Identifying Mechanisms Used by Adherent-Invasive 
*Escherichia coli* Associated with Crohn Disease to Evade the 
Immune System

By

Juan Cristobal Ossa

A thesis submitted in conformity with the requirements 
for the degree of Master of Science 
Graduate Department of Institute of Medical Science 
University of Toronto

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Identifying Mechanisms Used by Adherent-Invasive *Escherichia coli* Associated with Crohn Disease to Evade the Immune System

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Master of Science

Institute of Medical Science
University of Toronto

2012

**Background:** Adherent-invasive *Escherichia coli* (AIEC) is a pathogen isolated from the ileum of patients with CD. IFNγ is a key mediator of immunity, which regulates inflammatory responses to microbial infections. Previously, we showed enterohemorrhagic *E. coli* prevents STAT1 activation.

**Aims:** To determine; 1) whether activation of STAT1 by IFNγ was prevented following AIEC infection, and 2) define the mechanisms used.

**Methods:** Human epithelial cells were infected with AIEC strains or other pathogenic and commensal *E. coli* strains. Following infection, cells were stimulated with IFNγ. Activation of STAT1, was monitored by immunoblotting.

**Results:** AIEC strains prevented STAT1 phosphorylation in response to IFNγ. Effect required live bacteria with active protein synthesis. A bacterial product was responsible for blocking STAT1 signalling and interfered with downstream signalling cascades.
Conclusion: Suppression of epithelial cell STAT1 signal transduction by AIEC strains represents a novel mechanism by which the pathogen evades host immune responses to the infection.
Dedicated to my parents, Mr. Carlos E. Ossa and Mrs. Maria I. Alemparte
Acknowledgements

First of all, I would like to say that without the effort and support of many people, this work could not have been completed.

I am enormously grateful to my supervisor, Phil Sherman, for his constant support and encouragement to maintain excellence and curiosity in the area of medical science. Moreover, I am thankful for his patience and mentorship during this period.

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Dissemination of Work Arising from this Thesis

Publications:

Ossa Juan C., Ho N.K., Wine E., Yeung N., Gray-Owen S.D., Sherman P.M. Adherent-invasive *Escherichia coli* associated with Crohn disease prevents the activation of signal transducer and activation of transcription (STAT) 1 induced by interferon-gamma- in human intestinal epithelial cells. Manuscript in Preparation

Ho. N.K., Ossa Juan C., Sousa A., Sherman P.M. Enterohemorrhagic *Escherichia coli* O157:H7 Shiga Toxins Inhibit Interferon-gamma Mediated Cellular Activation. Submitted to Cellular Microbiology

Ho. N.K., Ossa Juan C., Sousa A., Sherman P.M. Microarray comparison of the genomic immune responses to two enteropathogenic *Escherichia coli* in epithelial cells. Manuscript in Preparation

Wine E., Ossa Juan C, Gray-Owen, S.D., Sherman, P.M. Adherent-invasive *Escherichia coli* target the epithelial barrier. Gut Microbes 2:80-84, **2010**


Oral Presentations:


**Ossa J.C., Ho N.K., Wine E., Yeung N., Gray-Owen S.D., Sherman P.M.** Oral presentation “Adherent-Invasive *Escherichia coli* blocks interferon gamma-induced STAT1 phosphorylation in human intestine epithelial cells” University of Toronto GI Research Day, Toronto, Ontario, Canada (June 2010)


Poster Presentations:

**Juan C. Ossa**, Nathan Ho, Eytan Wine, Nelly Leung, Scott D. Gray-Owen, Philip M. Sherman. Poster presentation “Interferon gamma activation of STAT1 is blocked by Adherent-Invasive *Escherichia coli* associated with Crohn disease” Institute of Medical Science Scientific Day, Toronto, Ontario, Canada (May 2011)
Juan C. Ossa, Nathan Ho, Eytan Wine, Nelly Leung, Scott D. Gray-Owen, Philip M. Sherman. 

**Poster of Distinction** “Adherent-Invasive *Escherichia coli* associated with Crohn disease blocks interferon gamma-induced STAT1 phosphorylation in human intestine epithelial cells” CDDW Toronto, Ontario, Canada (March 2010) 

Juan C. Ossa, Nathan Ho, Eytan Wine, Nelly Leung, Scott D. Gray-Owen, Philip M. Sherman. 

Poster presentation “Interferon gamma-induced STAT1 phosphorylation is blocked by adherent-invasive *Escherichia coli* in human intestine epithelial cell lines” DDW New Orleans, USA (May 2010)
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<td>AIEC</td>
<td>Adherent invasive <em>Escherichia coli</em></td>
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<td>CD</td>
<td>Crohn disease</td>
</tr>
<tr>
<td>CEACAM</td>
<td>Carcinoembryonic antigen-related cell-adhesion molecule</td>
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<tr>
<td>CFTR</td>
<td>Cystic fibrosis transmembrane conductance regulator</td>
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<tr>
<td>DAEC</td>
<td>Diffusely adherent <em>Escherichia coli</em></td>
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<tr>
<td>EAEC</td>
<td>Enteroaggregative <em>Escherichia coli</em></td>
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<tr>
<td>EHEC</td>
<td>Enterohemorragic <em>Escherichia coli</em></td>
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<tr>
<td>EIEC</td>
<td>Enteroinvasive <em>Escherichia coli</em></td>
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<tr>
<td>EPEC</td>
<td>Enteropathogenic <em>Escherichia coli</em></td>
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<tr>
<td>ETEC</td>
<td>Enterotoxigenic <em>Escherichia coli</em></td>
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<tr>
<td>ExPEC</td>
<td>Extraintestinal pathogenic <em>E. coli</em></td>
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<tr>
<td>Gb3s</td>
<td>Globotriaosylceramide</td>
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<tr>
<td>GWAS</td>
<td>Genome wide association studies</td>
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<td>IBD</td>
<td>Inflammatory bowel disease</td>
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<tr>
<td>IEC</td>
<td>Intestinal epithelial cell</td>
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<td>IFNGR</td>
<td>Interferon gamma receptor</td>
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<tr>
<td>IFNRA</td>
<td>Interferon alpha receptor</td>
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<tr>
<td>IFNα</td>
<td>Interferon alpha</td>
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<tr>
<td>IFNβ</td>
<td>Interferon beta</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>IFN(\gamma)</td>
<td>Interferon gamma</td>
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<tr>
<td>IFN(\lambda)</td>
<td>Interferon lambda</td>
</tr>
<tr>
<td>IFN(\omega)</td>
<td>Interferon omega</td>
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<tr>
<td>IRGM</td>
<td>Immunity-related GTPase family M</td>
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<td>JAK</td>
<td>Janus kinases</td>
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<tr>
<td>LEE</td>
<td>Locus of enterocyte effacement</td>
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<tr>
<td>Lpf</td>
<td>Long polar fimbriae</td>
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<tr>
<td>LTs</td>
<td>Heat labile toxin</td>
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<td>MAMPs</td>
<td>Microbe-associated molecular patterns</td>
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<td>MHC I and II</td>
<td>Major histocompatibility complex I and II</td>
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<td>MSMD</td>
<td>Mendelian susceptibility to mycobacterial disease</td>
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<td>NOD2</td>
<td>Nucleotide-binding oligomerization domain containing 2</td>
</tr>
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<td>PRR</td>
<td>Pattern recognition receptor</td>
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<td>SNP</td>
<td>Single nucleotide polymorphism</td>
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<td>SOCS</td>
<td>Suppressors of cytokine signalling</td>
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<td>STAT</td>
<td>Signal transducer and activator of transcription</td>
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<td>STs</td>
<td>Heat stable toxin</td>
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<td>Stx</td>
<td>Shiga toxin</td>
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<td>TGF(\beta)</td>
<td>Transforming growth factor beta</td>
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<td>TIR</td>
<td>Translocated intimin receptor</td>
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<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>TLR</td>
<td>Toll like receptor</td>
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<td>TNFα</td>
<td>Tumour necrosis factor alpha</td>
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<tr>
<td>UC</td>
<td>Ulcerative colitis</td>
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<tr>
<td>UTI</td>
<td>Urinary tract infection</td>
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<td>XBP1</td>
<td>X-box binding protein</td>
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Chapter I – Introduction
Introduction

Inflammatory Bowel Disease

Idiopathic inflammatory bowel disease (IBD) is comprised of two distinct clinical entities; Crohn Disease (CD) and Ulcerative Colitis (UC), which are both characterized by chronic inflammation of the intestine (Kaser et al., 2010). Patients with IBD present with chronic remitting and relapsing inflammation in the gastrointestinal tract. Gut inflammation seems to arise from an altered interaction between luminal intestinal microbes, the epithelium, and the underlying mucosa, influenced by the host harboring specific genetic susceptibility loci and by the environment (Khor et al., 2011).

Patients with IBD classically present with symptoms of diarrhea, abdominal pain, rectal bleeding, weight loss, undernutrition, and diminished health-related quality of life (Podolsky, 2002; Abraham & Cho, 2009). IBD patients are prone to suffer from other chronic inflammatory conditions beyond the GI tract, including: ankylosing spondylitis, pyoderma gangrenosum, and primary sclerosing cholangitis (Larsen et al., 2010). Later in life, IBD patients present with an elevated risk of developing colon cancer (Ullman & Itzkowitz, 2011).

CD differs from UC regarding both the location and extent of intestinal inflammation. In CD, the inflammation can affect anywhere along the length of the entire gastrointestinal tract in a discontinuous fashion, but more commonly involving the terminal ileum and proximal colon. In contrast, in UC, inflammation is confined only to the colon extending in a continuous manner from the rectum proximally (Podolsky, 2002). With respect to pathology, inflammation in CD is discontinuous and often transmural, with the presence of non-caseating granuloma formation in approximately 30% of cases. In UC, inflammation is superficial, extending proximally from the rectum in a continuous fashion that extends to the cecum, and is confined only to colonic mucosa (Herésbach et al., 2005; Cho, 2008). Only CD patients develop stricturing disease, fistula formation, and perianal involvement, whereas these complications are not seen in UC (Baumgart & Sandborn, 2007).
Medical treatment of IBD include the use of aminosalicylates, antibiotics, immunosuppressives (azathioprine, methotrexate), corticosteroids, and monoclonal antibodies directed to blocking molecules involved in pro-inflammatory signalling pathways indicative of these diseases (Baumgart & Sandborn, 2007). Although the treatment for IBD seems to have improved since the introduction of monoclonal antibodies, such as anti-tumour necrosis factor (TNF)α, their use often still leaves patients requiring surgery, with the known increased risk for disease recurrence in those with CD (Burger & Travis, 2011).

Before the era of anti-TNF therapy, reports indicated that roughly half of patients with CD required an intestinal resection during their lifetime (Wolters et al., 2004). In UC, 10-30% patients (with even higher rates in children who tend to have more extensive and severe colitis than is observed in those with onset of illness as adults) will require a colectomy as a treatment for either ongoing refractory symptoms of bloody, mucoid diarrhea or the complications of UC (Peyrin-Biroulet et al., 2009; Cosnes et al., 2011). To date, there is no cure for IBD; therefore, the elucidation of IBD pathogenesis and the development of new therapeutic approaches remains a focus of intensive investigation by research groups around the world.
Epidemiology of IBD

Clinical manifestations of IBD generally start in early adulthood and then persist in a remitting and relapsing manner throughout life, with important consequences of decreased quality of life, an impaired ability to perform in a job, and an increase in disability (Cosnes et al., 2011). The incidence and prevalence of IBD is increasing worldwide, with the highest rates reported in Canada, United States, United Kingdom, and Northern Europe, reaching an incidence of 10-15 per 100,000 people of new cases per year and a prevalence of 50-200 per 100,000 people (Rubin et al., 2000; Vind et al., 2006; Loftus et al., 2007).

In Canada, there are also reports related to a recent increase in the incidence of pediatric IBD (Benchimol et al., 2009). Increasing burden of illness due to IBD appears to be due to an increase in rates of CD rather than UC, which seems to have remained relatively stable (Benchimol et al., 2010; Cosnes et al., 2011). Moreover, the increase in IBD incidence is now being reported in countries, such as in Japan, China, and South Korea, where CD and UC were previously quite unusual. These epidemiological data support the theory that the pathogenesis of IBD could be related to temporal changes towards a western-lifestyle that include changes in diet, sanitation, and more industrialization all of which can alter the intestinal microbiota (Thia et al., 2008).

The peak incidence for CD is at 20-30 years of age, and for UC at 30-40 years (Loftus et al., 2002). However, there seems to be another peak in IBD that is seen in the pediatric population (Benchimol et al., 2010). With respect to gender, in adults, UC is more common in men than in women, whereas conversely more women than men have CD. For unknown reasons, the opposite gender predilection has been observed for both UC and CD in pediatric populations (Cosnes et al., 2011).

Epidemiological studies report a higher incidence of IBD in the northern parts of the United States and Canada compared to southern regions. This phenomenon is also seen in France, possibly adding the potential for an environmental association for IBD (Bernstein et al., 2006; Nerich et al., 2006; Sonnenberg, 2009).
Interesting data also comes from ethnic migration studies where persons of Jewish ancestry, known to have an increased risk for IBD, have an increased risk of developing IBD if they live in areas with a high incidence of IBD, compared to those residing in low prevalence areas of the world. Moreover, Jews born in Asia tend to have less IBD compared to those born in either northern Europe or North America (Shapira & Tamir, 1992).

**Etiopathogenesis of IBD**

Classically, the cause of IBD was thought to have a genetic cause based on data showing an increased IBD risk in relatives of patients presenting with the disease (Orholm *et al.*, 1991), and an increased risk of developing IBD in specific ethnic populations i.e.; Ashkenazi Jews (Lowe *et al.*, 2009). Moreover, data from studies performed in monozygotic twins show a phenotypic concordance, of 30% for CD and 15% for UC, with prevalence rates much higher in monozygotic as compared to dizygotic twins; both pieces of evidence arguing for a genetic influence in the development of IBD, which is likely higher in CD than in UC (Brant, 2010).

Indeed, with the advancement of sophisticated genetic studies, using candidate gene mapping techniques, researchers found an association of major histocompatibility complex class II alleles related to IBD (Stokkers *et al.*, 1999). Later on, using linkage mapping studies and genome wide association studies (GWAS) where, through the identification of chromosomal segments shared among affected relatives, compared to non-affected is shown to be greater than that which can expected by chance. Researchers have found many susceptibility genes associated with IBD and especially CD (currently >100), including genes that code for bacterial recognition (NOD2), autophagy (ATG16L1 and IRGM), and immune signalling pathways (IL-23, Th17 pathway) (Van Limbergen *et al.*, 2009; Cho & Brant, 2011). Regardless of formidable current advances related to the genetic associations of IBD, it seems that genes account for < 25% of predicted hereditability in IBD (Franke *et al.*, 2010). Thus, these observations confirm the complexity of disease pathogenesis, and the importance of considering other factors, including microbes and environmental factors as potentially involved in disease pathogenesis.
Data from epidemiological studies provide clear support for a role for the environment as an important risk factor related to the development of IBD (Bernstein, 2010), and that the majority of the component of this environmental risk is constituted by the intestinal microbiota (Maloy & Powrie, 2011). Microbes are known to modulate host immune composition and function, which are the main targets of the inflammatory responses seen in IBD (Round & Mazmanian, 2009).

The intestinal human microbiome harbors more than 100 trillion individual organisms at a density of $10^{11}$ to $10^{14}$ cells per gram of luminal contents in the large intestine (Hill & Artis, 2010). With the development of sophisticated molecular techniques to analyze microbial composition in human samples of feces and in mucosal biopsies, using DNA bar coding and 454 pyrosequencing of 16S ribosomal RNA gene segments, it is possible to now identify bacteria and archaea species in numbers as high as 15,000–40,000 individual species (Frank & Pace, 2008). These organisms increase in abundance and complexity from the stomach and duodenum ($10^2$-$10^3$ organisms/gram of luminal content), where mainly aerobic microorganisms are found, towards the cecum and colon ($10^{11}$-$10^{12}$), where mostly strictly anaerobic organisms are present (Sartor, 2008).

More than 99% of the intestinal microbiota is composed of species contained in the 4 bacterial divisions: Firmicutes, Bacteroidetes, Proteobacteria, and Actinobacteria; Firmicutes is the dominant (64%) followed by Bacteroidetes representing (23%) of normal species. *Escherichia coli* is part of Enterobacteriaceae, which is a minor component of the Proteobacteria division (8%) (Frank & Pace, 2008).

Collectively, the microbiota and its interaction with the intestinal epithelial cell (IEC), is responsible for many physiological functions that are important to mammalian biology (Ley *et al.*, 2006). Indeed, the intestinal microbiome is implicated in food metabolism, maintaining the epithelial homeostasis in the intestine, promoting regeneration of blood vessels and enteric nerve function, supporting resistance to infection, and in promoting the normal development and regulation of the immune system (Hill & Artis, 2010).
Lessons learned from studies in mice, confirm that the gut microbiota is a requisite for the normal development and differentiation of local and systemic immune components (Round & Mazmanian, 2009). For example, *Bacteroides thetaiotaomicron* induces the expression of angiogenin, an antimicrobial peptide present in the intestinal epithelium through the activation of Toll-like receptors (TLR) expressed on Paneth cells (Vaishnava *et al.*, 2008), or the presence of segmented filamentous bacteria in the intestine, which induce the differentiation of Th17 - a lymphocyte subtype known to be implicated in the intestinal immune response against pathogens i.e. *Citrobacter rodentium* (Mangan *et al.*, 2006; Ivanov *et al.*, 2008). By contrast, evidence from mice not exposed to any microorganism (germ free animals) have underdeveloped mucosal and systemic immune systems with decreased cellularity of Peyer’s patches, lamina propria, mesenteric lymph nodes, and spleen, and diminished mucosal immune function (Cebra, 1999). Taken together, IBD likely develops in a genetically predisposed host with alterations in the immune system and changes in either the composition or function of the intestinal microbiota.

**Interaction between the gut microbiota and the host immune system**

The intestinal microbiota, residing in the intestinal lumen in the outer mucus layer overlying surface epithelial cells (Wells *et al.*, 2010), allows a constant interaction between the host and microbes. The results from these interactions can result in either mutual benefits or in adverse effects, which could contribute to remitting and relapsing inflammation that is characteristic of IBD (Abraham & Medzhitov, 2011).

IEC lining the intestine provide an anatomical and functional barrier that separates the luminal microbiota from the intestinal associated lymphatic tissue. IEC actively defend the host against infection and prevent the passage of pathogens into the underlying tissues. On the other hand, the innate and adaptive immune systems have a role to defend against enteric pathogens while maintaining tolerance to the commensal microflora (Sansonetti & Medzhitov, 2009).

In order to defend against enteric pathogens, IEC use pattern recognition receptors (PRR) to recognize MAMPs expressed on the surface of microbes. Upon recognition of MAMPs by
these PR receptors, the activation of cytokine and chemokine signalling pathways results in antimicrobial killing (e.g. antimicrobial peptides, phagocytosis, autophagy, and reactive oxygen and nitrogen species), unfolded protein responses, and initiation of adaptive T- and B-cell responses against pathogens (Abraham & Medzhitov, 2011). Simultaneously, immune-tolerance is achieved by limiting the exposure of PRR to microbes (e.g. mucin production by goblet cells, IgA secretion by plasma cells in the mucosa, and regulation of epithelial barrier function by IEC to prevent microbe translocation). In conjunction with these responses, other innate and adaptive immune responses act to down-regulate and suppress gut inflammation via the secretion of anti-inflammatory cytokines, such as IL-10, TGF-β, and by the induction of regulatory T cells (Abraham & Medzhitov, 2011).

**Bacterial involvement in Inflammatory Bowel Disease**

Since the earliest description of IBD, and especially in CD the presence of non-caseating granuloma, made clinicians consider an infectious cause (Crohn, 1965). Even today, there is no clear infectious etiology demonstrated. There are still many observations from both clinical studies and rodent experiments that support the potential involvement of bacteria in the pathogenesis of IBD (Man et al., 2011). Nowadays, it is well known that recurrent CD can be prevented by the diversion of the fecal stream, by performing a surgical loop-ileostomy (Rutgeerts et al., 1991). Patients experience a significant clinical improvement in symptoms and histology for 3 to 6 months, but symptoms recur quickly following reinfusion of fecal contents back into the lumen of the diverted intestinal segment (D'Haens et al., 1998).

The use of antibiotics is also associated with an improvement in the clinical outcome of patients with CD. For instance, this was demonstrated in a meta-analysis in which the use of broad spectrum antibiotics improved both clinical symptoms and laboratory markers of inflammation with an odds ratio of 2.25, compared to placebo (Rahimi et al., 2006). Moreover, the use of the anti-anaerobic antibiotic; metronidazole or other irnidazole compounds decreases the postoperative recurrence rates of CD after ileal resection (Rutgeerts et al., 1995; Rutgeerts et al., 2005).
These clinical observations are supported by lessons learned from mouse infection models developed in order to understand the pathogenesis of IBD. In these models, specific pathogenic agents are used to trigger intestinal inflammation (e.g., *Citrobacter rodentium*, *Salmonella enterica*, and *Helicobacter hepaticus*). However, it has been shown that the intestinal commensal microbiota have an essential role in the initiation and perpetuation of intestinal inflammation (Nell *et al.*, 2010). For example, in germ free mice lacking IL-10/−, an important anti-inflammatory cytokine, mice do not develop intestinal inflammation unless there is the presence of an intestinal microbiota (Sellon *et al.*, 1998). Moreover, mice lacking Recombination activating protein 1 (RAG1−/−), which is essential for the development of mature T and B cell lymphocytes, upon CD4+ transfer, the induction of intestinal inflammation depends on both T cells and the presence of an intestinal microbiota (Niess *et al.*, 2008).

**Evidence of dysbiosis in patients with IBD**

Dysbiosis is a term used to describe changes in the composition of the intestinal microbiota, where there is an imbalance between the “commensal/protective” and “harmful” bacteria living in the intestine (Sartor, 2011). Analyzing luminal samples, mucosal biopsies, and fecal samples from patients with IBD, multiple studies have found that patients with CD, UC, and pouchitis have a decreased microbial diversity (Sartor, 2008), where the phyla Firmicutes and Bacteroidetes are underrepresented, compared to controls (Frank *et al.*, 2007). Most studies also report an increased representation of Enterobacteriaceae, particularly *Escherichia coli*, found in feces and in mucosal biopsy samples obtained from patients with CD (Swidsinski *et al.*, 2002; Baumgart *et al.*, 2007). One *E. coli* isolate, called 13I, has been found to invade the intestinal mucosa (Sasaki *et al.*, 2007), other isolates have been found to be present in granulomas and adjacent to ulcers and fistulas in CD patients (Cartun *et al.*, 1993; Ryan *et al.*, 2004).

The decrease of Bacteroidetes includes a reduced number of *Bacteroides fragilis*, shown to be protective against inflammation in a mouse model of enterocolitis using the murine enteropathogen, *Helicobacter hepaticus* (Mazmanian *et al.*, 2005) and also to be an important inducer of IgA in the intestine of mice (Yanagibashi *et al.*, 2009). The reduction in Firmicutes is
due to an underrepresentation of Clostridium XIVa and IV groups within the Lachnospiraceae subgroups, where *Faecalibacterium prausnitzii*, is a major representative, and has shown to be decreased in the ileum and colon of patients with CD, and protective against recurrence of the disease (Sokol *et al.*, 2008).

Currently, it is not clear if dysbiosis is a primary or a secondary change in IBD (Sartor, 2010). The evidence of increased mucosal bacteria in patients with different disease activity and the changes seen towards normalization after the use of corticosteroids (Swidsinski *et al.*, 2008) suggest a secondary cause. On the other hand, genetic polymorphisms in patients with IBD are found to be associated with dysbiosis (Frank *et al.*, 2010). In this study, patients with IBD and polymorphisms in either *NOD2* or *ATG16L1* genes associated with ileal CD were shown to be significantly associated with changes in the microbial composition of mucosal intestinal samples, compared to non-inflamed controls. Mice experiments support these findings, showing that the presence of *NOD2*+/+, compared to mice lacking *NOD2*−/−, is fundamental for the establishment of an intestinal microbiota both in early development and throughout later life (Rehman *et al.*, 2011).

Although the question of whether dysbiosis is causative or resulting in gut inflammation is still not answered, the alteration between protective versus harmful bacteria in patients with CD suggests a potential involvement of microbes in the etiology and the pathogenesis of IBD.

**Host factors that affect commensal bacteria in IBD**

Patients with CD and their relatives both have an increased permeability in their intestines, suggesting a mucosal barrier function defect, which could allow the translocation of microbes into the underlying mucosa. This was demonstrated by decreased exclusion of large macromolecules in patients with CD and their unaffected first-degree relatives (Hollander *et al.*, 1986), suggesting a primary genetic defect in barrier function. The molecular basis for this defect could be secondary to an up regulation in the pore forming intercellular tight junction protein claudin-2, and to redistribution in claudins 5-8, that normally seal the tight junctions in the gut epithelium (Zeissig *et al.*, 2007). However, there is ongoing controversy regarding if
these changes correspond to a primary defect or whether it is occurring secondary to the underlying intestinal mucosal inflammation.

Alterations in the epithelial mucus layer have been suggested to be present in UC patients, mainly with alterations in the structure and composition of the phospholipid content (Braun et al., 2009). The potential significance of these observations is supported by the spontaneous colitis that develops in mice deficient in Muc2 (Heazlewood et al., 2008).

Defects in the production of antibacterial peptides by Paneth cells in the intestine are another host factor that clearly causes a predisposition to gut inflammation. Such changes have been confirmed in CD where patients with ileal disease have abnormal production of α-defensin 5 (Wehkamp et al., 2005), and there are reduced levels of β-defensin-2 in colonic disease (Aldhous et al., 2009).

Other interesting data supporting the role of microbes involved in the pathogenesis of IBD come from GWAS studies where single nucleotide polymorphisms (SNP) in genes involved in key host defense mechanisms against pathogens, are present in CD compared to the healthy individuals (Khor et al., 2011). For instance, polymorphisms in the gene of nucleotide oligomerization domain 2 (NOD2), an intracellular pattern recognition receptor (PRR), that binds muramyl dipeptide present in the peptidoglycan of both Gram-positive and Gram-negative bacteria (Man et al., 2011), is associated with decreased α-defensin production by Paneth cells (Wehkamp et al., 2004), decreased proinflammatory cytokine production in immune cells (Hedl et al., 2007; Brosbol-Ravnborg et al., 2009), and results in impaired bacterial killing and less efficient autophagy (Cooney et al., 2009).

Other polymorphisms found in association with CD are in the genes of ATG16L1 and IRGM, two molecules with important functions in autophagy, a cellular process involved in the degradation of both bacteria (when it is also referred to as xenophagy) and cellular components (Kaser & Blumberg, 2011). Mutations in ATG16L1 result in abnormal Paneth cells with an altered composition of granules (Cadwell et al., 2008), and defective epithelial killing of salmonella infection in epithelial cells (Kuballa et al., 2008). Moreover, NOD2 is a requisite for recruitment of ATG16L1 for autophagy (Travassos et al., 2009). This important discovery gives important clinical significance to mutations in NOD2 in a proportion of patients with CD.
Mucosa-associated bacteria in Crohn disease

Utilizing fluorescent *in-situ* hybridization (FISH) and electronic microscopy as complementary experimental approaches, studies have found the presence of bacteria on the mucosa or within the submucosa of the intestine in patients with CD (Swidsinski *et al.*, 2002).

Several microorganisms have been postulated as a potential cause for the development of IBD (Naser *et al.*, 2004; Abubakar *et al.*, 2008), although there is still insufficient evidence to support one specific organism as an etiologic agent. The most extensively studied pathogen is the *Mycobacterium avium* subspecies *paratuberculosis* (Map), a pathogen that causes granulomatous enteritis, called Johne Disease, in ruminants (Harris & Barletta, 2001). Different groups using slow growing culture, PCR of the insertional element IS900, FISH, or serology have recovered Map from intestinal tissues of CD patients, but in ranges varying from 0-100% (Autschbach *et al.*, 2005; Behr & Schurr, 2006). A trial of 2 years with triple antibiotic therapy (clarithromycin, rifabutin, and ethambutol) directed against Mycobacteria in patients with CD failed to achieve a clinical response (Selby *et al.*, 2007). More recently, two studies found no association between the presence of Map and mutations in NOD2 (Bernstein *et al.*, 2007; Bentley *et al.*, 2008).


**Adherent Invasive Escherichia coli**

**Introduction**

*Escherichia coli* is the most abundant facultative anaerobe of the human intestinal microbiota. This microorganism colonizes the intestinal tract within hours of life and thereafter shares mutual benefits with the host (Nataro & Kaper, 1998). Normally, commensal *E. coli* does not cause disease, except in certain conditions, such as seen in a severely immunocompromised host or when there is a break in the intestinal barrier epithelia, such as occurs in the setting of peritonitis (Kaper *et al.*, 2004).

Commensal *E. coli* normally reside in the outer mucus layer in the lumen of the colon. However, some *E coli* have acquired specific virulence genes, which allow the organism to colonize other niches and cause disease. The groups of *E. coli*, highly efficient in the acquisition of virulence genes, conform to a pathotype and are able to cause at least three distinct clinical syndromes: enteric/diarrheal disease, urinary tract infections (UTIs), and sepsis/meningitis (Kaper *et al.*, 2004). The different pathotypes of *E. coli* can be grouped into clones, following their respective antigens: O antigen (polysaccharide-PS), and the H antigen (flagella). A serogroup refers to when the O antigen is shared. If the antigen H is also shared, this defines a serotype.

The types of *E. coli* commonly causing enteric disease include: enteropathogenic *E. coli* (EPEC), enterohemorrhagic *E. coli* (EHEC), enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAEC), enteroinvasive *E. coli* (EIEC), and diffusely adherent *E. coli* (DAEC). All are extracellular pathogens, with the exception of EIEC, which shares many features with *Shigella* including the ability to invade and replicate within epithelial cells. Generally pathogenic *E. coli* strains first adhere to and colonize the mucosal site of infection, evade host defenses, multiply and then induce damage in the infected host (Croxen & Finlay, 2010).
As an example of disease pathogenesis, EPEC and EHEC, which cause infantile diarrhea and hemorrhagic colitis and associated hemolytic-uremic syndrome, respectively, are both attaching and effacing (A/E) pathogens that efface the microvilli and recruit host cell F-actin into pedestals immediately beneath the bacterial attachment site (McDaniel et al., 1995). Similar to other E. coli, EPEC and EHEC use fimbriae/pili for initial attachment, and then the outer membrane protein intimin for intimate attachment.

Intimin binds to the translocated intimin receptor, Tir, which subsequently recruits F-actin to form adhesion pedestals. EPEC and EHEC, in order to translocate Tir and exert disease pathogenesis, use the locus of enterocyte-effacement (LEE) that encodes a type III secretion system on a pathogenicity island integrated into the bacterial chromosome. Through this biological syringe, the pathogens inject a large repertoire of factors, referred to as effector proteins, which subvert cell signalling and induce damage in the infected host cell (Croxen & Finlay, 2010). For example, EPEC secretes EspF, involved in mitochondrion-induced apoptosis, inhibition of phagocytosis, and disruption of the integrity of intercellular tight junctions (Croxen & Finlay, 2010).

In addition to secreting multiple effectors EHEC, similar to EPEC, also elaborates two phage encoded toxins, called Shiga toxin (Stx) I and II. These toxins are released by phage-mediated lysis in response to stress, and then bind to their receptor, globotriaosylceramide (Gb3s) that is present on Paneth cells in the human intestine (Schuller et al., 2007) and in the glomerular endothelium of the kidney (Lingwood et al., 2010). Upon internalization, Shiga toxins induce apoptosis and necrosis in colonic epithelial cells and in vascular endothelial cells in the glomerulus of the kidney (Croxen & Finlay, 2010).

ETEC causes traveller’s diarrhea and secretes toxins as a pathogenic effector following bacterial attachment via pili/fimbriae to enterocytes in the small bowel. These toxins are heat-stable enterotoxins, a and b (STs), and the heat-labile enterotoxin (LT). STa activates guanylyl cyclase in enterocytes, with a consequent increase in cyclic GMP that activates the cystic fibrosis
transmembrane conductance regulator (CFTR), inducing Cl− and water effluxes into the lumen of the small intestine (Croxen & Finlay, 2010). LT, on the other hand, acts like cholera toxin to increase levels of intracellular cAMP and activation of protein kinase A that also opens the CFTR channel and thereby promotes ion and water secretion (Croxen & Finlay, 2010).

**Adherent Invasive *Escherichia coli* in Crohn disease**

In a study performed in France, it was shown that adherent *E. coli* is abnormally increased in early and chronic ileal lesions of patients with CD, with the frequency in detection rates ranging between 50 and 100% of the total number of aerobes and anaerobes. This finding led to the possibility of a particular bacterium being associated with the development of CD (Darfeuille-Michaud et al., 1998). Initially, the French group showed that this *E. coli* adheres to intestinal epithelial cells, with the adhesion being mannose resistant, meaning that this *E. coli* used adhesins other than the type 1 pili commonly expressed by commensal *E. coli*. Moreover, this CD-associated *E. coli* was probed for virulence factors seen in other *E. coli* strains causing diarrheal diseases, but failed to match any of the known virulence factors (Darfeuille-Michaud et al., 1998).

In subsequent studies, one strain, LF82 isolated from the terminal ileum of a patient with CD, had the ability to invade epithelial cells and induce the elongation of microvillar extensions from epithelial cells, engulfing the bacteria in a process called macropinocytosis. Using inhibitors of both actin (cytochalasin D) and microtubule (colchicine) function, bacterial invasion was inhibited, in a dose dependent manner (Boudeau et al., 1999). Moreover, strain LF82 replicates and survives more than 24 h in infected epithelial cells and murine macrophages *in vitro*, inducing the secretion of large amounts of TNFα without inducing host cell apoptosis (Glasser et al., 2001).

Considering the pathogenic effects of these bacteria and the lack of known pathogenic features encountered in the classic pathogenic *E. coli*, these newly recognized CD-associated organisms
were named Adherent-Invasive *E. coli* (AIEC) (Darfeuille-Michaud, 2002). A follow-up study, from the same group in France, reported the presence of AIEC in 36% of ileal lesions in CD patients after surgical resection, compared to just 6% of healthy controls (Darfeuille-Michaud *et al.*, 2004). Years later, other research groups from around the world subsequently independently confirmed these novel findings (Baumgart *et al.*, 2007; Kotlowski *et al.*, 2007; Sasaki *et al.*, 2007; Eaves-Pyles *et al.*, 2008; Martinez-Medina *et al.*, 2009a). Moreover recently, an Italian group confirmed the presence of AIEC in the intestinal mucosa of pediatric patients with CD (Negroni *et al.*, 2011) thus supporting the importance of an association of AIEC with the pathogenesis of at least some cases of CD.

**Virulence mechanisms employed by AIEC**

In order to adhere and colonize epithelial cells, AIEC, strain LF82 employs a type 1 pili variant expressing a fimH adhesin at the tip of the pilus (Boudeau *et al.*, 2001). This adhesin binds D-mannose residues present in epithelial cell surfaces and in secreted glycoproteins, thereby allowing bacterial adherence to a large variety of cells, including epithelial and immune lineages (Sokurenko *et al.*, 1994). *Figure 1.1* outlines the general mechanisms employed by AIEC to exert pathogenicity.

Subsequently, using ileal biopsies derived from patients with CD, researchers discovered that AIEC strain LF82 adheres to the carcinoembryonic antigen-related cell-adhesion molecule (CEACAM)-6 a glycosylated receptor over-expressed in CD patients, compared to controls (Barnich *et al.*, 2007). Moreover, the infection of epithelial cells with AIEC or stimulation with proinflammatory cytokines, TNFα or IFNγ, induces over-expression of CEACAM-6, allowing more adherence by AIEC (Barnich *et al.*, 2007). These results were later validated by using a humanized mouse, called CEABAC10, which overexpress human CEACAM-6 in the intestine, showing that challenge with AIEC produces a severe colitis in mice compared to inoculation with the non-pathogenic *E. coli* K12 strain (Carvalho *et al.*, 2009).
In order to invade epithelial cells, AIEC takes advantage of the localized stress response seen in CD, where the expression of an endoplasmic reticulum chaperone protein, called GP96, acts as a receptor for the protein OMP-A present in outer membrane vesicles secreted by AIEC (Rolhion et al., 2010). This finding has clinical validity since there is a known genetic polymorphism associated with IBD in the X-box binding protein 1 (XBP1) gene, a key transcription factor involved in cell stress responses (Kaser & Blumberg, 2011). Other genetic polymorphisms AIEC uses to favor its pathogenicity are previously described mutations in NOD2, ATG16L1, and IRGM where defects in autophagy allow AIEC non-restricted replication inside the host cell cytoplasm (Lapaquette et al., 2009; Brest et al., 2011).

AIEC strains from serogroup O83, including strain LF82, form bacterial biofilms as a mechanism for persistent colonization, as seen initially in enteroaggregative E. coli (Martinez-Medina et al., 2009b). Moreover, AIEC also disrupts the epithelial barrier function in a model of polarized intestinal epithelia, inducing a redistribution of the tight junction adaptor protein zona occludens-1 (Wine et al., 2009) and redistribution of F-actin and E-cadherin from the apical junctional complex (Sasaki et al., 2007), increasing the likelihood of bacterial translocation into the submucosa and subsequent macrophage infection. Furthermore, AIEC induces proinflammatory cytokine secretion, including CXCL-8 (IL-8) and CCL20, by intestinal epithelial cells with consequent leukocyte and dendritic cell translocation in a co-culture model (Eaves-Pyles et al., 2008).

*In vitro* experiments performed using murine macrophages and AIEC, strain LF82 show the ability of the organism to survive and replicate within macrophages. In order to do this, AIEC expresses the htrA gene coding for the HtrA protein, which is induced by acid stress inside the phagolysosome and allows bacterial intracellular replication (Bringer et al., 2005). Another important virulence mechanism AIEC uses is expression of the dsbA gene that encodes DsbA, a periplasmic oxidoreductase essential for bacterial survival inside macrophages (Bringer et al., 2007).
Recently, (Chassaing et al., 2011) showed that AIEC, strain LF82 uses long polar fimbriae (Lpf) in order to translocate across M cells overlying Peyer’s patches. This interaction was significantly increased in samples derived from Nod2–/– mice.

**Suggested origin and genetic analysis of prototypic AIEC, strain LF82**

All *E. coli* strains can be divided into four different phylogenetic groups: A, B1, B2, and D (Herzer et al., 1990). In most of the studies reporting the presence of AIEC in patients with CD, these strains belong to the B2 and D phylogroup of *E. coli* (Miquel et al., 2010). Analyzing the genome sequence of strain LF82, it clusters and shares variable homology with all the B2 extraintestinal pathogenic *E. coli* (ExPEC) causing urinary tract infection, meningitis, and avian colibacillosis (Russo & Johnson, 2000). Interestingly, LF82 lacks any of the specific virulence genes identified in this group, but features highly conserved homology to plasmids contained in *Salmonella enteritidis* and *Yersinia pestis*, both highly invasive bacterial pathogens, suggesting that LF82 may have evolved from the B2 group by acquisition of these genes through horizontal gene transfer (Miquel et al., 2010).

Searching for predicted coding sequences (CDS), AIEC strain LF82 has 2.6% of the genome not found in any *E. coli* previously sequenced. Moreover, 0.3% of CDS have no homology with genes identified in any pathogenic bacteria. From these CDS, LF82 harbors four potential pathogenicity islands, with genes coding for different virulence factors involved in biofilm formation, bacterial adhesion (*fimH, ompA*, and *ompC*), epithelial invasion (*lpf*), and a type VI secretion system that could inject proteins across the bacterial cell membrane and also present in both *Vibrio cholerae* and *Pseudomonas aeruginosa* (Pukatzki et al., 2009). In addition, this study found genes involved in iron acquisition (a process linked to virulence in many other pathogenic bacteria) (Nairz et al., 2010), serum resistance, proteases, and propanediol catabolism, the latter being indispensable for the utilization of carbon. This gene is also found in *Salmonella* where it is known to be required for bacterial growth in the intestine and survival inside macrophages (Miquel et al., 2010).
In summary, AIEC, strain LF82 seems to have evolved from the B2 group of *E. coli* by acquiring more virulence genes that are harbored by other intestinal pathogens, making this strain a highly sophisticated bacterium, and a good candidate organism for inducing gut inflammation in patients with CD (*Figure 1.1*).
AIEC organized in biofilm layers favour permanent colonization (1). Bacteria adheres to epithelial cells (e.g., CEACAM6) (2) and invade epithelia (e.g., GP96) (3). Inside cells, AIEC replicates (4). Parallel to this, the bacteria induces changes in apical junctional complexes (5) allowing translocation of bacteria. In the submucosa, AIEC invades immune cells (e.g., macrophages) (6) and induces proinflammatory cytokine and chemokine response (7), classical features in of patients with CD.
Interferon signalling as a host response to infection

Introduction

The interferons (IFN’s) are a large cytokine family initially described in 1957 as antiviral agents (Isaacs & Lindenmann, 1957). Since the first description, a large variety of functions have been attributed to IFN’s including: a) provide key innate immune defense against viral infection and to a variety of other microbial pathogens, b) antitumour effects, and c) modulation of host immune responses (Takaoka & Yanai, 2006). The different interferons interact with specific receptors, composed by a pair of heterologous subunits. Interferon upon ligation of the receptor activates the JAK-STAT signal transduction pathway and other signalling cascades in the cell, thereby inducing the transcriptional regulation of thousand of genes (Stark et al., 1998; Hertzog et al., 2011).

There are three major classes of interferons, arranged according to receptor specificity and sequence homology (Bonjardim et al., 2009). Type I interferons (IFNa/β/ω) transduce intracellular signals through the common receptor IFNAR1/2 and are primarily secreted from plasmacytoid dendritic cells, monocytes, macrophages, B lymphocytes, and NK cells (Commins et al., 2009). In the presence of viral infection, viral RNA or viral proteins are recognized by these cells, through their PR receptors, Toll-like receptors (TLRs) 3/7/9, and retinoic acid-inducible gene I (RIG-I)-like receptors, which activate intracellular signalling leading to the secretion of type I interferons (Bonjardim et al., 2009). The antiviral effects consist of blocking viral replication within the infected cell, protecting uninfected cells from further infection, and stimulating innate antiviral immunity by inducing cytotoxic (CD81) lymphocytes and NK cells (Takeuchi & Akira, 2009). Also, IFNa induces the upregulation of class I MHC antigens and triggers antitumour activity (Commins et al., 2009). Moreover, the direct and indirect tumour suppression activity of IFNa/β is one of the major therapeutic applications of recombinant interferon type I (Platanias, 2005).

Interferon γ (IFNg) is the only component of type II interferon responses. IFNg interacts with the IFNGR1/2 for the transduction of its signal and is mainly secreted by T and NK cells and, to
a lesser degree, by other cells such as B cells and by professional antigen-presenting cells (APC) (monocytes/macrophages and dendritic cells) (Schroder et al., 2004; Commins et al., 2009). IFNγ production is stimulated by the cytokine IL-12 and by chemokines secreted by APC at the initial encounter with pathogens that serve as a bridge between the infection and innate immunity responses, where NK cells that secrete IFNγ activate macrophages (Schroder et al., 2004). Also, the combination of IL-12 and IL-18 in APC’s induces the secretion of IFNγ (Munder et al., 1998). The role IFNγ has in innate immunity is discussed in more detail below.

The relatively recently described type III interferons are composed of IFNλ1, IFNλ2, and IFNλ3, which are also called IL-29, IL-28A, and IL-28B, respectively. They share common features with type I interferons regarding antiviral, antiproliferative, and antitumour activities (Takaoka & Yanai, 2006). Unlike other interferons, the receptor for type III IFN is a heterodimeric receptor composed of a short IL10-Rb chain (also called IL-10R2) and a long chain IL-28Ra (also called IL-28R1) (Kelly et al., 2011). These interferons are secreted by dendritic cells, macrophages, subtypes of T cells, and hepatocytes in response to viral infection.

Interestingly, recently described is a SNP in IFNλ3 that is associated with failure to respond to IFNα treatment of hepatitis C virus infection. As a result, there are phase I trials in humans with IFNλ1 and IFNλ3 (Kelly et al., 2011) currently underway. Another interesting function exclusive to this interferon group is that IFNλ shifts immature DCs toward a cell program with the ability to produce forkhead box protein 3 (Foxp3)–expressing CD4+ CD25+ Treg cells in mice (Mennechet & Uze, 2006).

**IFNγ and host immunity**

IFNγ is a key mediator of host immunity and inflammation that serves to protect the host against microbial infections. It is also known to have a role in tumour surveillance and controlling the differentiation of naive CD4+ T cells into Th1 effectors (through IL-12), which mediate cellular immunity against both viral and intracellular bacterial infections (Schoenborn & Wilson, 2007). However, when over-produced IFNγ is involved in the pathogenesis of a variety of autoimmune
diseases including: multiple sclerosis, diabetes mellitus, and, possibly, IBD (Hu & Ivashkiv, 2009; Strober & Fuss, 2011).

IFNγ signals through the Janus kinase (JAK)-signal transducer and activator of transcription 1 (STAT1) intracellular signal transduction pathway in order to achieve the transcription of IFNγ dependent genes (Stark et al., 1998). The JAK/STAT signalling pathway is composed of JAKs (1-3 and Tyk2) and STAT’s (1-6, including STAT5A and STAT5B) (Schroder et al., 2004).

STAT signalling

The 7 members of the STAT family are involved in many different cytokine pathways (Table 1.1), with STAT1 serving as the major protein activated by IFNγ. However, it is also known to be involved in the signal transduction of other cytokine pathways, showing the subsequent complex cross-talk that these molecules exert between different cytokine pathways (Darnell et al., 1994; Hu & Ivashkiv, 2009). For instance, fibroblasts stimulated with IFNγ require a constitutive IFNα/β subthreshold signal in order to be able to respond to IFNγ (Takaoka et al., 2000). Macrophages infected with Listeria monocytogenes induce secretion of type I interferon that downregulates the type II interferon (IFNγ) response, known to be indispensable for macrophage activation and intracellular killing of the pathogen (Rayamajhi et al., 2010).
Table 1.1. **STAT family of proteins.** Adapted from (Commins et al., 2009)

<table>
<thead>
<tr>
<th>STAT Protein</th>
<th>Cytokine</th>
</tr>
</thead>
<tbody>
<tr>
<td>STAT1</td>
<td>IFNα/β* IFNγ* Epidermal growth factor, platelet-derived growth factor, M-CSF, IL6, IL11</td>
</tr>
<tr>
<td>STAT2</td>
<td>IFNα/β* IFNλ</td>
</tr>
<tr>
<td>STAT3</td>
<td>IL6 (IL6 family cytokines, including IL6, oncostatin M, and LIF) trigger STAT3 though the gp130 receptor) IL5, IL10, epidermal growth factor, human growth factor</td>
</tr>
<tr>
<td>STAT4</td>
<td>IL12 (essential endogenous mediator of TH1 differentiation)</td>
</tr>
<tr>
<td>STAT5A and STAT5B†</td>
<td>Prolactin IL2, IL3, IL7, GM-CSF, erythropoietin, thrombopoietin</td>
</tr>
<tr>
<td>STAT6</td>
<td>IL4 (essential endogenous mediator of TH2 differentiation)</td>
</tr>
</tbody>
</table>


†Two distinct genes that are 90% identical.
The STAT1 molecule after transcription undergoes splicing with the resulting formation of 2 isoforms: STAT1α (91 kDa) and STAT1β (84kDa). The transactivation domain (TAD), required for activation of the molecule, is present in the α isoform. The β isoform lacks most of the TAD, but both isoforms contain a SH2 domain, a DNA binding domain, and an N-terminus domain (Figure 1.2) (Najjar & Fagard, 2010).

Following stimulation of cells with IFNγ, the cytokine binds to the IFNγ receptor 1 and 2 present on the exterior surface of the plasma cell membrane and activates the receptor associated JAK1 and JAK2 to create a docking site for latent STAT1 present in the cell cytoplasm. (Figure 1.3) STAT1 is then activated by phosphorylation of tyrosine residue 701, dimerized, and then translocated to the nucleus where it binds DNA in a regulatory segment called the gamma-activated sequence (GAS) to stimulate the transcription of STAT1 target genes (Stark, 2007; Najjar & Fagard, 2010). In order to increase the transcriptional activity of IFNγ, after activation by phosphorylation of tyrosine 701, MAP kinases phosphorylate STAT1 at the serine residue at position 727 (Hu & Ivashkiv, 2009).

On the other hand, there are different feedback mechanisms with a negative effect on the signalling that function as negative regulators of the IFNγ/Jak1,2/STAT1 signalling pathway. The suppressors of cytokine signalling (SOCS) 1 and SOCS 3 are induced proteins with the ability to block the kinase activity of JAK in the receptor, compete with the STAT1 docking site, and trigger proteasome degradation of JAK by ubiquitination (O'Shea & Murray, 2008). Another important regulator of STAT1 activity occurs in the nucleus, where histones are responsible for STAT1 acetylation on lysine residues, with the subsequent removal of tyrosine 701 phosphorylation, and return of the molecule to the cell cytoplasm (Kramer et al., 2009).
Figure 1.2 STAT1 molecule domains. Adapted from (Najjar & Fagard, 2010)
IFNGR1 and IFNGR2 are isolated in the absence of stimulation and have JAK1 and JAK2 constitutively bound to the respective inactive chains of the receptor. Upon the binding of IFNγ to the receptor, the receptor becomes activated (1). This induces the autophosphorylation of JAK2, which in turn phosphorylates JAK1, and then the two kinases phosphorylate the IFNR subunits to form STAT1 binding sites (2). STAT1 binds via its SH2 domain and is phosphorylated on tyrosine 701 (3). The phosphorylated STAT1 proteins dissociate from the receptor and form a reciprocal homodimer which translocates to the nucleus (4). The transcriptional activity of STAT1 homodimer is enhanced by serine phosphorylation at position 727, and binds to specific GAS elements of IFNγ-inducible genes to stimulate their transcription (5).
Role for IFNγ in the innate host defense against intracellular pathogens

When a pathogen enters the host, one of the first cells of the innate immune system it encounters is the macrophage (Schroder et al., 2004). Different activators or stimuli, with IFNγ being one of the most important components, are required for macrophages to mount an appropriate microbiocidal response that finally results in phagocytosis and killing of the pathogen (Boehm et al., 1997) are required. The microbiocidal response induced by IFNγ includes the transcription of genes coding for nitric oxide (NO) and for other reactive nitrogen intermediates (RNI), as well as genes for the NADPH-dependent phagocyte oxidase (NADPH oxidase) system and the formation of reactive oxygen species which destroy the microbe cell wall (Bogdan et al., 2000; Schroder et al., 2004).

IFNγ also promotes the destruction of intracellular pathogens by inducing the expression of the receptor FcRγI, a high affinity receptor which binds extracellular pathogens via IgG in the adaptive phase of the immune response, triggering cell mediated cytotoxicity in myeloid cells (Erbe et al., 1990). IFNγ is also known to prime macrophages in response to LPS, present in the cell wall of Gram negative bacteria, allowing a rapid and robust response to LPS, which activates macrophage microbiocidal effector functions (Kamijo et al., 1993). Moreover, recently it was shown that IFNγ induces a series of small GTPases in mice that protects them against bacterial infection (Listeria and mycobacterium) by inducing phagocyte oxidase, antimicrobial peptide production, and autophagy effectors responsible for the killing of intracellular bacteria (Kim et al., 2011).

Other genes are involved in the production of complement proteins (C2, C4, and Factor B) secreted by macrophages after IFNγ activation to opsonize pathogens for subsequent phagocytosis. At the same time, the specific receptor for binding opsonized pathogens (Mac-1) is also under the effect of IFNγ stimulation (Schroder et al., 2004). Moreover, the induction of genes related to the production of chemoattractants for monocytes/macrophages (CCL2) and T cells (CXCL9) and the vascular adhesion molecules (ICAM-1 and VCAM-1) allow these cells to extravasate into tissues and, thereby, enhance the host immune response to infection (Schroder et al., 2004; Hu & Ivashkiv, 2009).
Mutations in the IFNγ signalling pathway - lessons learned from mice and humans

All mice deficient in STAT1 die after 1 week of inoculation with *L. monocytogenes*, compared to no mortality in wild-type animals challenged with the same pathogen (Meraz *et al.*, 1996). Moreover, this study showed macrophages derived from STAT1+/− mice had a deficient upregulation of key immunomodulatory molecules after induction with IFNγ or IFNα, demonstrating the important function of STAT1 in the crosstalk of the interferon signalling pathway and their key roles in human immunity. Several other studies generated mutations at other levels of the IFNγ signalling pathway which created defects in the induction of IFNγ, its production, or in responses to the cytokine. All defects show increased susceptibility to infection caused by intracellular bacteria, including mycobacteria, Salmonella, protozoans (*Leishmania major*, *Toxoplasma gondii*), and viruses (vaccinia virus, Theiler’s murine encephalomyelitis virus) (Schroder *et al.*, 2004; Schoenborn & Wilson, 2007).

The animal data are confirmed by mutations found in humans related to the IFNγ pathway, where mutations at various levels of the pathway (IL-12p40 subunit, IL-12Rβ1, INFGR1/2, and STAT1) each predispose individuals to mendelian susceptibility to mycobacterial disease (MSMD), a rare congenital syndrome that is characterized by a severe and disseminated infection caused by weakly virulent mycobacterial species (e.g. Bacille Calmette–Guerin (BCG) or other environmental mycobacteria) and Salmonella infections (Zhang *et al.*, 2008; Sologuren *et al.*, 2011). STAT1 mutations, other than those predisposed to intracellular bacterial infections, confer susceptibility to recurrent and severe viral infections (e.g. herpes simplex encephalitis), confirming the important role this molecule has in interferon type I and II responses and their role in immunity against virus infections (Zhang *et al.*, 2008; Chapgier *et al.*, 2009).

Inactivation of STAT1 signalling by pathogens and evasion of the host immune response

Some pathogens, have evolved sophisticated strategies to subvert the host immune system; including both innate and adaptive components, in order to exert pathogenicity (Finlay & McFadden, 2006). In the context of intestinal inflammation, several enteric bacterial pathogens have the ability to benefit from mucosal inflammation by increasing their ability to cross the
epithelial barrier, invade epithelial cells, and express molecules that modulate the host inflammatory response (Pedron & Sansonetti, 2008).

STAT1 is an essential component of the innate and adaptive immune systems in response to infections, (Chapgier et al., 2009; Najjar & Fagard, 2010). On the one hand, the cell host, in order to defend against pathogens, uses pattern recognition receptors (PRR) to recognize MAMPS expressed on the surface of microbes. Upon recognition of MAMPS by these PR receptors, the activation of multiple transcription factors is triggered, including: interferon-regulatory factors (IRFs), NF-κB, and mitogen-activated protein kinases (MAPKs) which induce transcription of type I/III IFN and pro-inflammatory cytokines (e.g. IL-6 and TNFα) (Roy & Mocarski, 2007; Bonjardim et al., 2009). On the other hand, IFNγ is produced by T helper lymphocytes, and is also produced by other cell types in response to infection (Schroder et al., 2004).

Pathogens are able to inhibit at every step of STAT1 activation. For instance, in the cytoplasm STAT1 can be degraded in the proteasome, trapped in high molecular weight complexes, changed to an inactive isoform (βSTAT1), blocked in its ability to undergo nuclear translocation, or not become tyrosine phosphorylated. At the level of the nucleus, microbial pathogens can block signalling by exporting STAT1 back into the cytoplasm, dephosphorylate active STAT1, inhibit transcriptional activity, or induce increased expression of SOCS-1 and SOCS-3 (Najjar & Fagard, 2010). The net result is inducing the down-regulation of the JAK/STAT pathway involved in IFN I and II responses as an effective way to evade host immune responses (Najjar & Fagard, 2010).

The mechanisms exerted by viruses are the most studied; however, the mechanisms employed by bacterial pathogens are not as well characterized and remain incompletely understood.
Chapter 2 – Hypothesis and objectives
**Rationale:** Microbes can interact with the intestinal epithelium by either activating or subverting signalling events in the host. As one example, our laboratory has previously demonstrated the ability of enterohemorrhagic *E. coli* (EHEC) O157:H7, but not enteropathogenic *E. coli* E2348/69, to block the tyrosine phosphorylation of STAT1 after stimulation with IFNγ (Ceponis et al., 2003). There is increasing evidence that AIEC is involved in the pathogenesis of at least some cases of CD. However, it is not known how AIEC causes intestinal inflammation in CD.

**Thesis Objectives:** The present study addressed whether AIEC could block STAT1 tyrosine phosphorylation in epithelial cells in response to IFNγ stimulation, and, if subversion of signalling was observed, to delineate the mechanisms underlying this observation.
Chapter 3 – Methods and Results
Introduction:

Chronic inflammatory bowel disease (IBD) is comprised of two distinct clinical entities; Crohn Disease (CD) and Ulcerative Colitis (UC), which are both characterized by chronic inflammation of the gastrointestinal tract (Kaser et al., 2010). The resulting inflammation arises from an altered interaction of microbes and the intestinal mucosa, influenced by the host harboring specific genetic susceptibility loci and by the environment (Xavier & Podolsky, 2007). Evidence, from both clinical observations and basic science research, support a role for microbes and their crosstalk with the innate immune system involved in the development of IBD (Sartor, 2008). Moreover, results from genome-wide association studies (GWAS) have found multiple susceptibility genes associated with IBD, including genes that code for bacterial recognition, autophagy and immune signalling pathways (Cho, 2008).

Microorganisms have been previously postulated as a potential cause for the development of IBD (Naser et al., 2004; Abubakar et al., 2008), although there is still insufficient evidence to support one specific organism as its cause. Escherichia coli is an abundant commensal bacteria, which is found in the intestinal lumen. However, some strains of E. coli have acquired specific virulence genes, which allow them to colonize the intestinal tract and cause disease in humans (Kaper et al., 2004). The recent discovery of an increased presence of adherent E. coli in the ileum of patients with CD (Darfeuille-Michaud et al., 1998) led to the possibility of a particular bacteria being associated with disease development. This E. coli adheres to and invades intestinal epithelial cells in vitro and infected macrophages without inducing cell death (Boudeau et al., 1999; Glasser et al., 2001). Interestingly, AIEC does not have any of the known virulence factors described in classic pathogenic E. coli strains (Darfeuille-Michaud et al., 1998). Given these characteristics, this E. coli was coined adherent invasive E. coli (AIEC) (Darfeuille-Michaud, 2002). A follow-up study from the same group reported the presence of AIEC in 36% of ileal lesions in CD patients after surgical resection compared to 6% of healthy controls (Darfeuille-Michaud et al., 2004). Others groups subsequently confirmed these novel findings (Baumgart et al., 2007; Kotlowski et al., 2007; Sasaki et al., 2007; Eaves-Pyles et al., 2008; Martinez-Medina et al., 2009a; Negroni et al., 2011).
In order to colonize and invade epithelial cells, AIEC binds the carcinoembryonic antigen-related cell-adhesion molecule (CEACAM)-6 through its type 1 binding pili (Barnich et al., 2007). Furthermore, its long polar fimbriae target Peyer’s patches isolated from human and mice (Chassaing et al., 2011).

Interferon γ (IFNγ) is a key mediator of immunity and inflammation, involved in pathogenesis of CD, as well as in many other autoimmune diseases (Hu & Ivashkiv, 2009). Following stimulation of cells with IFNγ, the cytokine binds to the IFNγ receptor present on surface of the plasma cell membrane and activates the receptor associated JAK1 and JAK2 to create a “docking site” for latent STAT1 present in the cytoplasm. STAT1 is then activated by phosphorylation of tyrosine 701 residue, dimerizes, and then translocates to the nucleus to bind DNA in a regulatory segment, called the gamma-activated sequence (GAS) and thereby stimulates transcription of STAT1 target genes (Stark, 2007).

The STAT family consists of 7 members, and STAT1 is the major protein activated by IFNγ. STAT1 activation leads to the transcription of genes encoding antiviral proteins, microbiocidal molecules, phagocytic receptors, chemokines, cytokines, and antigen-presenting molecules (Hu & Ivashkiv, 2009). Mice deficient in STAT1 die of induced microbial and viral infections, and human mutations in the IFNγ pathway are predisposed to severe mycobacterial and salmonella infections (Meraz et al., 1996; Zhang et al., 2008; Sologuren et al., 2011).

Our laboratory has previously demonstrated the ability of enterohemorrhagic E. coli (EHEC) O157:H7 to block the phosphorylation of STAT1 after stimulation with IFNγ (Ceponis et al., 2003). The present study addressed whether AIEC also prevents the activation of STAT1 in epithelial cells in response to IFNγ stimulation.
Methods:

Bacterial cultures: *E. coli* strains used in this study are summarized in Table 3.1. Bacteria were held on 5% blood agar plates at 4°C and cultured in static, non-aerated Penassay broth (Difco Laboratories, Detroit, MI) for 18h at 37°C. Prior to infection of tissue culture cells, 1 ml of bacteria were centrifuged at 8,000 G for 5 min and the pellet washed with antibiotic-free cell culture medium and resuspended in 0.25 ml of culture medium to a concentration of ~ 1 x 10⁹ colony forming units (CFU) per ml.

In some experiments, AIEC, strain LF82 was heat-killed either by boiling at 100°C for 60 min or fixed in cold methanol for 1h at 4°C and then resuspended in tissue culture medium. In order to confirm death of bacteria, sheep blood agar culture plates were used to culture both boiled and methanol treated bacterial suspension.

To determine the requirement for new bacterial protein synthesis in the inactivation of STAT1, the bacteriostatic antibiotic chloramphenicol, was used (100 µg/ml) in some experiments at varying times (30min - 6h) after challenge of epithelial cells.

Bacterial culture supernatants: To study bacterial-derived secreted factors contained in culture supernatant (CS), 1.0 ml of bacteria grown overnight was incubated for 24h in antibiotic- and FBS-free cell culture medium at 37°C and 5% CO₂ and then passed through a 0.45μm filter (Pall, Mississauga, Ontario, Canada). To confirm the absence of viable bacteria, 0.1 ml of CS was plated onto sheep blood agar plates and cultured overnight at 37°C. The filtrate was applied at varying dilutions onto Caco2-bbe cells. In some experiments, a culture supernatant preparation was subjected to boiling at 100°C in an Eppendorf tube for 30 min to assess the inactivation of an inhibitory effect.

Culture supernatants from AIEC, strain LF82 were incubated with proteinase K (Sigma Aldrich, Oakville, Ontario, Canada) conjugated to agarose beads (10 - 1000 µg/ml) for 1h with shaking at 37°C. As negative controls, culture supernatants were incubated with agarose beads without proteinase K and pre-incubated with bovine serum albumin (BSA; 5%). After incubation, the
Table 3.1 *Escherichia coli* strains employed in this thesis.

<table>
<thead>
<tr>
<th><em>Escherichia coli</em>; serotype</th>
<th>Description</th>
<th>Isolation source</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>LF82; O83:H1</td>
<td>AIEC</td>
<td>Ileum CD patient (France)</td>
<td>(Darfeuille-Michaud <em>et al.</em>, 2004)</td>
</tr>
<tr>
<td>O83; O83:H1</td>
<td>AIEC</td>
<td>Ileum CD patient (Germany)</td>
<td>(Eaves-Pyles <em>et al.</em>, 2008)</td>
</tr>
<tr>
<td>UM146</td>
<td>AIEC</td>
<td>CD patient (Manitoba)</td>
<td>(Sepehri <em>et al.</em>, 2010)</td>
</tr>
<tr>
<td>CL-56; O157:H7</td>
<td>EHEC</td>
<td>Hemorrhagic colitis, HUS</td>
<td>(Philpott <em>et al.</em>, 1998)</td>
</tr>
<tr>
<td>E2348/69; O127:H6</td>
<td>EPEC</td>
<td>Infant diarrhea</td>
<td>(Ismaili <em>et al.</em>, 1999)</td>
</tr>
<tr>
<td>HB101; O:rough</td>
<td>Non pathogenic</td>
<td>Commensal</td>
<td>(Philpott <em>et al.</em>, 1998)</td>
</tr>
</tbody>
</table>
agarose beads were removed by centrifugation (3,000 g for 1 min) and the culture supernatant applied directly onto Caco2-bbe cells.

**Eukaryotic cell culture:**

The epidermoid carcinoma of the larynx derived cell line, HEp2 (ATCC Catalogue No. CCL-23) was cultured in Eagle’s Minimum Essential Medium (MEM) supplemented with 10% FBS, 1.8% sodium bicarbonate, 2.5% penicillin-streptomycin, and 1.2% Fungizone (all reagents were obtained from Invitrogen, Burlington, Ontario, Canada). The colorectal carcinoma derived cell line T84 (ATCC Catalogue No. CCL-248) was grown in a 1:1 mixture of Ham's F12 medium and Dulbecco's modified Eagle's (DMEM) supplemented with 10% (vol/vol) FBS, 2% sodium bicarbonate, and 0.6% L-glutamine, and 2% penicillin-streptomycin. The embryonic intestine derived cell line Intestine 407 (ATCC Catalogue No. CCL-6) was cultured in MEM supplemented with 10% FBS and 2% penicillin-streptomycin. The colorectal adenocarcinoma derived cell line Caco2, clone C2Bbe1 (ATCC Catalogue No. CCL-2102) was cultured in DMEM supplemented with 10% FBS, 1.5% sodium pyruvate (1mM), 0.28% human transferrin (0.01 mg/ml), and 2% penicillin-streptomycin.

All tissue culture epithelial cells used in this study were grown at 37°C with 95% air and 5% CO2. HEp2, T84, and Intestine 407 cells were seeded (~2.5 x 10^6 cells) as a cell monolayer in 6-well cell culture plate (Falcon, New York, NY) and then grown to cell confluence. Caco2-bbe cells were seeded (~1.8 x 10^4 cells) into either 96-well plates (Falcon) or 24-well plates (Costar, Mississauga, Ontario, Canada) for viability and invasion assays, respectively.

To better mimic polarized intestinal epithelium, Caco2-bbe cells were also seeded (~1.3 x 10^5 cells) into polyester membrane Transwell-clear inserts (Corning, Corning, NY) with a membrane diameter of 1.2 mm and membrane pore size of 0.4 µm. and then grown until cell confluence and achieving a transepithelial electrical resistance, measured using chopstick electrodes and a Voltmeter, of >500 Ω/cm^2 (Donato *et al.*, 2010).
All epithelial cells were infected using a Multiplicity of Infection (MOI) of 100:1. After 6 h, cells were washed once with PBS, and then cell media replaced with ± interferon gamma (IFNγ) (R&D Systems, Minneapolis, MN) 50 ng/ml for 30 min at 37°C. Before whole cell extraction, cells were washed twice with cold phosphate buffered saline (PBS) (Sigma Aldrich, Oakville, Ontario, Canada).

In order to assess the impact of AIEC infection in other cell signalling pathways, in some experiments, human epidermal growth factor (EGF) (Sigma Aldrich) 20-100 µM for 30 min at 37°C, was used to stimulate cells following bacterial challenge. Later whole cell protein was blotted for ERK phosphorylation.

**Gentamicin protection assay:** To assess bacterial invasion, confluent Caco2-bbe cells grown in 24-well plates were infected with AIEC, strain LF82, at an multiplicity of infection (MOI) of 100:1, for 5h at 37°C in 5% CO₂. After challenge, cells were washed 3 times with sterile PBS (Invitrogen), and incubated in medium containing gentamicin (100 µg/ml, Sandoz, Quebec, Canada) for 1.5 hr at 37°C to kill extracellular bacteria (Darfeuille-Michaud et al., 2004). After incubation with the antibiotic, Caco2-bbe cells were washed 3 times with sterile PBS and lysed with Triton X-100 (1%) in deionized water for 5 min, and viable intracellular bacteria enumerated by culture on LB plates for 24 h at 37°C. Bacterial internalization into Caco2-bbe cells was calculated as a percentage of viable internalized bacteria relative to the number recovered from untreated cells and challenged with LF82, considered as 100%.

In separate experiments, 30 min prior to the start of the internalization assay, Caco2-bbe cells were incubated with either colchicine (Sigma Aldrich, Oakville, Ontario, Canada ) at 0.005-0.5 µg/ml or cytochalasin D (Sigma Aldrich) at 0.01-1 µg/ml (Darfeuille-Michaud et al., 2004). In some experiments, the inhibitors were maintained for 6h at 37°C in 5% CO₂ during the course of bacterial challenge. Cells were then washed twice with sterile PBS and stimulated with IFNγ (50 ng/ml, 30 min). Whole cell extracts were obtained and stored at -80°C for immunoblotting.

**Western blotting:** Whole-cell protein extracts were obtained from infected epithelial cells, as previously described (Ceponis et al., 2003). Briefly, cells were washed twice with cold PBS,
resuspended in PBS and scraped with a rubber policeman into an Eppendorf tube. Cells were then pelleted at 13,500 G in a Microfuge® centrifuge (Block Scientific, Bohemia, NY) for 10 sec at 4°C. Subsequently, the pellet was resuspended in 0.15 mL of RIPA buffer (1% NP-40, 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate in PBS) supplemented with 50 mM NaF, 150 mM NaCl, 1 mM Na3VO4, 20 µg of phenylmethylsulfonyl fluoride per ml, 15 µg of aprotinin per ml, 2 µg of pepstatin A per ml, and 2 µg of leupeptin per ml (all obtained from Sigma Aldrich) by vortexing and left for 10 min at 4°C. The lysate was then separated by centrifugation at 13,500 G for 10 min at 4°C and the supernatant, containing whole cell protein extracts, separated using precast 10% or 12.5% polyacrylamide gels (Ready Gel; Bio-Rad Laboratories, Hercules, CA).

Gels were electrophoresed at 100 V for 1 to 1.5 h, and migrated proteins then transferred onto nitrocellulose membranes (BioTrace NT; Pall Corporation, Ann Arbor, MI) at 100 V for 1.0 to 1.5 h at 4°C. The nitrocellulose membranes were subsequently incubated in Odyssey blocking buffer (LI-COR Biosciences, Lincoln, NE) for 1 h at 20°C. The blocking buffer was decanted and membranes incubated with either rabbit anti-native STAT1 primary antibody (Cell Signalling, Beverley, MA; 1:1,000 dilution in Odyssey buffer), rabbit anti-phospho (Tyr 701)-STAT1 primary antibody (Cell Signalling; 1:1,000 dilution), rabbit anti-IRF-1 primary antibody (Santa Cruz Biotechnology, Santa Cruz, CA; 1:1,000 dilution) or rabbit anti-phospho-ERK1/2 primary antibody (Cell Signalling; 1:1,000 dilution) and mouse anti-β-actin primary antibody (Sigma Aldrich,1:5,000 dilution) at 4°C overnight on a shaker. Primary antibody solutions were then decanted off and the membranes washed (4X; 5min) with PBS plus 0.1% Tween.

The membranes were incubated with one of the following secondary antibodies: IRDye 800 goat anti-rabbit immunoglobulin G (IgG) (Rockland Immunochemicals, Gilbertsville, PA; 1:20,000 dilution) or Alexa Fluor 680 goat anti-mouse IgG (Molecular Probes, Eugene, OR; 1:20,000 dilution) at RT for 1h on a shaker. Nitrocellulose membranes were again washed four times with PBS-Tween with a final wash in PBS without Tween. Bands were detected by scanning the nitrocellulose membranes on an Odyssey system (LI-COR Biosciences) with both 700-nm and 800-nm channels, at a resolution of 169 µm.
Isolation of mRNA and quantitative reverse transcriptase PCR (qRT-PCR): Caco2-bbe cells grown in Transwells for 10 days were challenged apically with AIEC, strain LF82 for 6h. After 4 h, the basolateral medium was changed to medium containing IFNγ (50 ng/ml; 2 h). Monolayers were then rinsed twice with cold PBS and scraped in 0.8 mL of TRIzol (Invitrogen). RNA extraction was undertaken, following the manufacturer’s instructions. Samples were standardized to 1 µg of mRNA using a Nanodrop 2000c spectrophotometer (Thermo Scientific, New York, NY) and then treated with DNaseI (Invitrogen) and converted to cDNA using a iScript cDNA synthesis kit (Bio-Rad). A dilution of 1:10 of cDNA was used to perform q-RT-PCR. Samples were mixed with iQ SYBR Green supermix and 500 nM of a primer pair.

Primer sequences for IRF-1 and β-actin were as follows: IRF-1 - F, 5’-CGAATCGCTCCTGCAGCAGA; R GCCCAGCTCCGGAACAAACA-3’ (Andersen et al., 2008); β-actin – F, TGCGTGACATTAAGGAGAAG; R, AGGAAGGAAGGCTGGAAGAG (Donato et al., 2010). The qRT-PCR reaction was performed in a CFX1000 thermocycler (Bio-Rad) at 60°C annealing temperature. Changes in expression were quantified using 2^ΔΔCt values for comparison against β-actin mRNA expression.

Cell cytotoxicity assay: Lactate dehydrogenase (LDH) release into the tissue culture medium of bacterial challenged Caco2-bbe cells was quantified using Cytoscan™ (G-Biosciences, St. Louis, MO). Briefly, supernatants of Caco2-bbe cells challenged with bacteria (MOI 100:1) for 6 h were transferred to a new 96-well plate and incubated with a commercial substrate mixture in the dark for 30 min at room temperature. A stop solution was then added and absorbance read at 490 nm in a spectrophotometer (Victor3 Reader, Perkin Elmer, Woodbridge, Ontario, Canada).

The level of LDH release from infected epithelia was calculated as a percentage, compared to total LDH activity measured in lysates of Caco2-bbe cells.
Statistics: Results are expressed as means, ± standard error of the mean (SEM). Comparison of results between multiple groups was performed by using analysis of variance (one way-ANOVA) and Tukey-Kramer multiple comparisons test for results between the different experimental groups. Software used for analysis was InStat3 and Microsoft Excel 2007. Differences with a p value of < 0.05 were considered statistically significant.
Results:

**Adherent-invasive E. coli (AIEC), strain LF82 prevents the activation of STAT1 induced by IFNγ in T84 cells:** Infection of T84 monolayers for 6 h and subsequent stimulation with IFNγ for 30 min demonstrated that in uninfected cells there was a positive band on the immunoblot confirming tyrosine residue phosphorylation (Figure 3.1, Panel A). By contrast, cells challenged with either EHEC O157:H7, strain CL56 or AIEC, strain LF82 resulted in loss of STAT1-induced tyrosine phosphorylation following exposure to IFNγ.

To determine the specificity of this subversion of signalling response, challenge with AIEC was compared to infection of epithelia with enteropathogenic E. coli (EPEC), strain E2348/69 (serotype O126:H7) or the laboratory E. coli, strain HB101. As shown in Figure 3.1, Panel B, only AIEC, strain LF82 decreased tyrosine phosphorylation of STAT1 in response to IFNγ. Integrated intensity values confirmed a significant decrease of STAT1 tyrosine phosphorylation only in T84 cells challenged with EHEC and AIEC (Figure 3.1, Panel C).
Figure 3.1: Adherent-invasive *Escherichia coli* (AIEC), strain LF82 prevents the IFNγ-induced tyrosine phosphorylation of STAT1 in T84 cells. T84 epithelial cells were infected with either enterohemorrhagic *Escherichia coli* (EHEC) serotype O157:H7, strain CL-56 or AIEC, strain LF82 serotype O83:H1 (MOI 100:1). After 6 h, cells were incubated in the presence or absence of IFNγ (50 ng/ml) for 30 min at 37°C in 5% CO₂. Whole cell protein extracts were separated by SDS-PAGE (1 h, at 110 volts at room temperature and then transferred in the same conditions at 4°C) and positively stained bands were detected by infrared imaging.

**Panel A:** IFNγ mediated STAT1 tyrosine phosphorylation was inhibited in T84 cells following infection with either EHEC O157:H7 or AIEC, strain LF82 in upper blot. Bottom blot shows native STAT1.

**Panel B:** A reduction of P-STAT1 in response to IFNγ stimulation was not observed when T84 cells were challenged with either enteropathogenic *E. coli* (EPEC) E2348/69 (serotype O126:H7) or the laboratory *E. coli*, strain HB101.

**Panel C:** Densitometry of the integrated intensities for tyrosine phosphorylation of STAT1, in response to interferon-γ and presence of bacteria, (expressed as a percentage reduction, compared to uninfected, interferon-gamma treated cells). Only LF82 significantly reduced P-STAT1 (68%), compared to EPEC and HB101 (16 and 8%, respectively). The number of separate experiments is noted as the number at the base of each bar histogram. Values are reported as means, ± SEM. ANOVA with Tukey post test, **p<0.001. The western blots shown are a representative taken from at least three independent experiments.
FIGURE 3.1

Panel A
Panel B

<table>
<thead>
<tr>
<th>IFNγ</th>
<th>LF82</th>
<th>EPEC</th>
<th>HB101</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

kDa

42  p-Stat1
91  β-actin
Panel C

Integrated intensity (relative to control)

IFNγ: + 5 5 6 3

LF82  EPEC  HB101
AIEC, strain LF82 inactivation of STAT1 tyrosine phosphorylation in response to IFNγ occurs in multiple epithelial cells lines: To assess if the effect in T84 cells was cell line dependent, three other human epithelial cell lines were challenged with AIEC, strain LF82. As shown in Figure 3.2, AIEC prevented the activation of STAT1 in response to IFNγ in Intestine 407 (panel A), HEp-2 (panel B), and Caco-2 clone Bbe cell lines (panel C).

AIEC, strain LF82 decreases tyrosine phosphorylation of STAT1 in a time-dependent manner: Caco2-bbe cells were subjected to varying times of challenge with AIEC. Subversion of STAT1 signalling was not observed at 1 h and 3 h after pathogen challenge (Figure 3.3). Only after 6 h of infection, there was an inactivation of STAT1 tyrosine phosphorylation in response to IFNγ. Interestingly, there was also a decrease of the α-subunit of native STAT-1 in either the absence or presence of IFNγ. Taken together, these results indicate that after 6 h of AIEC, strain LF82 challenge there was degradation of α-STAT1 protein, which was accompanied by a corresponding loss of tyrosine phosphorylation.
Figure 3.2: AIEC, strain LF82 inactivation of STAT1 in response to IFNγ occurs in multiple epithelial cells types.

Intestine 407 (Panel A), HEp2 (Panel B) and Caco2-bbe (Panel C) tissue culture epithelial cells were challenged with either EHEC O157:H7 or AIEC, strain LF82 (MOI 100:1) for 6 h. And then incubated in the presence of IFNγ (50 ng/ml) for 30 min at 37°C in 5% CO₂. Whole cell protein extracts were separated by SDS-PAGE and positively stained bands detected by infrared imaging (Odyssey). The western blot shown is a representative taken from at least three independent experiments performed with each cell line.
Figure 3.2
Figure 3.3: AIEC, strain LF82 decreases tyrosine phosphorylation of STAT1 in Caco2-bbe cells exposed to IFNγ in a time-dependent manner.

Caco2-bbe cells exposed to AIEC, strain LF82 for 1, 3 or 6 h at 37°C in 5% CO₂ and then challenged for 30 min with IFNγ (50 ng/ml) showed a decrease in P-STAT1 and in the α subunit of native STAT1 after 6 h of infection. Whole cell protein extracts were separated by SDS-PAGE and positively stained bands detected by IR imaging (Odyssey). The western blot shown is a representative one, taken from at least three independent experiments.
Figure 3.3

| LF82 (h): | - | - | 1 | 3 | 6 | 1 | 3 | 6 |
| IFN$_{\gamma}$ | - | + | - | - | - | + | + | + |

- p-Stat1
- β-actin
- n-Stat1
- β-actin
IFNγ-induced tyrosine phosphorylation of STAT1 is prevented by different AIEC strains isolated from patients with Crohn disease: Intestine 407 cells were challenged with three different AIEC strains, with results confirming the inactivation of STAT1 tyrosine phosphorylation (Figure 3.4, Panel A; results quantified in Panel B).
Figure 3.4: Inactivation of STAT1 tyrosine phosphorylation induced by IFNγ occurs in response to multiple AIEC strains.

Panel A: Intestine 407 cells incubated in the presence of IFNγ (50 ng/ml) for 30 min at 37°C in 5% CO₂ prior after challenge with AIEC strain, LF82, AIEC strain O83:H1 (Germany isolate) and AIEC, strain UM146 (from Univ. Manitoba, Winnipeg, Canada) (MOI 100:1). Each of the AIEC strains originally isolated from a patient with Crohn disease showed no activation of STAT1. Whole cell proteins were separated by SDS-PAGE and positively stained phosphotyrosine bands detected by infrared imaging (Odyssey).

Panel B: Integrated intensities for tyrosine phosphorylation indicate that AIEC strains inhibited the tyrosine phosphorylation of STAT1 in Intestine 407 cells incubated with interferon-γ. Only strains LF82 and O83 significantly reduce P-STAT1 (57% and 59%, respectively), whereas UM146 did not reach statistical significance (48%). The number at the base of each bar histogram indicates the number of separate independent experiments. Values are reported as means, ± SEM. ANOVA with Tukey post test, *p<0.05. The western blot shown is a representative taken from at least three independent experiments.
FIGURE 3.4

Panel A
Panel B

Integrated intensity (relative to control)

IFNγ: 6 5 5 4 4

LF82  O83  UM 146
Live bacteria, with intact protein synthesis, are required for the inactivation of STAT1 induced tyrosine phosphorylation: By western blotting, chloramphenicol treatment of AIEC blocked subversion of STAT1 signalling, but only when the antibiotic was added either before or early during the time course of infection (Figure 3.5, Panel A). Using methanol-fixed or heat-killed bacteria (Figure 3.5, Panel B) resulted in loss of the ability of the pathogen to block STAT1 tyrosine phosphorylation responses to IFNγ.

Bacterial invasion is not required to prevent the activation of STAT1 in response to IFNγ: To determine whether AIEC, strain LF82 must invade the host cell cytosol to block STAT1 signalling, the cytoskeleton inhibitors cytochalasin D and colchicines were employed. Gentamicin protection assays demonstrated a dose-dependent decrease of AIEC internalization using both drugs (Figure 3.6, Panel A). By western blotting, using the highest doses of inhibitors where bacterial invasion was abolished, AIEC, strain LF82 still prevented STAT1 tyrosine phosphorylation (Figure 3.6, Panels B and C).
Figure 3.5: Live AIEC, with *de novo* protein synthesis, are required to prevent STAT1 tyrosine phosphorylation in response to interferon-γ.

**Panel A:** Intestine 407 cells challenged with strain LF82 and incubated with the protein synthesis inhibitor and bacteriostatic antibiotic chloramphenicol (Chlor.) (100 µg/ml) for varying periods of time and then challenged with IFNγ (50 ng/ml) for 30 min at 37°C in 5% CO₂. Immunoblotting of proteins separated by SDS-PAGE showed decreased activation of STAT1 only if strain LF82 was exposed to the antibiotic for prolonged periods of time. Methanol-fixed LF82 (MOI 100:1) did not prevent STAT1 activation following stimulation of tissue culture cells with IFNγ.

**Panel B:** Intestine 407 cells challenged with EHEC, AIEC, strain LF82 or heat killed (100°C for 1 h) (MOI 100:1) and applied to cells. Stimulation with IFNγ for 30 min at 37°C in 5% CO₂. Subsequent tyrosine phosphorylation of STAT1 was detected by immunoblotting of proteins extracted from cells. The western blot showed is a representative of at least three independent experiments.
Figure 3.5

Panel A and B

<table>
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<tr>
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</tr>
<tr>
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IFNγ; - | + | + | + | + | + | + | + | + |

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<th>HK</th>
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<tr>
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<td>-</td>
<td>+</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
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</table>

kDa

91

42

p-Stat1

β-actin
Figure 3.6: AIEC, strain LF82 internalization into Caco2-bbe cells is not required to prevent the activation of STAT1 in response to IFNγ.

Panel A: Treatment of Caco2-bbe cells with either cytochalasin D or colchicine for 30 min prior infection and present up to 5 h of infection reduced LF82 internalization in a dose-dependent manner. Viable internalized bacteria were enumerated from lysed epithelial cells after incubation with gentamicin (100 mg/ml for 90 min at 37°C and 5% CO2) to kill extracellular bacteria. Western blots of whole cell protein extracts from Caco2-bbe cells treated with either cytochalasin D (1.0 µg/ml, Panel B) or colchicine (0.5 µg/ml, Panel C) for 30 min prior to LF82 (MOI 100:1) challenge for 6 h at 37°C in 5% CO2 demonstrated that reduced bacterial internalization did not prevent STAT1 phosphorylation in response to IFNγ; n=1.
Figure 3.6

Panel A

Percentage of viable internalized bacteria related to untreated
Panel B and C

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<td>+</td>
</tr>
<tr>
<td>β-actin</td>
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Subversion of cell signalling is mediated by a secreted product of AIEC, strain LF82: To determine if a bacterial secreted factor in the basolateral compartment was responsible for the observed inhibitory effect, we applied bacteria to the basolateral compartment of the Transwell, showing no inactivation effect (**Figure 3.7, Panel A**) But when live LF82 or its culture supernatants were applied apically to polarized Caco2-bbe cells, they were shown to block tyrosine phosphorylation of STAT1 (**Figure 3.7, Panel B**). The culture supernatant was resistant to heat inactivation, but when subject to proteolysis in the presence of proteinase-K there was a correspondent loss of biological function (**Figure 3.7, Panel C**).

Disruption of STAT1 tyrosine phosphorylation by AIEC, strains LF82 suppresses downstream signal transduction events: The activation of interferon related factor (IRF)-1 was tested in Caco2-bbe cells challenged with AIEC, strain LF82 and then stimulated with IFNγ. Whereas IFNγ stimulated cells showed an increase of IRF-1 activation, cells challenged with AIEC, strain LF82 had reduced activation of IRF-1 (**Figure 3.8, Panel A**). Caco2-bbe cells stimulated with IFNγ showed a 23.6 fold increased expression of IRF-1 mRNA compared to untreated cells, whereas cells challenged with AIEC, strain LF82 or its CS had a markedly reduced level of mRNA IRF-1 expression in response to IFNγ (**Figure 3.8, Panels B and C**).
Figure 3.7: A factor secreted into culture supernatant is responsible for blocking STAT1 signalling.

Panel A: Caco2-bbe cells grown in Transwells, apical infection versus basolateral infection (MOI 100:1) (6 h at 37°C and 5% CO₂) and subsequent stimulation with IFNγ (50 ng/ml) for 30 min. Whole cell protein extracts separated by SDS-PAGE and positively stained phosphotyrosine bands detected by infrared imaging (Odyssey). The immunoblot shown is representative of at least three independent experiments.

Panel B: AIEC, strain LF82, grown for 24 h in Caco2-bbe cell culture medium and then sterilized with passage through a microfilter (0.45 µm) and then applied to apical compartment of Caco2-bbe cells grown in Transwells for 6 h and subsequent stimulation with IFNγ (50 ng/ml) for 30 min. Whole cell protein extracts were separated by SDS-PAGE and positively stained phosphotyrosine bands detected by infrared imaging (Odyssey). The western blot shown is a representative of at least three independent experiments.

Panel C: Culture supernatants from AIEC, strain LF82 boiled for 45 min at 100°C, or incubated with proteinase K (10 - 1000 µg/ml) or with BSA 5% conjugated to agarose beads, then applied for 6 h to apical compartment of Caco2-bbe cells in Transwells (37°C and 5% CO₂). Subsequent stimulation with IFNγ (50 ng/ml) for 30 min. Whole cell protein extracts were separated by SDS-PAGE and positively stained phosphotyrosine bands detected by infrared imaging (Odyssey). The western blot is representative of two separate experiments.
Figure 3.7

Panel A and B

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</tr>
<tr>
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**IFNγ:**

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**CS**

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**p-Stat1**

**non-specific band**

**β-actin**
Panel C

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p-Stat1

β-actin
Figure 3.8: AIEC, strain LF82 inactivation of STAT1 tyrosine phosphorylation interferes with down-stream signal transduction events.

Panel A: Western blot analysis of whole cell protein extracts taken from Caco2-bbe cells challenged with LF82 (MOI 100:1) for 6 h 37°C in 5% CO₂ demonstrated that LF82 inhibited interferon related factor (IRF)-1 activation in both the absence and presence of IFNγ (50 ng/ml). Western blot representative of three independent experiments.

Panel B IRF-1 mRNA quantification by qRT-PCR, AIEC LF82 infection (MOI 100:1) for 6 h at 37°C in 5% CO₂ on apical compartment of Transwells and stimulation with IFNγ (50 ng/ml) on the basolateral side, at 4 h of infection until the end of experiment, reduces significantly the increased expression of IRF-1 in response to IFNγ. The data presented are from three separate experiments. Values are reported as means, ± SEM. ANOVA with Tukey post test, **p<0.001.

Panel C: IRF-1 mRNA quantification by qRT-PCR. AIEC strain LF82, EPEC infection (MOI 100:1) or Culture Supernatant of LF82 applied apical compartment of Transwells for 6 h at 37°C in 5% CO₂ and stimulation with IFNγ (50 ng/ml) on the basolateral side, at 4 h of infection until the end of experiment, Only AIEC LF82 and its CS reduces significantly the increased expression of IRF-1 in response to IFNγ. n =1.
Figure 3.8

Panel A and B
Panel C

Gene Expression (fold increase)

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**AIEC infection does not affect cell viability or other cell signalling cascades:** An LDH release assay in Caco2-bbe cells after bacterial challenge for 6 h at a MOI 100:1 demonstrated no significant increase in LDH release, compared to untreated cells (Figure 3.9, Panel A), indicating that the lack of reaction in response to IFNγ stimulation was not due to cell death.

Using both Intestine 407 and Caco2-bbe cells, stimulation with epidermal growth factor demonstrated a dose-dependent increase in ERK phosphorylation (Figure 3.9, Panels B and C), indicating that AIEC inactivation of STAT1 is not occurring due to a generalized effect on cell signalling pathways.
Figure 3.9 Infection of AIEC does not interfere with all cell signalling processes and does not affect epithelial cell viability.

Panel A: Viability of Caco2-bbe cells measured by LDH activity assay as a marker for cytotoxicity. LDH activity after 6 h in untreated, EPEC, LF82 and LF82 CS challenged cells. The level of cytotoxicity was calculated as a percentage compared to the total LDH activity measured in the same number of seeded Caco2-bbe (~13,000 cells) treated with 10x lysis buffer. No significant differences in the treatment groups compared to untreated. n=3

Panel B and C: AIEC, strain LF82 does not affect the epidermal growth factor (EGF)-induced phosphorylation of ERK. Intestine 407 cells (B) and Caco2-bbe cells (C) were challenged with LF82 (MOI 100:1) and then incubated with varying concentrations of epidermal growth factor for 30 min at 37°C in 5% CO₂. Whole cell protein extracts were separated by SDS-PAGE and positively stained phosphotyrosine bands detected by infrared imaging (Odyssey). The western blots presented are a representative of at least two independent experiments for each cell line.
Figure 3.9

Panel A
Panel B and C

<table>
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<th>-</th>
<th>20</th>
<th>50</th>
<th>100</th>
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**LF 82**

- P-ERK1/2
- β-actin

**LF 82**

- P-ERK1/2
- β-actin
Chapter 4 – Discussion
Discussion:

In the course of my graduate studies, I have shown for the first time that AIEC associated with Crohn disease is able to evade one of the major parts of the human innate immune system by blocking the IFNγ/JAK/STAT1 signal transduction pathway. The effects were observed in multiple human intestinal epithelial cell lines; first using a model of non-polarized epithelial monolayers (T84, Intestine 407 and Hep2) and then validating some of the results in a polarized epithelium (Caco2-bbe), with the purpose of better mimicking the human intestinal epithelium. The results obtained are consistent with the evidence that human pathogens have developed multiple strategies to evade host immune responses (Finlay & McFadden, 2006). In the context of intestinal inflammation, several enteric pathogens have demonstrated the ability to dampen the host inflammatory response, which could facilitate their survival and growth (Pedron & Sansonetti, 2008).

I have shown that AIEC as well as EHEC, but not EPEC or the commensal strain HB101, were able to block the phosphorylation of STAT1 after IFNγ stimulation. It is unlikely that the AIEC ability to subvert the IFNγ pathway is mediated by any of the classical E. coli virulence factors since this strain lacks known bacterial virulence factors (Darfeuille-Michaud et al., 1998). Interestingly, inactivation of STAT1 tyrosine phosphorylation by EHEC O157:H7 has also been reported as independent of the type III secretion system and intimin (Ceponis et al., 2003). Moreover, we have recently demonstrated that EHEC O157:H7 exerts this effect dependent on Shiga toxins 1 and 2 secreted by the bacteria, indicating that AIEC acts differently because it dose not contain the phages encoding for these multimeric toxins (Ho, Ossa et al. In Preparation).

The interferon signalling cascade is a key component of the immune system, composed by three major subgroups; α/β (type I), γ (type II) and λ (type III) (Zhang et al., 2008). The major function for type I and III interferons is to generate responses in the host immune system in order to resist viral infections (Zhang et al., 2008).
IFN\(\gamma\) also plays a role in protecting the host against viral infection, but it is also involved in the defence against infection by invasive bacterial pathogens, including Mycobacteria, Salmonella and *Listeria monocytogenes* (Schroder *et al.*, 2004; Zhang *et al.*, 2008).

Pathogens inhibit different steps of STAT1 activation (Najjar & Fagard, 2010). For instance, in the cytoplasm STAT1 can be degraded in the proteasome, trapped in high molecular weights complexes, changed to its inactive isoform (\(\beta\)-STAT1), blocked in its ability to undergo nuclear translocation or not become tyrosine phosphorylated (Najjar & Fagard, 2010). At the level of the nucleus, microbial pathogens can block signalling by exporting STAT1 back into the cytoplasm, dephosphorylating active STAT1, inhibiting transcriptional activity or inducing the expression suppressors of cytokine signalling (SOCS)-1 and SOCS-3, which are members of a family of inducible proteins that down-regulate the JAK/STAT1 signal transduction pathway by either binding to the IFN\(\gamma\)R to block STAT1 phosphorylation or by competing with STAT1 for phosphorylation sites (O'Shea & Murray, 2008; Najjar & Fagard, 2010).

To elucidate the mechanism employed by AIEC, LF82 to decrease STAT1 tyrosine phosphorylation, the bacteriostatic antibiotic chloramphenicol was used to show that live bacteria, with *de novo* protein synthesis were required to exert the suppressive effects. Having in mind the invasive properties of AIEC, the gentamicin protection invasion assay was employed to demonstrate that LF82 internalization into epithelial cells was not required to subvert the interferon-\(\gamma\) signal transduction pathway. This finding led me to consider the possibility that a secreted factor from bacteria could be responsible for the observed effects. To address this possibility, bacteria-free culture supernatants were tested to show that a factor secreted into the tissue culture medium could mediate inactivation of STAT1 signalling. Further characterization of the culture supernatants indicated that the secreted factor was a protein, as evidenced by the use of proteinase K to abolish the observed effects. Further characterization of the culture supernatants showed that the inhibitory factor was resistant to heat which indicates the possibility of a peptide or small molecule.
Using the Caco2-bbe polarized epithelial cell line, only apical challenge with AIEC, strain LF82 prevented tyrosine phosphorylation of STAT1 in association with a decreased level of the αSTAT1 subunit. This finding suggested that, at least in Caco2-bbe cells, the decrease in STAT1 phosphorylation could have to do more with protein abundance of native STAT1, rather than reduced tyrosine phosphorylation. Interestingly, viruses from Paramyxoviridae family (mumps, parainfluenza) are known to secrete proteins into the cytoplasm of infected host cells that cause the degradation of STAT1 by recruiting a cellular E3-ubiquitin-ligase with subsequent ubiquitination and degradation in the proteasome (Ulane & Horvath, 2002; Yokosawa et al., 2002). Similarly, Leishmania donovani infection of murine macrophages induces the degradation of α-STAT1 through cellular ubiquitination and subsequent proteosome degradation (Forget et al., 2005).

In order to elucidate the consequences of the inactivation of STAT1 signalling, and verify the subversion of the IFNγ pathway, I studied the expression of interferon related factor 1 (IRF-1), which is a downstream target that becomes upregulated upon IFNγ stimulation and is known to be required for iNOS induction in macrophages in response to IFNγ and is also an important cell cycle regulator with roles in growth inhibition and apoptosis (Kamijo et al., 1994; Tamura et al., 2008). Using western blot analysis, I showed that IRF1 expression was inhibited in Caco2-bbe cells by AIEC and its culture supernatants. These findings were verified using a complementary qRT-PCR analysis, which found that IRF1 gene expression, was significantly down-regulated when the cells were exposed to AIEC or its culture supernatant prior to IFNγ stimulation. Thus, these experiments confirm the downstream effect of AIEC in inactivating the IFNγ signal transduction pathway and its important potential consequences.

As mentioned earlier in this thesis, mutations in the IFNγ signalling pathway, including mutations in STAT1, predispose patients to suffer from severe intracellular infections. These gene mutations exert a hypofunction of the STAT1 molecule, where dominant mutations impair STAT1 phosphorylation or reduce its binding to DNA in response to IFNγ, and recessive mutations cause a loss in native STAT1 expression (Zhang et al., 2008).
On the other hand, recent evidence shows that another dominant gene mutation in STAT1 predisposes patients to chronic extracellular fungal infections by fungi in a condition referred to as chronic mucocutaneous candidiasis disease (CMCD). This mutation enhances STAT1 function and impairs immunity provided by IL-17 and the Th17 T cell lineages (Liu et al., 2011). Taken together, these findings show that an imbalance in the activity of STAT1 (either too little or too much) disrupts host immune function, thereby predisposing affected patients to chronic infections.

The concept of immunity balance is also supported by a recent publication where the use of daily low doses of recombinant interleukin-2 was shown to ameliorate symptoms of intractable steroid resistant chronic graft-versus-host disease after hematopoietic stem cell transplant in which there is a dysfunction of T regulatory (Treg) cells and overactivity of T effector (Teff) cells. The low doses of IL-2 induced an expansion of Treg (Koreth et al., 2011). On the other hand, high doses of IL-2 are associated with chronic infections and increased risk of cancers due to an overexpansion of Tregs (Bluestone, 2011).

To confirm that AIEC infection did not alter cell viability or alter other important molecular signalling cascades, I showed by using an LDH assay that cell viability was not significantly affected after infection with these pathogens, and that after EGF stimulation; the ERK signalling pathway was not affected. In summary, these data demonstrate that the CD-associated AIEC pathogen is able to invade epithelial cells and selectively evade the immune system without causing cell death, thereby allowing bacterial replication and potential further spread to other sites beyond the surface epithelium.
Chapter 5 – Conclusions and Future Directions
Conclusions:

The findings arising from the research undertaken during the course of my graduate studies and which are presented in this thesis add to the body of current knowledge by describing a new interesting pathogenic feature to AIEC evading one important aspect of the immune response against pathogens.

Taken in the context of CD, the observed effects on the inactivation of the STAT1 pathway supports the hypothesis that AIEC evasion of the immune system could enhance bacterial survival and promote its long-term colonization in the gut. Previously, in collaboration with others in the laboratory, I showed that AIEC disrupts epithelial barrier function after 16 h of bacterial challenge (Wine et al., 2009). Others have shown, using a co-culture Transwell model, that AIEC induces the secretion of pro-inflammatory chemokines and cytokines by epithelia cells, with consequent leukocyte and dendritic cell translocation (Eaves-Pyles et al., 2008). AIEC invades macrophages in vitro and survives intracellularly for more than 24 h without inducing cell death (Glasser et al., 2001; Bringer et al., 2005; Bringer et al., 2007). These in vitro findings are supported by animal models of AIEC infection and by data obtained from human intestine (Barnich et al., 2007; Carvalho et al., 2009; Chassaing et al., 2011).

Taken together, it is plausible to consider that in patients with CD the presence of AIEC colonization has important consequences in the balance of the immune system, thereby contributing to the acute and chronic mucosal inflammation invariably seen in patients with CD. A proposed model for how AIEC associated with CD could be evading the host immune system is presented in Figure 5.1.
Figure 5.1 Model proposing how AIEC evades the IFNγ signalling pathway in order to enhance its pathogenicity.

AIEC organized in a biofilm favors permanent colonization (1). Bacteria adhere to epithelial cells (for example, via type 1 pilus mediated binding to CEACAM6) (2) and invade into intestinal epithelia (e.g., GP96) (3). Inside cells, AIEC replicates (4) and induce changes in apical junctional complexes (5) allowing translocation of bacteria. In the submucosa, AIEC invades into immune cells (including macrophages and dendritic cells) (6) resulting in the induction of proinflammatory cytokine and chemokine responses (7). Simultaneously, a secreted bacterial factor - likely protein in nature –results in the inactivation of the IFNγ/Jak/STAT1 signalling in epithelial and immune cells (8), thereby promoting AIEC survival and contributing to acute and chronic inflammation seen in CD.
Future Directions:

To compliment the results presented in this thesis, I am interested in assessing the translocation of STAT1 protein to the nucleus, which is required for the transcription of thousand of genes under IFNγ stimulation after binding to the GAS (Haspel & Darnell, 1999). For this purpose, the electrophoretic mobility shift assay (EMSA) could be employed, as in previous studies (Ceponis et al., 2003; Forget et al., 2005). Based on data presented in this thesis, showing AIEC suppression of IFNγ down-stream signalling, I predict that there will be an absence of STAT1 binding to DNA in samples infected with AIEC for 6 h followed by stimulation with the cytokine.

Interestingly, inactivation of STAT1 was seen in response to the three different AIEC strains used, even though the strain UM146 also decreased the activation of STAT1 (but not reaching statistical significance), all belong to the same phylum group of E. coli (B2), and are genetically similar to the extra-intestinal E. coli (ExPEC) causing urinary tract infections and meningitis (Miquel et al., 2010). These AIEC strains are also known to harbour common potential virulence factors, such as a type VI secretion system (t6ss) (Krause et al., 2010; Miquel et al., 2010; Nash et al., 2010), and also present in other pathogens, such as Vibrio cholerae and Pseudomonas, allowing pathogens to secrete toxin proteins either into the external medium or directly into eukaryotic cells (Cascales, 2008), which both could mediate the subversion of IFNγ signal transduction pathway. To asses this hypothesis, future studies could be undertaken to identify the type 6 secretion autoaggregation system (T6SS) associated gene sequences, which are located in the pathogenicity islands (PAI) I and III in the genome of strain LF82 (Miquel et al., 2010). The lambda red recombination technique (Datsenko & Wanner, 2000) could be used to delete specific T6SS gene sequences to create an LF82 isogenic mutant (Δt6ss).
After confirming that the gene of interest has been deleted, by selecting antibiotic resistant clones, and the viability and growth properties of the isogenic mutant, one would then compare the impact of the gene deletion on the inactivation of STAT1 signalling, compared with wild type, parental strain LF82. If the effect is lost in Δ*t6ss*, then the final confirmation of importance of the type 6 secretion system should be transcomplementation of the *t6ss* gene back into the isogenic mutant by using plasmid vectors (Guzman *et al.*, 1995), with an expected recovery in the inactivation of IFNγ/Jak1,2/STAT1 signalling.

For further characterization of bacterial proteins secreted into tissue culture medium, size exclusion column chromatography could be used to isolate proteins/peptides by molecular mass (Irvine, 2001) and the various size fractions then used to test for the ability to suppress the signalling pathway. Secreted proteins then could be further characterized by both mass and surface charge by using a 2-dimension gel (2D gel). To obtain the molecular composition of 2D-mapped proteins, bands can be excised from the 2D gel, and then analyzed by a technique denominated protein fingerprinting using mass spectrometry (Henzel *et al.*, 1993).

From my results using Caco2-bbe cells, one can also suggest a role for an AIEC secreted factor in inducing the ubiquitination of the αSTAT1 molecule, as a mechanism to evade the immune system. Forget *et al.* (2005) used the proteasome inhibitors MG-132 and clasto-lactacystin-lactone (c-lactacystin) 1 h prior infection of murine macrophages with *Leishmania* to show the restoration of αSTAT1 protein in cell lysates after infectious challenge. For similar experiments using AIEC, I would first propose to test cell viability in the presence of the inhibitors (either MG-132 or c-lactacystin, or both) by incubating cells for the time course of infection and then assessing the STAT tyrosine phosphorylation response to IFNγ stimulation. After confirming cell viability, I would incubate Caco2-bbe cells with the inhibitors for varying time periods (1–4 h) and then follow the same protocol used in this thesis for infection with AIEC, strain LF82 and IFNγ stimulation to test whether the STAT1 signalling cascade was still subverted by the CD-associated bacterial pathogen. This experimental approach could be employed to define the involvement of the proteasome in the degradation of αSTAT1 after AIEC, LF82 challenge of human epithelial cells.
A limitation of my studies relates to the focus on an *in vitro* experimental setting. An interesting future aspect of this research program would be to test the effects of AIEC in macrophages and then extend the findings to both an animal model of IBD and to humans with Crohn disease. In addition, it will be important to assess the role of AIEC in subverting signalling in response to IFNα/β and to the other cytokines that are known to activate STAT1 (Table 1.1). It is also of interest to assess the role of AIEC infection involving other STAT molecules, especially STAT3, known to be activated in patients with CD, and other host response factors to microbial infection, such as TLR and NLR activation and the subsequent activation of the transcription factor NF-kB.

An extension of my studies would be to undertake host gene expression profiling in response to AIEC infection using, for example, a microarray technique. Such an approach would provide a more integrative and high level understanding of which host genes are regulated in response to AIEC infection. Such information would guide future targeting of select signalling pathways involved in the host response to AIEC infection in the human host, with the potential to ultimately design therapeutic targets for use in intervening in chronic gut inflammation.
Chapter 6 – References Cited
References:


