D.L., Y.M. Kuo, A. Roher, T. Martin, and R.J. Lukas. 2000. Alterations of Alzheimer's disease in the cholesterol-fed rabbit, including vascular inflammation. Preliminary observations. *Annals of the New York Academy of Sciences* 903:335-344.

Stan, R.V. 2004. Multiple PV1 dimers reside in the same stomatal or fenestral diaphragm. *Am J Physiol Heart Circ Physiol* 286:H1347-1353.

Stan, R.V., M. Kubitza, and G.E. Palade. 1999. PV-1 is a component of the fenestral and stomatal diaphragms in fenestrated endothelia. *Proc Natl Acad Sci U S A* 96:13203-13207.

Stan, R.V., D. Tse, S.J. Deharvengt, N.C. Smits, Y. Xu, M.R. Luciano, C.L. McGarry, M. Buitendijk, K.V. Nemani, R. Elgueta, T. Kobayashi, S.L. Shipman, K.L. Moodie, C.P. Daghlian, P.A. Ernst, H.K. Lee, A.A. Suriawinata, A.R. Schned, D.S. Longnecker, S.N. Fiering, R.J. Noelle, B. Gimi, N.W. Shworak, and C. Carriere. 2012. The diaphragms of fenestrated endothelia: gatekeepers of vascular permeability and blood composition. *Dev Cell* 23:1203-1218.

Stenman, J.M., J. Rajagopal, T.J. Carroll, M. Ishibashi, J. McMahon, and A.P. McMahon. 2008. Canonical Wnt signaling regulates organ-specific assembly and differentiation of CNS vasculature. *Science* 322:1247-1250.

Stewart, P.A., and M.J. Wiley. 1981. Developing nervous tissue induces formation of blood-brain barrier characteristics in invading endothelial cells: a study using quail--chick transplantation chimeras. *Developmental biology* 84:183-192.

Stuart, R.O., and S.K. Nigam. 1995. Regulated assembly of tight junctions by protein kinase C. *Proc Natl Acad Sci U S A* 92:6072-6076.

Sukumaran, S.K., M.J. Quon, and N.V. Prasadarao. 2002. Escherichia coli K1 internalization via caveolae requires caveolin-1 and protein kinase Calpha interaction in human brain microvascular endothelial cells. *J Biol Chem* 277:50716-50724.

Sun, C.M., J.A. Hall, R.B. Blank, N. Bouladoux, M. Oukka, J.R. Mora, and Y. Belkaid. 2007. Small intestine lamina propria dendritic cells promote de novo generation of Foxp3 T reg cells via retinoic acid. *The Journal of experimental medicine* 204:1775-1785.

Sun, J., M.E. Hobert, Y. Duan, A.S. Rao, T.C. He, E.B. Chang, and J.L. Madara. 2005. Crosstalk between NF-kappaB and beta-catenin pathways in bacterial-colonized intestinal epithelial cells. *Am J Physiol Gastrointest Liver Physiol* 289:G129-137.

Suzuki, K., B. Meek, Y. Doi, M. Muramatsu, T. Chiba, T. Honjo, and S. Fagarasan. 2004. Aberrant expansion of segmented filamentous bacteria in IgA-deficient gut. *Proc Natl Acad Sci U S A* 101:1981-1986.

Thomson, A.W., and P.A. Knolle. 2010. Antigen-presenting cell function in the tolerogenic liver environment. *Nat Rev Immunol* 10:753-766.

Vaishnava, S., C.L. Behrendt, A.S. Ismail, L. Eckmann, and L.V. Hooper. 2008. Paneth cells directly sense gut commensals and maintain homeostasis at the intestinal host-microbial interface. *Proc Natl Acad Sci U S A* 105:20858-20863.

Van der Sluis, M., B.A. De Koning, A.C. De Bruijn, A. Velcich, J.P. Meijerink, J.B. Van Goudoever, H.A. Buller, J. Dekker, I. Van Seuningen, I.B. Renes, and A.W. Einerhand. 2006. Muc2-deficient mice spontaneously develop colitis, indicating that MUC2 is critical for colonic protection. *Gastroenterology* 131:117-129.

van Sorge, N.M., P.A. Zialcita, S.H. Browne, D. Quach, D.G. Guiney, and K.S. Doran. 2011. Penetration and activation of brain endothelium by Salmonella enterica serovar Typhimurium. *The Journal of infectious diseases* 203:401-405.

Varol, C., A. Vallon-Eberhard, E. Elinav, T. Aychek, Y. Shapira, H. Luche, H.J. Fehling, W.D. Hardt, G. Shakhar, and S. Jung. 2009. Intestinal lamina propria dendritic cell subsets have different origin and functions. *Immunity* 31:502-512.

Varol, C., E. Zigmond, and S. Jung. 2010. Securing the immune tightrope: mononuclear phagocytes in the intestinal lamina propria. *Nat Rev Immunol* 10:415-426.

Velcich, A., W. Yang, J. Heyer, A. Fragale, C. Nicholas, S. Viani, R. Kucherlapati, M. Lipkin, K. Yang, and L. Augenlicht. 2002. Colorectal cancer in mice genetically deficient in the mucin Muc2. *Science* 295:1726-1729.

Vittet, D., T. Buchou, A. Schweitzer, E. Dejana, and P. Huber. 1997. Targeted null-mutation in the vascular endothelial-cadherin gene impairs the organization of vascular-like structures in embryoid bodies. *Proc Natl Acad Sci U S A* 94:6273-6278.

Voedisch, S., C. Koenecke, S. David, H. Herbrand, R. Forster, M. Rhen, and O. Pabst. 2009. Mesenteric lymph nodes confine dendritic cell-mediated dissemination of Salmonella enterica serovar Typhimurium and limit systemic disease in mice. *Infect Immun* 77:3170-3180.

Warner, N., and G. Nunez. 2013. MyD88: a critical adaptor protein in innate immunity signal transduction. *J Immunol* 190:3-4.

Wehkamp, J., N.H. Salzman, E. Porter, S. Nuding, M. Weichenthal, R.E. Petras, B. Shen, E. Schaeffeler, M. Schwab, R. Linzmeier, R.W. Feathers, H. Chu, H. Lima, Jr., K. Fellermann, T. Ganz, E.F. Stange, and C.L. Bevins. 2005. Reduced Paneth cell alpha-defensins in ileal Crohn's disease. *Proc Natl Acad Sci U S A* 102:18129-18134.

Wolburg, H., K. Wolburg-Buchholz, J. Kraus, G. Rascher-Eggstein, S. Liebner, S. Hamm, F. Duffner, E.H. Grote, W. Risau, and B. Engelhardt. 2003. Localization of claudin-3 in tight junctions of the blood-brain barrier is selectively lost during experimental autoimmune encephalomyelitis and human glioblastoma multiforme. *Acta Neuropathol* 105:586-592.

Wood, M.W., M.A. Jones, P.R. Watson, A.M. Siber, B.A. McCormick, S. Hedges, R. Rosqvist, T.S. Wallis, and E.E. Galyov. 2000. The secreted effector protein of Salmonella dublin, SopA, is translocated into eukaryotic cells and influences the induction of enteritis. *Cell Microbiol* 2:293-303.

Worbs, T., U. Bode, S. Yan, M.W. Hoffmann, G. Hintzen, G. Bernhardt, R. Forster, and O. Pabst. 2006. Oral tolerance originates in the intestinal immune system and relies on antigen carriage by dendritic cells. *The Journal of experimental medicine* 203:519-527.

Wyss, L., J. Schafer, S. Liebner, M. Mittelbronn, U. Deutsch, G. Enzmann, R.H. Adams, M. Aurrand-Lions, K.H. Plate, B.A. Imhof, and B. Engelhardt. 2012. Junctional adhesion molecule (JAM)-C deficient C57BL/6 mice develop a severe hydrocephalus. *PLoS One* 7:e45619.

Xu, C., Y. Shen, D.R. Littman, M.L. Dustin, and P. Velazquez. 2012. Visualization of mucosal homeostasis via single- and multiphoton intravital fluorescence microscopy. *J Leukoc Biol* 92:413-419.

Yeung, D., J.L. Manias, D.J. Stewart, and S. Nag. 2008. Decreased junctional adhesion molecule-A expression during blood-brain barrier breakdown. *Acta Neuropathol* 115:635-642.

Zarepour, M., K. Bhullar, M. Montero, C. Ma, T. Huang, A. Velcich, L. Xia, and B.A. Vallance. 2013. The Mucin Muc2 Limits Pathogen Burdens and Epithelial Barrier Dysfunction during Salmonella enterica Serovar Typhimurium Colitis. *Infect Immun* 81:3672-3683.

Zhang, Y.G., S. Wu, Y. Xia, D. Chen, E.O. Petrof, E.C. Claud, W. Hsu, and J. Sun. 2012. Axin1 prevents Salmonella invasiveness and inflammatory response in intestinal epithelial cells. *PLoS One* 7:e34942.