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Role of Autoimmune Susceptibility Gene, *PTPN2*, in Mediating Host-Pathobiont Interaction

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ABSTRACT OF THE DISSERTATION

Role of Autoimmune Susceptibility Gene, *PTPN2*, in Mediating Host-Pathobiont Interaction

by

Pritha Chatterjee

Doctor of Philosophy, Graduate Program in Biochemistry and Molecular Biology
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Declan F. McCole, Chairperson

The intestinal epithelium acts as a physical barrier between the luminal microbes and the immune cells in the lamina propria while also coordinating a very delicate equilibrium to maintain mucosal homeostasis. Dysregulation in the physical barrier leads to inflammatory conditions like inflammatory bowel disease (IBD). Single nucleotide polymorphisms (SNPs) in the gene protein tyrosine phosphatase non-receptor type 2 (*PTPN2*) have been implicated in IBD. These SNPs cause loss-of-function in the protein product of *PTPN2* – T-cell protein tyrosine phosphatase (TCPTP). Along with genetic susceptibilities, alterations in the gut microbiota and expansion of pathobionts such as adherent-invasive *Escherichia coli* (AIEC) have also been associated with IBD. Our previous work showed, mice deficient for the expression of *Ptpn2*, exhibit a microbial shift and pronounced expansion of a novel murine adherent-invasive *Escherichia coli* (*m*AIEC) strain. In this dissertation, we aimed to investigate how PTPN2 expression in intestinal epithelial cells (IEC) maintains equilibrium between the commensal bacteria and restricts

invading pathobionts like *m*AIEC. To do so, we used, tamoxifen-inducible, IEC-specific knockout mice (*Ptpn2*$^{\Delta IEC}$) and control floxed (*Ptpn2*$^{fl/fl}$) mice. I also used Caco-2 BBe colonic IECs which were genetically modified by CRISPR-Cas9 to carry the clinically relevant *PTPN2* SNP *rs1893217* (*PTPN2*-KI) or a complete knockout of the *PTPN2* gene (*PTPN2*-KO). Overall, in this dissertation we have demonstrated a critical role of intestinal epithelial PTPN2 in mucosal immunity as it promotes anti-microbial peptide defenses and enhances barrier function during infection from pathobionts like *m*AIEC. Additionally, we also revealed that epithelial PTPN2 is crucial for maintaining the immune-cytokine regulatory landscape of the gut to withstand pathobiont colonization. We also demonstrate a role for PTPN2 in regulating proteins that mediate AIEC entry into host cell. These finding provide an essential cell-specific role for this clinically relevant gene in the preservation of multiple aspects of mucosal barrier defenses.

Table of Contents

LIST OF FIGURES

LIST OF TABLES

CHAPTER 1

1 INTRODUCTION

The gastrointestinal tract is the largest mucosal surface which is in continuous contact with several different microorganisms[1]. The intestinal epithelium lines the mucosa and forms a cellular barrier between the microbiota of the lumen and the underlying immune cells, while maintaining uptake of nutrients as well as exchange of ions, water, and macromolecules[2]. This property of selective permeability ensures nutrient and water transport while serving as a barrier, thus, maintaining mucosal homeostasis[3].

1.1 Function of intestinal epithelial subtypes

1.1.1 Intestinal Stem cells

The intestinal epithelium is composed of several differentiated cell types reflecting either absorptive or secretory lineages[4]. The epithelium is challenged by numerous mechanical, chemical and biological stressors and renews every 4-5 days via replenishment from intestinal stem cells (ISC)[5]. The small intestine is characterized by crypt-villus structures, while the large intestine lacks villi but has longer crypt structures[Figure1]. Intestinal stem cells are located at the bottom of these crypts[6]. ISCs possess self-renewal properties and maintain epithelial self-renewal and proliferation under homeostatic conditions[4]. The leucine-rich repeat containing G-protein coupled receptor-5 positive (Lgr5+) ISCs give rise to new enterocytes and therefore, sustains epithelial regeneration[6]. The quiescent or slow cycling stem cells are present right above the stem cells and are a reserve population of ISCs that become activated upon injury[6].

This property of rapid regeneration makes the intestine a very convenient model for studying epithelial development and response to injury. Over recent years, the several studies have characterized the intestinal epithelial niche. The ISC niche comprises of distinct neighboring cells and various components that form the extracellular matrix surrounding the base of the crypt that are the major source of the stem cell niche factors. Our understanding of how these niche factors support ISC development and proliferation has given rise to three-dimensional (3-D) *in-vitro* cultures that model *in vivo* intestine. The wingless-related integration-3 (Wnt3) producing Paneth cells together with epidermal growth factor (EGF), R-spondin and noggin serve as minimal media for *in vitro* ISC culture by mimicking the ISC niche *in-vivo*. The expression of Wnt pathway is active as a gradient in the intestinal crypt, most prominent in the crypt and reduces upwards in the crypt-villus axis. Conversely, bone morphogenetic protein (BMP), a Wnt antagonist, is present in least amounts in the crypt but is in high concentrations at the crypt-villus axis. Noggin promotes ISC growth by binding to BMP ligands. The Wnt3 produced by Paneth cells is very crucial for ISC differentiation and proliferation. However, while Paneth cells were indispensable for organoid culture, it was dispensable *in vivo*, suggesting that the Wnt can be derived from other mesenchymal sources in an *in vivo* system. EGF and R-spondin positively regulate the Wnt signalling which in turn induces proliferation of intestinal crypt epithelial cells.

The organoids cultured in distinct differentiation media can give rise to differentiated epithelial cells surrounding a functional lumen that mimics the intestinal epithelium *in vivo*. Intestinal organoids have been a great resource for studying pathogenic bacterial infections, electrophysiology, cancer, and immunology studies, and to study the pathophysiology of infectious bacteria and viruses[7-9].

1.1.2 Paneth cells

Next to the stem cells, at the base of the crypt are the Paneth cells (PC). PCs are mostly present in the distal small intestine and secrete several types of anti-microbial peptides (AMPs) that modulates the gut microbiome and prevents pathobiont colonization[10]. The secretory Paneth cells are pyramidal shaped with basally situated nuclei and contain an extensive network of endoplasmic reticulum and Golgi bodies with distinct apical granules that secrete AMPs (Figure 1). The AMPs secreted by Paneth cells are lysozyme, Reg 3A (Reg3γ in mice) and α-defensins. The α-defensins (known as cryptdins in mice) are the most abundant AMPs present in the intestine of humans as well as mice[11]. Transgenic mouse models of human α-defensin 5 (HD5) and 6 (HD6) have enhanced survival upon challenge with the foodborne enteric pathogen, *S. typhimurium*[12, 13]. Interestingly, Paneth cell dysfunction has been strongly implicated in inflammatory bowel disease (IBD)[14]. Pediatric patients with Crohn's Disease (CD), one of the major subsets of IBD along with ulcerative colitis (UC), have reduced production of α-defensins[14]. Further, although Paneth cells *per se* are restricted to the small intestine, metaplastic Paneth cells are present in the distal large intestines of IBD patients[15]. Many studies observed microbial dysbiosis with loss of protective microbiome and enrichment in pro-inflammatory microbes in both mice and humans with Paneth cell abnormalities[14, 16, 17]. Apart from production of AMPs and maintenance of microbial populations, Paneth cell-derived factors such as Wnt3, Notch ligands, and epidermal growth factor (EGF) support the stem-cell niche, albeit specific deletion of Paneth cells *in vivo* in mice does not hamper ISC homeostasis[18, 19]. Overall, Paneth cells play a very important role in gut mucosal homeostasis.

1.1.3 Goblet cells

The luminal surface of the intestinal epithelium is lined by a hydrated gel-like mucus which forms the first line of defense against infiltration of microorganisms[20]. The mucus layer is mostly composed of complex glycosylated proteins called mucins secreted by epithelial goblet cells[20]. The small intestine has one layer of loosely attached mucus while the large intestine has 2 layers, a fixed adherent layer, and a looser layer of mucus on top. [20-22]. Goblet cell-derived factors, like trefoil factor 3 (TFF3) and contributes to epithelial integrity by crosslinking mucins in the mucus layer and acts as a signal that promotes epithelial repair and reduces apoptosis[23, 24].

Apart from producing mucus, goblet cells also play an important role in mediating communication between the luminal microbes and the immune cells in the lamina propria by forming goblet-cell associated passages (GAPs)[25]. GAPs are essential for the sampling of bacterial products to CD103+ dendritic cells in the lamina propria[25]. Under homeostatic conditions, GAPs are observed only in the small intestine, however, during inflammation, GAPs appeared in the colon and presented with intracellular bacteria. This suggests that GAPs can be exploited by bacteria to gain access to the lamina propria immune cells and further exacerbate inflammation[25,26].

Further, specialized goblet cells called sentinel goblet cells, present in the distal colon, endocytose bacteria by detecting microbial ligands via toll-like receptors (TLRs) and depending on the concentration of the bacterial ligands, secretes mucus which not only washes the bacteria away but also strengthens the mucus barrier[26, 27]. Therefore, goblet cells perform a vital role in the maintenance of mucosal homeostasis in the gut.

1.1.4 Micro-fold cells

The micro-fold cells (M-cells) are specialized intestinal epithelial cells (IEC) that are present in the follicle-associated epithelium overlaying Peyer's patches, that are important sites of antigen presentation to the underlying immune system[28]. M-cells mediate transcytosis of luminal antigens and intact micro-organism in a non-specific manner[28]. Antigen sampling by M-cells is likely a key initiator for the intestinal immunoglobulin A (IgA) response to commensals as mice with defective M-cell functioning display reduced secretory IgA[29]. Recent studies have demonstrated that M-cell glycoprotein2 (GP2) interacts with specific proteins in the pili of pathogenic bacteria such as *Salmonella* and adherent-invasive *E. coli* (AIEC) to mediate their transport across the epithelial barrier[30, 31]. Such interactions can be taken advantage of by pathobionts to mediate inflammation in susceptible hosts.

1.1.5 Tuft cells

Tuft cells are chemosensory epithelial cells that can respond to various luminal signals. Despite their low numbers in the intestine, several studies have elucidated a central role for Tuft cells in preventing helminth and protozoan colonization[32]. The tuft cells activate group 2 innate lymphoid cells (ILC2) in an IL-25 dependent manner[33]. Helminth infection causes production of succinate that binds to the G-protein coupled succinate receptor 1 (SUCR1) on tuft cells and stimulates them to produce IL-25. IL-25 in turn binds to ILC2s causing them to produce IL-13 leading to goblet and tuft cell expansion; followed by mucus secretion and smooth muscle hypercontractility which is a part of host protective “weep and sweep” response to helminth infection[33-35]. Upon stimulation, the tuft cells can also

produce acetylcholine, thymic stromal lymphopoietin (TSLP), β-endorphins, prostaglandin E2 (PGE2), and leukotriene C4 (LTC4) which are paracrine and endocrine signaling molecules. However, the exact impact of these tuft cell secretions on mucosal homeostasis has not been elucidated yet[36, 37]. Expectedly, alterations in intestinal tuft cells and its related cytokines have been observed in IBD, coeliac disease, duodenal ulcers, and obesity[38].

1.1.6 Enteroendocrine cells

Enteroendocrine cells (EECs) are scattered throughout the GI tract and play a central role in the production of gut hormones that are necessary for digestion, absorption, appetite, and insulin secretion[39, 40]. EECs are stimulated by products derived from food such as glucose, amino acids and fatty acids and produce gut hormones including glucagon-like peptide 1 (GLP-1), cholecystokinin and glucose-dependent insulinotropic polypeptide, which play a central role in metabolism[39]. The metabolites derived from gut microbiota such as short-chain fatty acids (SCFA), secondary bile acids and lipopolysaccharides can also modulate EECs[39]. These stimuli cause EECs to produce hormones (listed above) that regulate dietary intake and epithelial integrity[40].

In summary, gut epithelia are composed of several highly differentiated epithelial cell types that perform unique specialized functions in the host to promote gut mucosal homeostasis.

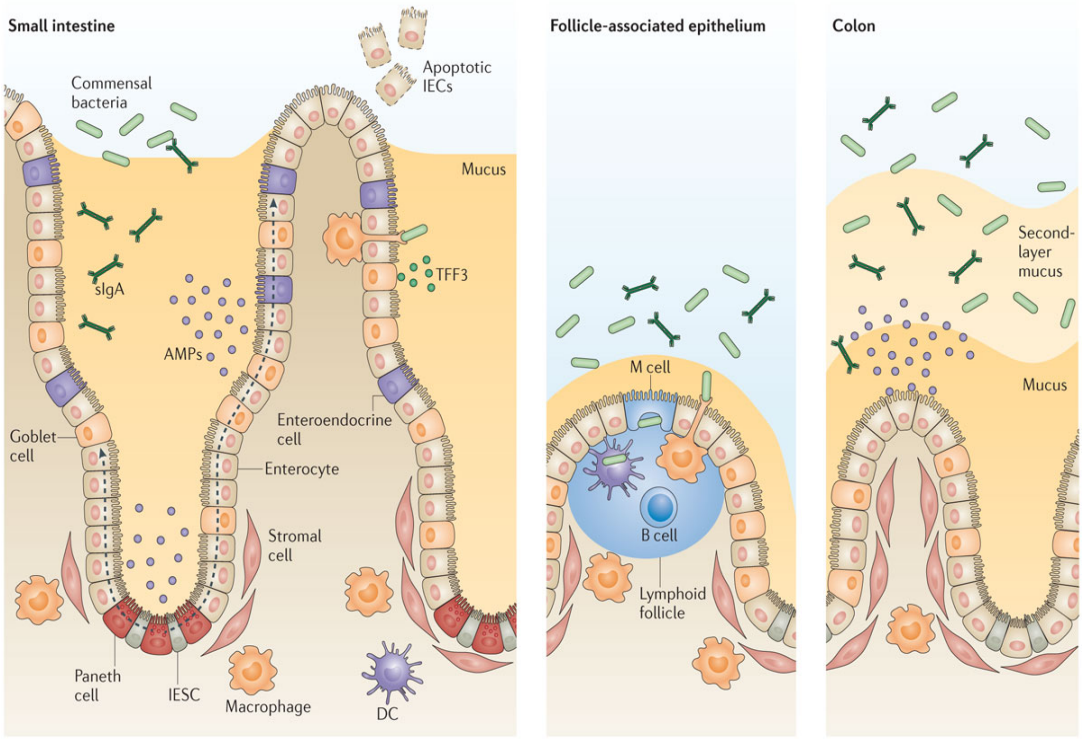

Figure 1: Diagram depicting the different types of intestinal epithelial cells and their localization in the colon and small intestine.

The intestinal epithelial cells form a barrier between the luminal microbes and the immune cells in the lamina propria. The intestinal stem cell (IESC) niche give rise to new enterocytes and migrate to the top of crypt-villus axis. The secretory cells like the Paneth cells and the goblet cells produce AMPs and mucus respectively that keeps pathogenic bacteria from interacting with the immune cells. Further, goblet cell derived TFF3, maintains epithelial integrity by crosslinking mucins and signals as a factor for epithelial repair. The M-cells are engaged in antigen presentation to the dendritic cells which promotes tolerance in the underlying immune system. Further, the enteroendocrine cells (EECs) are sense environmental stimuli and produce gut-hormones necessary for digestion and absorption of nutrients. The IgA produced by the B cells strengths epithelial barrier and prevents pathogenic bacteria from attacking the immune cells. Figure adapted from the article : “Intestinal epithelial cells: regulators of barrier function and immune homeostasis”. Nature Reviews Immunology (2014) [1].

1.2 Different pathways of intestinal permeability

The intestinal epithelial barrier maintains a very delicate interaction between microbes and the immune cells in the lamina propria. Unlike the skin epithelial barrier, destruction of which can be catastrophic, the epithelial barrier is much more flexible and maintains selective permeability. Intestinal permeability can be attributed to 3 distinct pathways: the pore pathway, leak pathway and the unrestricted pathway[41]. The intestinal epithelium must maintain a very delicate interaction wherein it restricts potentially pathogenic molecules from crossing while allowing selective permeability for absorption of nutrients and water from the gut lumen[3, 41]. Intestinal permeability is functionally determined by a conglomeration of transmembrane and apical membrane-associated proteins that act in concert to form a “tight junction” (TJ)[42]. The TJ proteins connect the intracellular spaces between adjacent epithelial cells and are the rate limiting step for paracellular passage of nutrients, water, and ions.[42]. Compromised intestinal barrier permeability has been associated with increased risk for several inflammatory conditions like Crohn’s disease (CD), ulcerative colitis (UC), celiac disease, graft versus host disease (GVHD)[41]. It was observed that patients in remission for CD that displayed elevated intestinal barrier permeability, had a higher probability of relapse[43]. Recently, a large cohort study showed that asymptomatic healthy relatives of patients with CD that had higher intestinal permeability were at 50% higher risk of developing the disease than relatives with normal barrier function[44]. These clinical data provide strong evidence that increased intestinal permeability is an early event in the development of chronic inflammatory conditions.

1.2.1 Pore Pathway

The claudin family of TJ proteins are key proteins that mediate epithelial permeability[45]. The claudins are tetra-spanning membrane proteins and are a family made up of at least 27 members[46, 47]. Each member has a selective role in maintaining either cation or anion permeability[47]. A well-studied member of the Claudin family – Claudin 2 – increases Na^+ and water flux, but larger molecules are unable to penetrate[42]. Claudin-2 maintains the intestinal pore-pathway and is elevated in inflammatory conditions like inflammatory bowel disease (IBD) and celiac disease[48, 49]. Interestingly, Claudin-1, a pore sealing member of the claudin family was found to also be increased in patients with IBD and this may reflect its role in countering the effects of claudin-2 [48]. Of note, claudin-2 overexpressing mice were protected against experimental colitis by reduced epithelial apoptosis, increased proliferation, and reduced inflammatory cytokines like IL-6, TNF, IFN-γ etc[50]. Recently, it was also demonstrated that claudin-2 is protective against colitis-associated cancer by virtue of promoting mucosal healing[51]. During inflammation, cytokines like IL-6, IL-13 and IL-22 can upregulate Claudin-2 expression and increase pore-pathway permeability[52-54]. Transgenic (Tg) mice overexpressing Claudin-2 in their IECs were protected against *Citrobacter rodentium* infections, a model for infectious colitis[54]. It has been shown that claudin-2 induced diarrhea by mediating water efflux which potentiated pathogen clearance[54]. Reciprocally and surprisingly, claudin-2 overexpression in mice resulted in increased susceptibility to T-cell mediated experimental colitis[55].

Another pore-forming member of the claudin family, claudin-15, has also been implicated in disease[56]. Interestingly, claudin-15 was shown to be indispensable for sodium and glucose homeostasis in mouse small intestine, while claudin-2 was involved

but was not required for Na+ recycling involved in glucose uptake [57]. Claudin- 3, 4 and 7 are pore-sealing Claudins and are reduced during inflammation[54, 58]. In active ulcerative colitis (UC), Claudins 4 and 7 were reduced in patients while 1 and 3 remained unchanged[58]. In active CD, Claudin-3 was reduced while 4 and 7 were unchanged. These data suggest that claudins have an important role in barrier permeability[46]. However, these studies suggest that while claudins have an important role in mediating intestinal permeability, the full scope of claudin roles in health and disease is yet to be fully identified.

1.2.2 Leak Pathway

Intestinal epithelial cells prevent free exchange of ions, fluid and other molecules between the lumen and the lamina propria[41]. However, a semipermeable barrier is maintained by the tight junction proteins (TJs) that allows paracellular passage of certain macromolecules[41]. Epithelial cells are connected by desmosomes and adherens junction proteins that maintain the structural integrity of the epithelial layer[3, 45]. The leak-pathway of permeability is not charge selective but can occupy molecules upto a maximum size of 100Å[54]. The functional characterization of the leak pathway can be assessed using a variety of probes including by administration of size-specific fluorescent probes like fluorescein isothiocyanate-dextran, 4 kDa (FITC)[42]. While much is known about the leak pathway and how its functional capacity can be increased by inflammatory and bacterial stimuli, there are still a number of features that are yet to be characterized [41, 42].

The TJ protein, myosin-light chain kinase (MLCK), can mediate the leak-pathway[59]. Constitutively active MLCK was sufficient to increase the leak pathway[59,

60]. Occludin, another membrane-spanning TJ protein, is endocytosed after TNF challenge and its internalization was correlated with an increase in leak pathway permeability[61]. Further, over-expression of occludin in IEC prevented TNF-induced leak pathway upregulation[62]. Interestingly, occludin-KO mice have normal intestinal permeability compared to controls[63]. These suggest that occludin may also have an important role in regulating leak pathway permeability although compensatory mechanisms likely are enacted following its deletion in rodent models[62]. Zonula-occludins (ZO-1) was the first TJ protein to be discovered. ZO family of proteins are scaffolding proteins[62] that organize TJs are essential to TJ stability and functional interactions with composite proteins as well as the cytoskeleton. ZO-1 is reduced in IBD patients, however, its specific role in intestinal permeability is not well-understood[64].

In-vitro studies have shown that loss of ZO-1 increases FD4 permeability. However, it was recently demonstrated that mice with IEC-specific knockout of ZO-1 showed only a modest increase in FD4 permeability but were hypersensitive to mucosal insults and failed to upregulate proliferation and repair after damage[60, 64]. These data suggest that while there is still a lot to uncover on how the leak pathway is mediated via specific TJ proteins, however, there is strong evidence that epithelial permeability is a crucial factor for intestinal homeostasis.

1.2.3 Unrestricted Pathway

The unrestricted pathway of intestinal permeability is not dependent on tight junction proteins[1, 2]. The unrestricted pathway is an indicator of severe epithelial damage and apoptosis[3]. At this stage, larger bacteria and proteins can acquire access to the

underlying immune system and further exacerbate inflammation. Probes like Rhodamine B-dextran 70kDa (RD70), can serve as functional assessment of the unrestricted pathway[3].

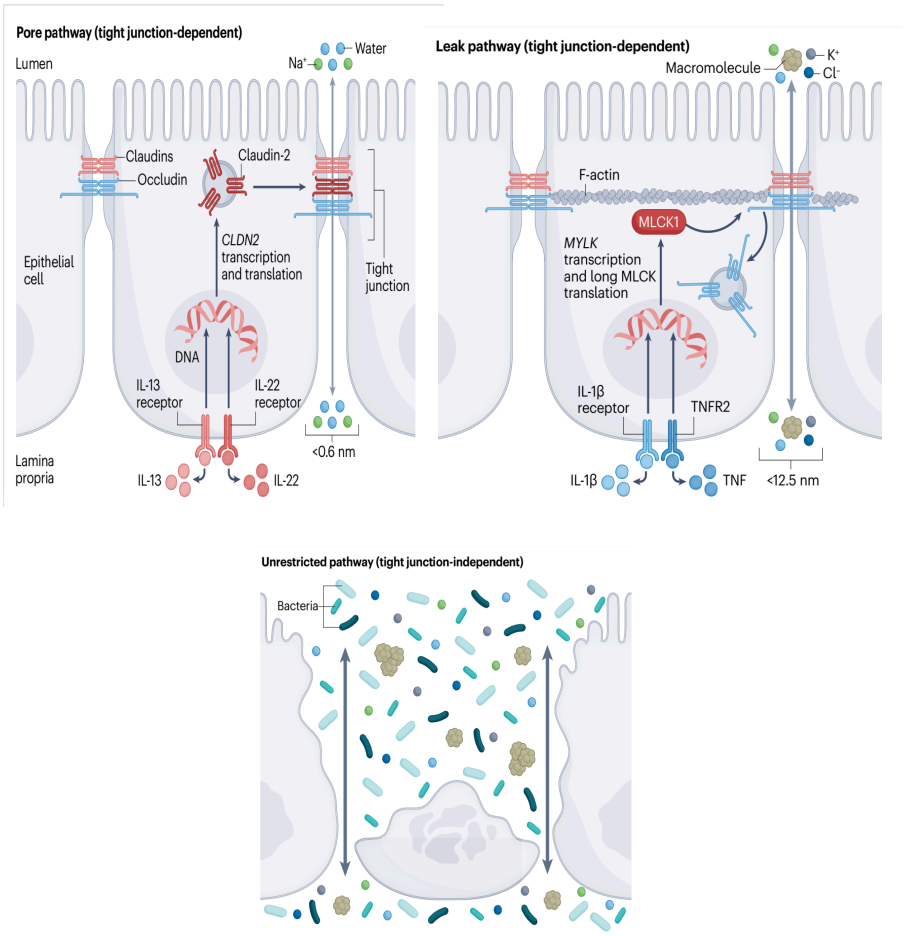

Figure 2: The 3 distinct pathways of intestinal permeability – pore pathway, leak pathway and unrestricted pathway.

The permeability via the pore-pathway is maintained by the protein claudin-2. The claudin-2 channels is size and charge selective. The expression of claudin-2 can be elevated by cytokines like IL-6, IL-22 and IL-13. The leak pathway allows molecules of a greater size (~12.5nm) to pass through the paracellular space to pass through in comparison to the pore-pathway. MLCK, a tight junction protein, can mediate leak pathway although its mechanism is not very well understood. Cytokines like TNF and IL-1β can increase leak pathway permeability. The unrestricted pathway is not tight-junction dependent and increase in unrestricted pathway indicates substantial damage to the intestinal barrier. Figure adapted from : “Paracellular permeability and tight junction regulation in gut health and disease.” Nature Reviews Gastroenterology & Hepatology, 2023 [41].

1.3 Cross talk between intestinal epithelial cells, the microbiome, and host immunity

Intestinal epithelial cells act as a partition between the trillions of microbes in the lumen and the immune cells in the lamina propria, while also maintaining a semi-permeable epithelial barrier that allows the regulated paracellular passage of water and ions. Several studies have demonstrated that specific bacterial species interact with host cells under homeostatic conditions to promote mucosal health, suggesting that the epithelial barrier acts as a sensor to components of the luminal microenvironment such as bacterial and dietary antigens[65]. The microbiome's interaction with host cells maintains mucosal homeostasis by educating the immune cells, promoting intestinal barrier functions and protecting against pathobiont overgrowth[66, 67]. Other than modifying specific bacterial interactions, microbial-derived metabolites have been shown to maintain intestinal barrier and immune cell functions[67]. Conversely, host IECs have been shown to maintain and promotes certain intestinal microbial niches[65].

Several studies have elucidated the role of IECs in sensing and influencing the gut microbiota to maintain intestinal homeostasis[68]. IECs express pattern recognition receptors (PRRs) that detect various commensals and pathogenic species in the local environment[65-67]. In response to bacterial sensing, IECs secrete factors that promote barrier function and prevent pathogenic bacterial infiltration[69, 70]. Some of these factors are antimicrobial peptides (AMPs), mucins, and barrier-forming proteins. Commensal bacteria such as *Akkermansia mucinophila* have been shown to interact with PRR toll-like receptor 2 (TLR-2) to promote barrier function by increasing mucous layer thickness and TJ protein expression[71]. *Lactobacillus rhamnosus* was shown to have a direct impact on

epithelial TJ proteins, ZO-1 and occuldin, by elevating their expression thereby tightening the epithelial barrier[72]. Other commensals like *Ruminococcus gnavus* can also stimulate mucin production from goblet cells while pathobionts such as AIEC can reduce mucus layer thickness[73]. Interestingly, a recent study demonstrated that the gut commensal *Bacteroides thetaiotaomicron* could dampen Indian hedgehog (IHH) signaling in intestinal epithelial cells thereby diminishing epithelial barrier integrity[74]. These studies suggest that specific commensal populations can interact (both directly and indirectly) with intestinal epithelial cells and elicit very different outcomes on epithelial barrier integrity depending on the context of the association and the presence or absence of inflammation.

In addition to direct bacterial interaction with host cells, metabolites produced by bacterial fermentation, known as short chain fatty acids (SCFA), are also important signals detected by IECs[75]. Metabolite-sensing receptors belonging to the family of G-protein coupled receptors (GPCR) – GPR41 and GPR43 - have been shown to influence IEC secretion of AMPs in the presence of SCFAs[76]. Several studies on GPR43 demonstrate its central role in metabolite sensing which maintains gut homeostasis and inflammasome activation in non-hematopoietic cells[77]. The aryl hydrocarbon receptor (AhR) can also detect bacterial metabolites like tryptophan and butyrate[78]. These metabolites can induce IEC differentiation from stem cells and promote intestinal barrier respectively. Indeed, butyrate is the major energy source for colonocytes and thus plays an essential role in overall enterocyte integrity and functional capacity in the colon.

Bacterial metabolites can interact with both IECs and directly with immune cells to influence host responses. GPR43 present in colonic ILC3s can sense SCFAs to produce IL-22 and induce AMP production from epithelial cells, as well as promoting a tighter barrier[79]. The SCFA, butyrate, has a role in promoting monocyte to macrophage

transition and AMP reprogramming in epithelial cells[80]. Recently, it was also demonstrated that xanthurenic (Xana) and kynurenic acid (Kya), intermediates in the tryptophan metabolism pathway, negatively correlated with intestinal inflammation and supplementation with both acids reduced inflammation through IEC and T-cell upregulation of AhR ligands[81].

The microbiome and immune cells have limited but tightly regulated contact. Futhermore, a growing body of research has demonstrated interaction of commensals with host cells are important for the development of host immunity. Intestinal bacteria like segmented filamentous bacteria (SFB) attach to the mucosal surface and modulate the immune system by interacting with the IECs[82]. SFBs were initially classified SFBs are present in the distal ileum and transfer antigens to intestinal epithelial cells via microbial adhesion triggered endocytosis (MATE)[83]. After attachment, IECs produce serum amyloid A (SAA), which has been shown to promote specific T effector subsets, like the T-helper 17 (Th17) cell responses, in the lamina propria[84, 85]. These Th17 cells along with the type3 innate lymphoid cells (ILC3) secrete IL-17 and IL-22 which are important cytokines for Paneth cell maturation and secretion of AMPs[83]. The direct enrichment of Th17 cells by SFB provides an enhanced protection from pathogenic bacteria such as *Clostridium rodentium* and *Salmonella spp*[84]. Along with important Th cell responses, SFBs are important for secretory immunoglobulin A (IgA)[86]. While SFB are found in mice at all ages, their detection in humans has proven more controversial with some studies indicating SFB is present in newborns and is rapidly depleted by three years of age[170]. Moreover, some studies indicate detection of SFB in adult patients with ulcerative colitis and have proposed roles for SFB in mediating pathogenic Th17 immune responses[171]. *Bifidobacterium adolescentis* also associates very closely with the gut epithelium and is

also reported to induce Th17 responses in mouse intestine albeit, the exact mechanism of induction is not very clear[87]. Many other studies have also pointed to the role of the commensal derived butyrate in the induction of peripheral Treg cell population[88-90]. Another study showed that *t*ransfer of naive transgenic T cells specific for commensal antigens into mice with a normal microbiota resulted in a robust $Foxp3^+$ Treg phenotype[91]. At weaning, the intestinal microbiota induces a vigorous immune response associated with the generation of $ROR\gamma t^+$ Treg cells in an SCFA and retinoic acid-dependent manner, and inhibition of this response leads to an immunopathology like colitis[92]. These studies suggest that intestinal epithelial cells act as an essential mediator of interactions between the microbiome and host immune cells.

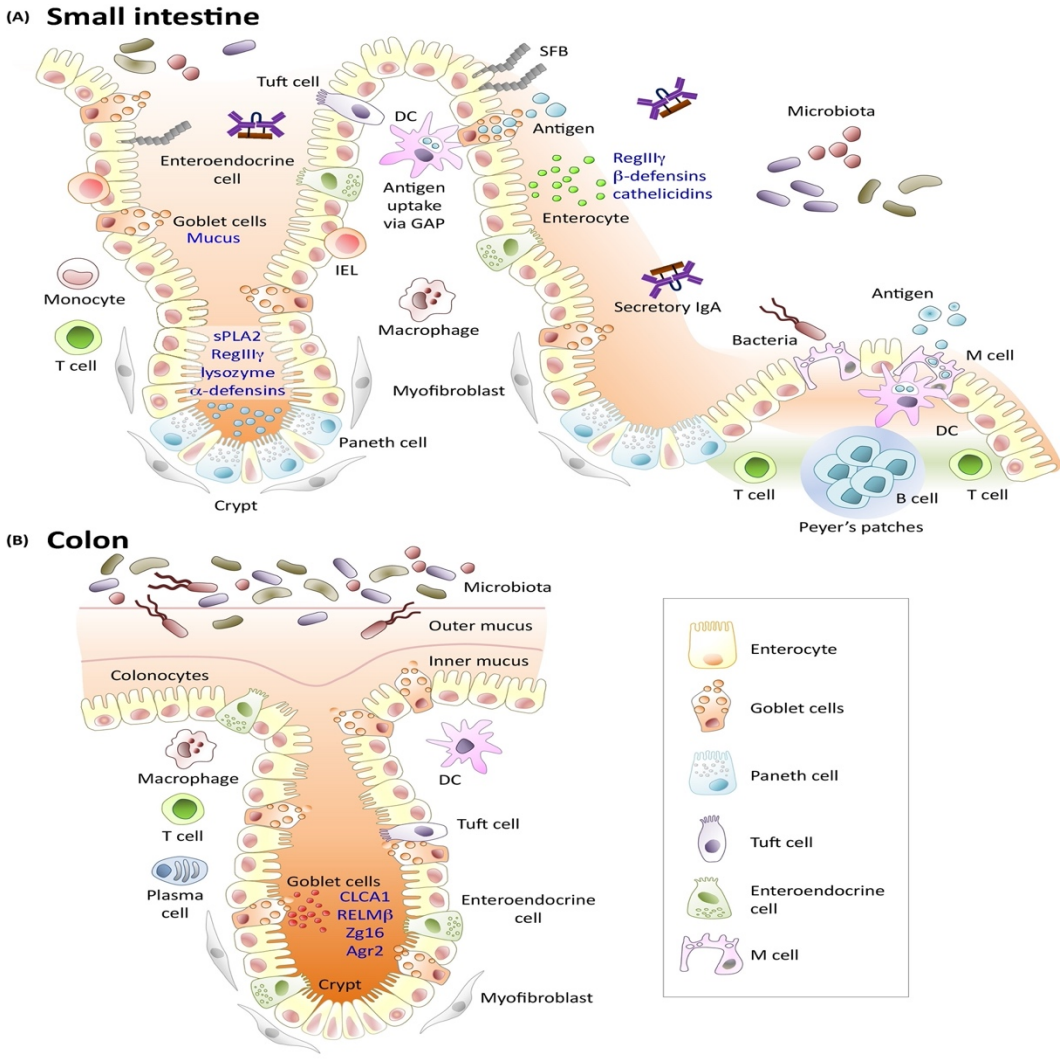

Figure 3: The epithelial cells are mediate interaction between the luminal microbes and host immunity.

Commensal bacterial species like segmented filamentous bacteria (SFB) interact with IECs and stimulate Th17 cells that produce cytokines like IL-17 and IL-22 at homeostatic levels. The IL-22 stimulates Paneth cells to produce anti-microbial peptides (AMP). SCFAs like tryptophan and butyrate modulate the epithelial barrier. Microbial metabolites can also induce anti-inflammatory cytokines from immune cells and therefore promotes healthy epithelial-mucosal crosstalk. Figure adapted from : "The intestinal epithelium: central coordinator of mucosal immunity." Trends in immunology, 2018 [54].

1.4 Inflammatory bowel disease

Inflammatory bowel disease (IBD), encompassing Crohn's disease (CD) and ulcerative colitis (UC), is a chronic relapsing condition thought to result from inappropriate immune responses to native gut microbes in genetically susceptible hosts[93]. Alterations in the microbiome, dysbiosis is closely linked to IBD, however, it is still unclear if it a primary event or secondary event[93]. Environmental factors like western diet, prolonged exposure to antibiotics, enteric infections and even pollution has been determined as triggers for a series of events that can initiate inflammation. Recent studies have identified that increased barrier permeability needs a trigger to lead to disease in first-degree relatives of CD patients[33]. As of data recorded up to 2017 (reported in 2021), IBD affects around 6.8 million people globally with increased incidence and prevalence worldwide compared to the past decade.[94].

1.4.1 Crohn's Disease

Crohn's Disease (CD) affects 100-300 people per 100,000 individuals in America[95]. It is a chronic inflammatory condition that can affect any part of the intestine and causes chronic diarrhea, abdominal pain, weight loss, rectal bleeding[93]. It is also characterized by granulomatous, transmural, and sometimes fistulizing inflammation[93]. The manifestation of CD has been attributed to several reasons like alterations in the gut microbiome, changes in diet, environmental factors, and genetic pre-disposition[93]. About 5-20% CD patients have 1st degree relatives with the disorder and an increased odds ratio was observed among individuals of Ashkenazi Jewish heritage[96-98]. The inflammatory response in CD is primarily mediated by T-helper 1 (Th1) or Th 17 immune

responses[93]. Early reports showed that healthy first-degree relatives of patients with CD display modest intestinal barrier function[99, 100]. Other studies have seen that the increase in barrier permeability was accompanied by loss of microbial diversity and lower metabolic activity[44]. Links between genetic risk factors and increased permeability have also been identified as first-degree relatives (FDRs) of patients carrying the CD-risk allele *NOD2* 3020insC displayed increased intestinal permeability[101]. More recently, a study showed that healthy relatives of patients with CD were two-threefold more likely to develop the disease than the relative with no defect in intestinal permeability[101, 102]. A seminal paper from the Canadian Crohns and Colitis Genetic, Environment and Microbial (GEM) project, identified that asymptomatic FDRs of CD patients who had increased intestinal permeability had a significantly greater risk of subsequently developing CD up to several years later. This paper confirmed increased intestinal permeability as an essential risk factor for development of CD, but increased permeability alone was not sufficient to induce disease, an additional 'trigger' was required. Overall, these studies confirm that increased intestinal permeability is an important and early risk factor in the pathogenesis of CD.

1.4.2 Ulcerative colitis (UC)

Ulcerative colitis (UC) is a chronic and debilitating subtype of IBD[103]. It can manifest in patients as bloody stools, fibrosis, and tissue damage because of mild to severe inflammation of the colon[104, 105]. A subset of UC patients may require surgery or colectomy[106]. Patients with UC display an exacerbated Th2 type cytokine response such as IL-5 and IL-13. Patients also display mucosal infiltration with neutrophils, macrophages, T and B lymphocytes, and plasma cells, formation of crypt abscesses, and extensive epithelial damage[93]. Treatment in UC has mainly focused on mucosal healing

as colectomy leads to complications in one-third of UC patients[107]. A defective epithelial barrier is also seen inpatients with active UC. It has been shown that the colonic mucosa of UC patients have markedly reduced epithelial barrier with alterations in tight junction proteins[108]. Another study showed that patients with early UC display minute leaks at apoptotic foci in the epithelium, in comparison to their controls[109]. Alterations in the expression of claudin family proteins have also been reported in UC. The proteins claudin-4 and 7 were downregulated in active UC patients while the cation pore-forming protein, claudin-2 was upregulated. Interestingly, the cytokine IL-13, present in high amounts in UC patients, promotes claudin-2 expression in IECs. Changes in these TJ proteins may partly explain the barrier defect seen in UC patients[58]. Further, given that UC patients display profuse watery diarrhea, the activation of proteins regulating the paracellular pathway of permeability further indicates the presence of a severe barrier defect in UC patients.

1.5 Protein tyrosine phosphatase type 2 (*PTPN2*)

Genome-wide association studies have identified over 240 genes that are associated with IBD[110]. The *PTPN2* (Protein Tyrosine Phosphatase Non-Receptor Type 2) locus is one such gene with single nucleotide polymorphisms (SNPs). SNPs in the PTPN2 locus, located on chromosome 18 locus (18p11), are significantly associated with IBD (CD and UC). However, they are also associated with type 1 diabetes mellitus, rheumatoid arthritis, and celiac disease[111-114]. The risk alleles that were identified to have elevated risk in IBD were: rs2542151 (5000bp from promoter start site), rs1893217 and rs7234029[115-118]. These SNPs in the chromosome 18 locus (18p11) which is the *PTPN2* gene region, led to reduced enzymatic activity of the *PTPN2* gene product, T-cell protein tyrosine

phosphatases (TCPTP)[116]. TCPTP is ubiquitously present in all cells and in humans has two isoforms with different sizes – 45 kDa and 48KDa. The 45 kDa (TC-45) can shuttle in and out of the nucleus and the 48kDa isoform (TC-48) is localized to the endoplasmic reticulum[119, 120]. Like other phosphatases, TCPTP deactivates tyrosine kinases such as epidermal growth factor receptor (EGFR), insulin receptor (IR), and several members of the Janus-kinase-signal transducer and activator of transcription (JAK-STAT) signaling pathway[120-123]. The phosphorylation and dephosphorylation of proteins regulates specific cell mechanisms that maintain homeostasis within cells. TCPTP negatively regulates signaling pathways activated by inflammatory cytokines such as IFN-γ, IL 6 and specifically dephosphorylates JAK1, JAK3, STAT1, STAT3, STAT5 and STAT6[124, 125]. Further, *PTPN2* expression was prominently elevated in the epithelium patients with active CD lesions which suggests a critical role of *PTPN2* during intestinal inflammation in IBD[126].

Previously, it has been demonstrated that loss of *PTPN2* in intestinal epithelial cells causes increase in intestinal permeability *in vitro*[125]. Loss of *PTPN2* increased paracellular permeability by reducing TER and increasing FD4 permeability. Further, administration of IFN-γ exacerbated these barrier defects[125]. Consistent with reduced TER, our lab has also observed a significant increase in claudin-2 protein in cells lacking *PTPN2*, which occurred in a STAT1-dependent manner[127]. In murine models, constitutive loss of *Ptpn2* (*Ptpn2*-KO) was accompanied by systemic inflammation, splenomegaly, diarrhea, and mice die within 5 weeks from birth. These mice displayed an increase in FD4 permeability and tight-junction remodeling compared to wildtype controls[127]. IEC-specific deletion of *Ptpn2* in mice ($Ptpn2^{\Delta IEC}$) did not increase in vivo FD4 permeability, however, *ex-vivo* analysis of small and large intestinal segments

mounted in Ussing chambers showed an underlying increase in FD4 permeability using this more sensitive approach[128]. Ex vivo analysis also confirmed an underlying decrease in TER, and this defect was exacerbated in mice challenged in vivo with inflammatory cytokines (IFN-γ, TNF, or IL-6)[128]. These results demonstrate that epithelial PTPN2 has a critical role in preserving epithelial barrier function.

PTPN2 also plays a critical role in modifying inflammatory responses in immune cells. T-cell specific loss of PTPN2 in mice leads to a loss of tolerogenic CD8+ T cell responses and promotion of auto-reactive and destructive responses[129, 130]. These mice also showed enhanced Th1 and Th17 responses but impaired Treg activation. This partly disagrees with our findings in whole-body Ptpn2-KO mice which exhibited reduced Th17 responses. An important difference between these mouse lines (whole-body vs. CD4 T-cell specific) is the age difference with Ptpn2-KO mice being 18-21 days old (pre-weaning) while T-cell specific Ptpn2-KO mice were more mature (6-12 weeks). The opposing effects on Th17 responses may reflect that the maturing Th17 system is important for homeostatic effects on the gut barrier and is dependent on epithelial-commensal interactions which also appear to be disrupted in whole-body Ptpn2-KO mice. Our lab has also demonstrated an essential role for PTPN2 in mediating crosstalk between epithelial cells and macrophages[131]. Loss of PTPN2 in macrophages led to increased FD4 permeability and polarization of macrophages to a more inflammatory – M1-like phenotype - while loss of PTPN2 in IECs caused M1 polarization and increased IL-6 production in co-cultured wild-type macrophages. Loss of PTPN2 in both cell types provoked an even stronger M1-like polarization of macrophages and further increased IEC monolayer permeability in co-culture[131].

PTPN2 also has a key role in preserving microbial populations. Loss of *Ptpn2* in mice led to microbial dysbiosis which was followed by expansion of pathobionts like adherent invasive *E. coli* (AIEC). In addition, IBD patient cohorts also exhibit pronounced dysbiosis associated with PTPN2 SNPs thus establishing clinical relevance of PTPN2 influencing gut microbiota[54, 56]. We have also demonstrated that both *Ptpn2*-KO and *Ptpn2*$^{\Delta IEC}$ mice have defective production of antimicrobial peptides such as lysozyme in mice[132, 133]. This may be an important event that creates the right environment for dysbiosis and expansion of disease-relevant commensals with pathobiont properties such as AIEC. Together, these studies highlight that PTPN2 is a key regulator of mucosal homeostasis and has a critical role in host health.

1.6 Microbial dysbiosis and adherent-invasive *E. coli* (AIEC)

The microbiota is a collective term given to all bacterial, viral, fungal, and archaeal species found within the human body. With the advent of 16S rRNA sequencing and other high-resolution techniques, scientists have been able to identify over a 1000 species of bacteria in the GI tract itself[134]. As discussed earlier, the microbes in the gut interact with host cells and play a crucial role in intestinal homeostasis. Unfavorable shifts in the microbial species – dysbiosis – have been associated with several inflammatory and chronic conditions including IBD[134].

Experimental models of IBD have demonstrated the central role of bacteria in disease pathogenesis. While interleukin-10 (IL-10) knockout mice develop a spontaneous colitis starting at approximately 8-10 weeks under specific pathogen free conditions (SPF), they do not develop colitis under germ free (GF) conditions [135]. Further, it has been

demonstrated that IL-10 KO mice colonized with *Helicobacter hepaticus*, a pathogenic bacterium, develop severe inflammation and colitis like features[135, 136]. Another example of this occurs with IL-2 deficient mice which develop spontaneous colitis when challenged with *E. coli* mpk (indigenous *E. coli* strain isolated from IL-2 knockout mice) but not *B. vulgatus*[137]. When both species were co-administered, the mice did not develop colitis. These studies indicate that bacteria have a crucial role in development as well as protection against IBD.

Patients with IBD display an overall reduced bacterial alpha-diversity (species richness), changes in certain taxa and diminished microbiome functional capacity[134, 135]. Data from both human and animal studies have suggested that the biggest changes in IBD occur in the phyla *Bacteroides*, *Firmicutes* and *Proteobacteria* which will be discussed in this dissertation[138].

Several metagenomic studies have demonstrated the reduced abundance of Firmicutes in IBD patients. Within the phylum Firmicutes, *Faecaulibacterium prausnitzii* and *Roseburia homonis* were more frequently reduced in CD patients than UC patients[139]. However, studies in patients with UC show normal levels of *F. prausnitzii*[140]. These are butyrate-producing bacteria and loss of these species may have an impact on epithelial barrier and epithelial cell function, particularly in the colon where butyrate is the major cellular energy source for colonocytes[140]. Another Firmicute, *Ruminococcus gnavus*, is increased in IBD patients[140]. These are mucin-degrading bacteria and loss of the protective mucous layer may reduce host defenses in inflammatory conditions and thereby exacerbate disease. As a mouse model of CD-like ileitis, *Tnf*$^{\Delta ARE}$ mice display increased numbers of the Firmicutes strain, segmented filamentous bacteria (SFB), that resulted in ileum inflammation[141]. This reflects a dual

role for SFB in that they are critical for maturation of protective Th17 responses in the gut but under certain conditions also appear capable of exacerbating inflammation.

The phylum *Bacteroides* is one of the dominant species, residing in the outer mucosal layer of adult colon[142]. Many complex plant polysaccharides in the human diet cannot be digested by the host enzymes. These carbohydrates serve as a major source of energy for many bacterial communities in the distal colon including *Bacteroides spp.* They can utilize a variety of polysaccharides and therefore have an important role in carbohydrate digestion (mostly plant derived polysaccharides), providing nutrition and vitamins to the host. *Bacteroides* spp[142]. is significantly lower in patients with CD and UC[142]. The levels were also lower in patients in remission compared to control groups[142]. However, a recent study has demonstrated that proteases from a member of the *Bacteroides* spp, *B. vulgatus, were* abundantly present in patients with clinically active UC[143]. Further, fecal transplantation of UC patients with the excessive *B. vulgatus* proteases induced colitis in germ-free mice. *In-vitro* studies with this strain showed that it can damage the epithelial barrier, partly due to its ability to attach and adhere to intestinal epithelial cells. Further, *B. thetaiotamicron* has been reported to also induce colitis in rats[144]. These data suggest that the role of *Bacteroides* in the pathogenesis of IBD is yet to be fully defined and may be determined by the underlying inflammatory, microbial, and nutritional context of their microenvironment.

The expansion of the phylum *Proteobacteria* in IBD has been well documented by several microbiome studies[145]. Members of the phyla *Escherichia coli*, *Campylobacter concisus* and *Helicobacter* can individually cause diarrhea, a core feature in IBD[145, 146]. In the past few years, a strain of *E. coli* named adherent-invasive *E. coli* (AIEC) has

been frequently isolated from patient biopsies and is a subject of great interest in IBD pathogenesis.

Mucosal-associated AIEC is present in 21-62% Crohn's disease patients[147]. However, it may be present in healthy individuals as well albeit at a much lower frequently and without causing inflammation[147]. Despite its prevalence in patients, no specific gene or molecular markers have been identified that are common to all AIEC that distinguish it from other E. coli [147]. Researchers have therefore mostly relied on phenotypic assays like IEC adherence/invasion and survival in macrophages *in vitro* to identify AIEC strains[14]. Some critical genes have been identified in AIEC that help it to potentiate disease. Some of these genes will briefly be discussed in this thesis.

The adhesin protein OmpC is used by AIEC to attach to intestinal epithelial cells. Although the host receptors or the exact mechanism of such attachment is unknown, studies from other labs have demonstrated that *OmpC* gene was upregulated in conditions of high osmolarity, similar to the environment in the gut[148]. Further, antibodies from OmpC were upregulated in CD patients. Chitinase 3-like-1 (CH3L1) is a host protein upregulated during colon inflammation [148]. AIEC adhesin protein ChiA can use *CH3L1* to gain entry inside host macrophages. Recently, it was demonstrated that anti-TNF medication alleviated the levels of *CH3L1*in IBD patient macrophages which subsequently reduced AIEC colonization[149].

AIEC do not have typical virulence genes like *Salmonella* and *Shigella spp*. AIEC lacks the Type III secretion system but carries genes that can utilize a variety of gut metabolites, which provides them with a colonization advantage[148]. AIECs have been shown to utilize metabolites like nitrates, L-serine and propanediol. The ability of AIEC to use these metabolites provided them with a fitness advantage in an inflamed host[150]. A

recent study showed that AIEC isolated from CD patients were enriched with *pduC* gene which provided AIEC competitive fitness to use 1,2 propanediol as an alternate carbon source. Further, AIEC-*pduC* protein product are important for production of IL-1β from mononuclear phagocytes (MNP) which drives Th17 inflammatory responses[151]. This demonstrates that a genetic unit of AIEC provides it with competitive fitness under inflammatory conditions and can incite an inflammatory response in the host.

A well characterized interaction between AIEC and epithelial cells is mediated by carcinoembryonic antigen-related cell-adhesion molecule 6 (CEACAM6) on the host side and FimH protein present on bacterial pili[152]. A murine homologue for CEACAM6 is absent, but transgenic mice that over-expressed CEACAM6 favored AIEC colonization and displayed features of inflammation[153]. CEACAM6 belongs to a highly mannosylated GPI-anchored protein located mostly on intestinal epithelial cells[154]. Interestingly, CEACAM6 is not produced in the ileum of healthy patients but was observed in several CD patients. CEACAM6 protein levels can be elevated by several inflammatory cytokines like IFN-γ and TNF[154]. Further, AIEC can also upregulate the production of CEACAM6 expression by HIF stabilization and indirectly by promoting cytokine release (TNF), suggesting that AIEC can in fact favor its own colonization by promoting expression of its preferential host cell receptor[155]. Another study showed that oligomannoseglycans exposed on early apoptotic cells are binding targets for AIEC specifically, oligomannosidicglycans, – Asn 197 and Asn 224 – are potential receptors for FimH adhesin [156]. These data demonstrate that AIECs have an affinity for binding to complex glucose residues and can take advantage of such proteins in the host to initiate invasion of host cells. This may be particularly exploited during active inflammation where epithelial damage is a feature and may provide a foothold for AIEC colonization.

1.7 Therapeutic options in IBD

Currently, there is no full cure for IBD, but different treatments including steroids, immunosuppressants, liquid diet or surgery can help in reducing symptoms[157]. Compounds like mesalamine (5-aminosalisylic acid) have been widely used for the treatment of mild-to-moderate forms of UC but are currently discontinued or not commonly used for CD as per local guidelines[158]. Patients with moderate to severe symptoms of IBD are frequently given systemic corticosteroids. However, steroids are not useful to maintain remission and one-third of the patients become dependent on steroid use[159]. Both mesalamine and corticosteroids target end-stage inflammation and are very non-specific. Biologics including anti-TNF antibodies have been a popular alternative to corticosteroids, however, about 50% of IBD patients do not respond well or lose responsiveness over time to the drug[160, 161]. Biologics are also more expensive than conventional therapeutics. Therefore, in the recent past small molecules that target specific inflammatory pathways have been developed that can maintain disease remission and control colonic inflammation[162].

The inflammatory cytokines involved in IBD are IL-1, IL-4, IL-6, IL-9, IL-13, IL-15, IL-17, IL-21, IL-23, IL-24, IL-31, and IL-34, all of which have been implicated in the immunopathogenesis of IBD[163]. The recognition and function of these cytokines are primarily mediated by the JAK-STAT signaling pathway. This observation led to the development of a new class of small molecule therapeutics known as JAK-inhibitors (JAKi). Tofacitinib, a pan JAK-inhibitor that preferentially targets JAK-1 and JAK-3, was the first FDA approved JAKi for rheumatoid arthritis and then ulcerative colitis[164]. In Phase II clinical trials with Tofacitinib, patients showed marked mucosal healing and endoscopic improvement/remission[165]. Tofacitinib is orally administered, non-

immunogenic, has a tolerable safety profile and is less expensive than biologics[165]. Studies from our lab have demonstrated that Tofacitinib can correct IFN-γ induced epithelial barrier dysfunction in cells and human organoids[166]. Further, we have also demonstrated that Tofacitinib can reverse barrier dysfunction caused by disruption of epithelial-macrophage crosstalk due to loss of PTPN2 expression in macrophages[167]. These results demonstrate the effectiveness of Tofacitinib is not just targeted at impairing immune cell functions, but that it directly modifies intestinal epithelium to strengthen the mucosal barrier and promote mucosal healing. However, it is still unclear how tofacitinib and other JAKi can impact host microbiome diversity.

Very surprisingly, Tofacitinib was completely ineffective in CD patients[168]. However, a selective JAK-1 inhibitor, Upadacitinib, was approved for treatment of moderate to severe CD in patients that were unresponsive to anti-TNF drugs[169]. This makes it the first JAK-inhibitor to be approved for use in CD. Therefore, JAK-STAT signaling plays a very important role in IBD pathogenesis and JAK-inhibitors have emerged as very strong therapeutics in treating moderate to severe forms of both forms of this disease (CD + UC). However, while current IBD therapeutics are primarily treating the endpoint of inflammation, there is still a real need for therapeutics that treat upstream targets - or early events - that give rise to inflammation. Moreover, there is an urgent need to establish treatment paradigms that are informed by individual pharmacogenomics i.e. a personalized medicine approach, particularly since no therapeutic option achieves greater than 50% efficacy across IBD.

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CHAPTER 2

2 Generation of a Novel Fluorescent murine Adherent-Invasive *Escherichia Coli*

2.1 ABSTRACT

Background: Dysbiosis of the gut microbiota and expansion of pathobionts such as adherent-invasive *Escherichia coli* (AIEC) are associated with the pathogenesis of inflammatory Bowel Disease (IBD). Mice deficient in expression of the IBD gene candidate, *Ptpn2*, exhibit pronounced expansion of a novel murine adherent-invasive *Escherichia coli* (*m*AIEC). The aim of this study was to generate a fluorescent *m*AIEC to study host susceptibility to *m*AIEC invasion, and to identify how loss of PTPN2 expression in host cells modifies *m*AIEC invasiveness.

Methods: *m*AIEC was isolated from the distal colon of a *Ptpn2* knockout (KO) mouse. *m*AIEC was made chemically competent and then transformed with a plasmid expressing mCherry fluorescent protein and a chloramphenicol resistance cassette. For bacterial adherence and invasion studies, parental Caco-2 BBe cells were infected with *m*AIEC-mCherry at multiplicity of infection (MOI) of 10 for 3 hours. For bacterial invasion studies, cells were infected with *m*AIEC-mCherry for 5 hours and washed with gentamycin. Cells were then fixed with paraformaldehyde and cell membrane was stained for F-actin. Wildtype C57BL/6J mice were infected with *m*AIEC-mCherry for four consecutive days. Putative virulence genes common to *m*AIEC, LF82 and another mouse AIEC, NC101 were identified.

Results: We previously demonstrated that *m*AIEC can adhere to and invade IECs. We next validated that *m*AIEC-mCherry adhered to (0.94 ±0.61 bacteria/cell compared to control *E. coli* K12; 0.14±0.03 bacteria/cell, *n*=4, *P*=0.0039) IECs. *m*AIEC-mCherry had comparable adherence (*n*=4, *P*=0.35) to IECs as control (unlabeled) *m*AIEC. Bacterial invasion of *m*AIEC-mCherry invasion of IECs was visually confirmed by fluorescence microscopy. We also visually confirmed that *m*AIEC-mCherry could invade murine macrophages.

Conclusion: Here, we validated a newly generated fluorescently tagged *m*AIEC to study interactions between this novel mouse AIEC with host cells.

2.2 INTRODUCTION

Inflammatory Bowel Disease (IBD) is a chronic condition in which the immune system mounts an abnormal response to normal constituents in the gastrointestinal (GI) tract[1-3]. The intestinal epithelium has a strategic position as a protective physical barrier to luminal microbiota and immune cells in underlying lamina propria[4,5]. Dysregulation within the epithelial layer can increase intestinal permeability, lead to abnormalities in interactions between the epithelium and luminal microbiota, and disturb the mucosal immune system, all of which are linked to the clinical disease course of IBD[6-8]

The exact cause of IBD is not yet known but it is largely accepted to be a multifactorial condition[9]. Several genome-wide association studies (GWAS) have identified polymorphisms in over 240 genetic loci associated with increased risk of onset of IBD[10,11]. Many of these loci are enriched in pathways that interact with the surrounding gut microbiome and modulate intestinal homeostasis[12]. Interestingly,

alterations in the intestinal microbiome are considered a major environmental factor in the pathogenesis of IBD[13]. Several IBD patient cohorts exhibit a significant shift in their gut bacterial communities, termed as dysbiosis[14,15]. The gut microbial community performs a range of important functions for the host including digestion of substrates, educating the immune system, and repressing harmful resident or invasive bacteria[16]. Our current understanding is that genetically predisposed hosts alter the intestinal environment to paradoxically favor resident putative pathogenic bacteria, “pathobionts”, such as adherent invasive *Escherichia coli* (AIEC), and reduce protective “commensal” bacteria[17]. However, a major gap in our understanding is how genetic susceptibility alters the gut microbial landscape and how these opportunist pathobionts hijack host defense machinery and manifest complex conditions like IBD.

One of the genes identified in IBD GWAS studies is the protein tyrosine phosphatase non-receptor type 2 (*PTPN2*)[18]. It encodes the T cell protein tyrosine phosphatase (TCPTP) which dephosphorylates members of the pro-inflammatory Janus-kinase signal transducer and activator of transcription (JAK/STAT) signaling pathway during the resolution of inflammation[19]. *In vitro* studies in our lab have demonstrated that transient loss of *PTPN2* in IECs causes elevated epithelial permeability, an early and critical feature in IBD pathogenesis[20]. Intriguingly, studies have also shown that IBD patients carrying *PTPN2* single nucleotide polymorphisms (SNPs) have bacterial dysbiosis and increased disease severity[21,22]. Furthermore, our lab has also studied the phyla levels of wildtype (WT), constitutive *Ptpn2* Heterozygous (Het), and Knockout (KO) mice. We have reported that *Ptpn2*-KO mice, and to a lesser extent Het mouse exhibited a reduced abundance of *Bacteroidetes* and conversely, increased abundance of *Proteobacteria* compared to WT mice. Of note, the greatest increase in abundance

occurred within the phylum *Proteobacteria* in *Ptpn2*-KO mice that we identified as a novel *Escherichia coli* that shared significant sequence overlap with the human AIEC, which was first isolated from ileal mucosa of IBD patients. We have labeled this novel mouse adherent-invasive *E. coli*, '*m*AIEC' (UCR-PP2)[23].

The initial IBD clinical AIEC isolate, LF82 was isolated from the ileum of a Crohn's disease patients, after which many clinical isolates of AIEC have been isolated from IBD patients. In this study, we will discuss the *m*AIEC's distinct genomic features and describe the generation of a fluorescent *m*AIEC strain to study its mechanism of infection *in vitro* and *in vivo.*

2.3 METHODS

Transformation of *m*AIEC

A competent *m*AIEC was generated by previously described methods[24]. Plasmids pKB4985 and pWSK30 were kindly provided by Dr. Casey Gries. For bacterial transformation, 90 µl of competent *m*AIEC cells and 2 µg of mCherry (pKB4985) or tdTomato (pWSK30) carrying plasmids were placed on ice for 20 minutes. Plasmid pKB4985 codes for mCherry and chloramphenicol resistance gene while the pWSK30 carried the tdtomato and ampicillin resistance genes. The mixture of cells and plasmid were heat-shocked at 42°C for 45 seconds and then placed on ice, again. Luria Bertolli (LB) medium was added to the mixture and incubated at 37°C with 200-250 rpm shaking for 1 hour. 100µl of the appropriate mixture was plated in LB agar plates supplemented with chloramphenicol (Sigma Aldrich, St. Louis, MO) at 20µg/ml or ampicillin (Sigma

Aldrich, St. Louis, MO) at 100 µg/ml placed in 37°C incubator for 16 hours. Bacteria transformed with mCherry displayed bright red colonies on LB agar plates.

Cell culture

Parental Caco-2 BBe brush border (between passages 4-5) were grown in Dulbecco's modified Eagle serum (DMEM, Invitrogen, Carlsbad, CA) supplemented with 1% L-glutamine (Invitrogen, Carlsbad, CA) and 10% fetal bovine serum (FBS; Gibco Waltham, MA). Cells were kept at 37°C in 5% CO_2 and 90% humidity and media was changed every other day after a wash in 1X $Ca2^{+}$/Mg^{2+}-free phosphate-buffered saline (PBS). Monolayers were sub-cultured every 4 days after trypsinization with 0.1% trypsin (EMD Millipore, Burlington, MA).

***m*AIEC Adherence Assay**

For adherence studies, *m*AIEC-mCherry or *m*AIEC-tdtomato was grown in LB medium supplemented with chloramphenicol or ampicillin respectively for 14-16 hours at 37°C 250 rpm shaking conditions. On the day of infection, the overnight culture was added to fresh LB medium supplemented with appropriate antibiotics and grown for 1 hour at 37°C and 250 rpm shaking conditions. For the adherence assay, Caco-2 BBe IECs were infected with bacteria for 3 hours at a multiplicity of infection (MOI) 10, followed by PBS wash. Cells were lysed with 1% Triton-X 100 and plated on LB agar plates supplemented with chloramphenicol (20µg/ml) or ampicillin (100 µg/ml). Bacterial titer per bacterial strain was quantified.

*m*AIEC Invasion Assay

Caco-2 BBe cells were seeded on 12-well plates at 5 X 10^5 cells per well and cultured until 70% confluent. The cells were then infected with *m*AIEC-mCherry or tdtomato at MOI-10. The cells were infected with bacteria for 3-5 hours followed by 1 hour of fresh media supplemented with 100µg/mL gentamicin (Sigma Aldrich, St. Louis, MO). Samples were washed thrice with PBS and then plated on LB agar plates with chloramphenicol (20µg/ml) or ampicillin (100 µg/ml).

Immunofluorescence

Peritoneal macrophages were isolated from mice as previously described[25]. Caco-2 BBe cells or macrophages were seeded on coverslips. Bacterial invasion assay was performed as described above. The cells were fixed with 4% paraformaldehyde (PFA) for 20 minutes at room temperature. Cells were washed with PBS thrice and then permeabilized with 0.3% Triton X-100 (Fischer Scientific, Waltham, MA) for 5 minutes and blocked with 5% BSA (Fischer Scientific, Waltham, MA) for 10 min at room temperature. Next, we stained the cells with Alexa Fluor 488-Phalloidin antibody (1:1000; Abcam #176753, Cambridge, MA) at room temperature for 90 minutes. The nuclei were stained with 4,6-diamididino-2-phenylindole (DAPI; Vector Laboratories, Newark, CA). Images were captured using Leica DM5500 microscope attached with a DFC365 FX camera using a 63X oil immersion objective or the inverted Zeiss Airyscan. DAPI was visualized using a 405 nm excitation laser and DAPI filter set. mCherry was visualized using a 561 nm excitation laser and mCherry filter set. Images were analyzed using ImageJ software.

Enumeration of *m*AIEC from fecal pellets

C57BL/6N mice were infected with 10^9 colony forming units (CFU)/mL of *m*AIEC^{red} by oral gavage for 4 consecutive days. Fecal pellets were collected daily and plated on LB agar plates supplemented with chloramphenicol (20µg/ml). Bacterial colonies were counted and expressed in CFU/gram/fecal sample.

Identifying unique genes in *m*AIEC

The genome of *m*AIEC was compared with the genome sequence of the human LF82 isolate. Putative virulence genes found in *E. coli*'s and other AIECs including LF82 were identified by comparing the known sequence of each gene from K12. Putative virulence genes common between *m*AIEC; another opportunistic mouse *E. coli* with some AIEC features, NC101; and the human LF82 AIEC; or genes unique to each isolate, were compared and the percent identity of each gene and predicted protein between the isolates was determined using EMBOSS Water.

2.4 RESULTS

mAIEC^{red} displays similar adherence and invasion in IECs as compared to wildtype mAIEC.

We successfully transformed the *m*AIEC strain with plasmids carrying the fluorescent gene mCherry or tdTomato. After this, we wanted to determine if the transformed strains displayed epithelial adherence and invasion as seen previously in the wildtype strain. To do so, parental Caco-2 BBe cells were infected with the wildtype *m*AIEC, *m*AIEC-mCherry, *m*AIEC-tdtomato or the non-invasive *E. coli* K12. We observed that *m*AIEC-mCherry and

*m*AIEC-tdtomato displayed similar adherence and invasion as the wildtype *m*AIEC strain (Figure 4 A, B). However, we see both *m*AIEC-mCherry and *m*AIEC-tdtomato have higher adherence and invasion than non-invasive *E. coli* K12.

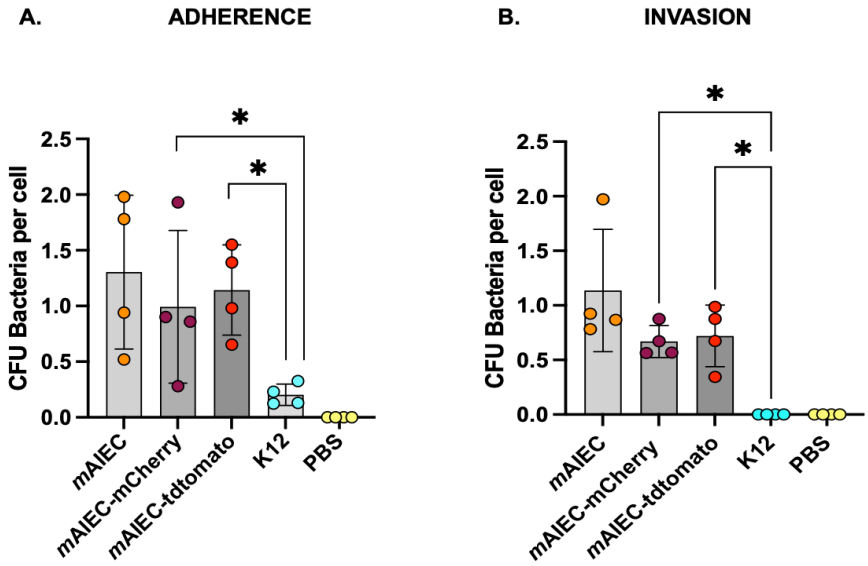


Figure 4: *m*AIEC-mCherry and *m*AIEC-tdtomato possess characteristics of epithelial adherence and invasion.

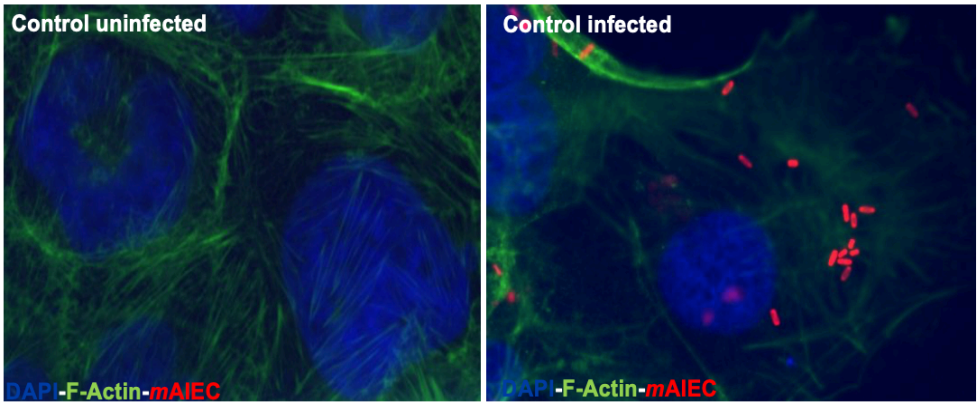
*m*AIEC-mCherry and *m*AIEC-tdtomato displayed similar (A) Adherence (B) Invasion of intestinal epithelial cells as wildtype *m*AIEC. Both *m*AIEC-mCherry and *m*AIEC-tdtomato have much higher adherence and invasion compared to non-invasive *E. coli* K12 ($P<0.005$; $n=4$).

mAIEC^{red} invasion of IECs and macrophages was confirmed by immunofluorescence.

After confirming *m*AIEC-mCherry and *m*AIEC-tdtomato's adherence and invasion potential, we next wanted to determine, if we can visualize *m*AIEC-mCherry/tdtomato invasion of IEC and macrophages by immunofluorescence microscopy. We stained the host-cell epithelial membrane with F-actin (green) while the nucleus was stained with DAPI (blue). We visualized the *m*AIEC-mCherry very clearly inside host cells (Figure 5 A). The fluorescent signals were bright and could be observed easily (Figure 5 A). However, *m*AIEC-tdtomato had a very weak fluorescent signal and therefore, could not be observed by immunofluorescence microscopy. Despite showing similar adherence and invasion properties as *m*AIEC, *m*AIEC-tdtomato was not selected for future experiments due to its weak fluorescence intensity. Instead, *m*AIEC-mCherry was chosen due to its bright fluorescence and adherence and invasion properties. We named this novel fluorescent *m*AIEC strain as *m*AIEC^{red}.

Further, we wanted to determine if *m*AIEC^{red} can invade macrophages. Peritoneal macrophages were isolated from C57BL/6N mice and infected with *m*AIEC^{red}. We visually confirmed *m*AIEC invasion of host macrophages (Figure 5 B).

A.



B.

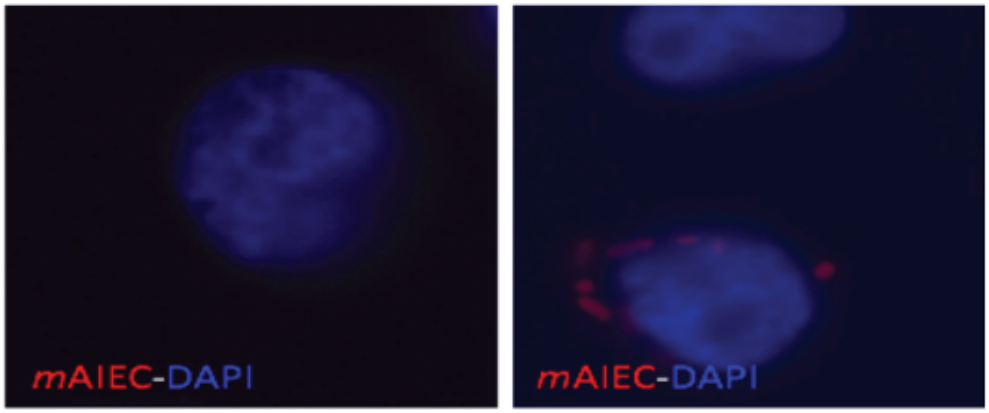


Figure 5: *m*AIEC^{red} invasion of intestinal epithelial cells and macrophages.

A. Caco-2 BBe cells or **B.** peritoneal macrophages were seeded on coverslips and infected with *m*AIEC ^{red} at MOI of 10 for 3-5 hours. Cell membrane of IECs was stained with F-actin (green) and nucleus of IEC and macrophages was stained with DAPI (blue). *m*AIEC^{red} is seen in red (n=3-5).

mAIEC persists in mice for approximately three weeks.

After validating *m*AIEC^{red} for *in vitro* invasion studies, we wanted to test if it can be used for *in vivo* studies in mice models. To do so, we used C57BL/6N mice and infected them with 10^9 cfu/ml *m*AIEC^{red} for four consecutive days. Fecal samples were collected daily and plated on chloramphenicol substituted LB agar plates. *m*AIEC^{red} colonies were enumerated. *m*AIEC^{red} persisted *in vivo* for upto 18 days (Figure 6).

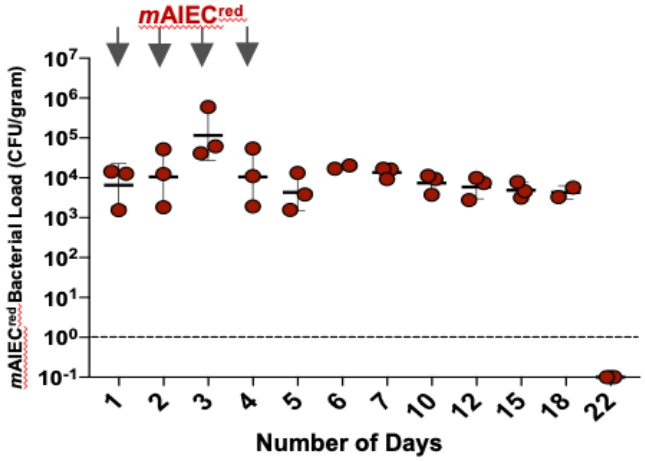


Figure 6: *m*AIEC^{red} could be detected from the fecal pellets of mice for upto 18 days post-infection in LB-chloramphenicol plates (n=3).

mAIEC harbors unique putative pathogenic genes.

Since the rRNA ITS region of *m*AIEC and the human AIEC LF82 had 100% sequence identity, we next wanted to identify how similar these bacteria are across their genomes. We sequenced the genome of our novel mouse AIEC and compared it with the published genome sequence of LF82 (GenBank CU651637.1). *m*AIEC showed approximately 90.3% sequence identity to the genome of the human AIEC LF82. We further probed for the presence in our *m*AIEC of putative virulence genes found in *E. coli's* and other AIECs including LF82. Putative virulence gene sequences and their predicted protein products were compared using EMBOSS Water. There were 14 total putative virulence genes that were used in this analysis. In particular, *m*AIEC showed two distinct differences: *m*AIEC *yfcU* and *tnpB* had very low % gene and protein identities when compared to the same gene and protein sequences from LF82 and NC101 (Table 1). Since analyses using BLAST determined that the closest matches to these genes and proteins were *yfcU* or *tnpB* for all three bacteria, this suggests that these proteins all have similar functions but that the sequences from our *m*AIEC are very different from those from LF82 and NC101 (Table 1).

We also identified unique virulence genes in *m*AIEC. Genes such as *Virb9*, *Virb4* relating to type IV secretion system, were present only in *m*AIEC and not LF82 or NC101 (Table 2).

Gene	Reference	Function	% gene identity with NC101	% query covered	% putative protein identity with NC101	% query covered	% gene identity with LF82 (Accession CU651637.1)	% query covered	% putative protein identity with LF82	% query covered
fimH	70	Terminal subunit of Type 1 pilus	*99.1	100	99	100	99.4	100	99.5	100
yfcU	71	Outer membrane usher protein	55.5	98	44.6	97	54.7	87	44.7	97
yadC	72	Fimbrial-like protein	*92.8	100	91.2	100	*93.2	100	91.2	100
csgE	73	Curli production assembly/transport component	99.7	100	100	100	99.7	100	100	100
yehA	74	Putative fimbrial-like protein	99.3	100	99.4	100	99.4	100	99.4	100
hcpA	73	Major exported protein	98.6	100	100	100	100	100	100	100
fimA	73	Type-1 fimbrial protein A chain	90.2	100	90.7	100	*91.4	100	91.3	100
ygiL	75	Fimbrial family protein	100	100	100	100	100	100	100	100
ftsh	76	ATP-dependent zinc metalloprotease	99.3	100	100	100	99.3	100	100	100
vat	77	Vacuolating autotransporter toxin	100	100	100	100	100	100	100	100
malX	73	PTS system maltose- and glucose- specific EIICB component	99.7	99	100	100	99.7	100	100	100
tnpB	78	IS200/IS605 family element transposase accessory protein	43.5	99	34.1	93	47.6	99	34.1	94
dsbA	73	Thiol:disulfide interchange protein	99.4	100	100	100	99.7	100	100	100
fliA	79	RNA polymerase sigma factor	98.9	100	100	100	99.8	100	100	100

Table 1: Summary of putative virulence genes between novel *m*AIEC, LF82 and NC101

Operon	*m*AIEC	LF82	NC101
CRISPR genes	Present	Present	-
Flagella associated genes	Present	Present	-
Fimbriae associated genes	Present	Present	-
Type II secretion system	Present	Present	Present
Type IV secretion System	Present	Not present	Not present
Iron-Sequestering genes	Present	Present	Present

Table 2: Summary of genes unique to *m*AIEC compared to human AIEC LF82 and murine AIEC NC101

2.5 DISCUSSION

We have previously demonstrated that AIEC is capable of both initiating and exacerbating disease[23, 25]. In this study, we generated a novel strain of fluorescent murine adherent-invasive *E. coli* (*m*AIEC^{red}) which can be used in both *in vitro* and *in vivo* model systems to study the role of AIEC in the development of IBD. We transformed the wildtype strain with two different plasmids, which did not affect *m*AIEC's properties of epithelial adherence and invasion. Surprisingly, *m*AIEC-tdtomato was weakly fluorescent, possibly due the plasmid construct, being improperly transcribed. However, transformation with the plasmid containing mCherry did not hamper AIEC's adherence/invasion characteristics and was sufficiently fluorescent. With *m*AIEC^{red} we can follow its dissemination *in vivo* or characterize host proteins that mediate its entry into host cells which we believe, will further our understanding of host-pathobiont interactions.

Confluence of faulty genetics and environmental variables perturb the immune-microbiome axis which lead to complex diseases. To also identify possible factors that contribute to *m*AIEC colonization in mice, we compared the genome sequence of the novel mouse AIEC to the human LF82 AIEC and another opportunistic mouse *E. coli* with some AIEC features, NC101. The genome of *m*AIEC showed >90% sequence identity to LF82. Intriguingly, two putative virulence genes, *yfcU* and *tnpB*, from our *m*AIEC were compared to those from LF82 and NC101. The *m*AIEC gene and putative protein sequences had very low percent identities when compared to the other two bacteria providing further evidence for the novelty of our strain and indicating potentially different gene variants or protein isoforms that may give rise to differences in pathogenicity (e.g., adherence/invasion) between *m*AIEC, LF82 and NC101.

Unlike *Salmonella* and *Shigella* spp, AIECs do not possess type 3 secretion system[26]. Our findings suggest that *m*AIEC may harbor genes relating to the type 4 secretion system (T4SS). Interestingly, the type 4 secretion system is seen in virulent strains of *Helicobacter* and *Brucella* spp[27]. Although, T4SS genes were absent in AIEC LF82 and NC101, more recent AIEC isolates from CD patients harbor the T4SS genes[28]. The study further showed that the T4SS was essential for biofilm formation on epithelial cells and promoted AIEC persistence in the gut. Although, we still need to validate the presence of T4SS genes in *m*AIEC, these findings shed light into one of the possible mechanisms of *m*AIEC pathogenesis.

In conclusion, in this chapter we describe the generation of a novel fluorescent AIEC. We also identify novel genes present in *m*AIEC that may impart additional virulence and fitness characteristics to *m*AIEC in a genetically susceptible host.

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CHAPTER 3

3 Intestinal Epithelial PTPN2 Limits Pathobiont Colonization by Promoting Immune-directed Antimicrobial Defense Mechanisms

3.1 ABSTRACT

Background and Aims: Loss of activity of the inflammatory bowel disease (IBD) susceptibility gene, protein tyrosine phosphatase non-receptor type 2 (*PTPN2*), is associated with altered microbiome composition in both human subjects and mice. Further, expansion of bacterial pathobionts, adherent-invasive *E. coli* (AIEC), is strongly linked to IBD pathogenesis. The mechanism by which the intestinal epithelial cells (IEC) maintain equilibrium between commensal microbiota and immune cells to restrict invading pathobionts is poorly understood. Here, we investigated the role of IEC-specific *Ptpn2* in regulating pathobiont colonization.

Methods: Tamoxifen-inducible, intestinal epithelial cell-specific knockout mice ($Ptpn2^{\Delta IEC}$) and control $Ptpn2^{fl/fl}$ were were infected with either non-invasive *E. coli* K12, or fluorescent-tagged *m*AIEC (mAIEC^{red}) for four consecutive days or administered PBS. Bacterial colonization of mouse intestinal regions was quantified. mRNA and protein expression were assayed in intestinal epithelial cells (IECs) or whole tissue lysates by PCR and Western blot. Tissue cytokine expression was determined by ELISA. Intestinal barrier function was determined by *in vivo* administration of 4 kDa FITC-dextran (FD4) or 70kDa Rhodamine-B dextran (RD70) fluorescent probes. Confocal microscopy was used to determine the localization of tight-junction proteins.

Results: *Ptpn2*$^{\Delta IEC}$ mice exhibited increased *m*AIEC^{red} - but not K12 - bacterial load in the distal colon tissue compared to *Ptpn2*$^{fl/fl}$ mice. The higher susceptibility to *m*AIEC^{red} infection was associated with reduced levels of anti-microbial peptides (AMPs). Ileal RNA expression of the alpha-defensin AMPs, *Defa5* and *Defa6*, as well as the AMP processing enzyme, matrix metalloproteinase 7 (MMP7), were significantly lower in *Ptpn2*$^{\Delta IEC}$ vs. *Ptpn2*$^{fl/fl}$ mice, after *m*AIEC^{red} but not K12 infection. Moreover, *Ptpn2*$^{\Delta IEC}$ mice displayed lower protein levels of AMP promoting cytokines, IL-22, IL6, IL-17A following *m*AIEC infection compared to *Ptpn2*$^{fl/fl}$ controls. *Ptpn2*$^{\Delta IEC}$ mice also exhibited increased FD4 but not RD70 permeability following infection with *m*AIEC^{red}. Surprisingly, but also with the non-invasive -K12 challenge also increased FD4 permeability in *Ptpn2*$^{\Delta IEC}$ mice compared to infected floxed controls. Permeability defects were associated with reduced membrane localization of zonula occludens 1 (ZO-1).

Conclusion: Our findings highlight that intestinal epithelial PTPN2 is crucial for mucosal immunity and gut homeostasis by promoting antibacterial defense mechanisms involving coordinated epithelial-immune responses to restrict pathobiont colonization.

3.2 INTRODUCTION

The intestinal epithelium has a strategic position as a physical barrier to trillions of luminal microbes and immune cells in the underlying lamina propria [4-6] while also coordinating a very delicate equilibrium to maintain mucosal homeostasis. Dysregulation of the physical barrier leads to very serious consequences including several autoimmune and systemic conditions including coeliac disease, graft-versus host disease, type 1 diabetes (T1D), and inflammatory bowel disease (IBD) [7-9].

IBD is a chronic and multifactorial condition affecting more than 6 million people worldwide [10, 11]. IBD clinically manifests as two sub-types, Crohn's Disease (CD) and Ulcerative colitis (UC)[12]. IBD involves a complex interplay of host genetics, gut flora, environmental factors, and the immune system. Genome-wide association studies (GWAS) have identified approximately 240 gene loci associated with IBD[13]. The single nucleotide polymorphism (SNPs) in *rs1893217* which is the gene encoding region of protein tyrosine phosphatase type 2 (*PTPN2*), has been associated with several chronic inflammatory conditions like type 1 diabetes (T1D), rheumatoid arthritis (RA) and both subtypes of IBD. The SNP is carried by around 16 % of the general population (non-IBD) and 19-20% of people with IBD, suggesting that there is an increased association of this SNP with IBD. Loss of *PTPN2* function leads to aberrant activation/proliferation of pro-inflammatory immune cells and causes hyper-responsiveness of the Janus Activated Kinase (JAK)- signal transducer and activator of transcription (STAT) pathway[14, 15]. Further, significant alterations in microbial communities – dysbiosis – has been observed in IBD patients including those carrying *PTPN2* SNPs[16-18].

Our current understanding of the pathophysiology of IBD is that that genetically pre-disposed hosts have an altered intestinal environment that favors the expansion of commensal microbes with pathogenic potential, "pathobionts" while reducing the number of protective commensal bacteria. One such pathobiont is adherent-invasive *E. coli* (AIEC), which was first isolated from the ileum of a CD patient[19]. AIEC can adhere to and invade intestinal epithelial cells, in addition to surviving within macrophages [20]. Previously, we have reported that whole-body constitutive *Ptpn2*-KO mice, exhibit a reduced abundance of *Bacteroidetes* and a greatly increased abundance of *Proteobacteria* compared to *Ptpn2* wild-type littermates. Of note, the greatest increase

in abundance within the phylum *Proteobacteria* in *Ptpn2*-KO mice was a novel *Escherichia coli* that shared significant sequence overlap with the human AIEC. We confirmed the adherent-invasive properties of this novel mouse adherent-invasive *E. coli* (*m*AIEC) strain, UCR-PP2, as well as its capacity to replicate in macrophages[21].

Intestinal epithelial cells (IECs) are physically connected to each other by tight junction (TJ), adherens junction (AJ) and desmosome protein conglomerates that hold neighboring epithelial cells in place to allow formation of a tightly regulated semi-permeable barrier while maintaining an intact yet flexible epithelial monolayer [22-25]. Tight junctions are the critical regulators of paracellular permeability and disruption of expression or localization of these proteins often leads to increased paracellular flux of electrolytes, water and macromolecules [26]. The epithelial monolayer is essential for intestinal health as it interacts with the commensal population in the luminal space and transmits specific signals that educates the underlying innate and adaptive immune cell population and therefore, maintains mucosal immune homeostasis[27-29]. However, increases in epithelial permeability or damage to the epithelial barrier can give access to potentially pathogenic bacteria to interact with the immune cells in the lamina propria, or in more severe cases to access the circulation and cause sepsis. Mucosal immune cells are critical in eliminating invading pathogens as they secrete cytokines and phagocytose bacteria, features essential for pathogen-clearance[30]. Regulation of gut luminal microbes is also dependent on specialized IEC subsets including goblet cells that secrete mucus, and Paneth cells that secrete several anti-microbial peptides that kill/ or inhibit microbial growth[31-33]. The intestinal barrier, therefore, is essential for mucosal homeostasis and increased intestinal permeability is an early and critical event in several inflammatory conditions including IBD[34, 35]. Previously, our lab has demonstrated that

Ptpn2-KO mice display increased intestinal permeability and displayed reduced anti-microbial peptide production[36-38]. These results point towards epithelial *Ptpn2* as an important contributor to maintenance of the intestinal barrier and anti-microbial defenses.

However, there remains a major gap in our understanding of how host-genetics alters the gut microbial landscape and how these opportunist pathobionts hijack host defense machinery and manifest complex conditions in a disease susceptible host. Thus, the main aim in this study was to assess the role of *Ptpn2* in the intestinal epithelium in mediating microbiome-immune cell crosstalk to prevent *m*AIEC colonization and preserve gut-homeostasis.

3.3 METHODS

Animal Procedures

Ethical Statement on Mouse Studies – All animal care and procedures were performed in accordance with institutional guidelines and approved by the University of California, Riverside Institutional Animal Care and Use Committee under Protocol #A2022001B.

Housing and husbandry of experimental animals

Tamoxifen-inducible IEC-specific *Ptpn2* knockout (*Ptpn2* $^{\Delta IEC}$) were generated as previously described[37]. Cre negative, floxed Ptpn2 (*Ptpn2*$^{fl/fl}$) littermates were used as controls. 5–6-week-old male and female mice were injected with tamoxifen via intraperitoneal injections at 1mg/ml in 100μl of corn oil for 5 consecutive days. 28-days from the last injection, mice were orally gavaged with 100μl of either PBS, *E. coli* K12 or

*m*AIEC^{red} (*m*AIEC strain-UCR PP2) at 10^9 cfu/ml for 4 consecutive days. All mice were 9–12 weeks-old at the time of sacrifice and housed in specific pathogen-free (SPF) conditions at the University of California, Riverside.

Bacterial infection and immunofluorescence studies

Bacteria from stocks frozen at –80°C in 1:1 vol/vol glycerol: LB were cultured overnight in Luria–Bertani (LB) broth supplemented with chloramphenicol at 37°C, 250–300rpm, and regrown the next day in fresh LB-chloramphenicol to exponential phase growth. Culture was pelleted, washed with phosphate buffered saline (PBS), and resuspended in PBS. The bacteria used were *m*AIEC^{red}, and K12 (a noninvasive *E. coli*, ATCC 25404).

Tissue RNA isolation and quantitative PCR.

Total RNA was extracted from intestinal segments from mice using TRIzol reagent according to the manufacturer's instructions (Invitrogen, Thermo Fisher Scientific). RNA purity and concentration were assessed by absorbance at 260 and 280 nm. One microgram of total RNA was transcribed into cDNA using qScript cDNA SuperMix (Quanta Biosciences). Two microliters of 5×-diluted cDNA were amplified using gene-specific primers (Table 3) and GoTaq Green, 2× mix. Gene-specific primers were used with the following conditions: initial denaturation 95°C for 5 minutes, followed by 30 cycles 95°C for 30 seconds (denaturation), 55°C for 30 seconds (annealing), and 72°C for 30 seconds (extension). The final extension was 72°C for 5 minutes. Mouse GAPDH was used as an endogenous control.

Table 3: Primer Sequences for Quantitative PCR

Gene	Temperature (℃)	Sequence
Gata3	62	Forwards: GGA AAG CTG GTT CGG AGG CA Reverse: GCC GAT TCA TTC GGG CTC AG
Il4	60	Forwards: CAT GGG AAA ACT CCA TGC TT Reverse: TGG ACT CAT TCA TGG TGC AG
Rorγt	62	Forwards: CCG CTG AGA GGG CTT CAC Reverse: TGC AGG AGT AGG CCA CAT TAC A
Ifnγ	60	Forwards: GCC ACG GCA CAG TCA TTG AA Reverse: CGC CTT GCT GTT GCT GAA GA
Il6	60	Forwards: AGTCCGGAGAGGAGACTTCA Reverse: TTGCCATTGCACAACTCTTT
Il22	60	Forwards: GCTCAGCTCCTGTCACATCA Reverse: CAGTTCCCCAATCGCCTTGA
Il17a	60	Forward: CATCACTGCCACCCAGAAGACTG Reverse: ATGCCAGTGAGCTTCCCGTTCAG
Il1β	60	Forwards: CCCAGAAATCAAGGAGCATT Reverse: TCACTCTTCACCTGCTCCAC
Defa5	60	Forwards: GAATATACCCTCCGCACGCA Reverse: TCTTTTGGCAGGCCAGTTCT
Defa6	62	Forwards: TGCAAGGTGAAGTTGCCAAG Reverse: GGTTCATAGCCCAGTGTCGG

In-vivo barrier permeability

Mice were gavaged with 80 mg/mL of fluorescein isothiocyanate (FITC)-dextran (4 kDa) (FD4) and 20 mg/mL of rhodamine B-dextran (70 kDa) (RD70). After 5 hours, blood was collected by tail bleed (germ-free mice) or retro-orbital bleed into serum collection tubes. Blood was centrifuged at 4°C, 1500 *g*, for 15 min, and serum was analyzed for FITC-dextran and rhodamine B-dextran concentration. Fluorescence of FITC and rhodamine in samples was determined by loading serum into a black plate and measuring excitation wavelengths of 495 nm and 555 nm, and emission wavelengths of 525 nm and 585 nm, respectively, with a SpectraMax iD3 plate reader (Molecular Devices), using established protocols[37, 39]. Standard curves for calculating fluorophore concentration in the samples were obtained by diluting the fluorophore stock in sterile MilliQ.

Isolation of IECs and western blot analysis

Isolated whole intestinal tissues were everted and incubated in Cell Recovery Solution (354253, Corning) on ice for 2 hours, then vigorously shaken by hand to release IECs. IECs were washed twice with ice-cold PBS, then lysed with radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, and 0.1% SDS) supplemented with 1× protease inhibitor (Roche), 2 mM sodium fluoride, 1 mM PMSF, and phosphatase inhibitors (2 mM sodium orthovanadate, Phosphatase Inhibitor Cocktail 2 and 3, MilliporeSigma) for at least 10 minutes on ice. Cells were homogenized on ice using the Q125 Sonicator (QSonica Sonicators), lysates centrifuged at 16,200*g* at 4°C for 10 minutes, and supernatants collected into new

microcentrifuge tubes. Protein concentration was determined using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific). Loading samples were prepared by mixing the same amount of total protein from each sample with Laemmli loading buffer (60 mM Tris-HCl pH 6.8, 2% SDS, 5% β-mercaptoethanol, and 10% glycerol), then boiling the samples at 95°C for 10 minutes. Protein (20 µg) was loaded on polyacrylamide gels, and after separation by gel electrophoresis, transferred onto polyvinylidene difluoride membranes. Nonspecific epitopes were blocked with 5% milk in Tris-buffered saline with 0.1% Tween-20 added for 1 hour at room temperature. Membranes were incubated overnight with primary antibody at 4°C, washed (×3) with Tris-buffered saline with 0.1% Tween-20, and incubated with horseradish-peroxidase–conjugated secondary antibody against the primary antibody species (Table 4) for 1 hour at room temperature. Immunoreactive proteins were detected with x-ray films (Labscientific, Inc, Highlands, NJ) using the SuperSignal West Pico PLUS chemiluminescence detection kit (cat. no. 34580; Thermo Fisher Scientific).

Table 4: Primary and Secondary Antibodies for Western-Blotting

Antibody	Host	Provider	Catalog Number	Dilution
CD45	Rabbit	Cell Signaling	72787	1:1000
CD3	Rabbit	Abcam	ab5690	1:1000
Claudin-2	Mouse	Invitrogen	32-5600	1:1000
Claudin-3	Rabbit	Invitrogen	34-1700	1:1000
Claudin 4	Mouse	Invitrogen	32-9400	1:1000
Claudin-7	Mouse	Invitrogen	37-4800	1:1000
EpCAM	Rabbit	Cell Signaling	42515	1:1000
E-Cadherin	Mouse	BD Biosciences	610181	1:1000
F4/80	Rat	Thermo Fisher Scientific	MA5-16624	1:1000
JAM-A	Rabbit	Invitrogen	36-1700	1:1000
Lysozyme	Rabbit	Abcam	76784	1:1000
MMP7	Rabbit	Abcam	232737	1:1000
Occludin	Rabbit	Thermo Fisher Scientific	71-1500	1:1000
TCPTP	Rabbit	Cell Signaling	58935	1:1000
ZO-1 Polyclonal	Rabbit	Thermo Fisher Scientific	61-7300	1:1000
β-Actin	Mouse	Sigma-Aldrich	A5316	1:1000
HRP-conjugated Anti-rabbit IgG	Goat	Jackson Immunoresearch	111-036-045	1:5000

Enzyme-Linked Immunosorbent Assay (ELISA)

Distal colon whole tissue was excised to perform ELISAs. IL22 and IL6 DuoSet enzyme-linked immunosorbent assays were obtained from R&D Systems (Minneapolis, MN) and performed according to the manufacturer's guidelines.

Cells

Caco-2 BBe1 cells and HT-29cl.9A IECs were cultured in Dulbecco's modified Eagles's Medium (DMEM) and McCoy's 5A medium, respectively. The medium was supplemented with 10% heat-inactivated fetal bovine serum (FBS), 1% L-glutamine, and 1% penicillin/streptomycin. Cells were cultured in 6-well plates for protein assay, or immunofluorescence assay. Insertion of SNP *rs1893217* (*PTPN2*-KI) and complete knockout of *PTPN2* (*PTPN2*-KO) in Caco-2 BBe cell lines was performed using CRISPR-Cas9 gene editing by Synthego. For *PTPN2* knockdown in HT-29 cells, lenti-viral particles containing scrambled shRNA (*PTPN2*-CTL) or PTPN2-specific shRNA (*PTPN2*-KD) were generated as previously described[40]. For barrier function analysis, cells were grown on trans-wells. Standard temperature and CO_2 conditions were maintained in the incubator.

Barrier-Function Assays

Polarized Caco-2 BBe cells were grown in 12-well trans wells. The media was changed to serum-free media overnight. The cells were exposed to multiplicity of infection (MOI) 10 AIEC-LF82 or PBS for 3 hours, after which transepithelial electrical resistance (TER) was measured using EVOM2 epithelial Voltameter (World Precision instruments) and

chopstick electrode set for EVOM2. 3 measurements were taken per transwell in ohms.cm^2.

Fluorescein isothiocyanate-dextran (4 Sigma-Aldrich, 4 kDa FITC-dextran; Sigma-Aldrich) was used to measure macromolecular paracellular permeability. Cells were washed twice and then equilibrated with PBS with $CacCl_2$-$MgCl_2$ for 30 minutes at 37° C. FD4 was prepared at a final concentration of 1 mg/ml and was added to the apical side. After 2 hrs, at 37° C, the basolateral solution was collected for measurements. Fluorescence measurements were taken using Molecular devices plate reader.

Immunofluorescence

Mouse intestinal segments were fixed in OCT-embedded frozen sections and cut into 5-µm sections, and the slides brought to room temperature, rinsed twice with PBST, and fixed in methanol (10 min at -20°C). Slides were incubated in blocking buffer (PBS + 2% donkey serum, 1% BSA, 1% Triton X, 0.05% Tween-20) for 1 hour before overnight incubation with F'ab donkey-anti-mouse antibody (Jackson ImmunoSearch Inc, West Grove, PA). The slides were rinsed with PBST, incubated with primary antibody (diluted in PBS + 5% normal donkey serum) for 30 min at room temperature, rinsed again with PBS before incubation with a biotinylated secondary anti-mouse antibody (Jackson ImmunoSearch Inc.) for 20 min, followed by an additional wash in PBST and subsequent incubation with Alexa Fluor 488-steptavidin, Alexa Fluor 647 secondary antibody (Jackson ImmunoSearch) for 30 min, and finally mounted with ProlongGold Antifade Reagent with DAPI (Thermo Fisher Scientific). Images were acquired with an inverted Zeiss 880 Airy-scan microscope.

Statistical analyses

We set critical significance level $\alpha = 0.05$ and analyzed our data using parametric statistics. Data are expressed as mean ± SD for *n* number of biological replicates. Between-group inferences were made by using 1-way or 2-way analysis of variance (ANOVA) followed by Tukey's or Holm-Sidak post-hoc test was performed in GraphPad Prism version 9 (GraphPad, USA).

3.4 RESULTS

Loss of intestinal epithelial Ptpn2 promotes mAIEC colonization.

Previous studies from our lab showed that constitutive *Ptpn2*-deficient mice exhibit expansion of a novel *m*AIEC and display reduced expression of anti-microbial peptides[21, 36]. To determine the specific role of epithelial *Ptpn2* in limiting *m*AIEC colonization, we used tamoxifen-inducible *Ptpn2* Villin-cre transgenic mice to induce specific deletion of the epithelial *Ptpn2* gene ($Ptpn2^{\Delta IEC}$) and control Cre-negative *Ptpn2* floxed mice ($Ptpn2^{fl/fl}$). Mice were treated with PBS; a non-pathogenic, non-invasive *E. coli*, K12; or the mCherry fluorescent-tagged *m*AIEC^{red} (Figure 7 A). We used *E. coli* K12 as a bacterial control to identify specific host responses after bacterial colonization with a non-invasive, non-pathogenic *E. coli*strain. *m*AIEC^{red} persistence was validated *in vivo* (Figure 6). *m*AIEC^{red} infected mice displayed a mild decrease in body weight in both $Ptpn2^{\Delta IEC}$ and $Ptpn2^{fl/fl}$ mice (Figure 7 B), however no changes in colon length were observed (Figure 7 C). Interestingly, we observed, $Ptpn2^{\Delta IEC}$ had higher *m*AIEC^{red} burden in the distal colon tissue

as compared to *Ptpn2* $^{fl/fl}$ mice (Figure 8 A). We also observed elevated colonization of *m*AIEC^{red} in the proximal colon tissue section albeit without reaching statistical significance (*P*=0.0192) (Figure 8 B). Expectedly, *m*AIEC^{red} displayed higher colonization of proximal colon in *Ptpn2* $^{\Delta IEC}$ mice compared to its K12 infected counterparts (Figure 8 A, B). Furthermore, we didn't observe any difference in bacterial load of *m*AIEC in the luminal contents between the 2 genotypes (Figure 9 C, D) suggesting that while the luminal bacterial *m*AIEC load remained similar between the genotypes, *Ptpn2*$^{\Delta IEC}$ mice were more susceptible to *m*AIEC colonization of IECs. The increased colonization of *Ptpn2* $^{\Delta IEC}$ was visually confirmed by immunofluorescence staining. Higher bacterial burden was associated with epithelial cells marked with epithelial cellular adhesion molecule (EpCam) as compared to the macrophages marked with ADGRE1 (F4/80) (Figure 8 C). Taken together, these data demonstrate that IEC-specific loss of *Ptpn2* makes the host susceptible to *m*AIEC infection.

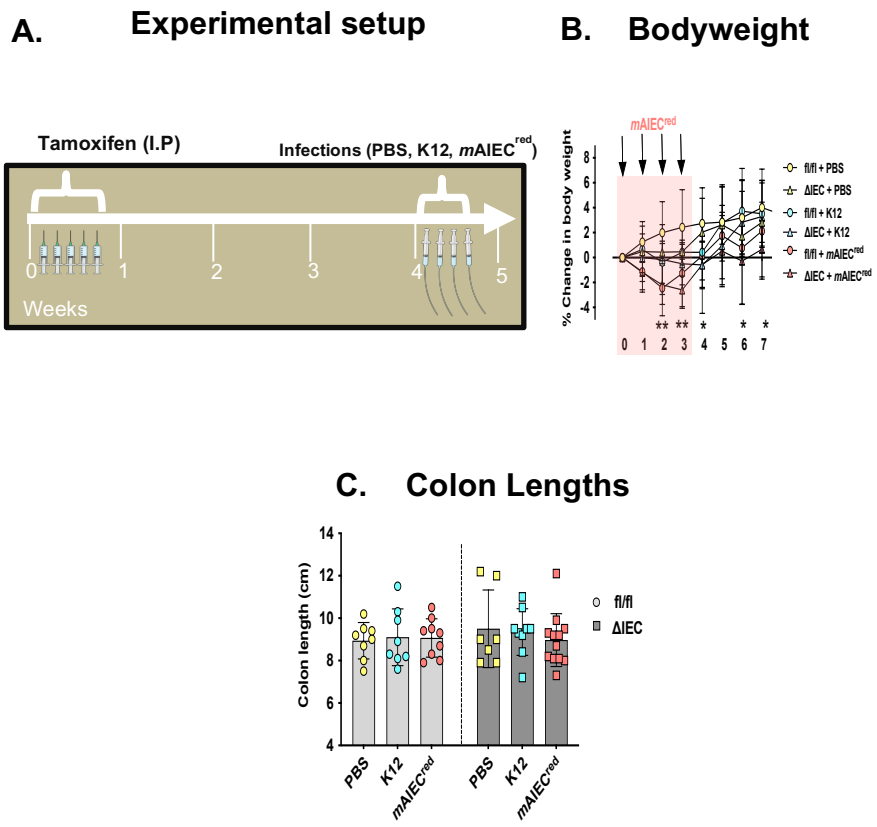

Figure 7: Loss of intestinal epithelial *Ptpn2* does not change colon length or bodyweight.
Ptpn2$^{fl/fl}$ or *Ptpn2*$^{\Delta IEC}$ were given (A) tamoxifen at 1mg/ml for 5 consecutive days. After a period of 28 days, the mice were given PBS, E. coli K12 or *m*AIEC^{red} (10^9 bacteria per mouse in 100 ul of cornoil) from day 0-3 (n=9-12). The mice were sacrificed, and bacterial colonies were enumerated at day 7. (B) *m*AIEC^{red} infected groups display mild loss of body weight (P=0.006). (C) No appreciable impact was seen in colon length (P>0.005; n=8-12).

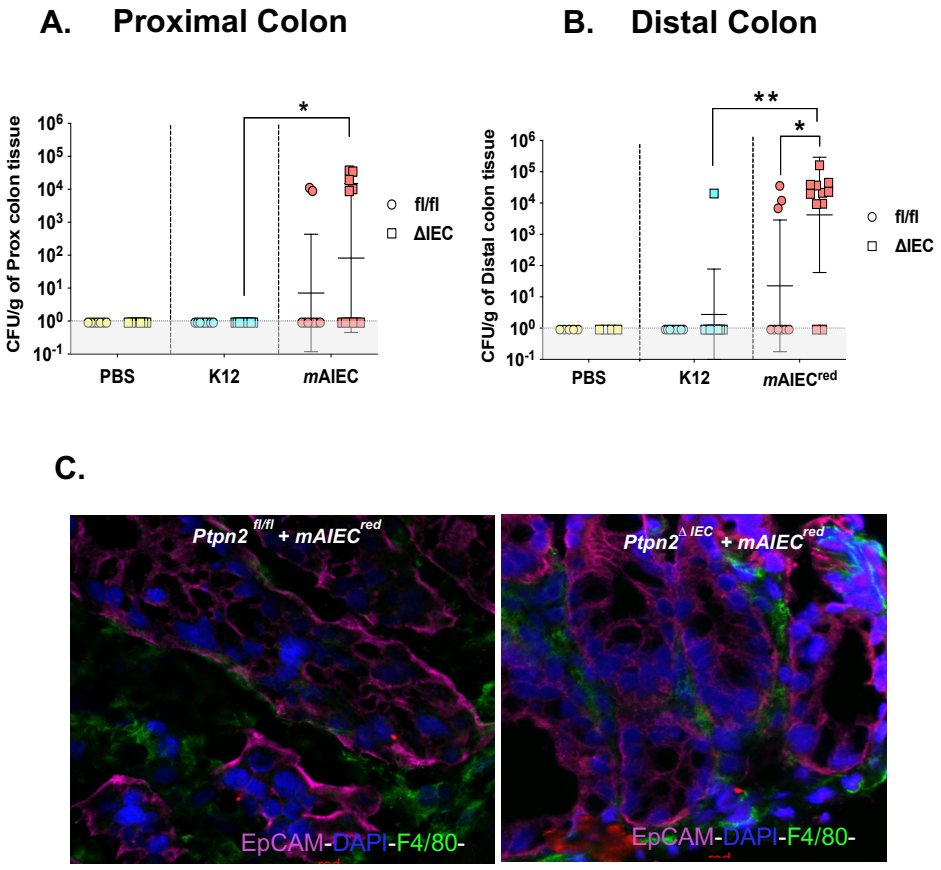

Figure 8: Loss of intestinal epithelial *Ptpn2* promotes *m*AIEC colonization. (A) Bacterial colonies were enumerated from proximal colon whole tissues, *Ptpn2*$^{\Delta IEC}$ - *m*AIEC^{red} mice have greater *m*AIEC^{red} invasion compared to *Ptpn2*$^{\Delta IEC}$ – K12 mice (*P*=0.0194; n-8-12). (B) *Ptpn2*$^{\Delta IEC}$ mice display higher *m*AIEC^{red} colonization compared to *Ptpn2*$^{fl/fl}$ controls (*P*=0.002;n=8-12). (C) Immunofluorescence imaging of *Ptpn2*$^{fl/fl}$ or *Ptpn2*$^{\Delta IEC}$ proximal colon section infected with *m*AIEC^{red}. Epithelial cells were identified by EpCam (magenta) staining, macrophages were marked by F4/80 (green), and *m*AIEC^{red} seen in red (n=3).

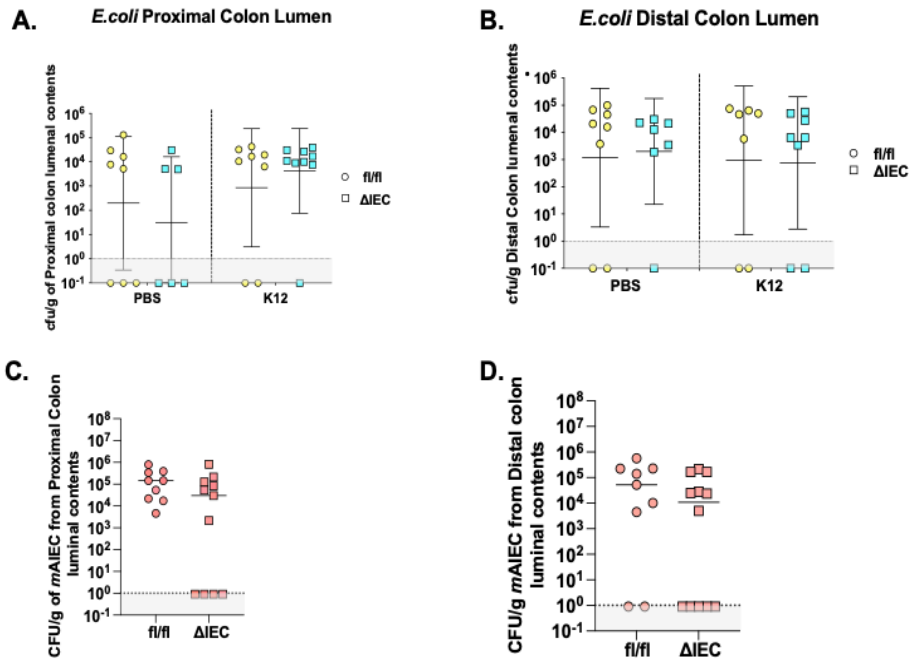

Figure 9: *Ptpn2*$^{fl/fl}$ and *Ptpn2*$^{\Delta IEC}$ have similar luminal content bacterial load.
Bacterial load was enumerated from *Ptpn2*$^{fl/fl}$ and *Ptpn2*$^{\Delta IEC}$ mice. (A), (B) Similar *E. coli* colonization was observed in the proximal colon, distal colon luminal contents between *Ptpn2*$^{fl/fl}$ and *Ptpn2*$^{\Delta IEC}$ mice (n=8-12). (C), (D) Comparable *m*AIEC colonization was observed between *Ptpn2*$^{fl/fl}$ and *Ptpn2*$^{\Delta IEC}$ *m*AIEC infected groups (n=9-12).

Ptpn2 – deficient epithelial cells display Increased Barrier Permeability post-bacterial infection.

AIEC such as LF82 has been previously shown to increase FD4 permeability in mice [41]. We also found that *m*AIEC infection exacerbated permeability increases in wildtype C57Bl/6 mice co-challenged with DSS [19]. To determine how AIEC challenge affected

intestinal barrier function in *Ptpn2*$^{\Delta IEC}$ mice, we assessed *in vivo* FD4 and RD70 permeability after bacterial challenge. Consistent with our previous data, no significant differences in FD4 permeability were observed between *Ptpn2*$^{fl/fl}$ and *Ptpn2*$^{\Delta IEC}$ groups treated with PBS (Figure 10 A)[37]. Surprisingly, *E. coli* K12 increased FD4 permeability in *Ptpn2*$^{\Delta IEC}$ mice compared to its floxed control, while *m*AIEC^{red} caused an even greater increase in FD4 permeability in *Ptpn2*$^{\Delta IEC}$ versus *Ptpn2*$^{fl/fl}$ mice (Figure 10 A). Moreover, no significant difference was observed in RD70 permeability between *Ptpn2*$^{fl/fl}$ and *Ptpn2*$^{\Delta IEC}$ mice or between treatments suggesting that the increase in FD4 permeability did not occur due to gut epithelial damage (Figure 10 B). These data demonstrate that loss of epithelial *Ptpn2* increases FD4 permeability following infection with invasive or non-invasive bacteria.

***Ptpn2*-deficient epithelial cells display alterations in barrier-forming proteins.**

Next, we investigated if AIEC challenged *Ptpn2*$^{\Delta IEC}$ mice displayed alterations in barrier forming proteins. In line with the FD4 results, IECs isolated from *Ptpn2*$^{\Delta IEC}$ mice infected with *m*AIEC^{red} had decreased E-cadherin and Occludin protein levels compared to their PBS-treated *Ptpn2*$^{\Delta IEC}$ counterparts. (Figure 11; Figure 12 A, B). Other barrier-forming proteins like JAM-A and Tricellulin remained unchanged between genotypes or treatment groups (Figure 11; Figure 12 E, F). We further investigated the levels of the claudin family of proteins which were essential for regulating the “pore” paracellular barrier pathway. Consistent with our previous studies, claudin-2 levels were elevated in *Ptpn2*$^{\Delta IEC}$ mice compared to the *Ptpn2*$^{fl/fl}$- PBS treated littermates (Figure 11; Figure 12 C). The increased

claudin-2 observed in *Ptpn2*$^{\Delta IEC}$ mice (PBS group) was reduced after K12 infection (Figure 11; Figure 12 C). Further, the increased claudin-2 in *Ptpn2*$^{\Delta IEC}$ was reversed after *m*AIEC infection, (Figure 11; Figure 12 C). Protein expression levels of claudin-7, a 'sealing' claudin that increases barrier properties, were also reduced in the *Ptpn2*$^{\Delta IEC}$ – *m*AIEC group compared to respective controls (Figure 11; Figure 12 D). Next, we determined the localization of the tight junction regulatory protein, ZO-1. The ZO-1 localization remained intact in all the *Ptpn2*$^{fl/fl}$ groups; however, small gaps in IEC apical membrane localization were seen in *Ptpn2*$^{\Delta IEC}$ – PBS and K12 group (Figure 13). Furthermore, ZO-1 localization was dramatically ablated in *Ptpn2*$^{\Delta IEC}$ – *m*AIEC group compared to controls (Figure 13).

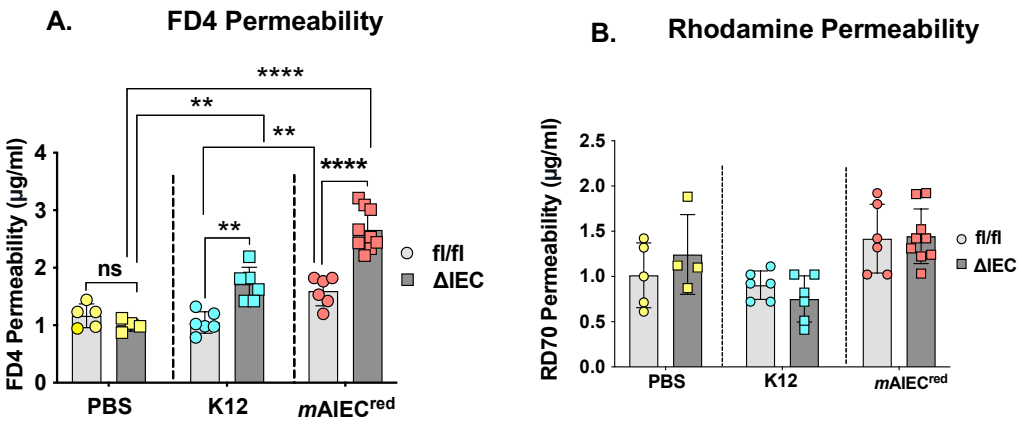

Figure 10: *Ptpn2* - deficient epithelial cells display increased barrier permeability and alterations in barrier-forming proteins post-bacterial infection.
Intestinal barrier permeability was measured using 4kDa FITC-dextran probe. (A) *Ptpn2*$^{\Delta IEC}$ infected with K12 display higher barrier permeability compared to *Ptpn2*$^{fl/fl}$-K12 controls (P= 0.0025), the barrier defect was further exacerbated after *m*AIEC^{red} infection (P< 0.0001) in *Ptpn2*$^{\Delta IEC}$ compared to floxed controls. The *Ptpn2*$^{fl/fl}$ – *m*AIEC^{red} group also display increased permeability compared to *Ptpn2*$^{fl/fl}$ – K12 mice(n=5-9). (B). No appreciable changes were observed in rhodamine permeability (RD70) between genotypes or treatment groups (n=5-9).

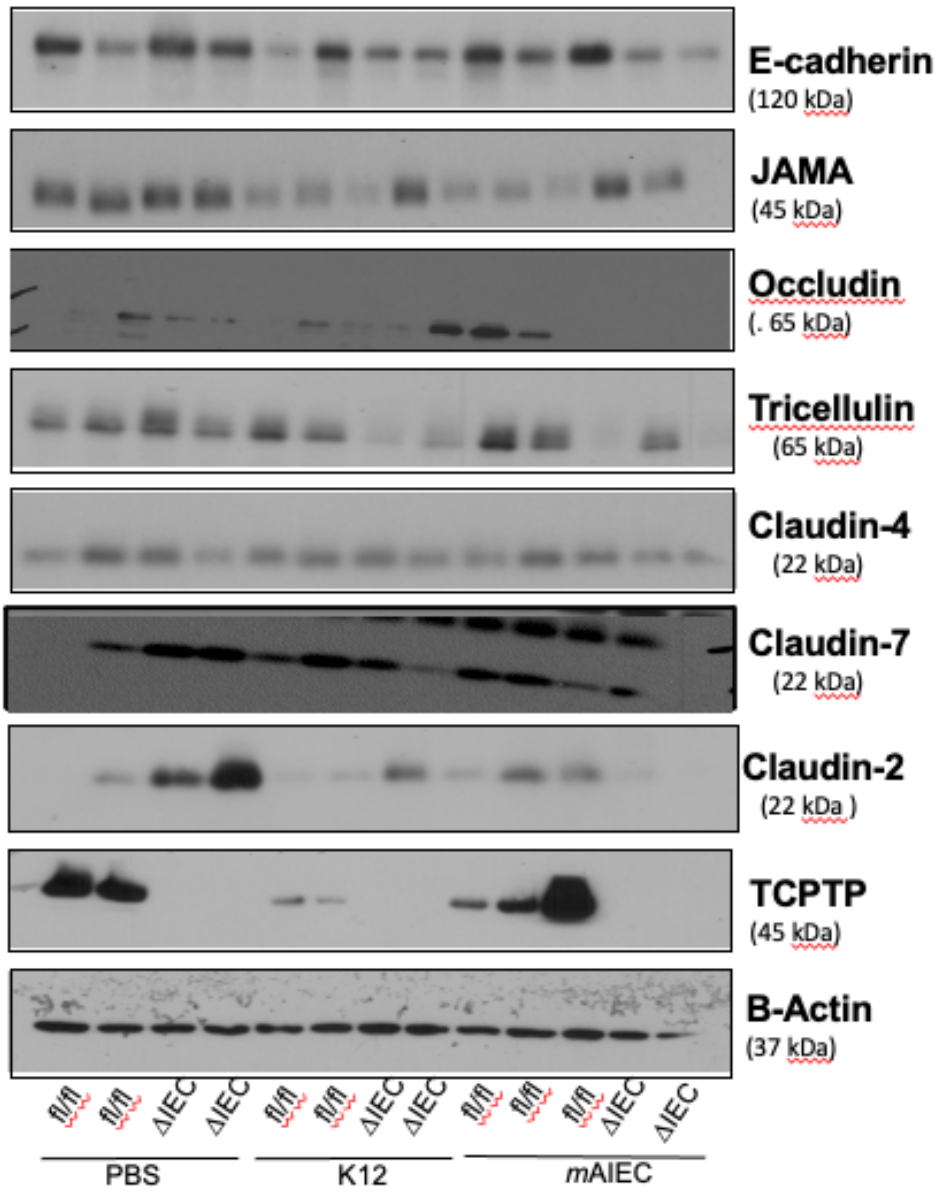

Figure 11 : Representative Western blot for barrier forming proteins. Western blot images for E-cadherin, Occludin, JAMA, Tricellulin, Claudin-7, Claudin-2 (n=4-8).

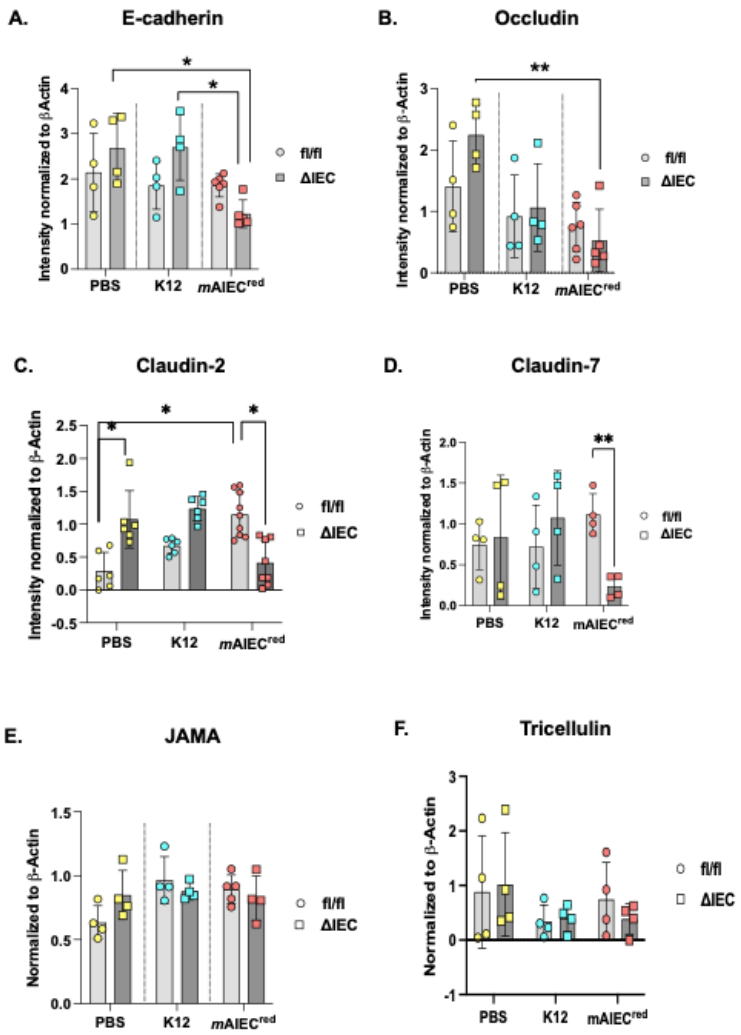

Figure 12: *Ptpn2*–deficient epithelial cells display Increased Barrier Permeability and alterations in Barrier-Forming Proteins post-bacterial infection.
(A) $Ptpn2^{\Delta IEC}$ - *m*AIEC^{red} mice show reduced E-cadherin expression compared to $Ptpn2^{\Delta IEC}$- PBS control (*P*= 0.0124) and compared to $Ptpn2^{\Delta IEC}$- K12 (*P*= 0.0142; n=4)). (B) $Ptpn2^{\Delta IEC}$ - *m*AIEC$^{red\ mice}$ display reduced Occludin levels compared to $Ptpn2^{\Delta IEC}$-PBS controls (*P*= 0.0030; n=4). (C) $Ptpn2^{\Delta IEC}$ display higher Claudin-2 protein expression compared to $Ptpn2^{fl/fl}$ controls (*P*= 0.0015; n=8). $Ptpn2^{\Delta IEC}$-*m*AIEC^{red} mice have reduced Claudin-2 levels compared to $Ptpn2^{fl/fl}$ -*m*AIEC^{red} controls (*P*= 0.004) whereas $Ptpn2^{fl/fl}$ - *m*AIEC^{red} display higher Claudin-2 protein expression compared to their respective PBS controls (*P*= 0.0025; n=8). (D) $Ptpn2^{fl/fl}$ - *m*AIEC^{red} display lower Claudin-7 protein expression compared to $Ptpn2^{fl/fl}$ - *m*AIEC^{red} controls (*P*= 0.0009; n=4). (E+F) No differences in expression of JAMA and Tricellulin between genotype or treatment (n=4).

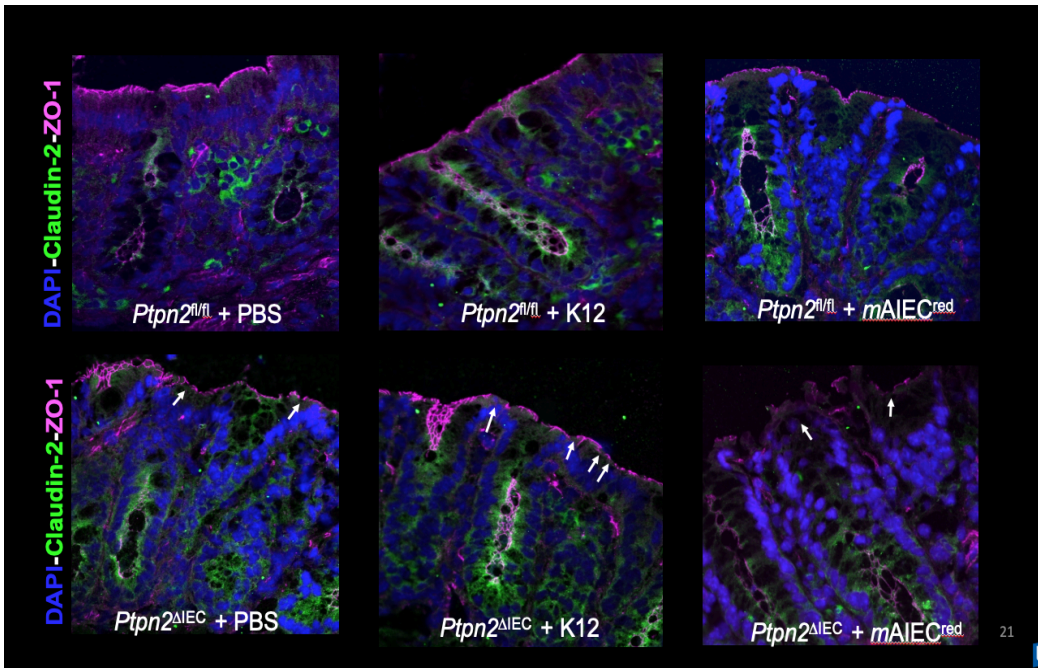

Figure 13: Representative images of IF staining from the of claudin-2 and Z0-1 in *Ptpn2*$^{fl/fl}$ and *Ptpn2*$^{\Delta IEC}$
Immunofluorescence staining of proximal colon whole tissue. Claudin-2 (green) localization was observed largely at the base of the crypt. *Ptpn2*$^{\Delta IEC}$ mice display gaps in ZO-1 localization (magenta). The gaps in ZO-1 increased in K12 infection in comparison with the PBS condition and was completely ablated after *m*AIEC^{red} infection (n=2-3).

Ptpn2 deficient mice exhibit reduced AMP production in response to mAIEC infection.

Given that *Ptpn2*$^{\Delta IEC}$ mice exhibited higher *m*AIEC^{red} - bacterial load in distal colon tissue, and our previous observations that whole body constitutive *Ptpn2*-KO mice have reduced AMP production and numbers of Paneth cells, we next investigated if the higher susceptibility to *m*AIEC^{red} infection was associated with altered host antimicrobial peptide defenses. [32]. Ileal IEC-mRNA expression of the α-defensins (*Defa5* and *Defa6*), was

significantly lower (Figure 14 A, B) in *Ptpn2*$^{\Delta IEC}$ vs. *Ptpn2*$^{fl/fl}$ mice, after *m*AIEC^{red} infection, but not K12 infection. Further, we found that protein levels of the AMP, lysozyme, were significantly lower in K12 infected *Ptpn2*$^{\Delta IEC}$ compared to K12 infected *Ptpn2*$^{fl/fl}$ mice (Figure 15; Figure 16 B), while no change in lysozyme was seen in the PBS- or *m*AIEC-infected groups (Figure 15; Figure 16 B). No difference was observed, in levels of regenerating islet-derived 3 gamma (Reg3ɣ), between genotypes or treatments (Figure 15; Figure 16 C). In addition, expression of the protease matrix metaloproteinase-7 (MMP-7) - which is responsible for the proteolytic cleavage of α-defensins – was significantly decreased in ileum and distal colon of *Ptpn2*$^{\Delta IEC}$ mice post *m*AIEC^{red} infection compared to *Ptpn2*$^{fl/fl}$ littermates (Figure 15; Figure 16 A). Together, these data demonstrate that intestinal epithelial *Ptpn2* is critical for AMP-mediated defenses in response to *m*AIEC infection.

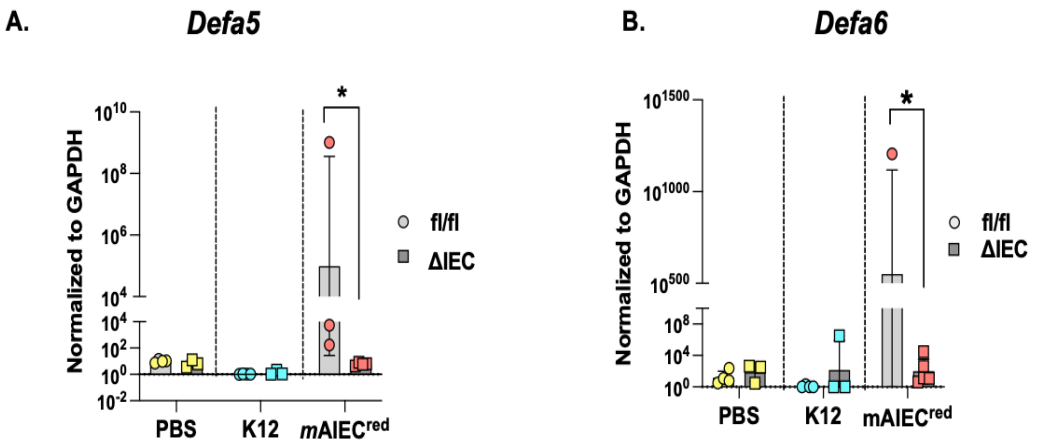

Figure 14: *Ptpn2* deficiency results in reduced α-defensin production in response to *m*AIEC infection.
Anti-microbial peptide expression was measured from ileum whole tissues. *Ptpn2*$^{\Delta IEC}$ - *m*AIEC^{red} display reduced (A) Defa5 (P= 0.0008; n=3) (B) Defa6 (P= 0.00168; n=3) in comparison to *Ptpn2*$^{fl/fl}$ - mAIEC^{red}.

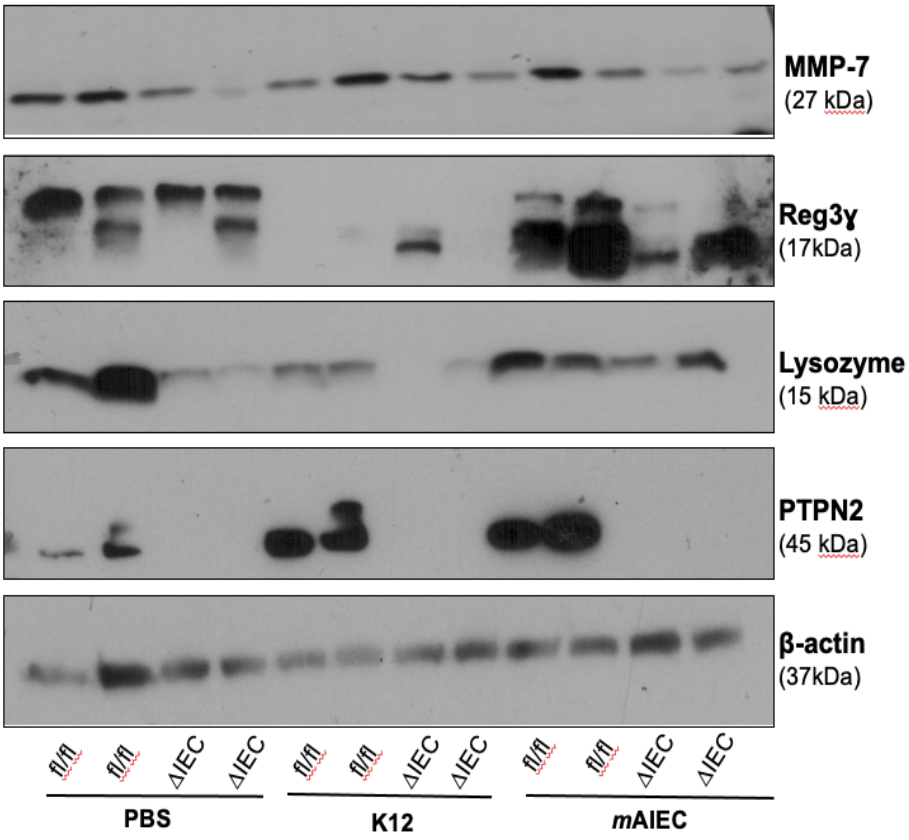

Figure 15: Representative western blot image for anti-microbial peptides from the ileum IECs (n=4-8).

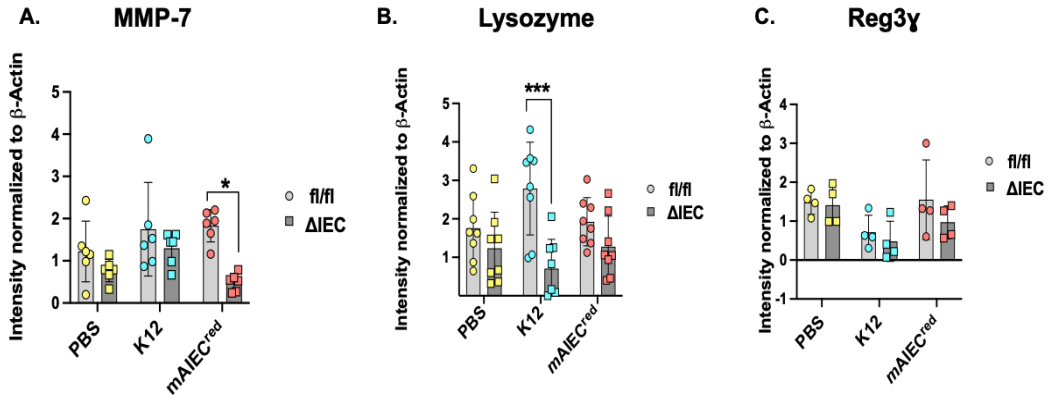

Figure 16: *Ptpn2* deficiency results in reduced AMP production in response to *m*AIEC infection. Quantification of AMP protein levels from western blot shows (A)MMP-7 protein levels were reduced *Ptpn2*$^{\Delta IEC}$ - *m*AIEC^{red} compared to *Ptpn2*$^{fl/fl}$ - *m*AIEC^{red} mice (*P*= 0.042; n=4). (B) *Ptpn2*$^{\Delta IEC}$- K12 mice have lower lysozyme expression compared to *Ptpn2*$^{fl/fl}$ - *m*AIEC^{red} mice (*P*= 0.0165; n=8) however, no differences were observed in lysozyme protein levels between *Ptpn2*$^{\Delta IEC}$ - *m*AIEC^{red} and *Ptpn2*$^{fl/fl}$ - *m*AIEC^{red} groups. (C) No differences were seen in Reg3ɣ protein levels (n=4)

Intestinal epithelial Ptpn2 loss compromises barrier modulating cytokine expression in response to mAIEC infection

Next, we determined the expression profiles of mucosal cytokines involved in promoting Paneth cell function and clearance of bacteria and bacterial products. Typically, the T-helper cells 1 (Th1) mount a cytokine reaction during bacterial invasion which is more inflammatory in nature, whereas the T-helper cells 2 (Th2) mount a repones against parasitic infestations. Additionally, mucosal cytokines are important for the appropriate functioning of epithelial cells by promoting secretion of AMPs and barrier promoting proteins and therefore, are essential in preservation of the epithelial barrier. We observed mRNA expression of *Il22*, *Il6* and *Il17* was significantly reduced in the proximal colon of *Ptpn2*$^{\Delta IEC}$ - *m*AIEC^{red} mice compared to its control littermates (Figure 17 A, B, C).

Interferon-gamma (*Ifn-γ*), *1l-1β* were not significantly reduced in these mice (Figure 17 D, E). Due to lower expression Th17 derived cytokines, we ran western-blots for the proteins cluster of differentiation-45 (CD45), a general marker for immune cells, and the T-cell marker CD3 . We observed lower expression for both CD45 and CD3 in *Ptpn2*$^{\Delta IEC}$ – *m*AIEC in comparison with its respective controls (Figure 19 A, B, C). Further, no changes were observed in expression of Type 2 cytokines *Il4* and *Il13*, or the Th-2 cell marker, GATA-binding protein 3 marker (*Gata3*) (Figure 20 A, B, C). Next, we validated the mRNA data by confirming that in colonic whole tissues, PBS treated *Ptpn2*$^{\Delta IEC}$ mice had reduced IL-22 and IL-6 protein in comparison to its *Ptpn2*$^{fl/fl}$ control, as determined by ELISA (Figure 18). We have also confirmed in previous studies that no appreciable differences were seen in *Il10r2* and *Il22r1* between *Ptpn2*$^{fl/fl}$ and *Ptpn2*$^{\Delta IEC}$ mice [41]. These data indicate that protective barrier and AMP-promoting cytokine responses are impaired in *Ptpn2*$^{\Delta IEC}$.

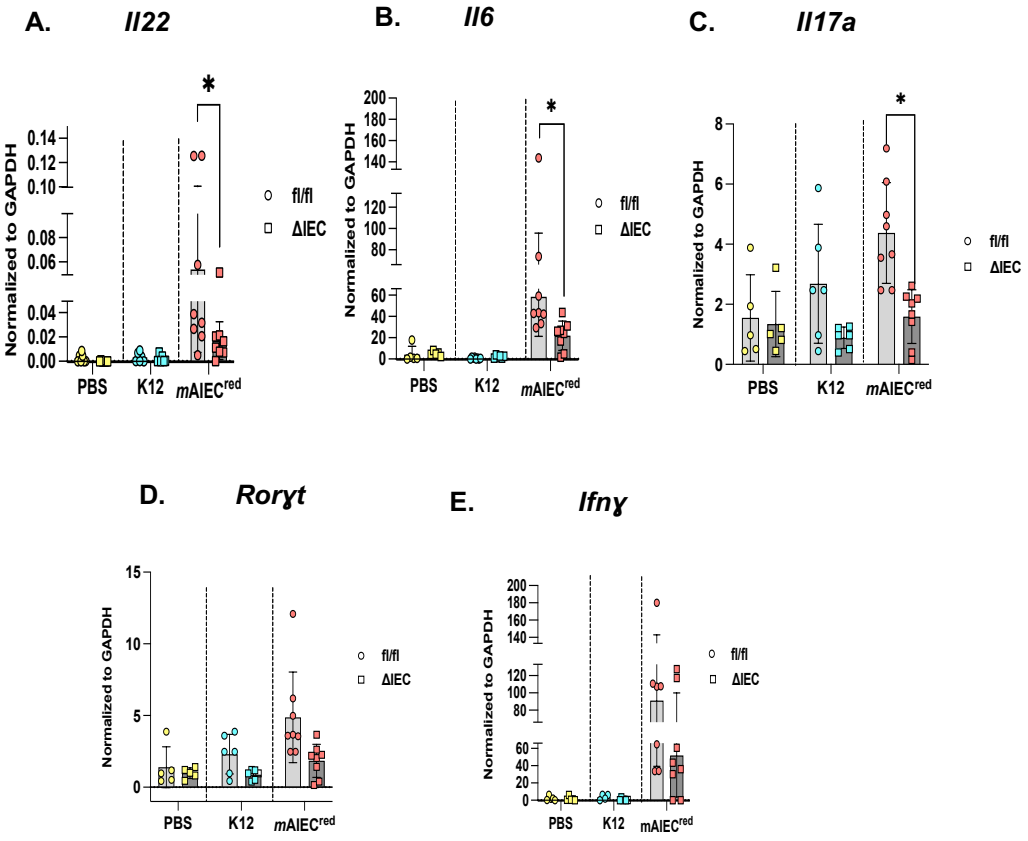

Figure 17: *Ptpn2* deficiency results in reduced cytokine expression in response to *m*AIEC infection

Cytokine expression profile was measured from proximal colon whole tissue sample. (A) *Ptpn2*$^{\Delta IEC}$ - *m*AIEC^{red} display lower cytokine levels compared to *Ptpn2*$^{fl/fl}$ - *m*AIEC^{red} for (A) IL-22 (P=0.0234) (B) IL-6 (P=0.0208) (C) IL-17A (P= 0.00216). No changes were observed between genotype or groups for (D) IFN-ɣ (E) IL-1β (n=6-8).

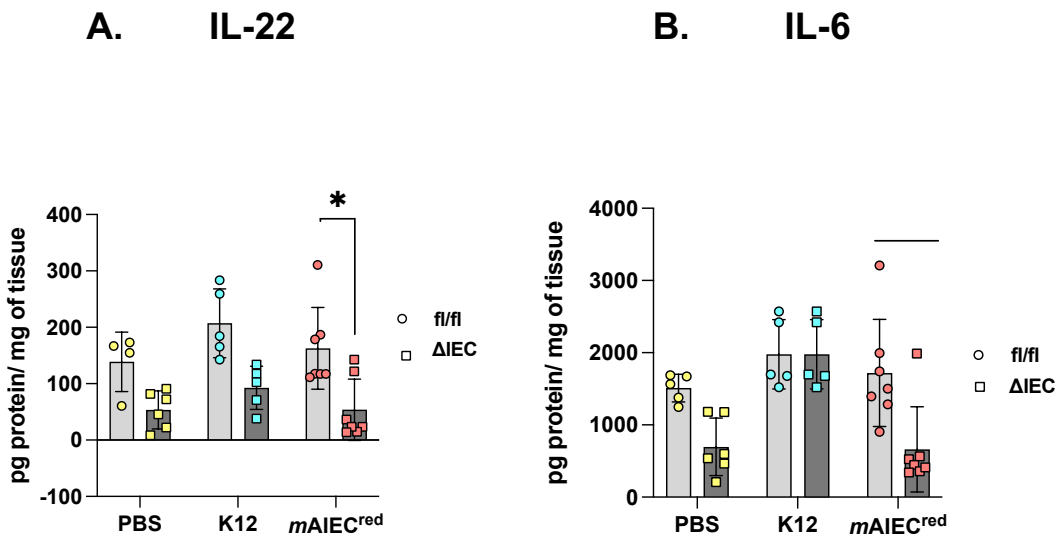

Figure 18: *Ptpn2* deficiency results in reduced IL-22 and IL-6 protein levels in response to *m*AIEC infection ELISA was performed from whole tissue distal colon samples. (A) *Ptpn2*$^{\Delta IEC}$ - *m*AIEC^{red} display lower IL-22 protein levels compared to *Ptpn2*$^{fl/fl}$ - *m*AIEC^{red} (*P*=0.0012; n=4-7). (B) IL-6 protein expression was also lowered between *Ptpn2*$^{\Delta IEC}$ - *m*AIEC^{red} and *Ptpn2*$^{fl/fl}$ - *m*AIEC^{red} groups (*P*=0.005; n=4-7)

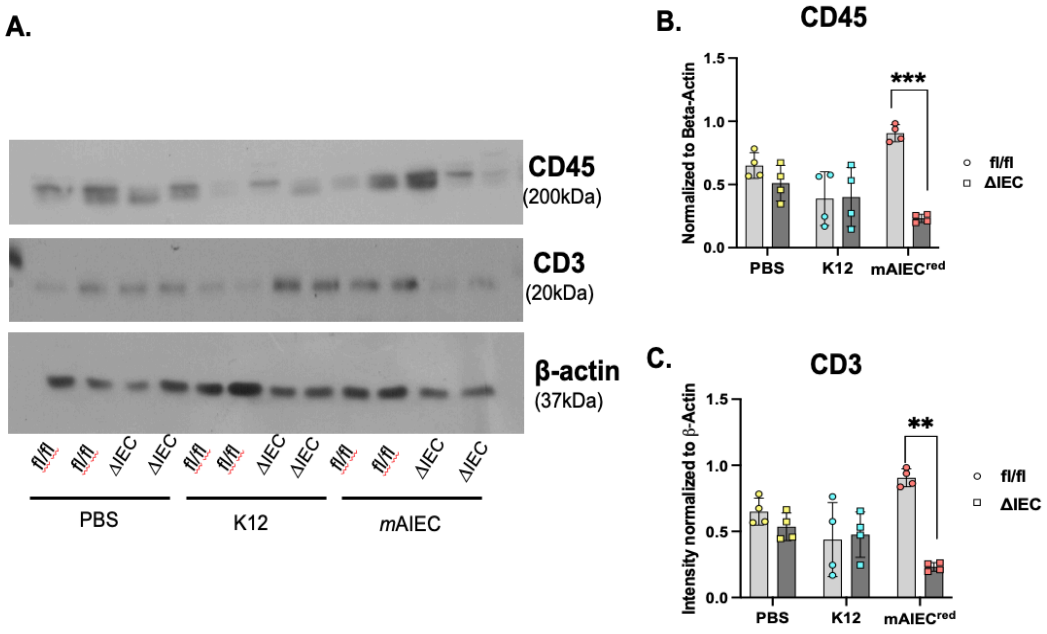

Figure 19: Loss of Epithelial *Ptpn2* led to reduced expression of CD3 and CD45 protein levels.
Ptpn2$^{fl/fl}$ and *Ptpn2*$^{\Delta IEC}$ whole tissue proximal colon was processed for western blot A. Representative western blot for CD3, CD45 protein expression. *Ptpn2*$^{\Delta IEC}$ mice display reduced (A) CD3 protein expression (P=0.00124; n=4) (B) CD45 protein expression after *m*AIEC infection compared to *Ptpn2*$^{fl/fl}$- *m*AIEC group (P<0.001; n=4).

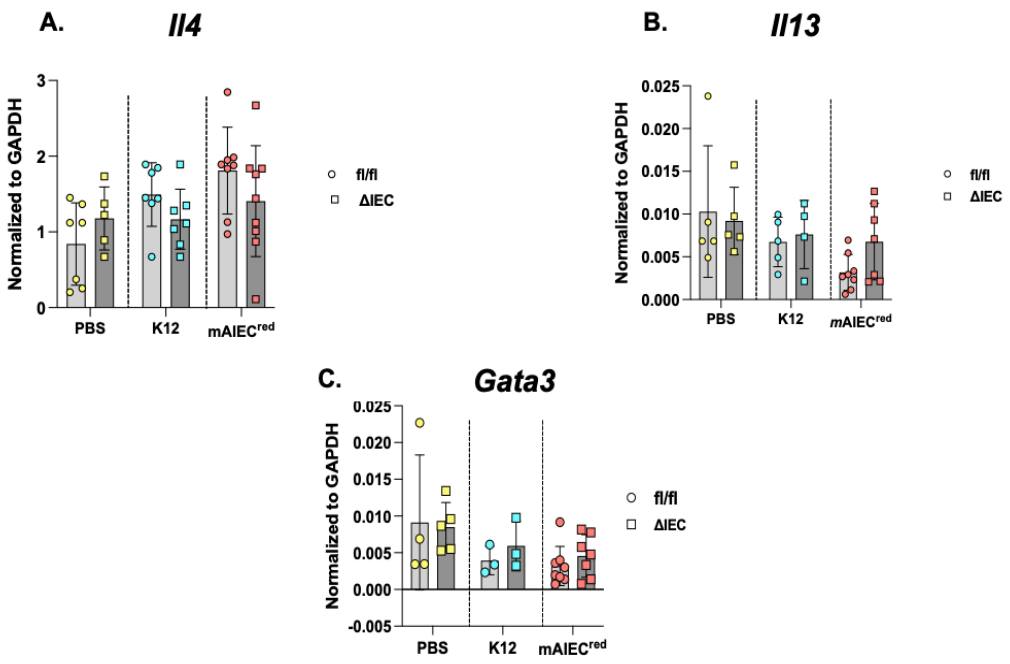

Figure 20: Loss of Epithelial *Ptpn2* does not change Th2 cytokine response. *Ptpn2*$^{\Delta IEC}$ and *Ptpn2*$^{fl/fl}$ whole tissue proximal colon samples were processed for mRNA quantification. *Ptpn2*$^{\Delta IEC}$ mice do not display any changes in (A) IL-4, (B) IL-13 or (C) GATA-3 response compared to control or infected littermates (n=6-8).

AIEC Deteriorates Intestinal Epithelial Barrier

Next, we wanted to investigate the functional consequences of AIEC invasion of human IECs carrying the clinically relevant *PTPN2* SNP *rs1893217* . Using CRISPR-Cas9 gene editing, the *PTPN2* SNP *rs1893217* was inserted (*PTPN2*-KI), or a complete knockout of the gene *PTPN2* (*PTPN2*-KO) was generated in Caco-2BBe IECs. A scrambled RNA was used as control (*PTPN2*-WT). We observed that the basal trans-epithelial electrical resistance (TEER) in the KI and KO cell lines was significantly lower than WT cells (Figure 3A). However, after challenging these cells with AIEC-LF82 (isolated from a CD patient), there was a significant drop in TEER of WT cells while the lower TEER in KI and KO cells persisted (Figure 21 A). Next, we investigated the changes in macromolecular paracellular permeability with FD4 assay. Interestingly, *PTPN2*-KI cell lines displayed comparable levels of FD4 permeability to the WT cells even though, they exhibited lower TEER than WT cells (Figure 23 B). The *PTPN2*-KO cell lines exhibited higher FD4 permeability in basal state compared to the WT and KI cells (Figure 21 B). Post-AIEC LF-82 challenge the FD4 permeability was significantly increased in both WT and KI cell lines compared to untreated controls (Figure 21 B). The permeability defect was further exacerbated in *PTPN2*-KO cell lines after AIEC challenge (Figure 21 B). Taken together, these results demonstrate that AIEC can worsen the underlying barrier defect in IBD-SNP carrying cell lines.

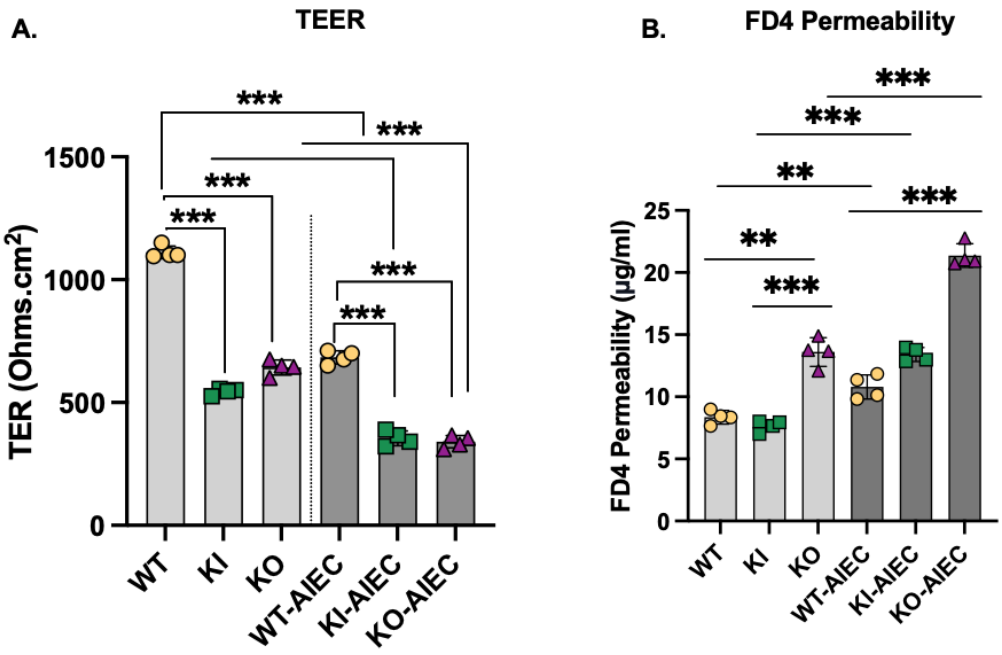

Figure 21: AIEC worsens barrier function in *PTPN2* deficient IECs.

A. Transepithelial electrical resistance (TEER) and **B.** Fitc- dextran 4 kDa (FD4**)** flux was measured across WT, KI and KO Caco-2 BBe monolayers. *PTPN2*-KI and KO cells display reduced TEER in comparison to WT controls (P< 0.005; n=4). Next, these cells were challenged with AIEC LF-82 at MOI of 10. The TEER is drastically reduced in WT cells after infection (P< 0.005; n=4) compared to untreated controls. However, the TEER WT-AIEC group remained higher than KI-AIEC and KO-AIEC groups (P<0.005; n=4). FD4 permeability was higher in basal *PTPN2*-KO cells compared to WT and KI groups (P<0.005). After AIEC challenge, permeability increased in WT, KI and KO groups compared to their respective untreated control group (P<0.005). The basal permeability defect is exacerbated in *PTPN2*-KO group post-AIEC challenge (n=4).

3.5 DISCUSSION

PTPN2 modulates intestinal microbial composition, anti-microbial peptide levels, and mucosal barrier permeability [37, 38, 42]. We have previously demonstrated that constitutive whole-body loss of *Ptpn2* results in expansion of AIEC and reduction in *Firmicutes* such as segmented filamentous bacteria (SFB) [19]. Further, we have demonstrated that loss of *Ptpn2* is critical for regulation of intestinal permeability through epithelial-macrophage crosstalk and preservation of barrier-forming proteins [40]. In this study, for the first time we demonstrate that intestinal epithelial *Ptpn2* is critical for immunity against *m*AIEC infection by promoting anti-microbial defense molecules, maintaining barrier integrity, and coordinating protective immune cell-mediated cytokine responses, highlighting the central role of *Ptpn2* in microbial-epithelial-immune homeostasis.

We confirmed that our fluorescent AIEC – *m*AIEC^{red} has similar properties of adherence, invasion and survival in macrophages compared to it wildtype counterpart[43].While the first AIEC was isolated from the ileum, several other AIEC strains have been discovered from both large and small intestinal regions[19, 44-46]. *m*AIEC is present in low levels in wild-type mice but displayed much higher abundance in constitutive *Ptpn2*-KO mice in both the small and large intestine [19]. Of note, in this current study, *m*AIEC^{red} preferentially colonized the distal colon versus more proximal regions of the intestine. While the reasoning for this has not yet been determined, it may reflect the impact of specific bacterial niches present in different regions of the intestine, coupled with a more favorable microenvironment, enabling *m*AIEC to achieve optimal colonization efficiency in the distal colon [19].

Paneth cells are specialized secretory cells present in the small intestine crypts that produce several AMPs to prevent bacterial colonization and growth. CD patients present with Paneth cell abnormalities and reduced expression of defensins[47]. In this study, we observed that *Ptpn2*$^{\Delta IEC}$ mice display reduced expression of the α-defensins, *Defa5* and *Defa6*, in response to infection with *m*AIEC. Further, these mice also display loss of MMP7, a protease that is important for activation of defensin family of proteins, and thus also serves as a Paneth cell marker[48]. Given, the critical function of AMPs in restricting bacterial colonization, these data align with the increased *m*AIEC colonization that we observed in the *Ptpn2*$^{\Delta IEC}$ mice. Despite the observed defects in Paneth cell antimicrobial peptide expression, are primarily localized in the small intestine, it is surprising that no bacterial colonization was observed in the ileum, the site of Paneth cells. It is appreciated that Paneth cell AMPs exert important effects not just in the ileum, but their loss can lead to dysbiosis in the colon[49].This may contribute to the region-specific variations in bacterial niches along the lower intestine and the colonization preference of *m*AIEC in the distal colon.

Enhanced intestinal barrier permeability is a risk factor for IBD patients[50, 51]. Newer evidence in the field has demonstrated that healthy relatives of patients with CD have significantly higher risk of developing the disease - up to several years later - if they present with increased intestinal permeability, suggesting that barrier loss is an early and crucial event in disease pathogenesis[34, 35]. Since the intestinal epithelium is semi-permeable, it maintains flux across the epithelial barrier through tight junction-dependent paracellular “pore” and “leak” pathways. The “unrestricted pathway” is generated by epithelial damage due to apoptosis or cell shedding. In this study we demonstrated that invasion of AIEC can exacerbate an underlying barrier dysfunction in PTPN2-deficient cell

lines. Further, our *in vivo* studies demonstrated that the unrestricted pathway – assessed by RD70 – remained unchanged, regardless of PTPN2 genotypes or by the bacterial challenge, we did observe increased paracellular flux via the leak pathway, as assessed by *in vivo* FD4 challenge. There was no observable difference in *in vivo* FD4 flux between the genotypes in the PBS condition, consistent with our previous reports although we did find region-specific increases in FD4 permeability in *ex vivo* Ussing chamber studies indicating a mild underlying paracellular flux in *Ptpn2*$^{\Delta IEC}$ mice that was exacerbated by cytokine challenge[37]. In our current study, we did make the intriguing finding that FD4 permeability was higher in the *Ptpn2*$^{\Delta IEC}$ mice infected with either K12 or *m*AIEC compared to infected *Ptpn2*$^{fl/fl}$ mice. This indicates that loss of PTPN2 in intestinal epithelium changes the dynamics of epithelial interactions with commensal bacteria and appears to confer increased pathobiont potential on both invasive and non-invasive commensals.

Functional changes in permeability following AIEC infection aligned with altered expression and localization of apical tight junction proteins. TJs include transmembrane proteins such as occludin, claudins and JAM-A, as well as regulatory proteins such as ZO-1. E-cadherin is a key component of the adherens junction which plays an important role in supporting tight junction development and overall stability. We observed that *m*AIEC infection of *Ptpn2*$^{\Delta IEC}$ mice decreased levels of, occludin and E-cadherin compared to *Ptpn2*$^{\Delta IEC}$ + PBS controls, however the effect of *m*AIEC was not significantly different between mouse genotypes. ZO-1 membrane localization was substantially ablated in the *Ptpn2*$^{\Delta IEC}$- *m*AIEC mice, while*Ptpn2*$^{\Delta IEC}$ – K12 mice displayed gaps in ZO-1 localization. Collectively, these changes in junctional protein expression or localization are consistent with the corresponding increases in FD4 permeability. Furthermore, the pore-forming, tight junction protein, claudin-2, was increased in *Ptpn2*$^{\Delta IEC}$ mice compared to their *Ptpn2*$^{fl/fl}$

controls. Claudin-2 is upregulated in inflammatory states and is shown to increase paracellular electrolyte permeability via the pore pathway, and also increases paracellular water flux[52, 53]. Interestingly, claudin-2 is also upregulated during infections with *C. rodentium* and it mediates diarrhea and clearance of the bacteria from the system, thus reflecting a protective role against infection [36]. Consistent with this study, we observed in *Ptpn2*$^{fl/fl}$ mice that claudin-2 was upregulated in response to *m*AIEC infection. Interestingly, the increased background claudin-2 in *Ptpn2*$^{\Delta IEC}$ mice did not mediate a predicted protective effect against infection. Indeed, claudin-2 levels were reduced in these mice to levels comparable to uninfected Ptpn*2*$^{fl/fl}$ mice. Thus, it appears that loss of PTPN2 in intestinal epithelium compromises multiple host defense mechanisms including AMP production, intestinal permeability, and claudin-2 mediated paracellular water flux.

To understand the mechanisms mediating these effects, we identified that PTPN2 loss compromised local mucosal cytokine responses involved in promoting AMP production (IL-22, IL-17A) and claudin-2 (IL-6, IL-22) expression. IL-22 has a very important role during pathogen infiltration in the host as it promotes AMP secretion from Paneth cells and upregulates claudin-2 from epithelial cells[36,57]. Previously, we have demonstrated *Ptpn2*$^{\Delta IEC}$ challenged with *C. rodentium* develop severe disease compared to their floxed counterparts and display reduced Reg3γ and IL-22 cytokines levels post-challenge [41]. In line with previous studies, we demonstrate that the loss of PTPN2 in epithelial cells leaves the host susceptible to infection with a commensal pathobiont and even confers mild pathological potential on a non-invasive commensal.

The intestinal epithelium plays a critical yet nuanced role in maintaining homeostasis between the subepithelial immune cells and the luminal microbes. The epithelial monolayer must strike a delicate balance to prevent invading microbes but allow

selective permeability necessary for nutrient absorption. Our findings highlight that intestinal epithelial PTPN2 is crucial for mucosal immunity as it promotes anti-microbial peptide defenses and enhances barrier function during infection from pathobionts such as AIEC, and non-invasive commensals. In addition, we identify a unique mechanism for epithelial PTPN2 in maintaining the immune-cytokine regulatory landscape of the gut to withstand pathobiont colonization. These findings provide an essential insight into a cell-specific role for this clinically relevant gene in the maintenance of multiple aspects of mucosal barrier defenses against commensal bacteria.

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CHAPTER 4

4 JAK-Inhibitor, Tofacitinib, Corrects CEACAM6 Over-Expression in *PTPN2* Deficient Intestinal Epithelial Cells

4.1 ABSTRACT

Background and Aims: A cohort of patients with inflammatory bowel disease (IBD) display expansion of the pathobiont, adherent-invasive *E. coli* (AIEC). Loss of the IBD susceptibility gene, protein tyrosine phosphatase type 2 (*PTPN2*), results in dysbiosis of the gut microbiota both in human subjects and mice. Further, constitutive *Ptpn2* knock-out (*Ptpn2*-KO) mice display expansion of AIEC compared to wildtype littermates. CEACAM6, a host protein, is exploited by AIEC to attach and gain entry into intestinal epithelial cells (IECs). Here, we investigate the role of IEC-specific *PTPN2* in restricting AIEC invasion.

Methods: Biopsies from IBD patients carrying heterozygous (CT) or homozygous (CC) *PTPN2* SNP (single nucleotide polymorphism) *rs1893217* were processed for immunohistochemistry. HT-29 cells were transfected with control shRNA (*PTPN2*-CTL), or a shRNA targeted towards *PTPN2* (*PTPN2*-KD). The *rs1893217* SNP was inserted (*PTPN2*-KI), or a complete knock-out of *PTPN2* (*PTPN2*-KO) was generated, with CRISPR-Cas9 gene editing in Caco-2 BBe cells lines. Adherence and invasion assay was performed with either AIEC LF82 or a novel fluorescent-tagged mouse adherent-invasive *E. coli* (*m*AIEC^{red}) at multiplicity of infection (MOI) of 10. Transepithelial electrical resistance (TER) and 4kDa FITC-dextran (FD4) permeability were measured to identify changes in paracellular permeability to ion flux and macromolecules respectively. IFN-γ was administered (100U/ml) for 24 hours and the pan-JAK inhibitor tofacitinib (50μM;

apically) for 1 hour. Protein expression was determined by western blotting and densitometry.

Results: CEACAM6 expression was elevated (colon and ileum) in IBD patients with *PTPN2* SNP *rs1893217* (CT, CC) compared to wildtype (TT) IBD patients. HT-29 and Caco-2BBe cell lines deficient in *PTPN2* express significantly higher levels of CEACAM6. Further, *PTPN2*-KI and *PTPN2*-KO cell lines also displayed greater adherence and invasion to AIEC LF82 and higher *m*AIEC^{red} invasion. CEACAM6 expression was further elevated after administration of IFN-γ in *PTPN2*–deficient cell lines compared to untreated controls. Silencing of STAT1 by siRNA targeted towards STAT1, reduced CEACAM6 protein expression by 40%. tofacitinib drastically reduced the elevated CEACAM6 protein expression in *PTPN2*-KI and KO cell lines compared to DMSO treated controls. **Conclusion:** Our finding highlights a crucial role for *PTPN2* in restricting pathobiont entry into host cells. Our study also describes a role for the FDA-approved drug, tofacitinib (Xeljanz) in correcting the JAK-STAT-mediated over-expression of CEACAM6, used by pathobionts as an entry portal into host cells. These findings suggest a role for JAK-inhibitors in mitigating AIEC colonization in IBD-susceptible hosts.

4.2 INTRODUCTION

Inflammatory bowel disease (IBD) encompassing Crohn's disease (CD) and ulcerative colitis (UC) is a chronic, relapsing condition characterized by inflammation of the gastrointestinal tract [1-3]. The immune system essentially mounts an abnormal response to the normal intestinal flora which results in abdominal pain, diarrhea, ulcers, and lesions. IBD is a multi-factorial condition that can be caused by a faulty immune-system, environmental factors, irregularities in the host microbiome and genetic predispositions [4,

5]. One or more of these factors acts in tandem to give rise to this autoinflammatory condition[6, 7].

Several genome-wide association (GWAS) studies have identified over 240 genes that has been associated with IBD[8]. The single nucleotide polymorphism (SNPs) in *rs1893217* which is the gene encoding region of protein tyrosine phosphatase type 2 (*PTPN2*), has been associated with several chronic inflammatory conditions like type 1 diabetes (T1D), rheumatoid arthritis (RA) and both subtypes of IBD[9, 10]. The SNP is carried by around 16 % of the general population (non-IBD) and 19-20% of people with IBD, suggesting that there is an increased association of this SNP with IBD. The SNP leads to the loss of function in the activity of T-cell protein tyrosine phosphatases (TCPTP), the protein product of *PTPN2*[11]. Among its substrates, it is a negative regulator of several members of the Janus Activated Kinase – signal transducers and activators of transcription (JAK-STAT) pathway activated by several mediators including the pro-inflammatory cytokines like IFN-γ and IL-6[12, 13]. Several JAK inhibitors have been approved for clinical use in rheumatoid arthritis and IBD[14-16]. Tofacitinib (Xeljanz, CP-690,550) is a pan-JAK inhibitor that has been approved for use in patients with moderate to severe UC[17-19].

Along with genetic susceptibilities, imbalance of microbial composition – dysbiosis – has been observed in many IBD patients[20]. Dysbiosis in IBD is associated with reduced microbial diversity accompanied by a decrease in beneficial commensals like Firmicutes, and a concurrent increase in Proteobacteria and Bacteroidetes[21]. A subset of patients also showed increased abundance of a particular B2 phylotype of *Escherichia coli* (*E. coli*) originally isolated from the inflamed ileal mucosa of CD patients[22]. The original LF82 strain had a unique ability to attach to and invade intestinal epithelial cells

(IECs) and survive within macrophages and therefore, was categorized into a new pathogenic group: adherent-invasive *E. coli* (AIEC)[23]. Multiple groups have identified several different AIEC strains associated with both CD and UC patients, highlighting its potential role in disease pathogenesis [24, 25]. Although the exact mechanism by which AIEC contributes to IBD is not known, an increasing number of studies have identified its role in the maintenance and/or induction of intestinal inflammation in genetically susceptible hosts[26-28].

IECs line the intestinal mucosa and act as a partition between the luminal bacteria and the immune cells in the lamina propria [29, 30]. The epithelial cells also maintain a tightly regulated and selectively permeable epithelial barrier. The intestinal epithelium is also responsible for absorption of nutrients and secretion of antimicrobial peptides (AMPs) by enterocytes or specialized ileal crypt IECs (Paneth cells) that prevent pathogenic bacteria from penetrating the mucosal surface to provoke immune cell-mediated inflammatory responses. However, pathobionts like AIEC can utilize certain host proteins in a susceptible host to colonize the intestinal mucosa. A well-described interaction between AIEC and the human host is mediated by carcinoembryonic antigen-related cell-adhesion molecule 6 (CEACAM6) and AIEC-LF82 fimbriae protein FimH[31]. CEACAM6 is a highly mannosylated GPI-anchored protein that is expressed on the apical surface of IECs and directly interacts with AIEC to enter epithelial cells[31].

Previously, our lab has demonstrated that *Ptpn2*-KO mice exhibit microbial dysbiosis with a profound decrease in Firmicutes levels and increased abundance of Proteobacteria compared to wildtype littermate controls[32]. This increase in Proteobacteria most prominently featured a dramatic expansion of a novel mouse adherent-invasive *E. coli* – *m*AIEC that genetically overlapped with the human AIEC LF82.

Having identified that loss of PTPN2 caused an expansion of AIEC, the aim of this study was to determine the role of intestinal epithelial *PTPN2* in limiting AIEC invasion of host cells.

4.3 METHODS

Cells

Caco-2 BBe1 cells and HT-29cl.9A IECs were cultured in Dulbecco's modified Eagles's Medium (DMEM; Corning, Tewksbury, MA) and McCoy's 5A medium (Corning, Tewksbury, MA), respectively. The medium was supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco, Waltham, MA), 1% L-glutamine (Invitrogen, Carlsbad, CA), and 1% penicillin/streptomycin (Corning, Tewksbury, MA) at 37°C incubator maintained at 5% CO_2/air mix. Cells were cultured in 6-well plates for protein assay, or immunofluorescence. Insertion of SNP *rs1893217* (*PTPN2*-KI) and complete knockout of *PTPN2* (*PTPN2*-KO) in Caco-2 BBe cell lines was performed using CRISPR-Cas9 gene editing by Synthego. For *PTPN2* knockdown in HT-29 cells, lenti-viral particles containing scrambled shRNA (*PTPN2*-CTL) or PTPN2-specific shRNA (*PTPN2*-KD) were generated as previously described[33]. For barrier function analysis, cells were grown on semi-permeable transwells.

Immunofluorescence

Caco-2 BBe IECs were seeded at 200,000 on coverslips in 12-well plates. After 24 hours, medium was deprived of serum and antibiotics. For bacterial fluorescence assay, cells were washed with PBS (x3) followed by fixation with 4% paraformaldehyde (PFA) for 20 min at room temperature. Cells were then permeabilized with 0.3% Triton X-100 (Fischer Scientific, Waltham, MA) for 5 min followed by blocking with 5% Bovine Serum Albumin (BSA; Fischer Scientific, Waltham, MA) for 10 min at room temperature. Cells were incubated with Alexa Fluor 488-Phalloidin antibody (1:1000) (Abcam #176553, Cambridge, MA) for 90 min at room temperature followed by nuclei staining with 4,6-diamididino-2-phenylindole (DAPI; Vector Laboratories, Newark, CA). For CEACAM6 staining, cells were fixed with 4% PFA and permeabilized with Triton X-100 for 30 minutes, followed by blocking with 5% BSA for 1 hour. Cells were then incubated anti-CEACAM6 antibody (1:100; Abcam #78029; Cambridge, MA) at 37°C for 3 hours. Cells were washed with 0.1% tween supplemented PBS (X3) after which they were incubated in Alexa-Fluor 488 (1:100; Jackson Research #711-586-154, West Grove, PA) for 1 hour. Images were captured using Leica DM5500 microscope attached with a DFC365 FX camera using a 63X oil immersion objective or the inverted Zeiss Airyscan. DAPI was visualized using a 405 nm excitation laser and DAPI filter set. mCherry was visualized using a 561 nm excitation laser and mCherry filter set. Images were analyzed using ImageJ software.

Protein isolation and Western blotting

For protein isolation from cells, the cells were washed with ice cold PBS and lysed in radioimmunoprecipitation assay (RIPA) buffer containing phosphatase and protease inhibitors (Roche, South San Francisco, CA). All samples were then sonicated for 30

seconds, centrifuged (10 min. at 13000 G at 4°C), and the supernatant transferred into fresh tubes. Protein concentration was detected using a BCA assay (Thermo Fisher Scientific, Waltham, MA). For Western blots, aliquots with equal amounts of protein were separated by electrophoresis on polyacrylamide gels, and the proteins blotted on nitrocellulose membranes. The membranes were then incubated in blocking buffer (5% milk, 1% BSA in tris-buffered saline with 0.5% Tween) for 1 hr. and incubated over night at 4°C with appropriate antibody (Table 1). The next day, the membranes were washed in tris-buffered saline with 0.5% Tween, incubated with HRP-coupled secondary antibodies (Jackson Immunolabs, West Grove, PA), washed again, and immunoreactive proteins detected using ELC substrate (Thermo Fisher Scientific, Waltham, MA) and X-ray films (GE Healthcare, Chicago, IL).

For STAT1 silencing, the cells were transfected with previously validated, STAT1-specific, or non-targeting control siRNA constructs (Dharmacon) using DharmaFECT transfection agents. In experiments with STAT1 siRNA and IFN-γ treatment, the culture media was replaced with serum-free medium 8 hr prior to addition of IFN-γ (100 IU; Roche). 24 hours post IFN-γ addition, cells were washed with PBS and collected with RIPA as previously described.

In experiments with tofacitinib, the cells were treated with tofacitinib (50 μM, MedChemExpress, Monmouth Junction, NJ). Control cells were treated with an equal amount of vehicle (dimethyl sulfoxide, DMSO, 0.5%, Sigma-Aldrich).

Immunohistochemistry of patient biopsies

Microscopy slides with paraffin-embedded and formalin-fixed (PEFF) intestinal biopsies from *PTPN2* genotyped patients (rs1893217) were provided by Dr. Dermot McGovern at Cedars Sinai Medical Center, Los Angeles, CA, USA. Twelve samples from colon (6 WT, 5 Het, 1 KO), and 12 samples from ileal segment (6 WT, 6 KO) were used in this analysis. Slides were deparaffinized, rehydrated and processed for immunohistochemistry as described before. Briefly, heat-induced antigen retrieval was performed for 20 minutes at ~96o C with sodium-citrate (pH 6) buffer. Primary antibody (Abcam – ab78029) diluted in PBS with 5% NDS at 1:200 dilution was incubated overnight at 4°C. Detection was done using the biotin-streptavidin de

tection system and signal developed by DAB reaction according to manufacturer's protocol (Cell Signaling Technology #8059, Beverly, MA,). Then, sections were counterstained with hematoxylin and slides were mounted with Permount® and visualized on a Leica microscope model DM5500B with DFC450C camera (Leica – Nussloch, Germany).

Statistics

Data are represented as the mean of a series of 'n' biological repetitions ± standard deviation (SD). Data followed a Gaussian distribution and variation was similar between groups for conditions analyzed together. Differences between groups were analyzed with one-way ANOVA and *P*-values below 0.05 were considered significant. No data points were excluded from statistical analysis. Statistical analysis was performed using GraphPad Prism version 9 (GraphPad, San Diego, CA).

4.4 RESULTS

PTPN2 regulates expression of CEACAM6 in IBD patients and in epithelial cells in vitro.

Intestinal biopsies from the ileum and colon of IBD patients genotyped for *PTPN2* loss-of-function SNP *rs 1893217* were stained for CEACAM6. IBD patients with "TT" wildtype allele were considered controls while, patients with heterozygous "CT" and homozygous "CC" where "C" is the minor allele carrying IBD associated SNP, displayed elevated CEACAM6 expression on IEC membranes (Figure 22 A). To further confirm these findings, we performed RNA-sequencing analysis on control (*PTPN2*-CTL) and *PTPN2*-KD HT-29cl.9A IEC cell lines. We observed CEACAM 6 RNA expression was the most abundantly increased gene in *PTPN2*-KD cells as compared to the control IECs (Figure 22 B). Further, we confirmed by western blotting that CEACAM6 protein expression was elevated in *PTPN2*-KD HT-29 cell lines in comparison to controls (Figure 22 C). Using CRISPR-Cas9 gene editing, the *PTPN2* SNP *rs1893217* was inserted (*PTPN2*-KI), or a complete knockout of the gene *PTPN2* (*PTPN2*-KO) was generated in Caco-2BBe IECs. A scrambled RNA was used as control (*PTPN2*-WT). We observed both KI and KO cells displayed higher CEACAM6 protein compared to the control cells (Figure 22 D). Further, CEACAM6 immunofluorescence staining on these cells revealed increased CEACAM6 fluorescence intensity in KI and KO compared to WT cells (Figure 23). These data indicate that loss of function of the IBD-susceptibility gene, *PTPN2*, promotes CEACAM6 protein expression in IECs.

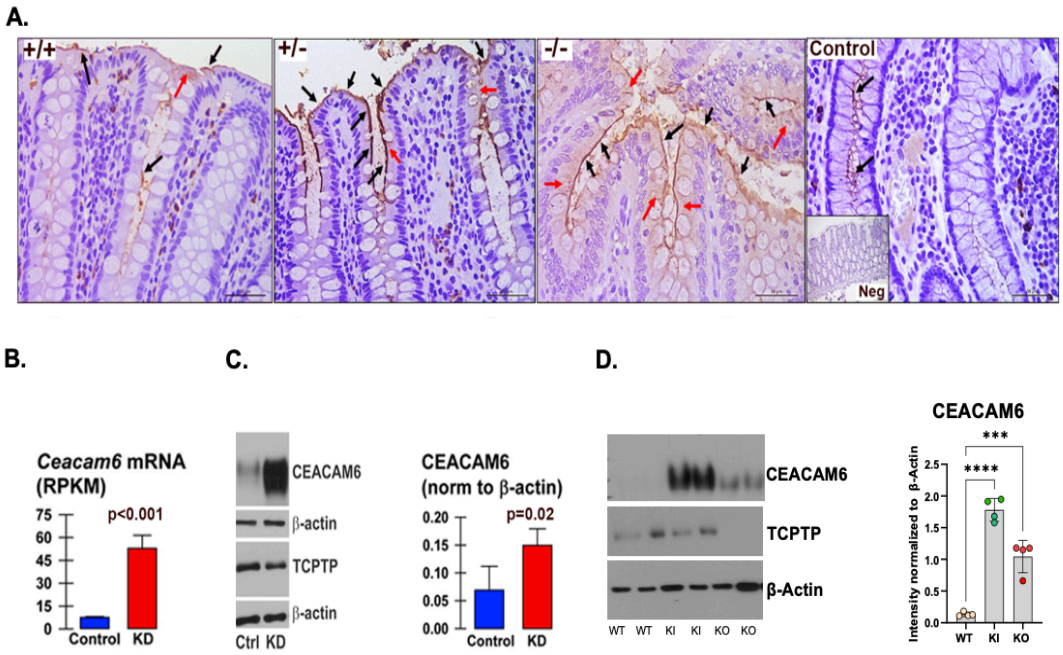

Figure 22: The *PTPN2* SNP rs1893217 promotes CEACAM6 protein expression.

A. Colonic biopsies from IBD patients homozygous for the wildtype allele (TT), heterozygous (CT) or homozygous (CC) where ”C” allele possess the *PTPN2* SNP rs1893217 that is associated with IBD susceptibility. The patient biopsies from variant carrying allele showed higher expression of CEACAM6 (n=1-5). B. RNA - sequencing analysis from *PTPN2*-CTL and *PTPN2*-KD HT-29 cells show higher CEACAM6 mRNA in the KD condition compared to the control condition (*P*< 0.*001;* n=2). C. Western blots from control and *PTPN2*-KD HT-29 cells confirm higher CEACAM6 protein expression in the KD cells (*P*<0.001; n=4). D. Caco-2 BBe *PTPN2*-KI and *PTPN2*-KO cells also display higher expression of CEACAM6 protein compared to controls(P<0.001; n=4) .

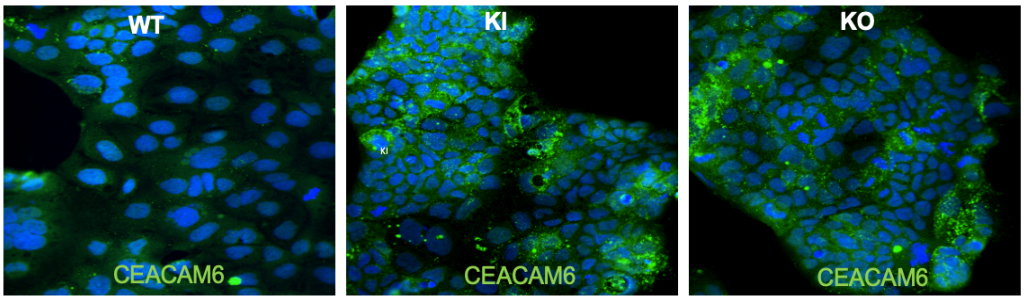

Figure 23: Higher intensity of CEACAM6 staining is visualized in Caco-2 BBe *PTPN2*-KI and *PTPN2*-KO cells compared to control cell lines (n=2).

Loss of Epithelial PTPN2 Increases Susceptibility to AIEC Invasion

To assess the functional consequences of increased CEACAM6 in *PTPN2*-deficient cells, we measured AIEC-LF82 adherence and invasion of IECs. AIEC LF82 showed increased adherence to *PTPN2*-KD cells in comparison to control cell lines (Figure 24 A). Further, *PTPN2*-KD cells also displayed higher invasion of AIEC-LF82 (Figure 24 B). Further, we used a fluorescent tagged AIEC strain (*m*AIEC^{red}) to visualize AIEC invasion of host cells. We observed, *m*AIEC^{red} challenge exhibited significantly higher invasion of *PTPN2*-KD cells compared to control cells (Figure 24 C). Interestingly, we observed, some intestinal epithelial cells (both in control and KD) were highly invaded by *m*AIEC^{red} as compared to the other cells. This phenomenon was more apparent in *PTPN2*-KD cells. We, therefore, quantified cells that demonstrated less than 50 bacteria per cell as having “low-level” invasion, while cells that displayed more than 50 bacteria/cell were designated as

experiencing a “high level” of invasion. *PTPN2*-KD cells displayed a significant increase in susceptibility to both parameters of AIEC invasion (Figure 24 D). To confirm a functional role for CEACAM6 in AIEC invasion, we pre-treated cells with a CEACAM6 blocking antibody. Anti-CEACAM6 greatly reduced *m*AIEC^{red} invasion of *PTPN2*-KD cells compared to untreated controls (Figure 25). Taken together, these results demonstrate loss of PTPN2 promotes AIEC invasion in host by promoting CEACAM6 protein expression.

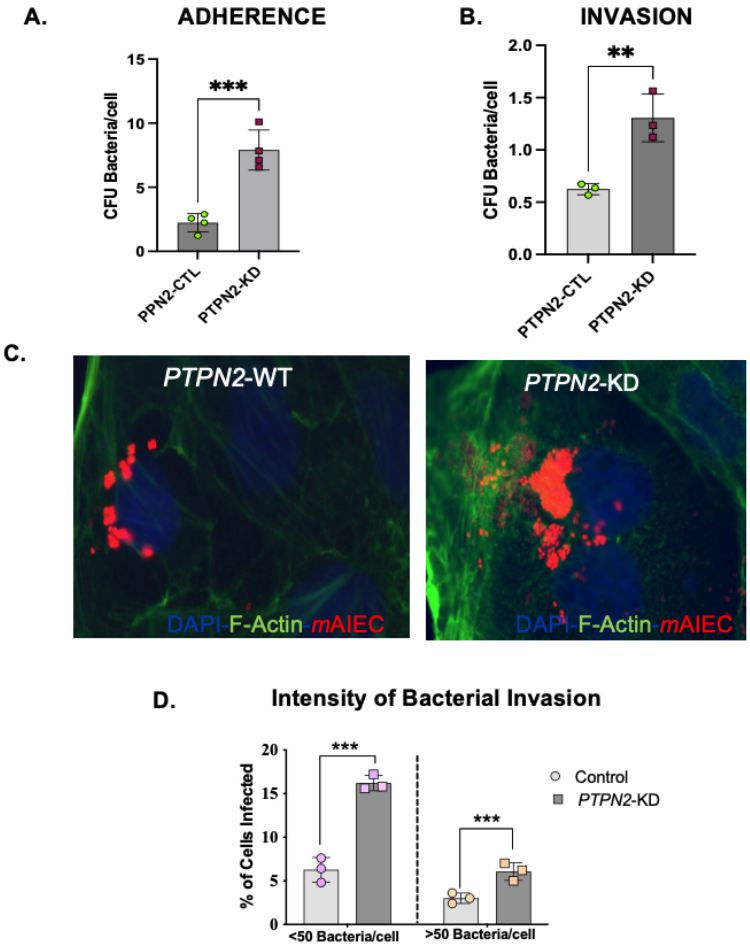

Figure 24: Loss of *PTPN2* in IECs increases susceptibility towards AIEC adherence and invasion.

Caco-2 BBe cells were transfected with either control shRNA (*PTPN2*-CTL) or *PTPN2* targeting shRNA (*PTPN2*-KD). AIEC LF-82 shows higher **A**. Adherence ($P<0.001$; n=3) and **B.** Invasion ($P<0.001$; n=3) of *PTPN2*-KD cells compared to controls. Control and KD Caco-2 BBe cells were seeded on coverslips and infected with *m*AIEC^{red} for 5 hours, washed with PBS and incubated with gentamycin and bacterial invasion was visualized via immunofluorescence microscopy **C.** Immunofluorescence showing increased *m*AIEC^{red} invasion of PTPN2-KD cells compared to control (n=4). **D.** *PTPN2*-KD cells display both high and low *m*AIEC^{red} invasion cell types. CEACAM6 antibody was used to block CEACAM6 protein (n=4). After which the bacterial invasion protocol was followed as usual.

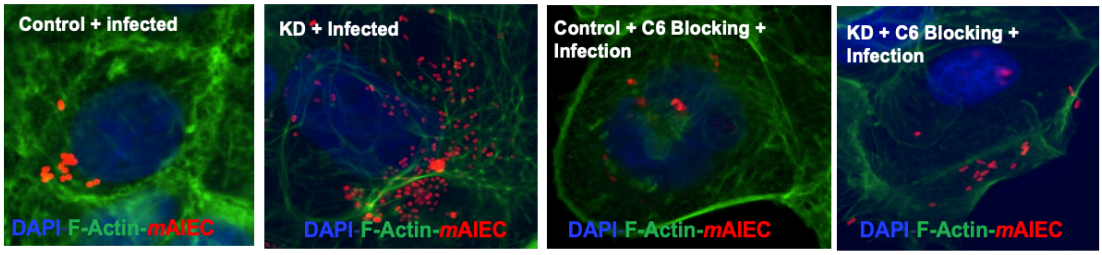

Figure 25: Reduced AIEC invasion in both control and KD cells after CEACAM6 blocking in comparison to the untreated controls(n=2).

IFN-γ Promotes CEACAM6 expression.

Previous studies have shown that CEACAM6 expression can be elevated by proinflammatory cytokines like IFN-γ[31]. Since PTPN2 is a negative regulator of the JAK-STAT signaling pathway and suppresses IFN-γ induced activation of this pathway, we assessed whether IFN-γ promotes CEACAM6 expression in epithelial cell lines. Expectedly, IFN-γ significantly upregulated phosphorylated STAT (p-STAT1) in *PTPN2*-KI and *PTPN2*-KO cells compared to WT cell lines (Figure 26 A). We also observed CEACAM6 expression in *PTPN2*-KI and *PTPN2*-KO cells was further upregulated after administration of IFN-γ (Figure 26 A, B). To determine if the elevated CEACAM6 was a consequence of higher STAT1 activation, we performed STAT1 silencing using siRNA constructs. We observed STAT1 and pSTAT1 expression was reduced by 95%. We also observed that CEACAM6 expression was reduced by 30% between untreated and IFN-γ treated *PTPN2*-KI cells and by 40% between untreated and IFN-γ treated PTPN2-KO IECs (Figure 27). These data suggest that CEACAM6 expression is regulated, at least in part, by STAT1 activation.

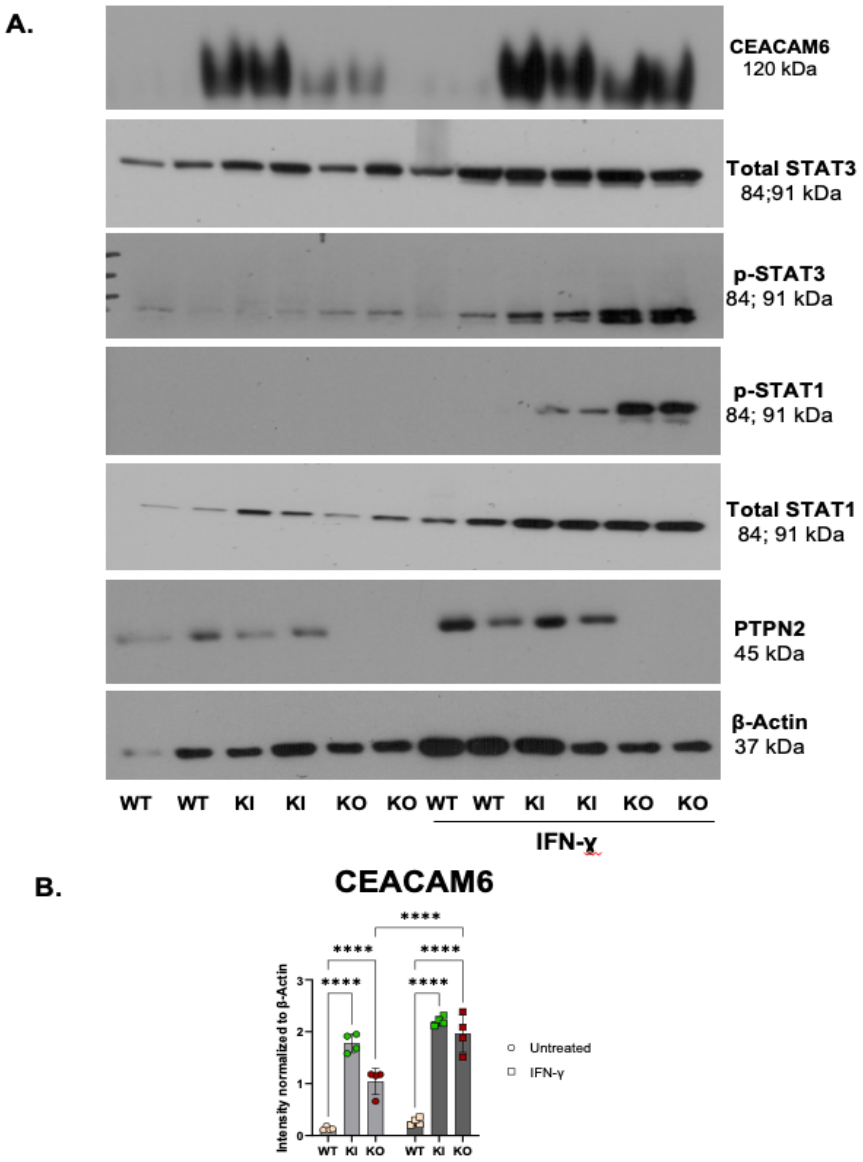

Figure 26: IFN-ɣ Promotes CEACAM6 Expression in *PTPN2*- deficient Caco-2 BBe cells

Caco-2 BBe cells, WT, KI and KO for *PTPN2* were treated with IFN-ɣ at 100U/ml for 24 hours. **A.** Representative western blot images depicting β-actin, *PTPN2*, phosphorylated (p-STAT1/3), STAT1 and CEACAM6. p-STAT1 protein expression in higher in *PTPN2*-KO cells and is further elevated after IFN-ɣ treatment. CEACAM6 protein expression was elevated at basal states for *PTPN2*-KI and KO cells and was further elevated after IFN-ɣ treatment (n=4). **B.** Densitometry analysis of CEACAM6 protein expression after administration of IFN-ɣ. CEACAM6 protein expression is significantly increased in IFN-ɣ treated *PTPN2*-KO cells compared to untreated controls (n=4).

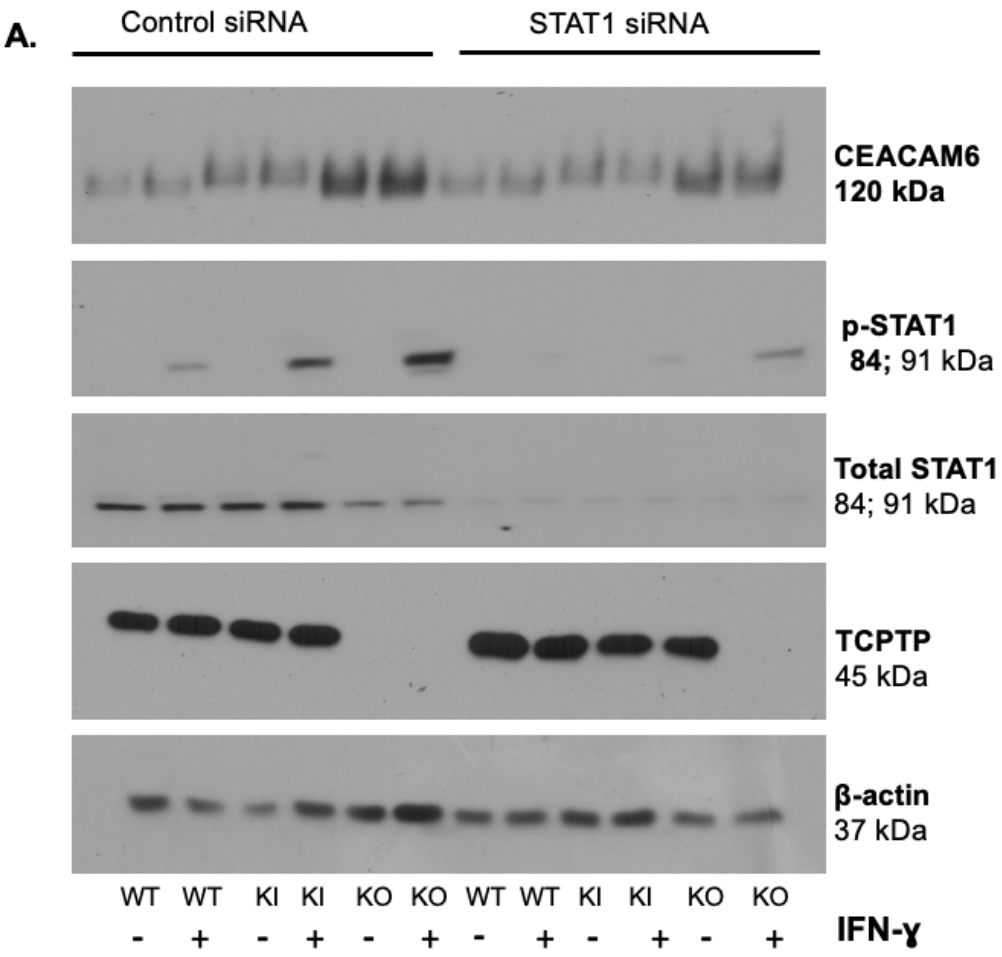

Figure 27: Silencing of STAT1 reduces CEACAM6 protein expression in *PTPN2*-deficient cell lines.

WT, KI and KO cells were treated with non-targeting control (siControl) and STAT1 specific (siSTAT1) siRNA. After 72 hours, IFN-γ was administered. **C.** Representative western blot images showing reduced total STAT1 and p-STAT1. CEACAM6 protein expression is reduced by 30% between *PTPN2*-KI control siRNA and STAT1 siRNA. Further, CEACAM6 protein expression is reduced by 40% between *PTPN2*-KO control siRNA and STAT1 siRNA (*n*=2).

Tofacitinib Rescues CEACAM6 Protein Overexpression

To test whether inhibition of STAT activation can indeed prevent elevated CEACAM6 expression, we treated Caco-2 BBe IECs with a pan-JAK inhibitor, tofacitinib. Like our previous findings with STAT1 siRNA, inhibition of JAK-STAT signaling pathway inhibited CEACAM6 protein expression (~70, 85%) in *PTPN2* KI and KO cell lines compared to control cells (Figure 28). These data demonstrate that tofacitinib, may have a therapeutic role in CEACAM6 protein suppression thereby potentially reducing AIEC colonization and the downstream exacerbated inflammatory response in *PTPN2*-deficient cells.

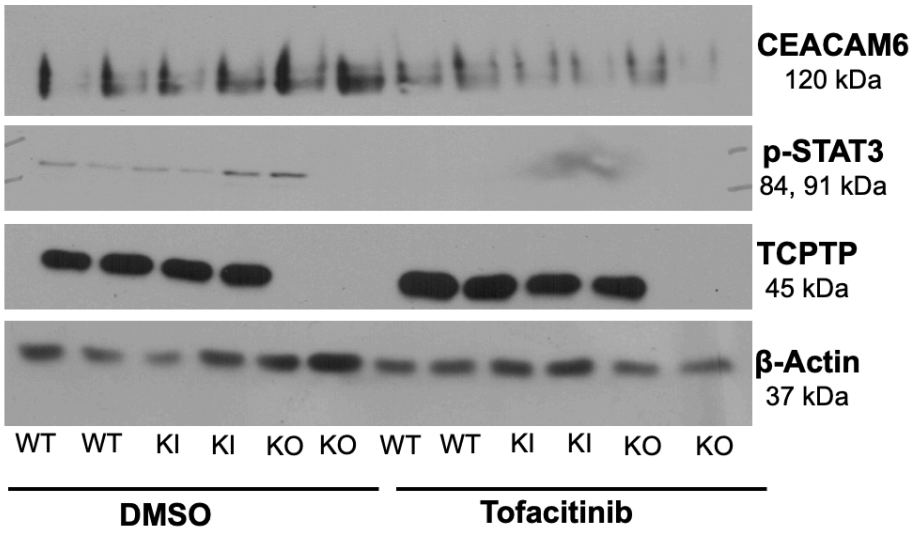

Figure 28*:* Tofacitinib alleviates CEACAM6 upregulation in *PTPN2*-deficient cell lines.

Caco-2 BBe WT, KI and KO cells were treated with vehical (DMSO) or Tofacitinib for 1 hour prior to harvesting the cells for protein. **A.** Representative western blot images showing reduced CEACAM6 protein expression in *PTPN2*-KI and KO cells after treatment with Tofacitinib compared to its respective DMSO controls (*n*=2).

CEACAM1 expression is higher in Ptpn2-KO mice but not Ptpn2$^{\Delta IEC}$ mice.

Since the murine homologue of CEACAM6 is absent, we determined if other proteins of the CEACAM family were involved in mediating AIEC entry into host cells. After screening, other CEACAMs we observed that CEACAM1 was upregulated in constitutive *Ptpn2*-KO mice compared to their wildtype (WT) and *Ptpn2* heterozygous (het) littermates (Figure 29 A). Additionally, staining the cecum section of these mice, further confirmed our findings that CEACAM1 was upregulated in *Ptpn2*-KO mice(Figure 29 B). Next, we determined if CEACAM1 is elevated in IEC-specific *Ptpn2* knockout mice. No differences, in the small or large intestine, were observed in CEACAM1 protein levels between the *Ptpn2*$^{fl/fl}$ and *Ptpn2*$^{\Delta IEC}$ mice (Figure 30 A, B, C) . Next, used the CEACAM1 KO mice to determine, if lack of CEACAM1 could prevent *m*AIEC colonization in mice. A mild drop in bodyweight was observed in both genotypes but no differences in *m*AIEC colonization between WT and CEACAM1 KO mice (Figure 31 A, B).

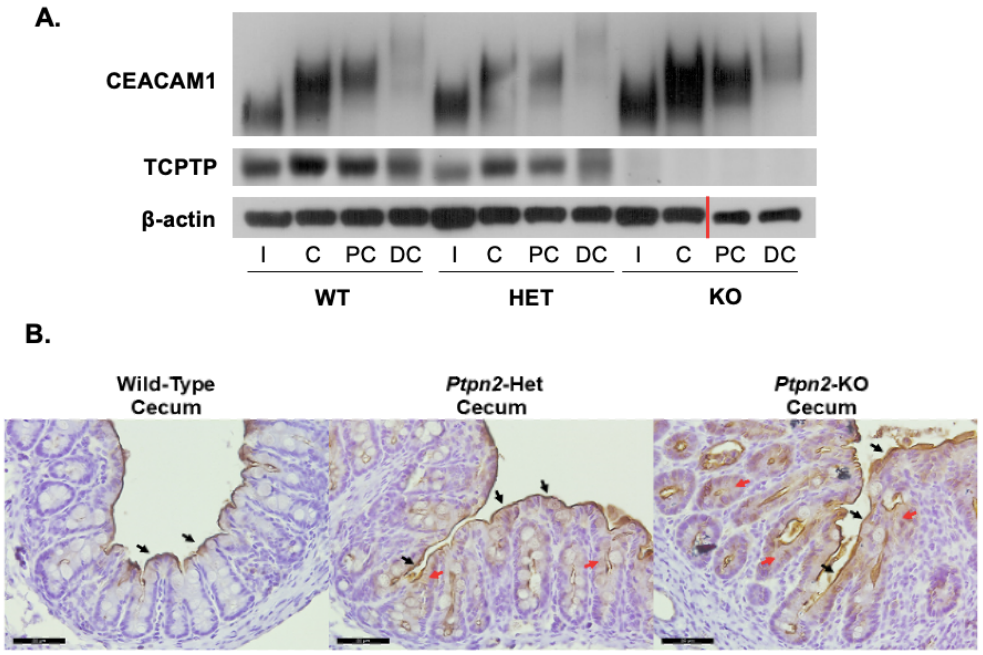

Figure 29: CEACAM1 expression is elevated in constitutive *Ptpn2*-KO mice.

A. Representative western blot images of CEACAM1 protein expression from IECs isolated from constitutive WT, *Ptpn2*-het and *Ptpn2*-KO mice. CEACAM1 protein is elevated in *Ptpn2*-KO compared to het and WT littermates(n=3). **B.** Immunohistochemistry staining from the cecum section shows higher CEACAM1 protein levels in *Ptpn2*-KO mice compared to WT and *Ptpn2*-Hets(n=3).

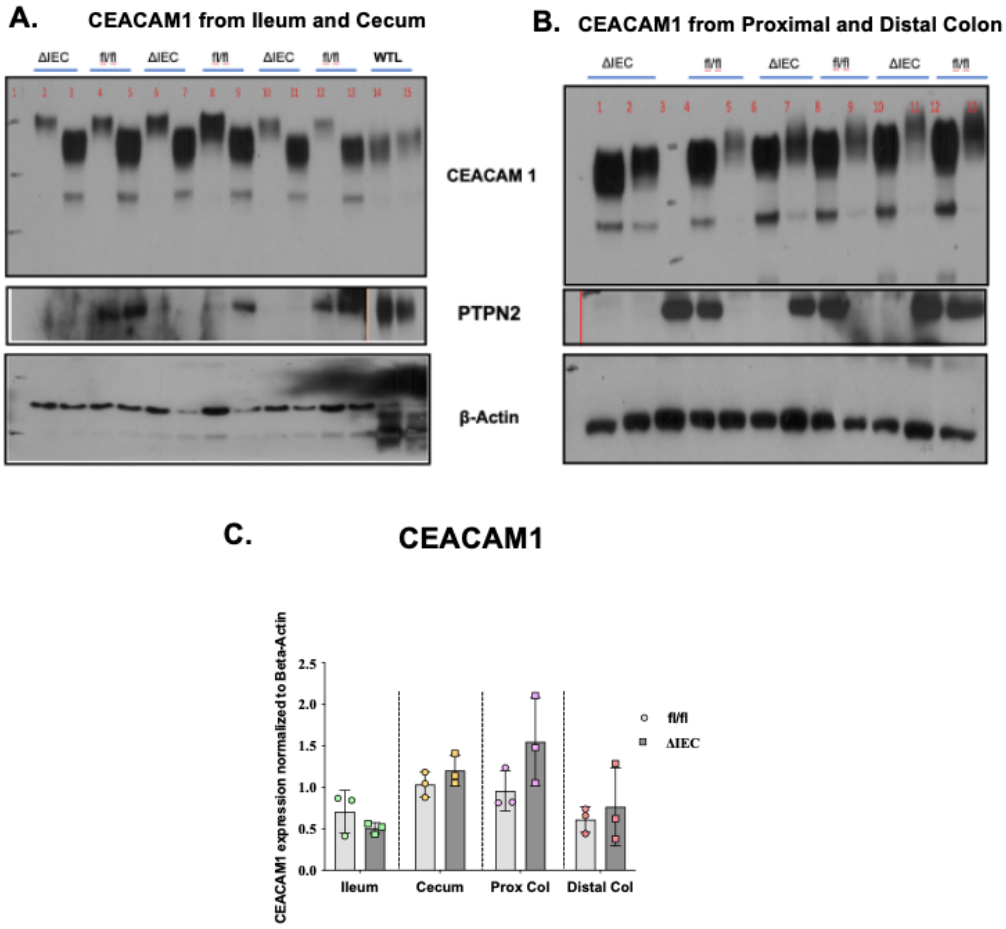

Figure 30: No differences were observed in CEACAM1 protein expression between *Ptpn2*$^{\Delta IEC}$ and *Ptpn2*$^{fl/fl}$ mice. **A.** Representative western blot images of CEACAM1 protein expression from ileum and cecum IECs isolated from *Ptpn2*$^{\Delta IEC}$ and *Ptpn2*$^{fl/fl}$ mice (n=3). **B.** Representative western blot images of CEACAM1 protein expression from the proximal colon and distal colon IECs isolated from *Ptpn2*$^{\Delta IEC}$ and *Ptpn2*$^{fl/fl}$ mice (n=3).**C.** CEACAM1 proteins levels were normalized to Beta-actin. No differences observed between groups (n=3).

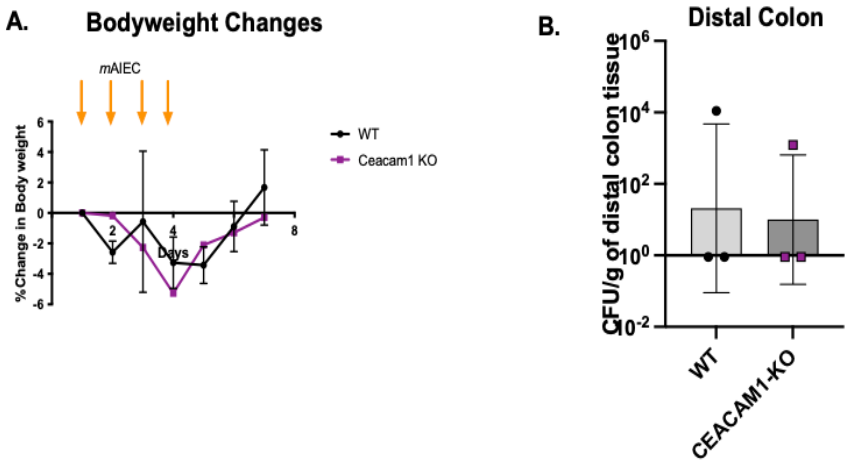

Figure 31: Loss of CEACAM1 does not limit *m*AIEC colonization.

Wildtype and CEACAM1 knockout mice were infected with 10^9 cfu/ml *m*IAEC. **A.** Modest drop in bodyweights were observed in both genotypes (n=3). **B.** Similar *m*AIEC colonization levels in the distal colon were observed in both WT and CEACAM1-KO mice (n=3).

4.5 DISCUSSION

Previously, our lab has described that loss of *Ptpn2* in mice causes expansion of the pathobiont, AIEC [36]. In this current study, we describe that the loss of epithelial *PTPN2* increases susceptibility to AIEC invasion by a mechanism involving upregulated expression of CEACAM6, a critical receptor for AIEC that mediates entry into IECs. We have also demonstrated increased AIEC invasion of IECs and its capability to exacerbate an underlying barrier dysfunction in *PTPN2*-deficient cell lines. Further, we have proposed a unique role of the UC approved drug, tofacitinib (Xeljanz) in alleviating CEACAM6 expression in *PTPN2*-deficient cell lines and potentially preventing AIEC colonization and barrier dysfunction. Taken together, these data highlight the central role of PTPN2 in microbial homeostasis, bacterial entry and preserving intestinal barrier function.

In this study, we show that CEACAM6 is significantly upregulated in *PTPN2* deficient colonic carcinoma cell lines, HT-29.cl19A and Caco-2 BBe. Further, IBD patients carrying heterozygous (CT) and homozygous (CC) *PTPN2* susceptibility variant *rs1893217* display increased CEACAM6 expression in the ileum and colon compared to wildtype (TT) controls. We validated the increased CEACAM6 is of functional consequence by demonstrating that *PTPN2*-deficient cell lines have increased AIEC adherence and invasion burden. Given that AIEC is increased in patients with IBD and IBD patients carrying the *PTPN2* SNP *rs1893217* display differences in their intestinal microbiome, our findings identify additional mechanisms of how PTPN2 loss-of-function contributes to inflammation in IBD[37, 38].

Previously, our lab has demonstrated the role of intestinal epithelial PTPN2 in intestinal barrier integrity[15, 35]. We have also demonstrated that mice lacking PTPN2 in macrophages (*Ptpn2*-LysMCre) are more susceptible to AIEC invasion and exhibit increased bacterial translocation to extra-intestinal organs[39]. Interestingly, macrophages derived from *Ptpn2*-LysMCre mice also displayed higher CEACAM1 expression and administration of the anti-CEACAM1 antibody, partially rescued the *m*AIEC translocation observed in these mice. Further, we also see increased CEACAM1 protein expression in the constitutive *Ptpn2*-KO mice compared to wildtype and heterozygous littermates. However, in this study, we observe no significant differences in CEACAM1 protein levels between $Ptpn2^{fl/fl}$ and$Ptpn2^{\Delta IEC}$ mice, although $Ptpn2^{\Delta IEC}$ display higher *m*AIEC tissue burden and increased intestinal barrier permeability compared to $Ptpn2^{fl/fl}$ mice (Chapter 3). Moreover, loss of CEACAM1 did not prevent *m*AIEC colonization IN CEACAM1-KO mice. While the exact cause of this discrepancy is unknown, it is possible that localized inflammation upregulates CEACAM1 expression which can be utilized my pathobionts to enter into specific host cells. Further, the CEACAM1 levels in the IECs remains to be determined in the *Ptpn2*-LysMCre. This would potentially explain the role of epithelial CEACAM1 in mediating AIEC entry into host cells. Further, CEACAM6 blocking antibody prevented AIEC invasion of human *PTPN2*-deficient macrophages demonstrating that CEACAM6 also played a role in AIEC uptake in macrophages as well.

However, increased CEACAM6 in the intestinal biopsies of *PTPN2*-SNP carrying patients and SNP carrying IEC lines, as well as the exacerbation of intestinal barrier defects in *PTPN2*-deficient IECs post AIEC challenge (see Chapter 3) demonstrates the clinical and physiological relevance of AIEC invasion in *PTPN2* deficient host.

CEACAM6 can be upregulated by several inflammatory cytokines and AIEC itself can upregulate CEACAM6 expression to favor its own colonization [31]. However, very few studies have investigated therapeutics that prevent AIEC colonization. Recently, a study demonstrated that anti-TNF medication, adalimumab, restricted AIEC replication in CD macrophages by inducing Flotillin (FLOT-1) and restricting chitinase 3-like 1 proteins (CHI3L1), an AIEC receptor, in macrophages [40]. In this study we show that tofacitinib was able to alleviate CEACAM6 expression in variant and *PTPN2*-KO IECs. IECs are AIEC's first port of entry inside the intestinal mucosa and checking its entry in the very first stages can prevent AIEC-induced inflammatory responses. Our findings are in line with another study where a mix of inflammatory cytokines increased the expression of CEACAM6 in IECs and this elevation was partially corrected by tofacitinib [41]. These findings demonstrate the possibility of other JAK-inhibitors as a potential therapeutic strategy to mitigate AIEC colonization in disease susceptible hosts.

The Phase II clinical trials of tofacitinib have mostly demonstrated its efficacy in mucosal healing compared to placebo controls [18, 19]. Our findings highlight the unique clinical benefits of tofacitinib in treating *PTPN2*-genotyped UC patients. Recently, a selective JAK1 inhibitor, upadacitinib, was approved for moderate to severe CD patients. Together these suggests that JAKi can used in IBD for not just mucosal healing as previously suggested but by also lessening of CEACAM6 upregulated by the JAK-STAT signaling pathway and potentially restricting AIEC invasion and subsequent barrier damage.

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CHAPTER 5

5 CONCLUSION

5.1 SUMMARY

In this dissertation we have showed that intestinal epithelial PTPN2 is critical for restricting AIEC colonization by promoting protective cytokine, anti-microbial and barrier enhancing responses to infection. We also demonstrate a role for epithelial *PTPN2* in regulating host protein, CEACAM6, that facilitates AIEC entry into host epithelial cells. We demonstrate that the clinically significant *PTPN2* SNP *rs1893217*, displayed a lower electrical resistance (= increased pore permeability) compared to wildtype cells and a higher macromolecular permeability after AIEC challenge compared to the unchallenged state. We also revealed that IBD patients carrying the SNP *rs1893217* exhibited increased CEACAM6 expression. We have therefore, revealed the cell-specific effect of an IBD-relevant gene in mediating several aspects of mucosal immunity.

In Chapter 2, we described the generation of a novel fluorescent strain of *m*AIEC–*m*AIEC^{red}. We demonstrated that it displayed similar levels of adherence, invasion in epithelial cells as the wildtype strain. Further, we demonstrated that *m*AIEC^{red} could be effectively detected by microscopy and efficiently isolated from *in vivo* mouse models as well. We also identified genes uniquely present in *m*AIEC, such as the type 4 secretion system-related genes, compared to the prototypical human AIEC LF82 strain and the putative mouse AIEC strain, NC101.

Next, in chapter 3, we demonstrated that *Ptpn2*$^{\Delta IEC}$ mice displayed higher *m*AIEC colonization in the distal colon compared to the *Ptpn2*$^{fl/fl}$ mice. Further, we show that

Ptpn2$^{\Delta IEC}$ mice have lower *Defa5* and *Defa6* and MMP7 anti-microbial response upon *m*AIEC challenge, in the ileum section of the intestine. Although, the Paneth cells in the ileum are the primary source of the AMPs, the mucus layer retains the secreted AMPs in both the large and small intestine[1]. The mucus layer associated with both the small intestine (SI) and large intestine (LI) has been shown to have high bactericidal activity albeit, the concentration of AMPs is much higher in the SI[1]. This could partially explain, why we see increased colonization of *m*AIEC in the distal part of the colon. Interestingly, we also observed lower lysozyme response in *Ptpn2*$^{\Delta IEC}$ group infected with *E. coli* K12 compared to the *Ptpn2*$^{fl/fl}$ K12 infected group. However, this response was not observed in the *Ptpn2*$^{\Delta IEC}$-*m*AIEC infected group. The significance of these findings remains to be determined. Concurrently, we also detected increased FITC-dextran (FD4) permeability in *Ptpn2*$^{\Delta IEC}$ mice post infection with *E. coli* K12. Interestingly, we observe the increased permeability between the *Ptpn2*$^{fl/fl}$ and *Ptpn2*$^{\Delta IEC}$ genotypes in response to the non-invasive *E. coli*, K12, was more pronounced in mice challenged with *m*AIEC. In line with the increased FD4 permeability in *Ptpn2*$^{\Delta IEC}$- *m*AIEC condition, we also observed lowered expression of barrier-associated proteins like occludin, ZO-1, claudin-7 and claudin-2 and E-cadherin in these mice compared their respective floxed controls. The reduced AMPs levels correlated with reduced IL-22 production in response to *m*AIEC, a cytokine critical for secretion of AMPs from Paneth cell. Interestingly, the cytokine IL-17 and IL-6 levels were also reduced. Taken together, these results demonstrate a vital role for PTPN2 in restricting pathobiont colonization and promoting protective anti-microbial and barrier strengthening responses to infection.

In Chapter 4, we observed that IBD patients carrying the *PTPN2* variant *rs1893217* displayed higher CEACAM6 protein expression compared to patients that were non-

carriers. We validated these results in Caco-2 BBe cell lines carrying the variant *rs1893217* (*PTPN2*-KI) or complete knockout of PTPN2 (*PTPN2*-KO). Both *PTPN2*-KI and *PTPN2*-KO cells exhibited higher expression of CEACAM6. We also demonstrated that the PTPN2 deficient cell lines were more susceptible to *m*AIEC invasion compared to control cells. We also revealed that the elevated CECACM6 in the *PTPN2* deficient cell lines was mediated, at least partially, by the IFN-γ/STAT1 pathway. Most importantly, we have revealed the effectiveness of tofacitinib, a pan-JAK inhibitor, in drastically reducing the levels of CEACAM6 in *PTPN2*-deficient cell lines. These findings reveal a novel role for IBD susceptibility gene, *PTPN2*, in regulating host proteins that can be exploited by pathogens to gain entry inside epithelial cells. Further, we have also, discovered a unique role for tofacitinib, in the case of *PTPN2* deficiency, in restricting CEACAM6 protein and potentially, limiting invasion of AIEC bacterium.

5.2 FUTURE STUDIES

Our findings in Chapter 3, we showed reduced antimicrobial peptides in the ileum section of the intestine but *m*AIEC had higher colonization in the distal colon segment of the intestine. As previously stated, the ileum section of the intestine has the highest concentration of AMPs and therefore, is difficult to colonize. In spite of lower AMP response by the *Ptpn2*$^{\Delta IEC}$ in the ileum, *m*AIEC still preferentially colonized the distal colon. Therefore, a major question that remains to be addressed is the microbial population in the different segments of the intestine between naïve *Ptpn2*$^{fl/fl}$ and *Ptpn2*$^{\Delta IEC}$ mice. These findings would reveal the microbial communities and possibly the metabolic landscape of the different regions of the intestine caused by the loss of intestinal epithelial *Ptpn2* and possibly explain, *m*AIEC's colonization preference in the distal part of the intestine. These

data can also answer if there an inherent expansion of *m*AIEC in *Ptpn2*$^{\Delta IEC}$ mice compared to *Ptpn2*$^{fl/fl}$ mice which will further, explain *m*AIEC's colonization preference to *Ptpn2*$^{\Delta IEC}$ mice.

In this chapter, we also demonstrate reduced mRNA and protein levels of IL-22 in *Ptpn2*$^{\Delta IEC}$ post *m*AIEC challenge. Given that IL-22 has a very critical role in production of AMPs and upregulating pore-forming host protein (claudin-2) to induce diarrhea to remove enteric pathogens, an important question would be to dissect its protective role during *m*AIEC infection. In the future, we would administer recombinant IL-22 in the *Ptpn2*$^{\Delta IEC}$ mice to check if the increased colonization and subsequent barrier defect can be rescued. In a similar study from our lab, we challenged the *Ptpn2*$^{\Delta IEC}$ with the enteropathogenic mouse *E. coli*, *Citrobacter rodentium*. Like our findings in chapter 3, this study also revealed lowered AMP response (Reg3γ and Reg3β) and IL-22 cytokine levels. The expression of IL-22 receptors, IL-10R1 and IL-22R1, were similar between the two genotypes. However, administration of IL-22 did not rescue the increased susceptibility to *C. rodentium* infection – possibly, due to intrinsic cell-mediated defects facilitated by the loss of *Ptpn2*. We may, therefore, also need to explore direct administration of AMPs like recombinant *Defa5* or *Defa6* to rescue the phenotype.

While the *in vitro* studies using STAT1 siRNA and tofacitinib have been promising, we would still need to include more replicates to generate a robust data set that meets statistical significance. Further, studies from other labs have demonstrated that CEACAM6 can be upregulated by the IL-6/STAT3 pathway[2]. Additionally, we have also determined interactions between CEACAM6 and STAT3 in the STRING software. Since, *PTPN2* is a negative regulator of *Stat3* mediated signaling, we would also determine if IL-6/STAT3 has a role in the overexpression of CEACAM6 in *PTPN2* deficient cell lines. While we

were able to rescue the CEACAM6 over expression in the *PTPN2*- deficient cell lines with the administration of tofacitinib, our next steps would be to verify if tofacitinib mediated CEACAM6 reduction can also rescue the increased susceptibility to *m*AIEC in the *PTPN2*-deficient cell lines.

Our findings with CEACAM1 in mice were conflicting. Macrophages derived from *Ptpn2*-LysMCre mice displayed higher CEACAM1 expression and administration of the anti-CEACAM1 antibody, partially rescued the *m*AIEC translocation observed in these mice[3]. Further, we also saw increased CEACAM1 protein expression in the constitutive *Ptpn2*-KO mice compared to their wildtype and heterozygous littermates. However, in this study, we observe no significant differences in CEACAM1 protein levels between $Ptpn2^{fl/fl}$ and $Ptpn2^{\Delta IEC}$ mice, although $Ptpn2^{\Delta IEC}$ display higher *m*AIEC tissue burden and increased intestinal barrier permeability compared to $Ptpn2^{fl/fl}$ mice (Chapter 3). Moreover, loss of CEACAM1 did not prevent *m*AIEC colonization in CEACAM1-KO mice. While the exact cause of this discrepancy is unknown, it is possible that localized inflammation upregulates CEACAM1 expression which can be utilized my pathobionts to enter specific host cells. Further, the CEACAM1 levels in the IECs remains to be determined in the *Ptpn2*-LysMCre. This would potentially explain the circumstances under which the epithelial CEACAM1 might mediate AIEC entry into host cells arising from the more pro-inflammatory phenotype in *Ptpn2*-LysMCre mice compared to the milder tissue landscape in $Ptpn2^{\Delta IEC}$ mice that only lack PTPN2 in the gut epithelium.

5.3 CONCLUSIONS AND IMPLICATIONS

In conclusion, we show that autoimmune risk gene *PTPN2* is involved in anti-microbial peptide, epithelial barrier, and cytokine defenses to prevent pathobiont colonization and preserve epithelial barrier function. Our findings might explain the microbial alterations observed in patients carrying the PTPN2 SNP *rs1893217* and identify, at least in part, why patients carrying this variant are more susceptible to IBD.

Data presented in this dissertation also sheds light into PTPN2's role in regulating the AIEC receptor, CEACAM6. We demonstrated the effectiveness of tofacitinib in alleviating CEACAM6 overexpression in *PTPN2*-deficient cell lines. Given the multi-factorial etiology of IBD, better incorporation of etiologic factors, i. e genetic polymorphism into the treatment regime, is an area of rapid growth with significant potential to greatly improve patient outcomes. Our data indicates a possibility for *PTPN2*-genotyped patients to benefit from JAKi (tofacitinib) regimens by not just accelerating mucosal healing, as previously described, but also restricting functional permeability defects and the upstream expression of epithelial proteins that mediate pathobiont entry into host cells thereby exacerbating inflammatory conditions.

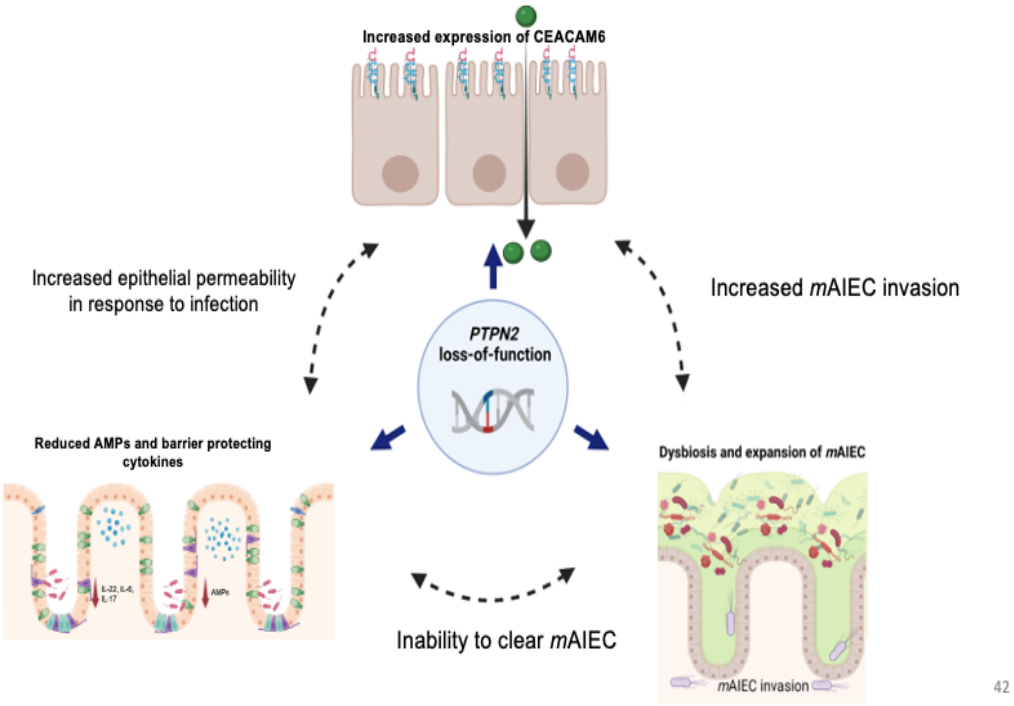

Figure 32: Role of intestinal-epithelial PTPN2, in mediating host-pathobiont interaction.

We had previously demonstrated that constitutive loss of *Ptpn2* in mice (*Ptpn2*-KO) led to microbial dysbiosis and expansion of *m*AIEC. In this dissertation, we demonstrated that IEC-specific loss of *Ptpn2* in mice ($Ptpn2^{\Delta IEC}$) causes susceptibility of *m*AIEC colonization. We also revealed that epithelial *PTPN2* regulates CEACAM6 production in human IECs, which mediates AIEC entry into host cells. We also demonstrate that epithelial barrier permeability is increased post *m*AIEC invasion. We also showed that loss of epithelial *Ptpn2* reduced production of anti-microbial peptide and protective cytokines post *m*AIEC challenge. These results demonstrate the critical role of epithelial PTPN2 in mediating host-pathobiont interaction.

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