UNIVERSITY OF CALIFORNIA, SAN DIEGO

STAT3 in Intestinal Epithelial Cells Regulates Barrier Function and Anti-bacterial Response

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by

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Introduction, in part, has been submitted for publication. Jongdae Lee is the primary investigator. Ji-Hun Mo, Adam N. Rucker, Shee-Eun Lee, Jose M. Gonalez-Navajas, Devorah Friedberg, Scott Herdman, Declan F McCole, Yolanda Aderson, Kim E. Barrett, Lars Eckmann, and Eyal Raz are the co-authors of this paper.

Materials and methods, in full, is a reprint of the material, as it appears in submitted for publication. Jongdae Lee is the primary investigator. Ji-Hun Mo, Adam N. Rucker, Shee-Eun Lee, Jose M. Gonalez-Navajas, Devorah Friedberg, Scott Herdman, Declan F McCole, Yolanda Aderson, Kim E. Barrett, Lars Eckmann, and Eyal Raz are the co-authors of this paper.

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Discussion, in part, has been submitted for publication. Jongdae Lee is the primary investigator. Ji-Hun Mo, Adam N. Rucker, Shee-Eun Lee, Jose M. Gonalez-Navajas, Devorah Friedberg, Scott Herdman, Declan F McCole, Yolanda Aderson, Kim E. Barrett, Lars Eckmann, and Eyal Raz are the co-authors of this paper.

ABSTRACT OF THE THESIS

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Inflammatory Bower Disease (IBD) is composed of ulcerative colitis (UC) and Crohn's disease (CD). Both genetic and environmental factors contribute to this pathogenesis, which has a common feature of compromised intestinal epithelial barrier in the small and large intestines. We investigated the role of STAT3 in intestinal epithelial cells ($STAT3^{IEC}$) and identified a novel function of STAT3 in maintaining intestinal homeostasis. $STAT3^{IEC}$ deletion in mice resulted in compromised barrier function and the loss of IEC polarity due to low tight junction protein levels (i.e. claudin-1, -3, and -5) *in*

vitro and *in vivo*. We further identified that STAT3 maintains tight junction protein level *in vitro* and *in vivo* by inducing ubiquitin-mediated degradation of SNAI, a transcriptional suppressor of claudins. STAT3 binds with GSK3b, which phosphorylates SNAI for ubiquination and results in degradation. Moreover, STAT3 is essential for host defense against enteropathogenic bacteria (i.e. *Citrobacter rodentium* and *E.coli*). $STAT3^{IEC}$ knock out mice are highly susceptible to *C. rodentium* infection due to impaired IEC barrier and antimicrobial peptide (i.e. regIIIg) induction, which is necessary for bacterial clearance. Collectively, STAT3 along with GSK3b forms a SNAI destruction complex in IECs to maintain claudin levels and thus results in proper barrier function and antibacterial defense.

1. Introduction

1.1. The Intestinal Microflora

The human gut harbors 10 to 100 trillion organisms (Xu and Gordon, 2003) This is roughly10 times more than the total cells in the human body (Guarner and Malagelada, 2003). The complex microbiota community in the intestine helps in processing host nutrients uptake and synthesizing essential molecules for the host metabolism. In return, the microbiota receives part of the energy and an inhabitable environment from the host. From an evolutionary standpoint, the host and the microbiota co-evolve together and develop a mutualistic relationship which enables the host to adjust easily to dietary change and digest food efficiently. Previous experiments show that conventional rodents with microbiota in the gut consume 30% less calories to maintain their body weight comparing to the germ-free rodents (Wostmann *et al.*, 1983). Moreover, the abundant energy resource provided by the host increases microbiota fitness; this in return also benefits the host because microbiota outcompetes the potential pathogens (Dethlefsen *et al.*, 2007). However, enteric pathogens have evolved a diverse array of virulence factors that allow them to surmount the intestinal epithelial barrier into the host. These factors enable the enteric pathogens to escape the highly competitive ecosystem of the gut lumen and access nutrients and replicate in the host (Hooper, 2009). We provide shelter and energy for intestinal microbiota in exchange of nutrient uptake and essential compound synthesis. Intestinal microbiota assists the host in nutrient uptake and

essential compound synthesis. In return, the gut provides the microbiota with energy and an inhabitable environment. This mutualistic relationship is essential for both the host's and the microbiota's survival. Its maintenance is dependent on keeping the virulence of microbiota in check through the actions of the innate and adaptive immune system.

1.2. Innate Immunity vs. Adaptive Immunity

The vast number of gut bacteria in close proximity to the host apical intestinal epithelial layer gives rise to how host immune system distinguishes between enteric pathogens and endogenous microbiota without potentially activating benign immune and inflammatory response. Both innate and adaptive immunity recognize foreign microorganisms as non-self and trigger the immune response to eliminate the pathogens. Innate immunity serves as first line of defense when encountering pathogens. Machrophages and dendritic cells both respond immediately when encountering the invading microorganisms. These innate immune cells act through phagocytosis and antigen presentation to naïve T-cells while inflammatory cytokines and various co-stimulatory molecules are secreted to induce inflammation and mediate the activation of adaptive immunity (Takeda and Akira, 2005; Kaser and Blumberg, 2008). The activated dendritic cells induce CD4+ T cells differentiation into T helper 1 (Th1) cells or Th2 cells. Th1 cells produce interferon-γ (IFNγ) and mediate the clearance of bacterial and viral infection, while Th2 cells produce IL-4 and IL-13 that are involved in the response against parasitic infection (Akira *et al.*, 2006). The cooperation between the innate immunity and adaptive immunity can effectively contain and destroy the foreign

pathogens. Consequently, the deregulation of the immune system leads to inappropriate immune response towards the commensal microbiota or the host itself giving rise to inflammatory bowel disease or autoimmune disease.

1.3. Barrier Functions

Both innate immune and adaptive immune systems are responsible for maintaining the intestinal homeostasis of the host. The host needs to mount an immune response towards the enteric pathogens but not rid of its symbionts. Thus, the host must remain constant immune survailance to control the number and composition of the bacterium but tolerant to microbiota which is essential for the host. Mucousal and epithelial layers are the first line of defense againsts the gut floras. Once the mucuosal layer is breached, the epithelial layer becomes the last line of defense before triggering symptomatic inflammation.

1.3.1. Mucousal Barrier

The mucosal immunity is tolerant to high numbers of symbiotic intestinal bacteria. Mucosal tolerance is maintained through localization of microbiota at the intestinal lumen side of the intestinal epithelium (Hooper, 2009). Intestinal goblet cells secrete a thick mucous layer that covers the entire intestinal epithelium impeding bacterial attachment while Paneth cells produce antimicrobial peptides (i.e. defensin and RegIIIg) restricting bacterial populations (Artis, 2008). Mucousal barrier consists of two layers. The inner mucousal layer is 100μm thick and is firmly adherent to the epithelium. It is highly concentrated with antimirobial peptides which preserves its sterility. The

outer mucousal layer which is about 700μm thick is has a signifcantly lesser antimicrobial peptide conentration which allows for bacterial presence. In other words, bacteria need to travel through 1000 bacterial cell lengths of thick viscous mucus packed with antimicrobial molecules to make contact with the epithelial sruface (McGuckin *et al.*, 2009). When mice is deficient in *Muc2,* which encodes for mucin glycogen production by goblet cells and paneth cells (Rousseau *et al.*, 2004), there is an increased bacterial colonization directly on the epithelium resulting to intestinal inflammation (Johansson *et al.*, 2008). Thus, the mucousal barrier functions in preventing both commensal bacteria and enteric pathogens from establishing infection or crossing the intestinal epithelial cell barrier.

1.3.2. Intestinal Epithelial Cell Barrier

Underneath the mucous layer is the intestinal epithelium that is composed of single columnar epithelial cells interlocking with one another and forming a long sheet of protective layer covering the entire intestinal surface. In the human gut, the intestinal surface area is about 100 m^2 (Ley *et al.*, 2006). One would be amazed in how a thin layer of intestinal epithelial cell (IEC) lining could protect the host from being infected by enteropathogens through maintaining the adherence and tight junctions of IECs (Round and Mazmanian, 2009). Both adherens junction proteins (i.e. E-cadherin, p120-catenin, β-catenin and α-catenin) and tight junction proteins (i.e. occludin, claudin, and ZO-1) are crucial in establishing IEC barriers to prevent bacterial infection (Hartsock and Nelson, 2008). The transmembrane glycoproteins, such as E-cadherin and β-catenin, initiate and maintain adherence junction, which is cell-cell contact. Tight junction proteins are

involved in two major functions: the gate function and the fence function. The gate function regulates the permeability of the ions and solutes through para-cellular junctions (Hartsock and Nelson, 2008). This separates the apical surface facing the intestinal lumen from the basolateral side facing the lamina propria, and creates two different compartments; thus, forming trans-electrical epithelial resistance (TEER). Also, the fence function segregates the membrane components from apical surface to basal lateral surface of the IEC. The polarized IEC thus has two separate domains and gives rise to different functions in each surface (Kazmierczak *et al.*, 2001). Adherence junction and tight junction prevent bacteria from surmounting the IEC barrier and most importantly, the polarity of IECs is also crucial in maintaining intestinal homeostasis (Lee *et al.*, 2006).

Patients with inflammatory bowel disease have shown the downregulation of junction proteins associated with the inflamed intestinal tissues. The disruption of the main adherence junctions and tight junction has been observed in IBD patients (Gibson *et al.*, 1988; Schmitz *et al.*, 1999). In actively inflamed IBD tissues, E-cadherin and a-cadherin are lost in the epithelial cells. Furthermore, leaky intestinal barriers have also been reported in IBD patients (Wyatt *et al.*, 1993). Both occludin and claudins are essential in primary sealing the epithelial cells and maintain fence function. Dysregulation of these TJs in IECs can increase enteropathogen infections resulting inflammation similar to the IBDs (Gassler *et al.*, 2001). Collectively, these suggest that there is a strong correlation between impaired barrier function in IBD and altered TJ structures.

1.4. Epithelial-Mesenchymal Transition

Epithelial-mesenchymal transition (EMT) originally occurs in embryogenesis and takes part in wound healing in adult life. However, EMT is also involved in the transition of stable carcinoma to invasive carcinoma, which epithelial cells gain motility and dissociated from the neighboring cells (Guarino, 2007). Normally, epithelia cells are polarized and tightly connected by tight junctions forming a uniformed epithelium sheets that covers the entire surface of the tissue. In contrast, when epithelial cells undergo EMT, they acquire fibroblastic-like characteristics and thus, do not form a stable cell-cell contacts which enable the cells to migrate away from the original tissues (Guarino, 1995).

SNAI (Snail-1) is a major inducer of EMT. SNAI is a transcription repressor that downregulates the polarized epithelium genes and upregulates mesenchymal genes. It mainly suppresses e-cadherins and claudins (Ikenouchi *et al.*, 2003; Carrozzino *et al.*, 2005) resulting to disruption of cell polarity and IEC barrier. Glycogen synthase kinase-3b (GSK-3b), a SNAI repressor, normally remains in its active form to export SNAI from the nucleus for proteosomal degradation in epithelial cells (Bachelder *et al.*, 2005). GSK3b physically interacts with and phosphorylates SNAI thereby tagging it for ubiquitanation which ultimately results in ubiquitin-mediated degradation of SNAI (Zhou *et al.*, 2004). SNAI is a major transducer of EMT. Recently, there has been report on EMT in CD patients (Bataille *et al.*, 2008). Thus, many upstream pathways of GSK3b are being targeted to control the phosphorylation of SNAI in prevent EMT from occurring.

1.5. Antimicrobial Peptide: RegIIIg

RegIIIg is an anitmicrobial peptide that contains conserved C-type lectin carbohydrate recognition domains (CRDs) and N-terminal secretion signal. RegIIIg and its human ortholog, HIP/PAP, are predominantly expressed in small intestines. RegIIIg and HIP/PAP are induced when encountering mucosal damage or bacterial stimulus (Cash *et al.*, 2006). Patients with IBD, characterized by chronic inflammation and increase mucosal adherence of commensal bacteria, showed an increase in HIP/PAP production (Swidsinski *et al.*, 2002). The microbiota in the gut induces the expression of RegIIIg that binds peptidoglycan and exhibits bacteriocidal activity through altering the permeability of the bacterial cell wall. RegIIIg targets more specifically towards Gram-positive bacteria because peptidoglycan is exposed on the Gram-positive bacterial surface; whereas, peptidoglycan is embedded in the periplasmic space of Gram-negative bacteria (Cash *et al.*, 2006). Paneth cells, a type of IECs, are located at the bottom of the crypt base and harbors abundant secretory antimicrobial granules, such as defensins and RegIIIg. RegIIIg remains inactive in the granules of Paneth cells until secretes into the intestinal lumen. The N-terminal segment contains inhibitory activity is then cleaved by trypsinresulting to the activation of RegIIIg in the intestinal lumen (Mukherjee *et al.*, 2009). Antimicrobial defense is crucial in restricting bacterial contact with the intestinal surface and preventing inflammatory disease, such as IBDs.

1.6. Citrobacter rodentium

Citrobacter rodentium is an ideal model to study pathogen-host immune interactions in the gut. The colon pathology associated with *C. rodentium* infection also has many features in common with mouse models of inflammatory bowel disease. Enteropathogenic *Escherichia coli (EPEC),* the human counterpart of *C. rodentium,* possess a locus of enterocyte effacement (LEE) pathogenicity island. EPEC is gram negative bacteria and utilizes type III secreting system (T3SS). EPEC causes watery diarrhea in infants from the developing countries (Nataro and Kaper, 1998). Both EPEC and *C. rodentium* utilize attaching and effacing (A/E) lesions to colonize the gastrointestinal tract of the host. A/E lesions are characterized by the destruction of brush-border microvilli and intimate attachment of the bacteria to the plasma membrane of the host. Enteric pathogens carry intimin b adhesin proteins and integrate their intimin receptors (Tir) into the host cell plasma membrane causing actin polymerization which forms pedestals beneath the adherent bacteria (Mundy *et al.*, 2005). Adherence of EPEC to IEC causes severe diarrhea in the host.

1.7. Pattern Recognition Receptors

Pattern Recognition Receptors (PRRs), includes Toll-like recoptors (TLRs) and nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs), are signaling receptors which cells use to discriminate between foreigner and self. PPRs recognize microorganism-associated molecular patterns (MAMPs) which are molecules that are highly conserved and unique to microorgansims (Takeda and Akira, 2005).

Toll-like receptors are type I integral membrane receptors that consist of Toll/IL1 receptor (TIR) domain in the extracellular domain that are functionally critical for MAMP recognition (Bowie and O'Neill, 2000). There are at least 13 TLRs identified in mammalian cells and they all recognize varieties of MAMPs derived from bacteria, fungi, protozoa, and viruses: TLR1, TLR2, and TLR6 recognize lipopeptides; TLR3, TLR7, and TLR8 recognize viral-RNA; TLR4 recognizes lipopolysaccharides (LPS); TLR 5 recognizes flagellin; TLR9 specializes in recognition of CpG DNA . In addition, TLRs are expressed on both immune cells (i.e. macrophages, dendritic cells, B cells, and specific T cells) and non-immune cells (i.e. fibroblast and epithelial cells) (Bowie and O'Neill, 2000). TLR3, TLR7, TLR8 and TLR9 are localized within the endosome, which is essential for recognition of invading pathogens (Takeda and Akira, 2005).

Nucleotide-binding oligomerization domain (NOD) proteins are intracellular receptors that recognize peptidoglycan, an important component for bacterial cell walls formation. The activation of NOD1 and NOD2 recruits the IκB kinase (IKK) complex resulting to the activation of NF-κB.

1.8. Toll-like Receptor Signaling Pathway

Once TLR is activated by the MAMPs, the TIR-domain containing adaptor, MyD88, recruits IRAK to the receptor. IRAK then activates TRAF6, leading to the activation of the IκB kinase (IKK) complex which consists of IKKa, IKKb and NEMO/IKKg. The IKK complex phosphorylates IκB resulting to the translocation of nuclear factor-κB (NF-κB) into the nucleus. TLR2 and TLR4 utilize TIRAP, a second TIR domain-containing adapter, involving MyD88 dependent signaling pathway. The

third TIR domain- containing adapter, TRIF is MyD88-indepenent, whereas the fourth TIR domain-containing adapter, TRAM, is specific to TLR4-mediated MyD88-independent/TRIF-dependent pathway (Takeda and Akira, 2005). The TLRs recognize invading pathogens and activate the innate immune response by initiating a variety of signaling pathways that ultimately lead to the activation of NF-κB resulting inflammatory cytokine and interferon inductions.

1.9. Type I Interferon Family

Type I Interferon (IFN) family consists 20 or more subtypes of IFNα, IFNβ, IFNκ, IFNω, IFNδ, and IFNμ, etc. All of them share homology in amino acid sequences, tertiary structures, and bind to the same receptor complex. IFNs have been known for their antiviral innate immunity through inhibiting viral replications. IFNs also inhibit cell growth and cell apoptosis. Type I IFN receptors comprise of two subunits IFNAR1 and IFNAR2, which interact with tyrosin kinase 2 (TYK2) and JAK respectively. When IFNAR is activated, tyrosin kinase phosphorylates JAK, in which then phosphorylate specific tyrosin residues in STAT proteins. Activated STAT proteins dimerize and form transcription factor complex with DNA binding proteins. They then translocate into the nucleus to regulate transcription (Du *et al.*, 2007). The intracellular domains of IFNARs and signal transducing molecules such as STATs may form multimolecular signal transduction complex which binds to different sets of IFN-regulated genes (IRGs). The classical transcription factor complexes identified in type I IFN signal transduction pathway is the IFN-stimulated gene factor (ISGF) 3 complex composed of STAT1, STAT2 and IRF9, which binds to IFN-stimulated response elements (ISREs) in the

promoters of IRGs. Moreover, there are STAT1:1 and STAT3:3 homodimers and STAT1:3 heterodimers, which binds to gamma-activated sequence (GAS) element that drives another sets of IRGs upon IFNAR stimulation (Noppert *et al.*, 2007). Besides JAK/STAT mediated IFN signaling pathway, type I IFN also mediated signaling through NF-κB pathway that involves STAT3, PI3K, and AKT. Upon stimulation, STAT3 acts as an adaptor to couple PI3K to the IFNAR1 subunit which enables IFNα to promote the dissociation between IκBα/ NF-κB complex. This dissociation causes the degradation of IκBα and results to NF-κB translocation into the nucleus. Both type I IFN signaling pathways activate and transcribed the genes that are involved in antiviral, cell survival, proliferation, and immune response (Du *et al.*, 2007). The properties of type I IFNs, such as anti-viral, anti-proliferative and immunoregulatory, make them candidates for therapeutic treatments for hepatitis B and C, hematological cancers and multiple sclerosis (Noppert *et al.*, 2007).

In addition, from our previous study, we found that IFNα also has a physiological and protective role in colonic injury and that it also cross-regulates other proinflammatory activities induced by TLR 9 (Katakura *et al.*, 2005). Dextran sulfate sodium (DSS) is administered orally to mice to induce acute colitis characterized by colonic epithelial cell death, mucosal edema, and the accumulation of neutrophils that are necessary to limit bacterial translocation to the adjacent tissues. Mice deficient in IFNα/βR are extremely susceptible to DSS-induced colitis. The administration of recombinant IFNβ to DSS-treated mice mimicked the anti-inflammatory effect on colonic inflammation induced by TLR 9 ligand (Katakura *et al.*, 2005). Thus, type I IFN produced by TLR activated cells (i.e. dendritic cells) facilitates the resolution of DSS-

induced colitis. While IFNs are beneficial to the host, they also induce side effects. The balancing signaling in order to generate an effective host response, while limiting adverse effects is crucial. IFN induction genes are regulated positively through IFN regulatory factors (IRFs)1, 3, 4, and 7 and negatively by IRF 2, 4, and 8. The selection of the subtypes of IFN produced will determine the potency and perhaps the nature of the effects. TLR 3, 4, 7, 8 and 9 induce type I IFNs (Noppert *et al.*, 2007).

1.10. Signal Transducers and Activators of Transcription

The STAT proteins (Signal Transducers and Activators of Transcription) are transcription factors that mediate cytokine driven signaling through tyrosine kinase (JAK) phosphorylation. The phosphorylated STATs dimerize and translocate into the nucleus where binding to specific DNA sites results in increased gene transcription (Bromberg and Darnell, 2000). STATs were discovered in the course of studying IFN receptors and were originally thought to be highly specific in individual signaling pathway. STAT proteins are involved in controlling cell cycle progression and apoptosis. In particular, STAT1 is believed to control cell growth arrest and promotes apoptosis; whereas, STAT3 and STAT5 promotes cell cycle progression and prevents apoptosis (Bromberg and Darnell, 2000).

Unlike other STAT proteins, STAT3 can be activated by different cytokines, growth factors and other stimuli. In addition, systemic deletion of STAT3 leads to embryonic lethality suggesting that STAT3 might represent the earliest STAT family proteins (Levy and Lee, 2002). Although STAT3 is important in early embryonic development, when STAT3 is silenced in different adult tissues, there is generally little or

no change in phenotypes. STAT3 can act as a transcriptional activator, as a transcriptional repressor, or as a signaling mediator depending on the target tissue.

STAT3 also plays a key role in human oncogenesis and chronic inflammatory disease (Yu *et al.*, 2007) . Recently, there has been a report that $STAT3^{IEC}$ activation leads to increase colitis-associated cancer (CAC) through IL-6 pro-tumorigenic effect (Grivennikov *et al.*, 2009). Moreover, the deletion of STAT3 in the bone marrow cells leads to IBD associated phenotype due to the lost of immune tolerance. This indicates that STAT3 normally regulates innate immune activation towards microbial antigens (Welte *et al.*, 2003). STAT3 polymorphism has been associated with UC and CD susceptibility genes (Barrett *et al.*, 2008). Yet, the role of $STAT3^{IEC}$ in colonic inflammation has not been examined. There still needs a tremendous effort in investigating the complex role of STAT3 in different tissues and piecing all the puzzles together (Levy and Lee, 2002).

1.11. Inflammatory Bowel Disease

Inflammatory Bowel Disease (IBD) mainly consists of Crohn's disease (CD) and ulcerative colitis (UC). IBDs can be caused by many reasons, genetic predisposition (mutations in NOD2, IL23R, STAT3, and AGT16L), life style, and environmental factors. IBD patients suffer from chronic inflammation and relapses. Although the pathology of both CD and UC are similar, their treatments are completely different. Crohn's disease usually develops from inflammation in the terminal ileum in the early stage and later on the mucosal lesions appears to spread over Peyer's patches. The inflammation is caused by nuetrolphil infiltration from the surface epithelial cells all the

way down to submucosal layer of the intestines creating granulomas. Crohn's disease occurs as patchy and segmental in the human gut. Unlike CD, ulcerative colitis is characterized by extensive superficial mucosal ulceration. The mucosal inflammation extends proximally from the rectum to a varying degree (Xavier and Podolsky, 2007). The number of goblet cells decreased, thus leads to decrease in mucin production in CD patients. Decrease in mucus production leads to diluted antimicrobial secretion and consequently leads to increase adherent bacteria on the surface of intestine.

1.12. Hypothesis

The gut microflora seems to be important components for the onset of the widespread IBD disease. The deletion of STAT3 in the bone marrow cells leads to abnormal myeloid cell (neutrophile and macrophage) development and gives rise to Crohn disease-like pathology (Yu *et al.*, 2007). STAT3 deficiency thus leads to the development of enterocolitis due to over production of IL-12p40. Although STAT3 polymorphism has been found in patients with UC and CD, the role of $STAT3^{IEC}$ in colonic inflammation has not been studied. Therefore, our goal is to identify the role of $STAT^{IEC}$ in the maintenance of intestinal homeostasis, which may fill in the piece of the puzzle of the role of STAT3 polymorphism in IBD patients.

Introduction, in part, has been submitted for publication. Jongdae Lee is the primary investigator. Ji-Hun Mo, Adam N. Rucker, Shee-Eun Lee, Jose M. Gonalez-Navajas, Devorah Friedberg, Scott Herdman, Declan F McCole, Yolanda Aderson, Kim E. Barrett, Lars Eckmann, and Eyal Raz are the co-authors of this paper.

2. Methods

2.1. Reagents and Antibodies

FITC-sulfonic acid was purchased from Invitrogen (Carlsbad, CA) and FITC-dextran 10K from Sigma (St. Louis, MO). Anti-IFNa and IFNb are from R&D Systems (Minneapolis, MN). Anti-phospho-STAT1, antiphospho-STAT3, anti-STAT1, anti-STAT3, b-catenin, GSK3b, SNAI, E-cadherin antibodies were obtained from Cell Signaling Technologies (Danvers, MA). Antib-actin, and anti-a-tubulin, anti-smooth muscle actin came from Sigma (St. Louis, MO). Anti-ubiquitin antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-CFTR and anti-IL-23p19 antibodies were obtained from Abcam (Cambridge, MA). The following antibodies were purchased from Invitrogen (Carlsbad, CA): anti-ZO-1, anti-occludin, anti-JAM-A and anti-IRFs. All the claudin antibodies were obtained from Lab Vision (Fremont, CA). Anti-*Citrobacter* antibodies were the generous gift of Gad Frankel (Imperial College, London, England), and anti-mouse RegIIIg antibodies and the RegIIIg expression construct were generously given by Lora Hooper (University of Texas, Southwestern Medical Center). The following antibodies are from eBiosciences (San Diego, CA): anti-CD4, anti-CD11b, anti-IL-17A and anti-CD11c. Anti-IL-12p40, anti-IL-6, anti-CD3, anti-CD28 and anti-IL-10 antibodies were obtained from BD Biosciences (San Jose, CA).

2.2. Cell Culture

All the IEC lines are of human origin. IEC are cultured in DMEM high-glucose (1 g/L) supplemented with 10% heat-inactivated FCS, 2 mmol/L Image -glutamine, and 10 μg/mL human apo-transferrin (Sigma Chemical Co., St. Louis, MO). HCA-7 cells were cultured in low glucose DMEM (Mediatech, Manassas, VA). HCA-7 cells are grown on microporous filter inserts (0.4 μl pore size, Transwell; Costar, Cambridge MA) to form a polarized epithelial cell monolayer. Unpolarized cells were also plated on Transwell plates but used when TER reached at least 300 Ωcm2 measured with Millicell-ERS Resistance System (Millipor, Billerica, MA).

2.3. RT-PCR, Confocal Imaging, Immunoblotting and Immunoprecipitation

The procedures were performed as described (Lee *et al.*, 2006). Unless otherwise mentioned, nuclei were stained with Hoechst 33258 and visualized in the blue channel (Invitrogen), and the specified antigens were visualized with an appropriate secondary antibody labeled with Alexa Fluor 488 (Invitrogen) in the green channel or with an appropriate secondary antibody labeled with Alexa Fluor 546 in the red channel. F-actin was visualized using phalloidin-Alexa Fluor 546 (Invitrogen) in the red channel.

2.4. Cell Proliferation and Apoptosis

BrdU incorporation (proliferation) in HCA-7 cells and in paraffinized colonic tissues, as well as the TUNEL assay in paraffinized colonic tissues were performed and analyzed according to the manufacturer's instructions (BD Biosciences, San Diego, CA). Apoptosis in HCA-7 cells was determined using the Vybrant assay kit according to the manufacturer's instruction (Invitrogen).

2.5. Permeability Test

FITC-sulfonic acid was used in permeability test as described (Resta-Lenert and Barrett, 2006), using a small molecular weight tracer, FITC-sulfonic acid (**FS,** MW 478, 200μg/ml) with a minor modification. FS (100μg/ml) was applied to apical chambers and, after 5 hrs of the incubation; the level of FS in the basolateral chambers was measured with a fluorometer (485nm excitation and 530nmemission wavelengths). Monolayer permeability is expressed as μg/ml/hr. FS is unable to permeate the cellular membrane at physiologic pH due to its lipophobicity and, therefore, must pass through the TJ space.

2.6. siRNA-mediated Knockdown in IEC

The procedure was performed using Dharmafect 4 according to the manufacturer's instruction (Dharmacon, Chicago, IL) or with Nucleofector (Amaxa, Germany). Non-targeting siRNA #2 (luciferase targeting siRNA) from Dharmacon was used as a control. STAT3 siRNA sequence: 5'- CAACATGTCATTTGCTGAA-3', SNAI

siRNA (Applied Biosystems, Cat. No.s13186), and GSK3b siRNA: 5'-TCCGAGGAGAACCCAATGTTTCGTATA-3'were used. Briefly, siRNA (5 µl of 20 µM solution) in 50 µl of Opti-MEM (Invitrogen) was mixed with 5 µl of Dharmafect 4 in 50 µl of Opti-MEM. After 30min incubation, the transfection mix was combined with 1X106 cells in culture medium (trypsinized before the transfection) and then plated in one well of a 12-well Transwell plate. After 6 hrs, the medium from the apical chamber was replaced with fresh medium and the cells were then left untouched for 3 days. TER was recorded daily and the medium was replaced every 48 hrs. For transfection with Nucleofector, V solution was used with the method S-030.

2.7. Generation of STAT3 IEC KO Mice

In order to generate STAT3 deficiency specifically in IECs in mice, STAT3flox/flox mice (a gift from Shizou Akira) were mated to villin-Cre mice, which are on a B57BL/6 background (Jackson Laboratory, Bar Harbor, ME). Genotyping was performed as described (Kobayashi *et al.*, 2003).

2.8. Purification of CD4+ T cells from Lamina Propria and Spleen

The procedures were performed as described (Mucida *et al.*, 2007).

2.9. Eradication of Commensal Bacteria by Antibiotic Treatment

Eradication of Commensal bacteria in STAT3IEC KO mice using a combination of the following antibiotics (Sigma) listed in the table below.

Vancomycin Hydrochloride 0.5g/L

Neomycin Hydrochloride 1g/L

Metronidozole 1g/L

Ampicillin Sodium Salt 1g/L

The antibiotics were given in the drinking water and, in addition, 200 µl of the solution was gavaged every day for one or two weeks. The eradication of colonic fecal bacteria was confirmed by CFU assay in BHI (Brain Heart Infusion) agar. Anaerobic bacteria were cultured in anaerobic chambers (BD BBL™ GasPak™, BD Biosciences).

2.10. EPEC Culture, Infection and Cell Counting

The procedures were performed as described elsewhere (Hodges *et al.*, 2006).

2.11. Citrobacter rodentium and Oral Infection

The procedures were performed as we have previously described 34. Briefly, *C. rodentium* was grown overnight in LB broth at 37°C, harvested by centrifugation, and resuspended in fresh PBS at a concentration of 2.5x109/ml. Mice (6-10 week old) were infected orally with 200 µl of the bacterial suspension ($5x10^8$ bacteria).

2.12. Preparing of recombinant Mouse RegIIIg

The procedure was previously described in detail (Cash *et al.*, 2006b).

2.13. Treatment of KO mice with recombinant RegIIIg

The procedure was performed after the KO mice were infected with *C. rodentium* as described above. The mice were then gavaged with 150µg of RegIIIg in 100 µl of buffer (25 mM MES, 50 mM NaCl, 2 mM CaCl2, pH 6) on days 1, 3, and 5. The control group was administered buffer alone.

2.14. Statistical Analysis

Student's t-Test was used in statistical analysis for paired samples.

2.15. PCR primers

Table 2.15.1: PCR Primers

Gene names	**Forward Primer**	**Reverse Primer**
hGAPDH	5'-CAT GTT CGT CAT GGG TGT GAA CCA -3'	5'-AGT GAT GGC ATG GAC TGT GGT CAT-3'
mGAPDH	5'-TGT GAT GGG TGT GAA CCA CGA GAA-3'	5'-GAG CCC TTC CAC AAT GCC AAA GTT-3'
mIL-6	5'-GAC TGA TGC TGG TGA CAA CC-3'	5'-GCT CCG ACT TGT GAA GTG G-3'

Table 2.15.1: PCR Primers Continued

Gene names	**Forward Primer**	**Reverse Primer**
mIL-12p40	5'-AAA CCA GAC CCG CCC AAG AAC-3'	5'-AAA AAG GCA ACC AAG CAG AAG ACA G-3'
mIL-23p19	5'-TCC GTT CCA AGA TCC TTC G-3'	5'-GAA CCT GGG CAT CCT TAA GC-3'
mIL-17f	5'- AGC AAG AAA TCC TGG TCC TTC GGA -3'	5'- CTT GAC ACA GGT GCA GCC AAC TTT -3'
mIL-22	5'- ACT TCC AGC AGC CAT ACA TCG TCA-3'	5'- AGC ACT GAC TCC TCG GAA CAG TTT-3'
hSNAI	5'- TAC AGC GAG CTG CAG GAC TCT AAT-3'	5'- AGG ACA GAG TCC CAG ATG AGC ATT-3'
hGSK3b	5'- TGG TCC GAG GAG AAC CCA ATG TTT-3'	5'- ACA CAG CCA GCA GAC CAT ACA TCT-3'
hRegIIIg	5'- ATG GGA GTG GAG TAG CAC TGA TGT-3'	5'- TGC CCT AGT CCT TGA ACT TGC AGA3'
mRegIIIg	5'- ACA CTG GGC TAT GAA CCC AAC AGA-3'	5'- ACC ACA GTG ATT GCC TGA GGA AGA-3'
mRegIIIb	5'- AAG ACA GAC AAG ATG CTG CCT CCA-3'	5'- ACA AGG CAT AGC AGT AGG AGC CAT-3'

Table 2.15.1: PCR Primers Continued

Gene names	**Forward Primer**	**Reverse Primer**
mTNFa	5'-CAT ATG TCA TCT TCT CAA AAT TCG AGT GAC-3'	5'-GGA TCC TCA CAG AGC AAT GAC TCC AAA GTA-3'
mIL-1b	5' GAA GAA GAG CCC ATC CTC TG-3'	5'-TCA TCT CGG AGC CTG TAG TG-3'
hIRF-1	5'-TCA TGA AGC TCT TGG AGC AG-3'	5'-TCA ATT TCT GGC TCC TCC TT-3'
hBcl-2	5'-TCA GCA ATA ATG GGA ACG GG-3'	5'-TCA TCA AAA GGT GGT GG-3'
hSTAT-1	5'-CTT ACC CAG AAT GCC CTG AT-3'	5'-CGA ACT TGC TGC AGA CTC TC-3'

Materials and methods, in full, is a reprint of the material, as it appears in submitted for publication. Jongdae Lee is the primary investigator. Ji-Hun Mo, Adam N. Rucker, Shee-Eun Lee, Jose M. Gonalez-Navajas, Devorah Friedberg, Scott Herdman, Declan F McCole, Yolanda Aderson, Kim E. Barrett, Lars Eckmann, and Eyal Raz are the co-authors of this paper.

3. Results

3.1. Type I IFN enhances IEC barrier function

Type I interferon (IFN-1) prevents colonic epithelial cell damage induced by dextran sulfate sodium (DSS) (Katakura *et al.*, 2005; Abe *et al.*, 2007). Therefore, we hypothesized IFNa is involved in the maintenance of epithelial barrier functions. To investigate the effect of IFNa in IEC barrier function, human intestinal epithelial cell line, HCA-7, was cultured on microporous filter transwells and stimulated with or without IFNa (10 ng/ml) for 24 hours. Transepithelial electrical resistance (TER) of HCA-7 cells was measured on day 0 and day 3 (n=6) (Figure 3.1.1A). Permeability assay was performed as mentioned in Material and Methods section with FITC-sulfonic acid (100µg/ml) (n=6) (Figure 3.1.1B). Increase in TER and the decline in permeability in HCA-7 cells were observed suggesting that IFNa enhanced IEC polarity and barrier function.

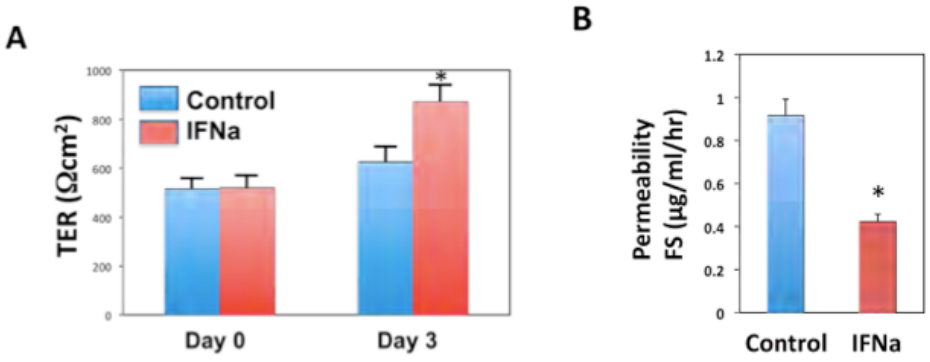

Figure 3.1.1: IFNa enhances barrier function of IECs.

We further examined the phenotypic differences of junction proteins between polarized and unpolarized HCA-7 cells through immunohistochemistry (IHC) ad confocal imaging (Figure 3.1.2). Proteins were stained with FITC and nuclei were stained with Hoechst. The upper and bottom images of each protein represent a XY section and a XZ section, respectively. Both tight junction proteins (ZO-1, Occludin, and CLD3) and adherence junction proteins (E-cadherin) were disorganized when IEC polarity is disrupted. In polarized conditions, these proteins locate laterally. The delocalization of the TJ and AJ proteins results in unpolarized IEC structural changes.

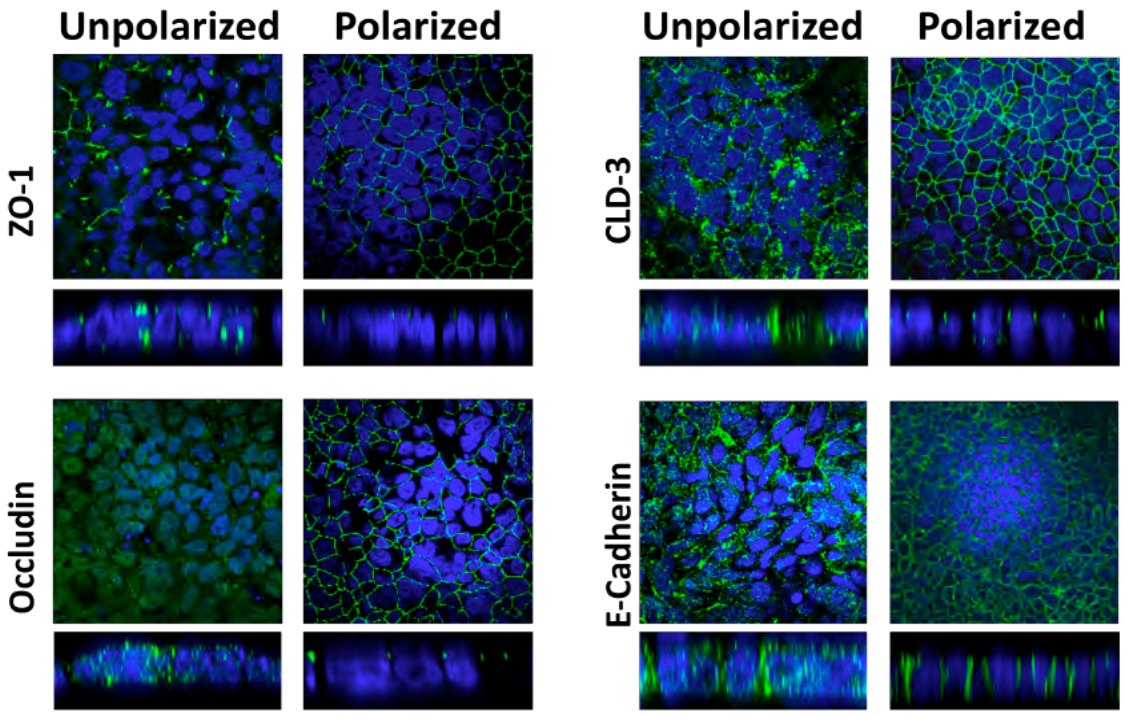

Figure 3.1.2: Characterizatoin of IEC polarity by confocal imaging

3.2. STAT3 is major transducer of IFN-I in polarized IECs

Previous investigations show that co-stimulation with IFNa and IL-21 can enhance STAT3 activation in healthy CD8+ and NK cells and inhibits tumor growth (Eriksen *et al.*, 2009). Thus, we wanted to see if there is a correlation between $STAT3^{IEC}$ and type I IFN in maintaining intestinal epithelium integrity. Polarized and unpolarized HCA-7 cells were stimulated with IFNa (10ng/ml) as indicated and total cell lysate was used for immunoblotting (IB) pSTAT1 and pSTAT3 (Figure 3.2.1A). Both STAT1 and STAT3 were activated in unpolarized HCA-7 cells, whereas, only STAT3 were activated in polarized cells under IFNa stimulation. Furthermore, polarized and unpolarized HCA-7 cells were stimulated with IFNa (10ng/ml) for 1 hr and gene inductions were measured by RT-PCR (Figure 3.2.1B). In unpolarized IECs, IRF-1 and STAT-1 have 12 and 40 fold induction respectively, while in polarized IECs, SLIM and BCL-2 have about 30 fold inductions upon IFNa stimulation. The results suggest that IFNa signaling activates different sets of genes between polarized and unpolarized IECs. In particular, STAT1 expression was abolished upon IFNa stimulation in polarized IECs suggesting proteosomal degradation is involved in this process.

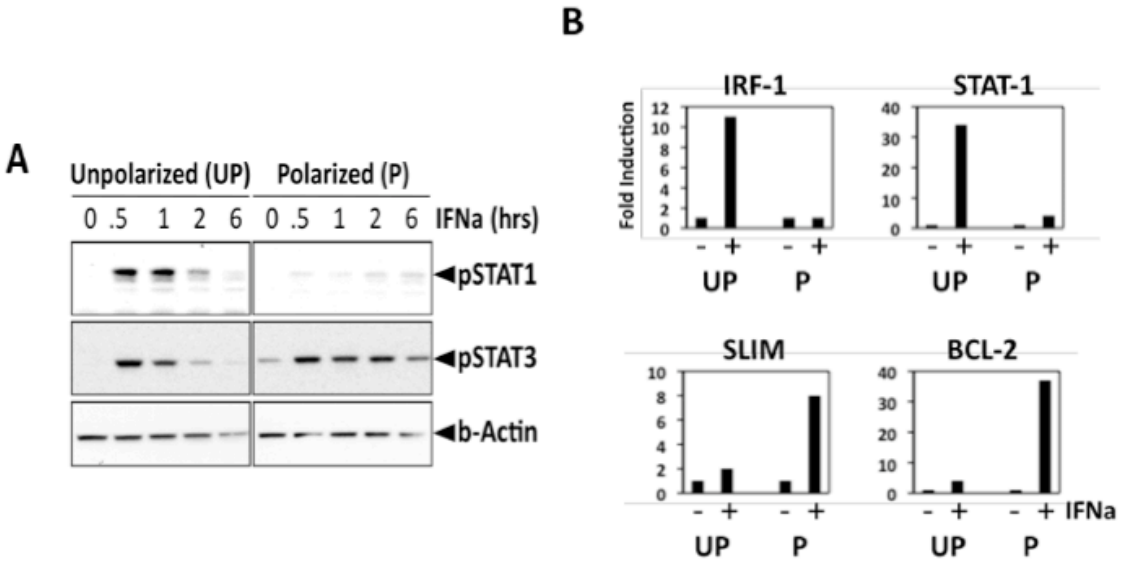

Figure 3.2.1: STAT3 mediates IFNa signaling in polarized IECs.

To test whether SLIM, an E3 ubiquitin ligase, mediates proteosomal degradation of STAT1, polarized HCA-7 cells were stimulated with IFNa in the absence or presence of protease inhibitor, MG132 (10μM) (Figure 3.2.2). Activation of STAT1 and STAT3 was measured by IB and ubiquination of STAT1 was measured by immunoprecipitation (IP) of STAT1 followed by IB with anti-ubiquitin (Ub) antibody. The addition of MG132 restored the STAT-1 activation and prevented ubiquitinated STAT1 from SLIM mediated proteosomal degradation upon IFNa stimulation. While in steady-state, polarized IECs expresses STAT3 upon IFNa stimulation and unlike STAT1, STAT3 is not subject to proteosomal degradation.

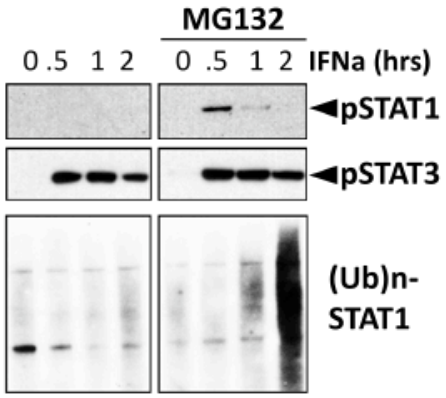

Figure 3.2.2: STAT1 is ubiquitinated in polarized IECs upon IFNa stimualtion

To confirm whether IFNa signaling through STAT proteins is different in polarized and upolarized cells, another human IEC line, T84 cells, was stimulated with IFNa (10ng/ml) and investigated for STAT proteins activation through IB (Figure 3.2.3A). The results were similar to experiments with HCA-7 cells. Upon IFNa stimulation, polarized T84 cells only activated STAT3, while IFNa treatment of unpolarized T84 cells activated both STAT1 and STAT3. When polarized T84 cells were treated with MG132, STAT1 was also subjected to ubiquitin-mediated proteolysis in polarized IECs upon IFNa treatment (Figure 3.2.3 B).

Next, we looked into the activation of IRFs (interferon regulatory factors) 1, 3, 7, and 9 that were involved in IFN-I induction in polarized and unpolarized HCA-7 cells (Figure 3.2.3 C). Nuclear lysates of polarized and unpolarized IECs in treatment of IFNa was collected and IB for activation of IRFs (nuclear translocation). The results showed that IFNa stimulation of HCA-7 cells resulted in nuclear translocation of IRFs in unpolarized cells but not in polarized cells. The results suggest IFN-I signaling is dependent on IEC polarity.

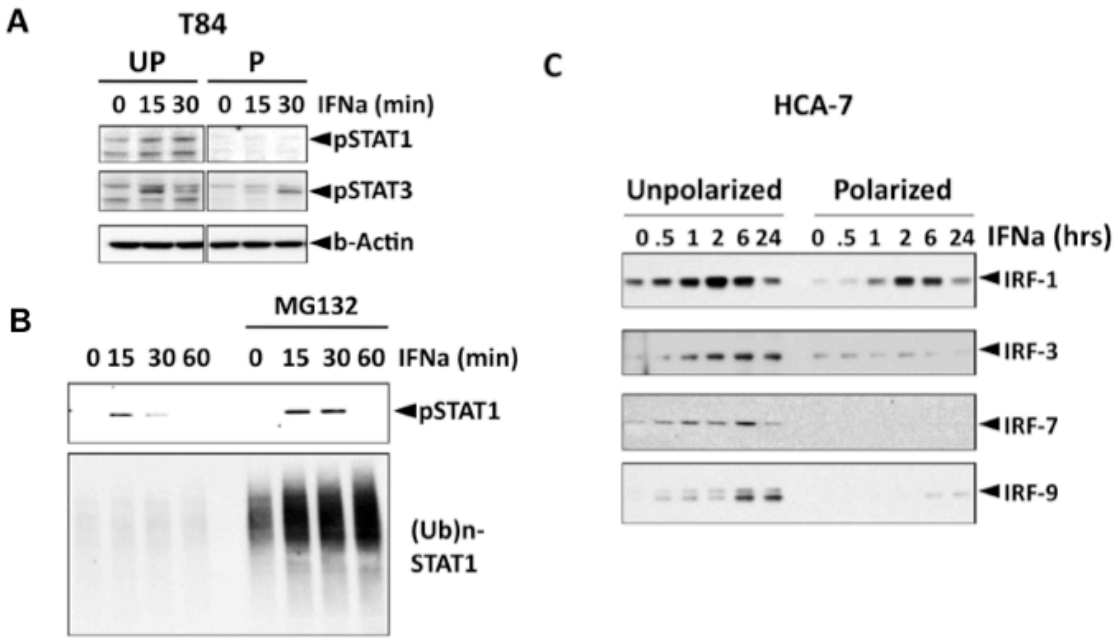

Figure 3.2.3: Type I IFN signaling is polarity dependent in IECs.

3.3. Type I IFN expression in myofibroblasts and CD11b^{+}cells in the colon

To identify the sources of IFN-1 that affects intestinal barrier function, IFNa and IFNb were located through IHC in frozen wild-type B6 colon cross sections followed by confocal imaging (Figure 3.3.1). IFNa co-localized with myofibroblast cellular makers, vimentin and smooth muscle actin (SMA), indicating IFNa was secreted mostly from myofibroblasts. IFNb was not detectible in this experiment (data not shown). IFNa was also found in CD11b^{+} macrophages in the colon.

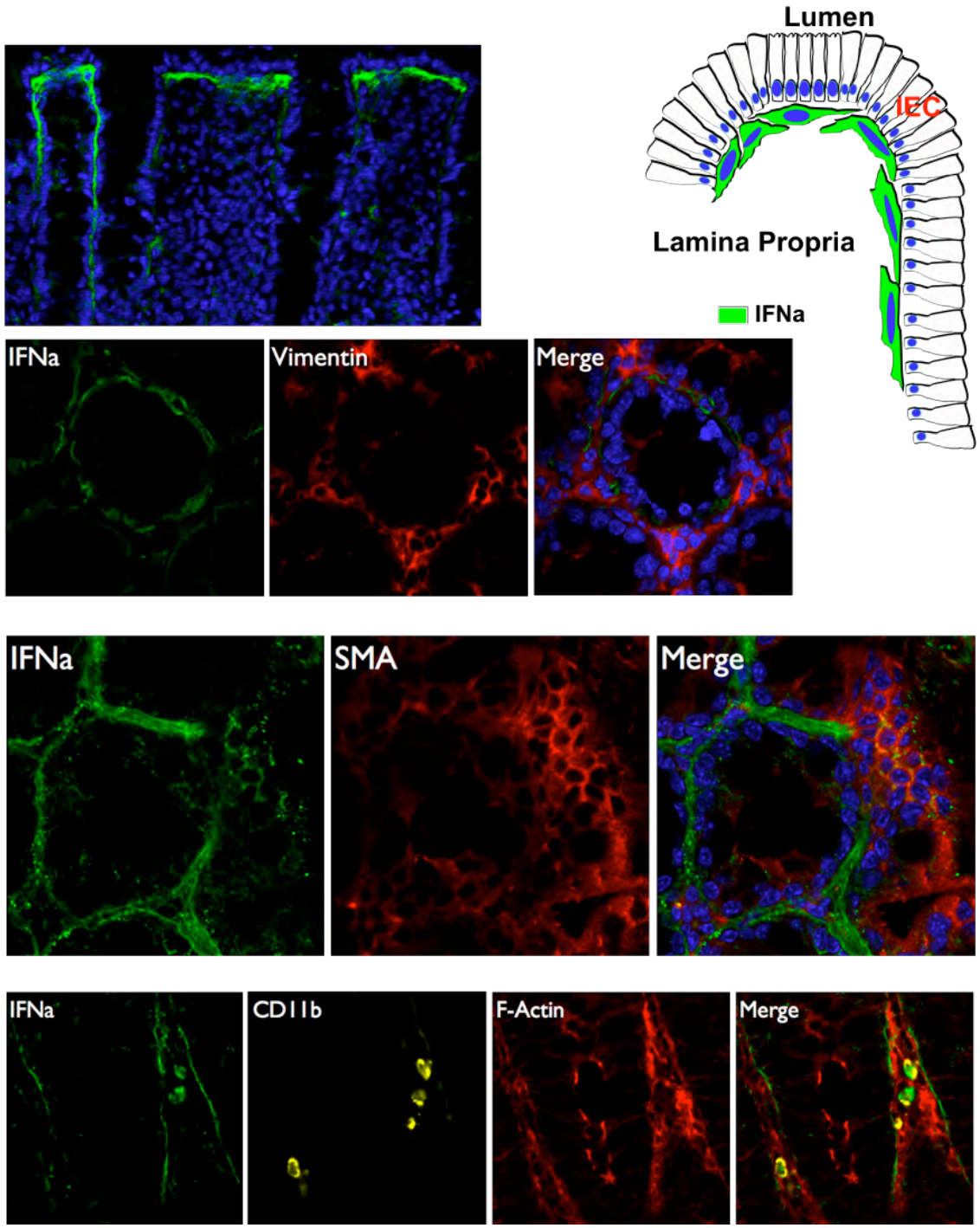

Figure 3.3.1: IFNa is strongly expressed in myofibroblasts and occasionally in CD11b^{+} macrophages

3.4. STAT3 regulates barrier functions in vitro and in vivo

The data presented above suggest that STAT3 is important in transducing IFNa signals in polarized intestinal epithelial cells. One of the major functions of polarized intestinal epithelium is to provide barrier functions through tight junction protein formations. These proteins are usually located at the apical side of the IECs to prevent intestinal epithelial leakage which increases the chances of possible bacterial infection. Previously, we have shown IFNa increased IEC polarity and tight junction proteins maintained IEC polarization. Therefore, we investigated the role of STAT3 in transducing IFNa signals in polarized epithelial cells *in vitro* and *in vivo.*

STAT3 was knocked down in HCA-7 by siRNA. STAT3 protein level was then measured 2 days post-transfection by IB (Figure 3.2.1A). HCA-7 cells transfected with control siRNA or STAT3 siRNA were cultured in a transwell plate and TER was measure daily after transfection (n=6). STAT3 knocked-down HCA-7 cells were unable to generate an intact IEC monolayer as indicated by the decrease in TEAR and increase in paracellular permeability compared to wild type HCA-7 cells(* represents P value < 0.05) (Figure 3.2.1B and C).

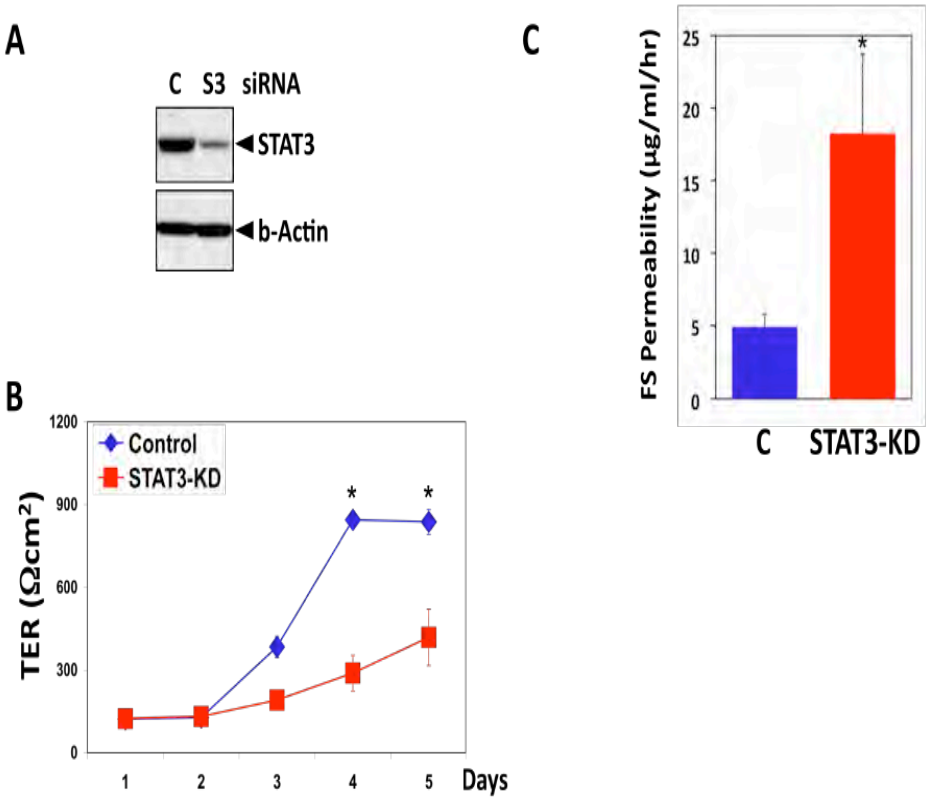

Figure 3.4.1: STAT3 is important for barrier function *in vitro*

Tight junction proteins are essential for the formation of tight monolayer epithelium that strengthens IEC barrier functions. Previous research shows upon cell to cell contact, a distinctive sets of genes involved in tight junction protein expression are activated; and the expression is peaked when IEC is polarized (Halbleib *et al.*, 2007).

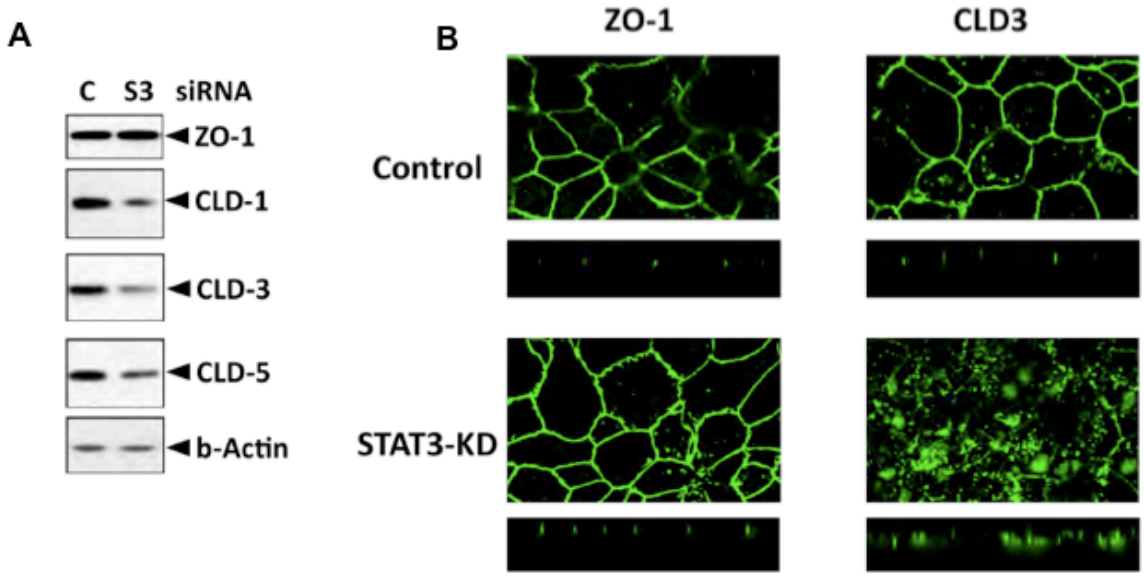

Figure 3.4.2: STAT3 maintains TJ expressions and localization *in vitro*

To determine whether $STAT3^{IEC}$ regulates TJ proteins, siRNA-mediated STAT3 KD was used to study tight junction protein levels by IB in HCA-7 cells 2 day post transfection (Figure 3.4.2A). $STAT3^{IEC}$ KD exhibited decreased tight junction protein levels (i.e. CLD-1, CLD-3, and CLD-5), whereas, intracellular scaffold protein (i.e. ZO-1) level did not change compared to control. Furthermore, IHC staining was done on the HCA-7 monolayer on the transwell plate to study the localization of tight junction proteins ZO-1 and CLD-3 by confocal imaging. The upper image for each protein is an XY plane and the bottom is an XZ plane (Figure 3.4.2.B). Confocal imaging revealed that CLD-3 failed to localize to apical tight junctions, while ZO-1 localization was not affected by low STAT3 cellular levels

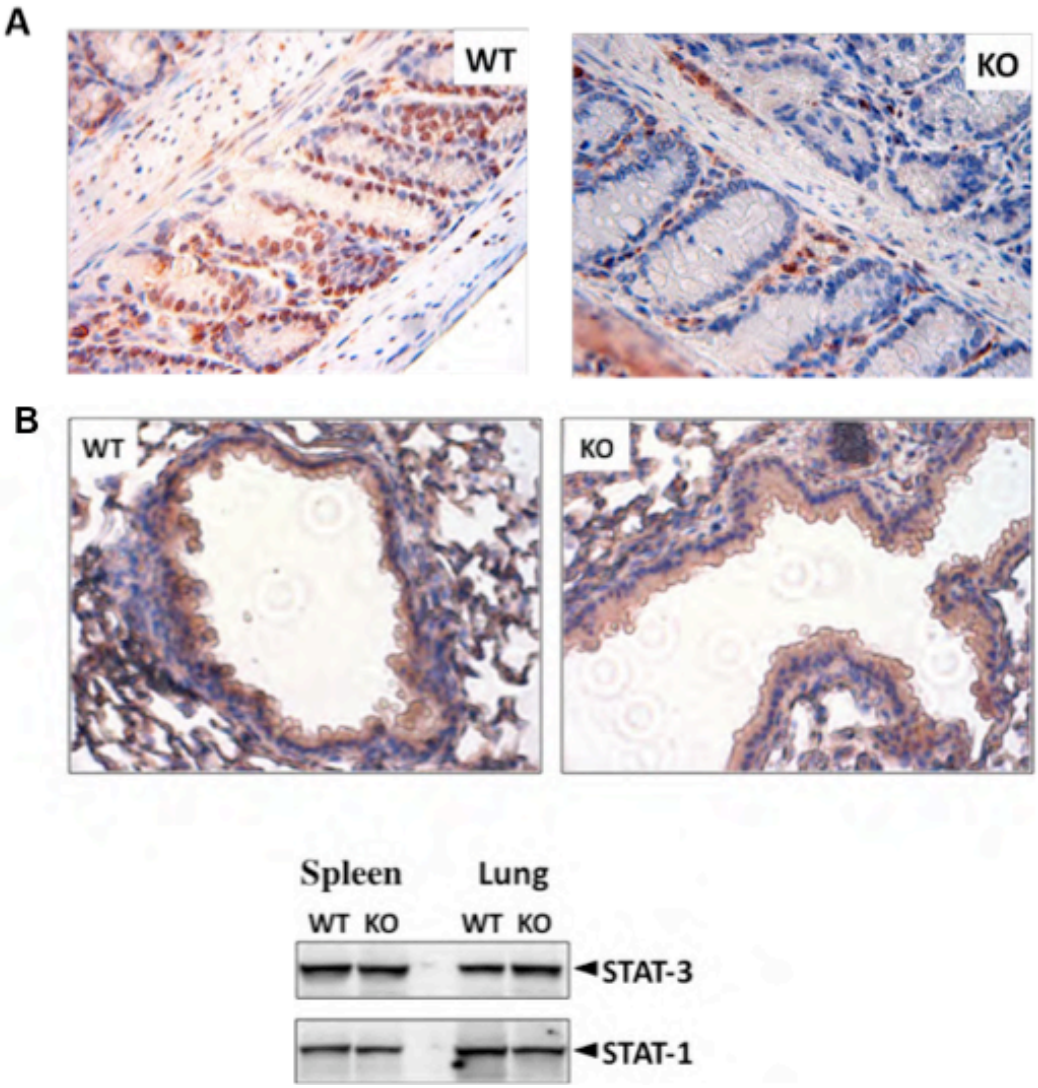

Figure 3.4.3: STAT3 deletion occurs specifically in the IECs.

To investigate the role of $STAT3^{IEC}$ *in vivo*, we generated mouse with STAT3 deletion specifically in the IEC ($STAT3^{IEC}$ KO) using *Cre-lox* system. LoxP transgenic mouse which have LoxP sequence inserted on the either side of the target gene (i.e. STAT3) was crossed with Cre transgenic mouse which carries tissue specific promoter (i.e. villin) that transcribes Cre recombinase. The double transgenic mouse was therefore created. Cre recombinase expressed only in specific tissue could then flank out the target

genes in between the LoxP sites resulting to gene deletion in that particular tissue (i.e. $STAT3^{IEC}$ KO) in mouse (Orban *et al.*, 1992). IHC and IB were used to identify STAT3 expression level in the colon, spleen, and lung tissues (Figure 3.4.3). STAT3 staining (brown) in the KO colonic tissues was absent in IECs, but is confined to mononuclear cells in the lamina propria underneath the IECs (Figure 3.4.3A). STAT3 was also expressed normally in the lung tissues (the upper panel, IHC), and both STAT1 and STAT3 levels were indifferent in WT and KO lungs and spleens (lower panel, IB) (Figure 3.4.3B) In addition, in agreement with our *in vitro* data, purified STAT3 ^{IEC} KO IECs also showed downregulation of CLD-1, -3, and -5 levels compared to WT IECs in IB (Figure 3.4.4).

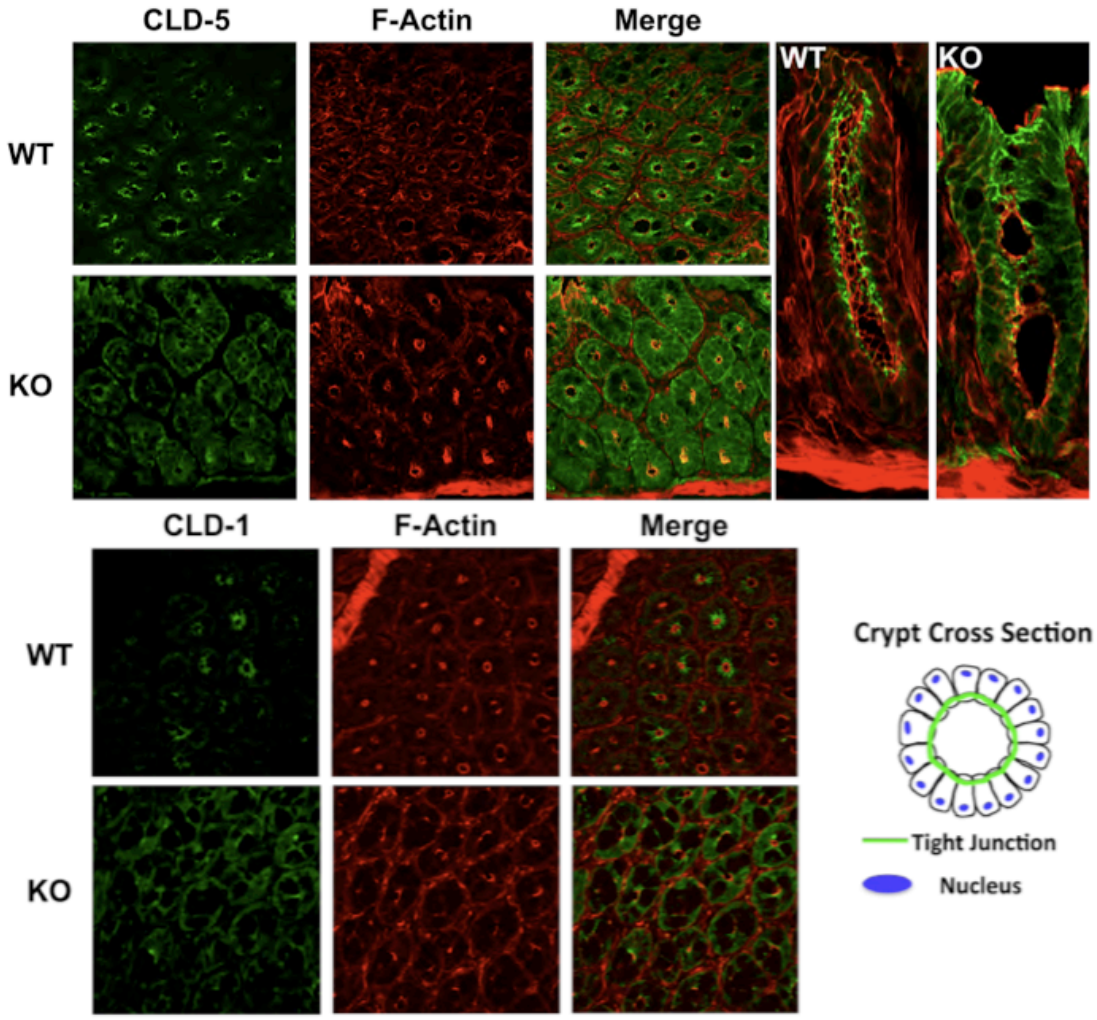

Figure 3.4.4: STAT3^{IEC} deletion distupts CLD-1 and -5 levels.

Normally, tight junctions are located in the apical side of the IECs to form a structurally uniform intestinal crypt. Confocal imaging of of CLD-1, -5 and CFTR (Cystic Fibrosis Transmembrane Conductance Regulator), an apical maker, show that they were not properly localized to the tight junction or apical membrane in the STAT3^{IEC} KO IECs (Figure 3.3.13.4.5 and Figure 3.4.6A), while ZO-1 localization was not affected (Figure 3.4.6B). Nuclei were stained in blue.

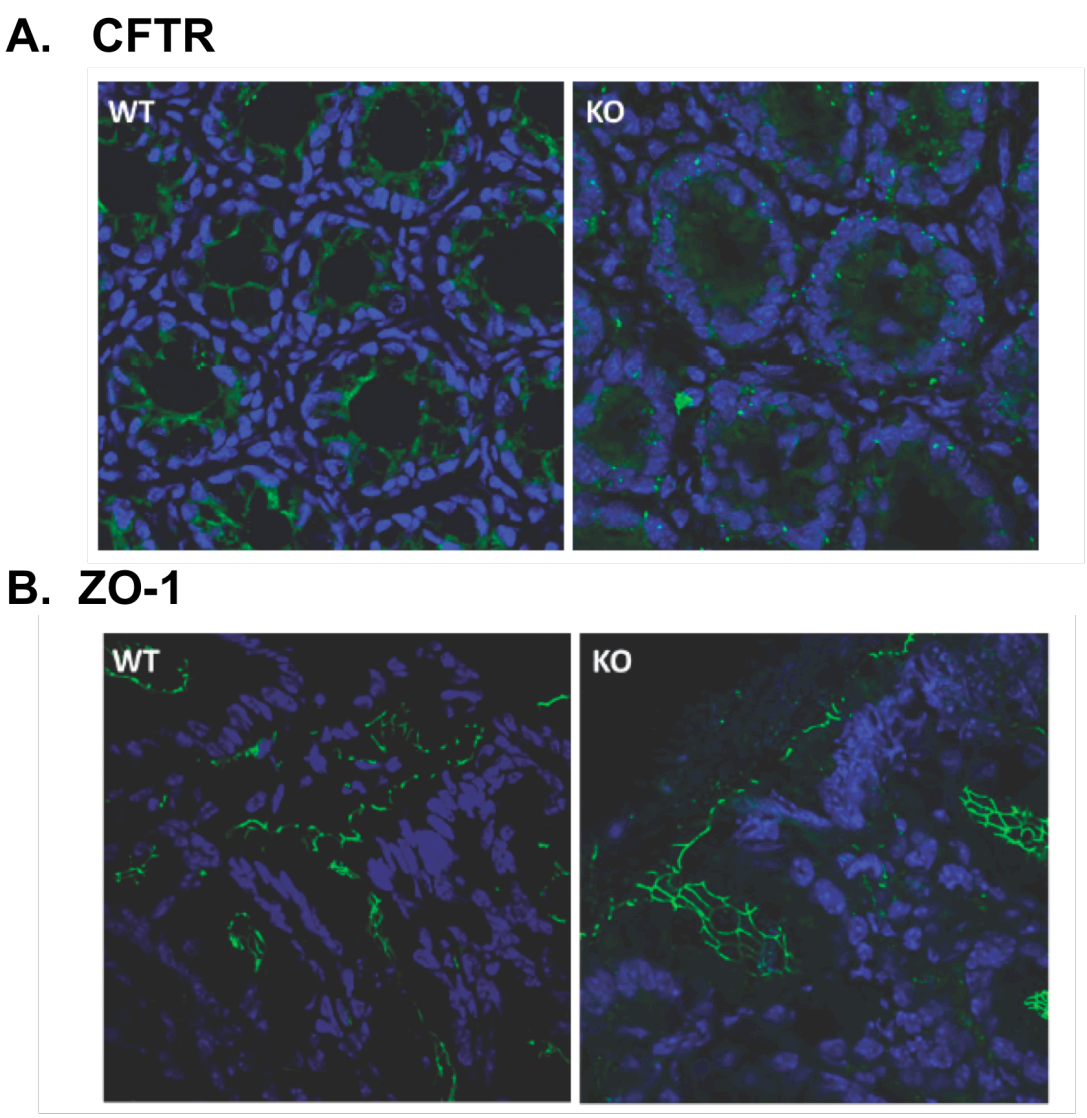

Figure 3.4.5: STAT3^{IEC} deletion disrupts IEC polarity.

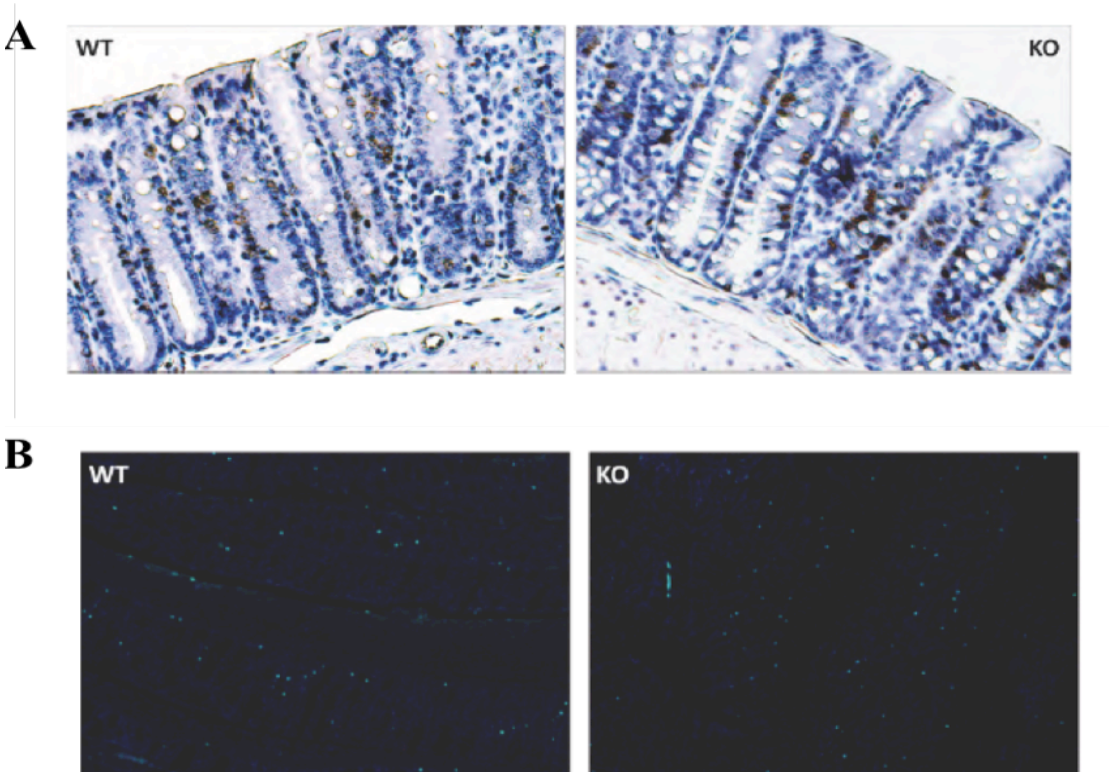

Figure 3.4.6: STAT3^{IEC} does not affect cell proliferation and apoptosis

In addition, STAT3 is known to mediate cell proliferation and apoptosis. Thus, IEC proliferation and apoptosis were measured. Both STAT3^{IEC} KO and WT B6 mice were sacrificed and the colons were paraffinized for staining 24 hours post injection with BrdU (2mg/mouse) using BrdU in situ staining kit (BD Biosciences) (Figure 3.4.6A). Nuclei were stained in blue and BrdU is stained in brown. TUNEL assay was performed on the paraffinized WT and KO colon tissues according to the manufacturer's instructions (BD Bioscience) (Figure 3.4.6B). Results reveal that STAT3^{IEC} KO did not affect IEC proliferation and cell death.

3.5. Barrier defect in STAT3^{IEC} KO mice activates innate immune response

Barrier leakage often results in the crossover of enteric bacteria into the lamina propria of the intestinal tissue triggering the onset of host immune response. We investigated whether compromising intestinal barrier due to STAT3^{IEC} deletion also resulted in the activation of immune response. The mRNA levels of indicated cytokines in the WT and KO colons were measured by RT-PCR (n=3) (Figure 3.5.1A). The transcript levels of pro-inflammatory cytokines (i.e. IL-6, IL-12p40, IL-23p19, and IL-17) in the colonic tissue were elevated.

Moreover, IL-17A induction level was measured in CD4+ T cells from WT and KO colons (n=3) upon co-stimulation of T cell receptors with anti-CD3 (2μg/ml) and anti-CD28 (1μg/ml) antibodies (Figure 3.5.1B). The supernatant was then collected and measured by ELISA (eBiosciences) after 24 hours. CD4+ T cells from KO colonic tissues and spleen expressed significantly higher IL17-A levels comparing to WT. These findings suggest that the induction of cytokines in STAT3^{IEC} KO mice maybe caused by microbial products reaching the lamina propria due to compromised epithelium barriers.

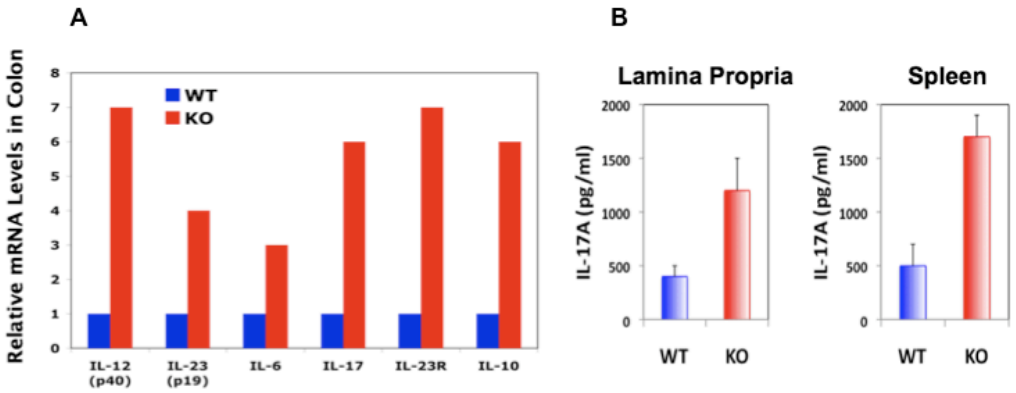

Figure 3.5.1: STAT3^{IEC} KO mice showed an elevated pro-inflammatory cytokine levels

To directly test whether cytokine induction in STAT3^{IEC} KO mice was caused by gut microbiota, KO mice were treated with antibiotics for one or two weeks to eradicate commensal bacteria and the levels of the indicated genes were measured by RT-PCR (Figure 3.5.2). Colony forming unit (CFU) count was performed to make sure the elimination of aerobic and anaerobic bacteria (data not shown). After two weeks of antibiotic treatment, the cytokine levels (i.e IL-1b, IL-10, IL-22 and TNF-a) in the KO colon were significantly decreased comparing to control KO colon. This suggests that commensal bacteria are indeed responsible for the induction of inflammatory cytokines in STAT3^{IEC} KO colons.

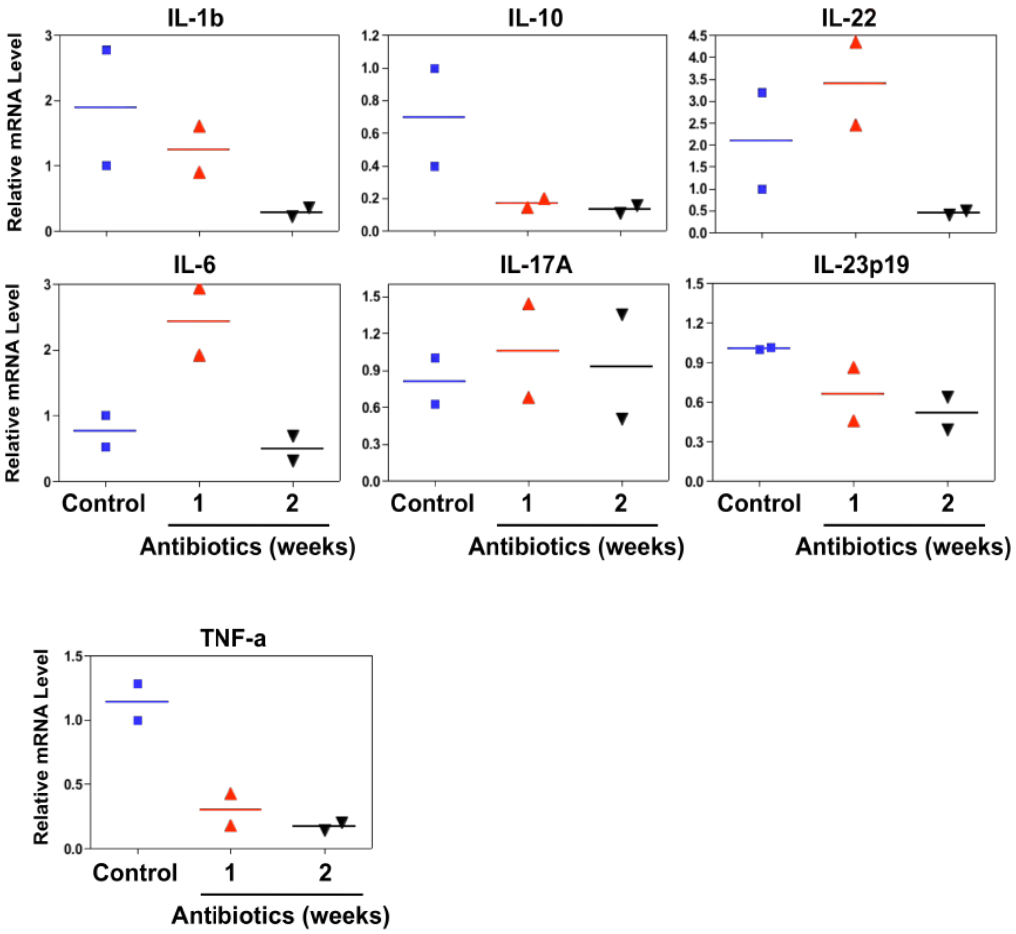

Figure 3.5.2: Commensal microbiota is reponssible for the onset of proinflammatory cytokine inductions in STAT3^{IEC} KO mice

3.6. STAT3^{IEC} is essential for host defense against enteropathogenic bacteria

Studies have suggested barrier defects often are observed in Crohn's disease patients and their first-degree relatives. Many speculate that leaky IEC barrier enables the bacterial antigen to cross over the epithelium layer and, thus triggering inflammation in the gut. The adherent-evasive *E.coli.* colonization of IECs has been observed in CD

patients (Darfeuille-Michaud *et al.*, 1998; Boudeau *et al.*, 1999). Therefore, we tested whether $STAT3^{IEC}$ is essential in preventing bacteria colonization in the gut. Enteropathogenic *E. coli* (EPEC) causes acute diarrhea by attaching and effacing (A/E) mechanisms that disrupts the polarity of human IECs (Gomes *et al.*, 1991). Based on our initial findings, EPEC preferentially targets tight junctions for attachment in HCA-7 cell. EPEC were located by confocal imaging and were concentrated on the tight junctions forming pedestal-like hollow tubes (indicated by the arrows) (Figure 3.6.1).

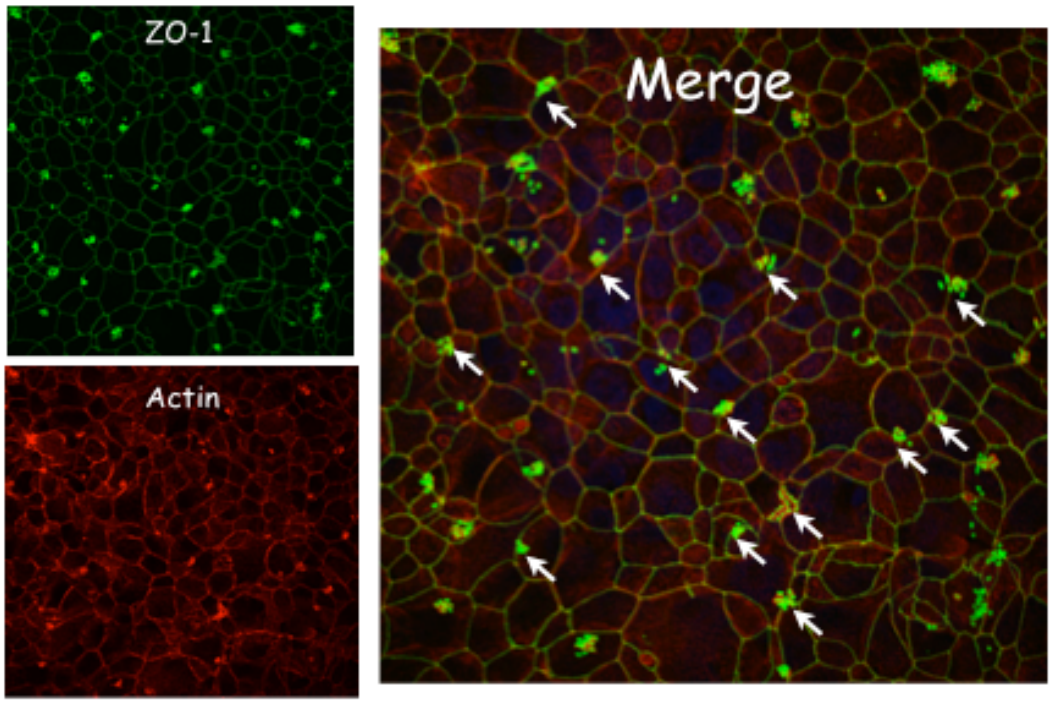

Figure 3.6.1: EPEC target tight junction in IECs.

We then hypothesized that $STAT3^{IEC}$ KO mice are more susceptible in enteropathogenic bacteria infections due to barrier defects. Both WT and $STAT3^{IEC}$ KO mice were infected with oral administration of Citrobacter rodentium, the murine counterpart of EPEC, and

monitored for mortality (n=10) (Figure 3.6.2). *C. rodentium* infection resulted in 80% mortality in KO mice, there were no death in WT mice.

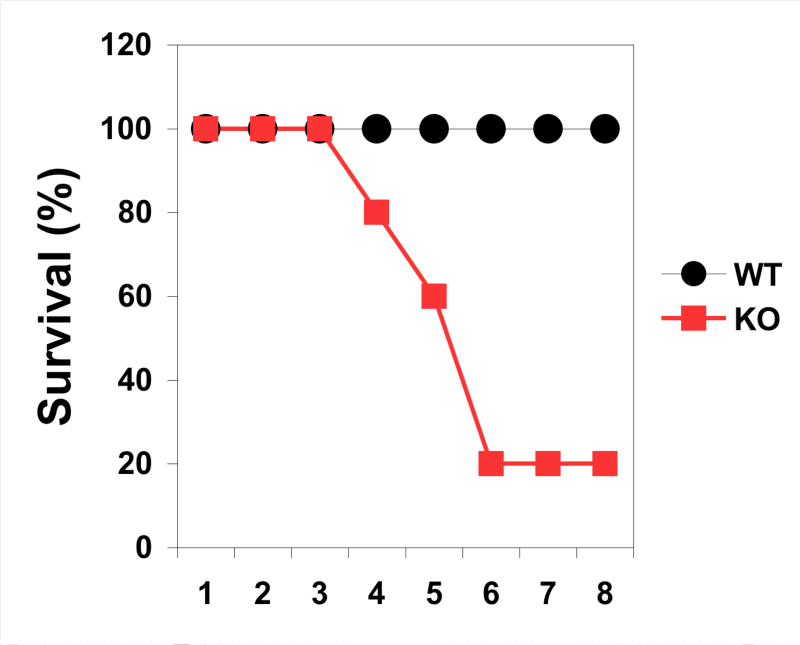

Figure 3.6.2: STAT3^{IEC} Protects mice against *C. rodentium* infection

The histological scores were measured in H&E staining of the colons from WT and KO mice infected with *C. rodentium* for four days (Figure 3.6.3A). KO colons exhibited severe inflammation and the destruction of the crypt architecture while neither inflammation nor crypt disruption was observed in the WT colons (Figure 3.6.3A). IHC followed by confocal imaging revealed massive colonization on IECs by *C. rodentium* in STAT3^{IEC} KO (Figure 3.6.3B). In contrast, little or no *C. rodentium* was detected in the WT IEC colons (Figure 3.6.3B). These data suggests that impaired IECs are more vulnerable to enteropathogenic infections. In addition, *in vitro*, unpolarized or polarized HCA-7 cells were infected with EPEC (5MOI, three hrs), and bacterial colonies were measured by confocal imaging and CFU counting (*$p<0.05$) (Figure 3.6.3C and D). As a

result, unpolarized IECs were more heavily colonized with EPEC in about 100 fold higher in CFU compared to polarized IECs.

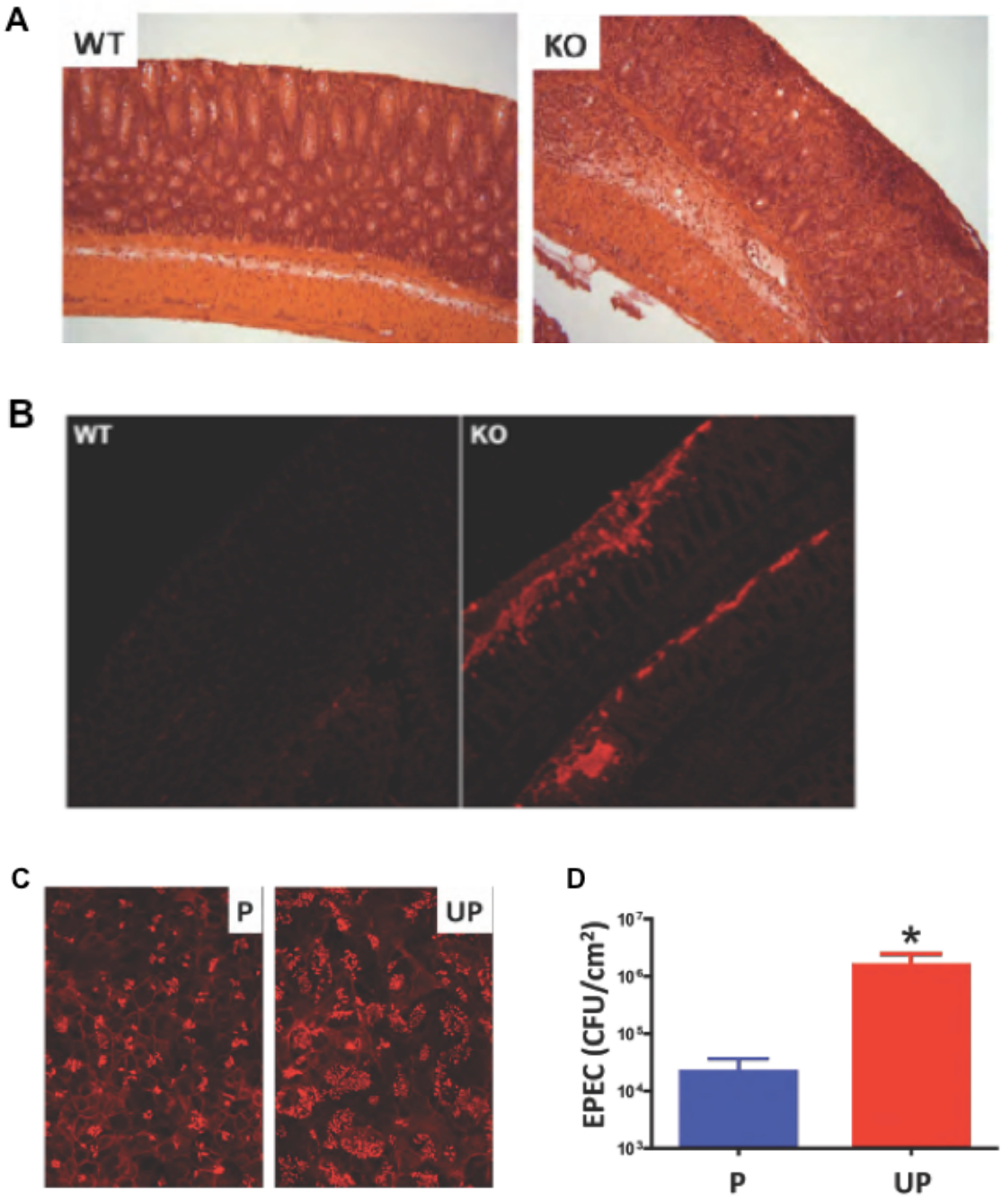

Figure 3.6.3: IEC polarity protects against bacterial infections.

3.7. STAT3^{IEC} is required for RegIIIg synthesis

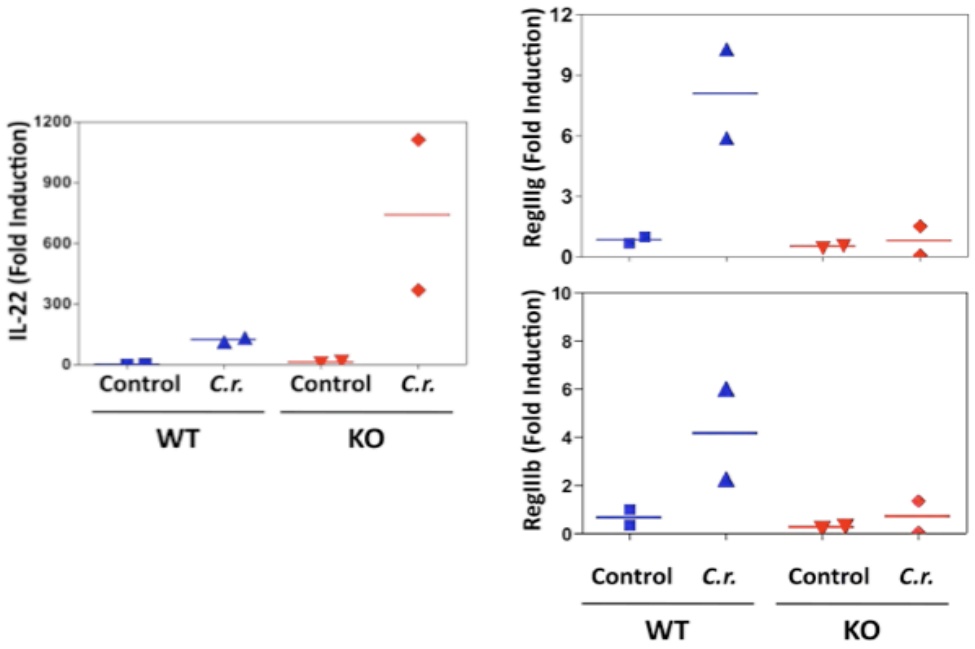

Figure 3.7.1: STAT3^{IEC} is required for antimicrobial peptide synthesis.

We next investigated why STAT3^{IEC} KO mice were more vulnerable to *C. rodentium* infection. IL-23 and IL-22 are essential for RegIIIg induction in order to obliterate *C. rodentium* colonization (Aujla *et al.*, 2008; Zheng *et al.*, 2008). Both cytokines are essential for STAT3 activation (Kolls *et al.*, 2008). Thus, we tested whether STAT3^{IEC} signaling was crucial in RegIIIg synthesis to clear out *C. rodentium* infection. To investigate whether IL-22 requires STAT3^{IEC} signaling for RegIIIg synthesis, WT and STAT3 KO mice were infected with or without *C. rodentium,* and IL-22 and RegIIIg and RegIIIb mRNA level were analyzed by RT-PCR 6 day post-infection (Firgure 3.7.1). To our amusement, there was a significantly higher IL-22 induction in

STAT3^{IEC} KO mice comparing to the WT mice treated with *C. rodentium*. In spite of the higher expression levels of IL-22, there was no induction of RegIIIb and RegIIIg in the KO mice while there were 5 and 7 fold increase in RegIIIb and RegIIIg expression respectively in the WT mice. The IHC further confirms that RegIIIg protein, stained in brown, was absent in STAT3^{IEC} KO colons indicating STAT3^{IEC} is indispensible for antimicrobial peptide synthesis upon IL-22 activation (Figure 3.7.2).

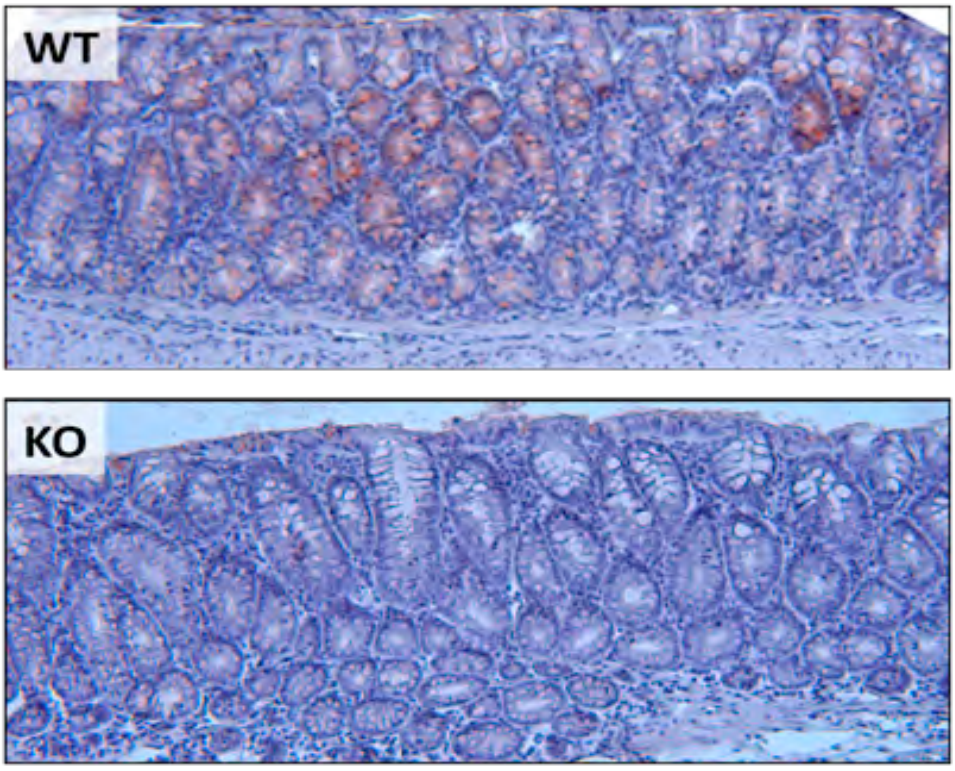

Figure 3.7.2: RegIIIg is absent in STAT3^{IEC} KO mice colon

To investigate the role of STAT3^{IEC} in human, both control and siRNA-mediated STAT3 KDHCA-7 cells were stimulated with IL-22 and checked for RegIIIg production. *In vitro* data confirmed STAT3 KD cells showed low induction levels of RegIIIg, while there were gradual RegIIIg inductions throughout each time point (Firgure 3.7.3).

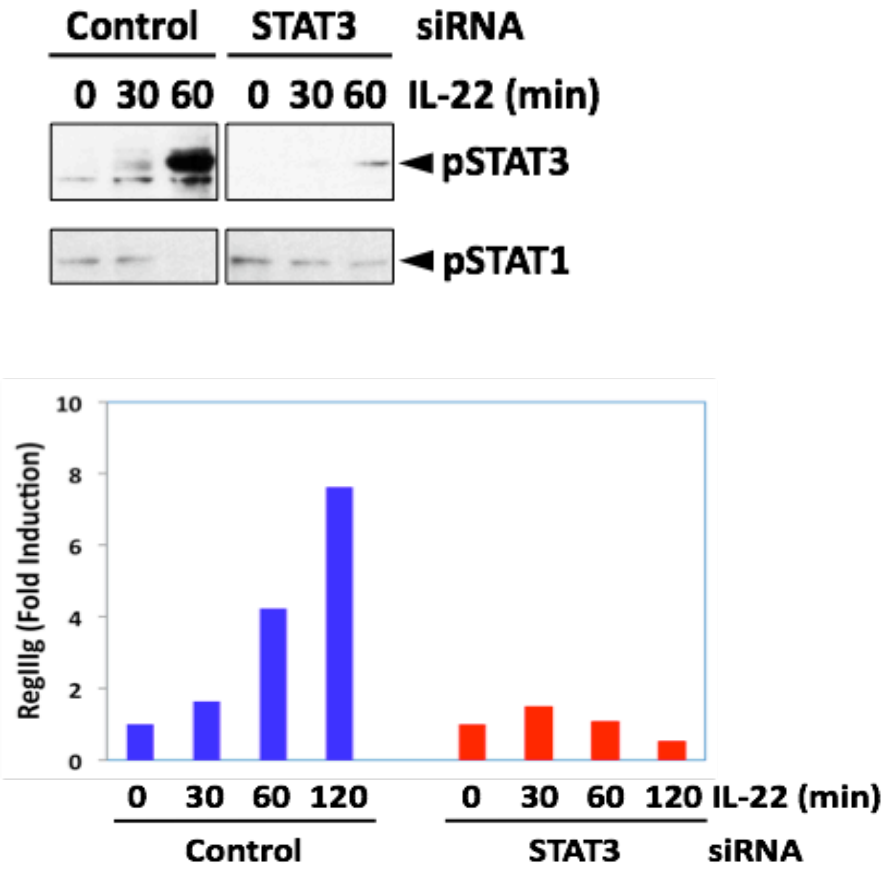

Figure 3.7.3: STAT3^{IEC} is essential for induction of the RegIIIg by IL-22

To test whether RegIIIg deficiency is responsible for the mortality in the C. rodentium infected STAT3IEC KO mice, we produced E. coli- refolded, and purified recombinant RegIIIg as previously described (Cash et al., 2006) to near homogeneity (Figure 3.7.4). RegIIIg monomers were located around17 kD. The upper band of the gel was also found to be RegIIIg by IB after an cation-exchange chromatography and it is likely misprocessed dimmer based on the moleculare weight. The purified proteins were separated on a SDS-PAGE gel and stained with Simply BlueTM Safe Stain (Invitrogen).

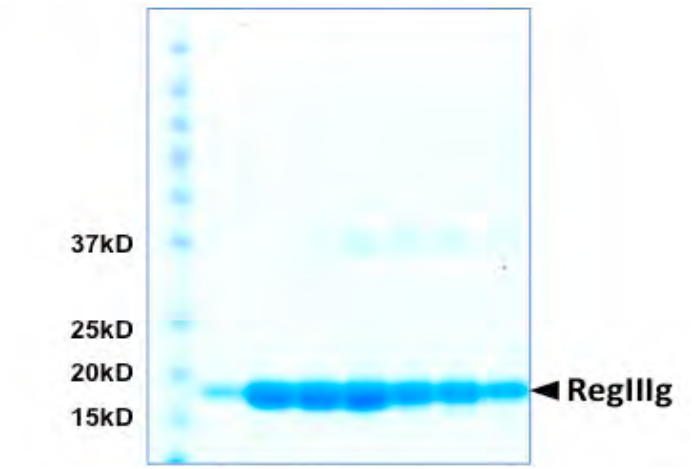

Figure 3.7.4: Expression, refolding, and purification of rec. mouse RegIIIg.

We found that Rec. RegIIIg exerted protective effect in STAT3IEC KO mice against C. rodentium infection, whereas, KO control mice treated with buffer only did not survive (Figure 3.7.5). The KO mice (n=4) infected with C. rodentium were treated with or without recombinant mouse RegIIIg (150g/mouse) through oral administration on days 1, 3, and 5 (Figure 3.7.5). However, it is not clear how RegIIIg clears C. rodentium in the gut since RegIIIg is known to target Gram-positive bacteria instead of Gram-negative bacteria. We found RegIIIg has very high bactericidal activity against the C.rodentium in vitro (Figure 3.7.6). C. rodentium were plated on Mconky plates together with RegIIIg over 24 hours in 37C incubator. Collectively, the data above indicate that the transcriptional activity of STAT3IEC provides an innate immune defense mechanism against enteropathogenic bacteria.

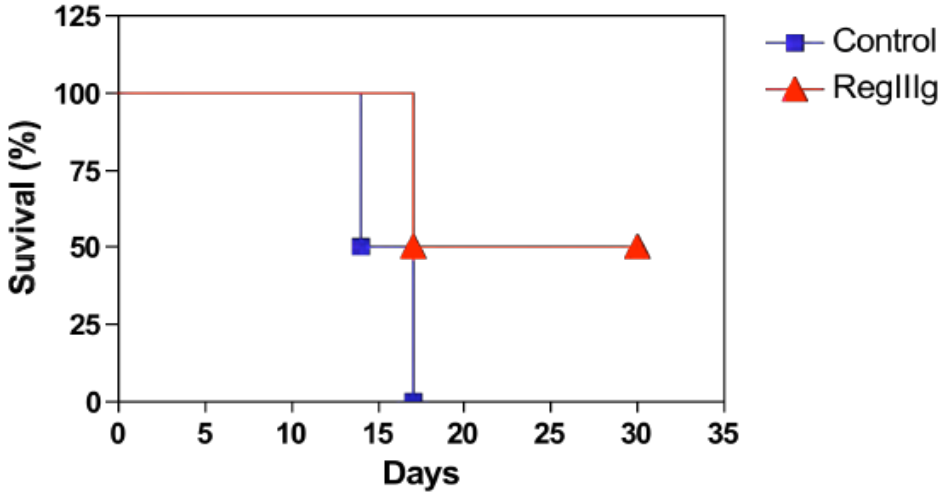

Figure 3.7.5: RegIIIg exerts protective effects in STAT3^{IEC} KO mice against *C. rodentium* infection.

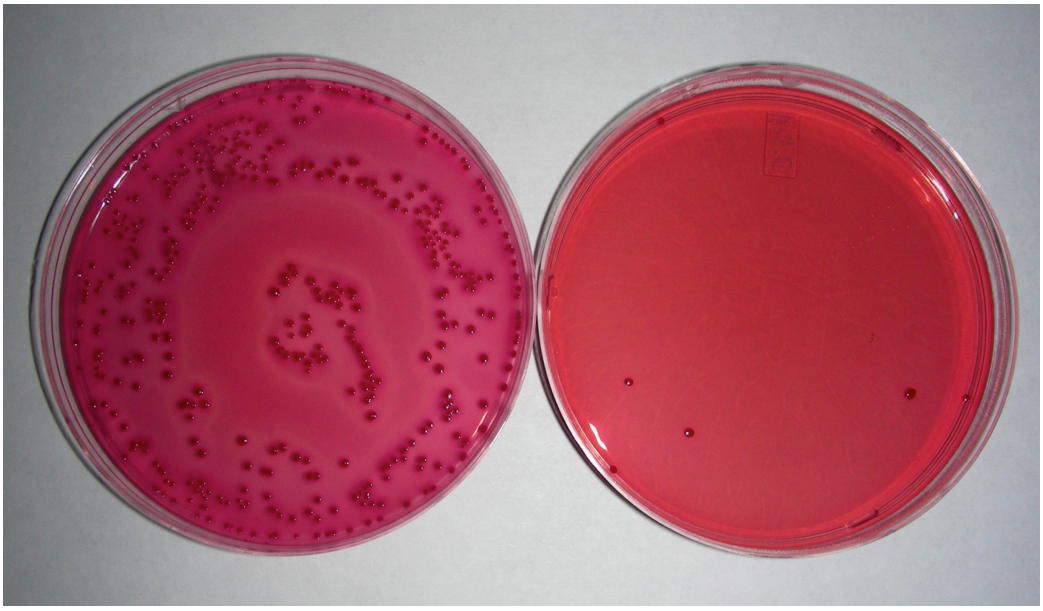

Figure 3.7.6:RegIIIg directly kills *C. rodentium*

3.8. STAT3^{IEC} maintains barrier function by ubiquitin-mediated degradation of SNAI via direct interaction with GSK3b

We next investigate how STAT3^{IEC} sustains the CLDs (claudins) and thus maintain barrier functions. Since CLDs promoter do not yield any STAT3 responsive elements (data not shown), we then asked whether STAT3 affects CLDs synthesis by regulating a master gene that controls the expressions of CLDs. Previous literature indicates that SNAI (Snail-1) induces EMT by suppressing transcription of E-cadherin and CLDs (1, 3, and 7 and occluding). Thus, we investigated whether STAT3 was responsible for downregulation of SNAI levels resulting in CLD expressions. IEC from WT and KO colons were isolated and IB with STAT3 and SNAI (Figure 3.8.1A). SNAI is elevated in STAT3-KO IECs (Figure 3.8.1A). STAT3 siRNA-mediated KD HCA-7 cells also exhibited an elevated SNAI levels (Figure 3.8.1B).

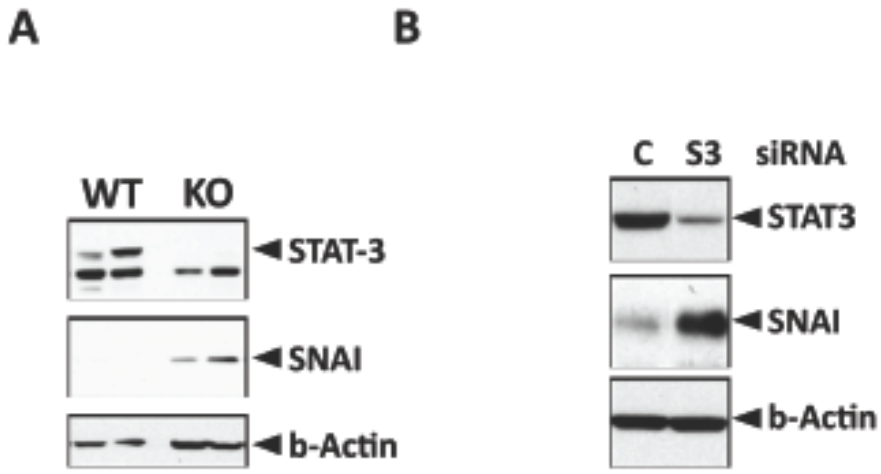

Figure 3.8.1:SNAI is elevated in STAT3 deficient IECs

Both in vivo and in vitro data suggest that STAT3 down-regulates SNAI in IECs and thereby up-regulates CLDs. Indeed, SNAI KD restored the levels of CLDs in HCA-7 cells (Figure 3.8.2A). TER and permeability were measured five days post SNAI siRNA-transfection and showed an enhanced barrier function (* p value <0.05) (Figure 3.8.2B and C). These data suggest SNAI inhibits polarization and enhances permeability in IECs.

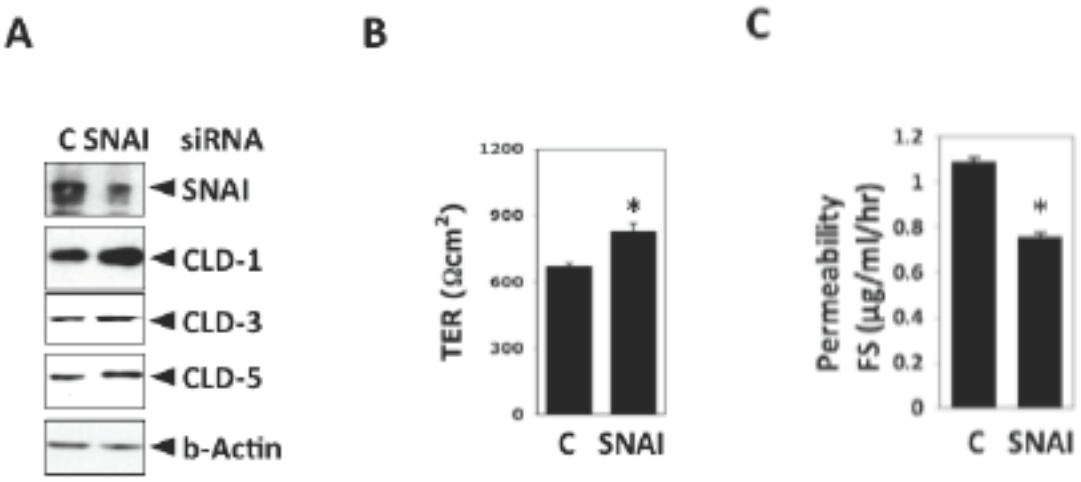

Figure 3.8.2: SNAI suppresses CLDs and disrupts barrier function.

Most importantly, SNAI siRNA-mediated transfection restored the CLD levels and barrier functions in STAT3-deficient IECs (Figure 3.8.3). HCA-7 cells were transfected with control, STAT3, or STAT3+SNAI siRNA (total siRNA level was adjusted with the control siRNA) (Figure 3.8.3A). TER and permeability were measured six days post-transfection (Figure 3.8.3B and C). Taken together, our data demonstrates that $STAT3^{IEC}$ sustains barrier function by downregulating SNAI.

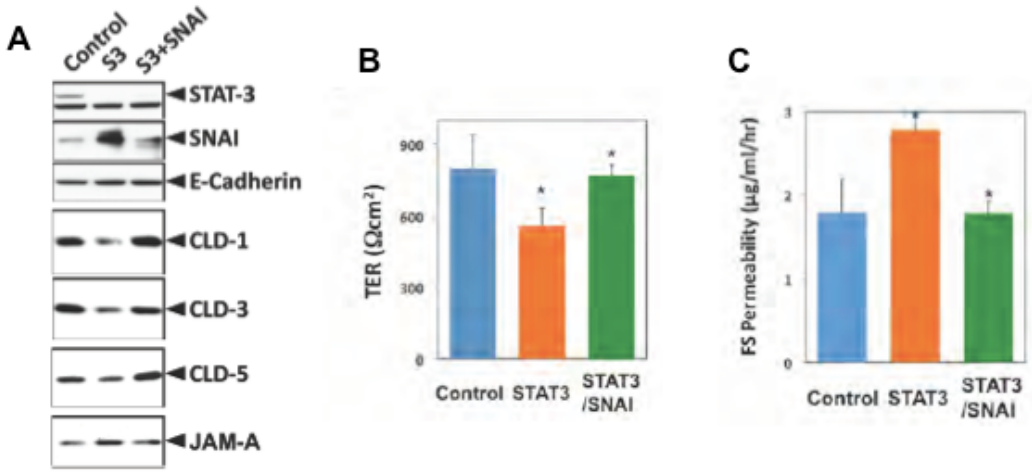

Figure 3.8.3:SNAI suppresses CLDs and disrupts barrier function

To investigate how STAT3 suppresses SNAI in IECs, we checked for the SNAI mRNA level between control and STAT3 KD HCA-7 cells. Since the SNAI mRNA level did not change significantly after STAT3 KD (data not shown), this suggests that STAT3 did not regulate SNAI post-transcriptionally.We then tested whether STAT3 regulates SNAI from post-translational modification. Previous studies suggest that GSK3b phosphorylates SNAI triggering ubiquitin-mediated proteolysis by binding to b-Trcp destruction box (Zhou *et al.*, 2004). Thus, we checked whether SNAI underwent steady-state proteosomal degradation in IECs. HCA-7 cells were incubated with MG132 (10µM) for five hrs to check for SNAI degradation. SNAI was immunoprecipitated and the blot was probed with either anti-ubitquitin or anti-SNAI antibodies (Figure 3.8.4). SNAI in IEC showed a steady-state degradation by proteosomes (Figure 3.8.4**),** suggesting the involvement of GSK3b in SNAI degradation process.

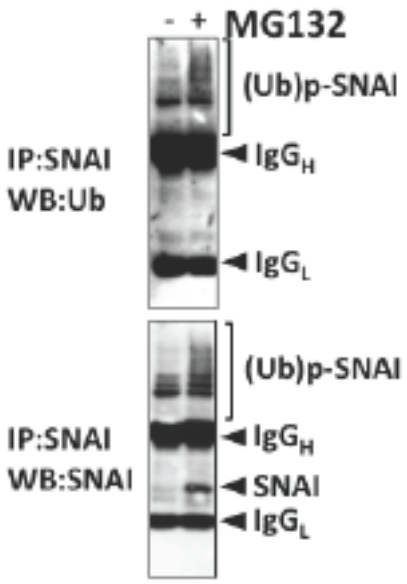

Figure 3.8.4:SNAI undergoes steady state proteosomal degradation

To test whether STAT3 directly interacts with GSK3b, IECs were isolated from WT B6 colons (n=2) and IP for GSK3b and IB STAT3 (Figure 3.8.5A). The presence of STAT3 in the GSK3b immunopricipitates was confirmed by IB. This suggests that STAT3 and GSK3b formed a direct physical interaction in IECs *in vivo* (Figure 3.8.5A).

In vitro data also showed an enhanced physical interaction between STAT3 and GSK3b in polarized HCA-7 cells upon IFNa (10ng/ml) stimulation (Figure 3.8.5B). STAT3 was immunoprecipitated and IB for the indicated proteins. Apparently, STAT3-GSK3b complex also included SNAI in the ubiquinated state (Figure 3.8.5C). Polarized HCA-7 cells were stimulated with IFNa (10ng/ml) over time, IP for GSK3b, and IB measured the indicated proteins. The presence of STAT3 and Ub-SNAI in the GSK3b immunoprecipitate directly confirms the formation of STAT3-GSK3b-SNAI destruction complex (Figure 3.8.5C).

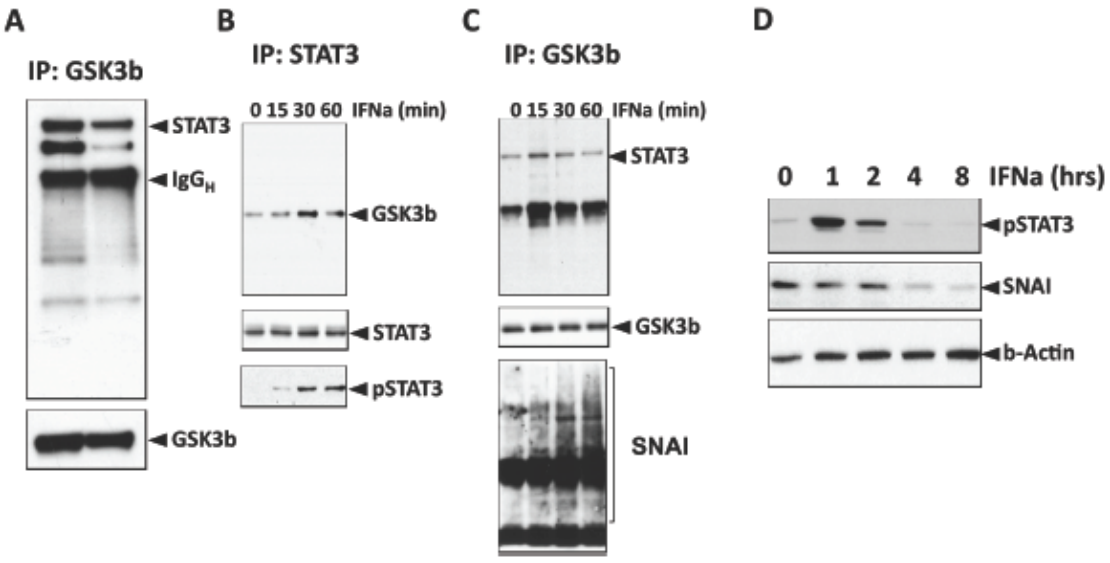

Figure 3.8.5:STAT3^{IEC} and GSK3b form a SNAI destruction complex.

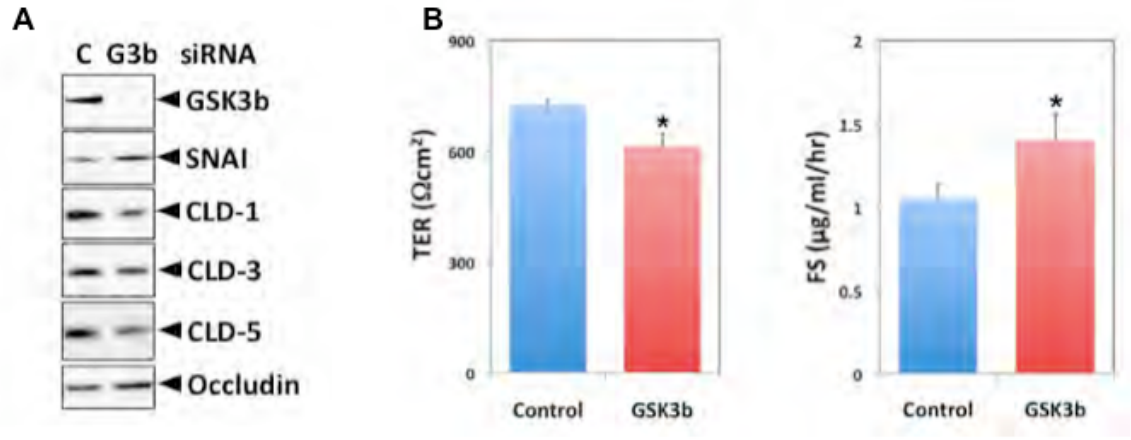

Figure 3.8.6:GSK3b KD enhances SNAI levels and reduces the levels of CLDs.

To confirm the link between STAT3 and SNAI, polarized HCA-7 cells were stimulated with IFNa (10ng/ml) and IB for the indicated proteins. Indeed, STAT3 activation downsregulated SNAI upon IFNa stimulation (Figure 3.8.5D). As expected with the results, we IB for the indicated proteins levels in HCA-7 cells 2 days after GSK3b siRNA trasfection (Figure 3.8.6A). GSK3b KD enhanced SNAI level while

reduced the levels of CLDs (Figure 3.8.6A) and led to the loss of barrier functions (Figure 3.8.6B). TER and permeability were measured six days post transfection (n=6).

Results, in part, have been submitted for publication of the material. Jongdae Lee is the primary investigator. Ji-Hun Mo, Adam N. Rucker, Shee-Eun Lee, Jose M. Gonalez-Navajas, Devorah Friedberg, Scott Herdman, Declan F McCole, Yolanda Aderson, Kim E. Barrett, Lars Eckmann, and Eyal Raz are the co-authors of this paper.

4. Discussion

STAT3 suppresses inflammation and the deletion of STAT3 in hematopoietic cells induces colitis in mice(Takeda *et al.*, 1999; Kobayashi *et al.*, 2003; Welte *et al.*, 2003). However, the role of $STAT3^{IEC}$ in intestinal homeostasis had not been investigated. Here we provide evidence that $STAT3^{IEC}$ controls an anti-inflammatory response in IECs in a manner that is distinct from the function of STAT3 in hematopoietic cells (suppression of proinflammatory cytokine production). $STAT3^{IEC}$ inhibits intestinal inflammation by sustaining the epithelial barrier and by inducing anti-microbial peptides. Our findings also indicate that the intracellular signal transduction pathways triggered by certain paracrine stimuli or microbes can be modified under conditions that disrupt IEC polarity, and consequently provoke intestinal pathology. Similar to what has been recently reported for TLR ligands (Lee *et al.*, 2006), IFNa signaling in IECs is modified when polarity is impaired. It would be interesting to identify whether these novel functions of $STAT3^{IEC}$, i.e., maintenance of barrier function via STAT3-GSK3b-SNAI complex and induction of anti-microbial peptides, are impaired in IBD patients with STAT3 polymorphism. Our data suggest that interventions that enhance barrier function (e.g. activation of STAT3) may inhibit bacterial colonization and reduce intestinal inflammation. It is not clear why $STAT3^{IEC}$ KO mice do not develop spontaneous colitis in spite of subclinical inflammatory manifestations.

Many IBD susceptibility genes have been discovered recently but the susceptible populations don't always develop colitis, indicating that either additional genetic disposition and/or environmental factors are needed for the development of disease. This

notion is supported by the mouse genetic studies, where mutations in NOD-2 (Kobayashi *et al.*, 2005; Maeda *et al.*, 2005), ATG16L1(Cadwell *et al.*, 2008), or other susceptibility genes produce no spontaneous colitis. In terms of $STAT3^{IEC}$ KO mice, although the leaky barrier causes inflammatory responses in the colon, STAT3 in other cell types such as innate and adaptive immune cells in the colon may be able to control inflammation to avoid colitis. In summary, we identified tow novel functions of STAT3 in IECs: maintenance of epithelial barrier by a posttranslational modification of SNAI and anti-bacterial response by transcriptional activation of anti-microbial peptides.

Discussion, in part, has been submitted for publication. Jongdae Lee is the primary investigator. Ji-Hun Mo, Adam N. Rucker, Shee-Eun Lee, Jose M. Gonalez-Navajas, Devorah Friedberg, Scott Herdman, Declan F McCole, Yolanda Aderson, Kim E. Barrett, Lars Eckmann, and Eyal Raz are the co-authors of this paper.

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