Characterising molecular mechanisms of Crohn’s disease-associated *Escherichia coli* that enable their survival and replication within macrophages

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor in Philosophy by

Dr Ahmed Tawfik

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Dedication

This thesis is dedicated to all members of my family, particularly my father and mother.
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Abstract

Mucosa-associated adherent, invasive *Escherichia coli* (AIEC), found in increased number in Crohn’s disease (CD) ileal and colonic mucosa, can survive and replicate within underlying host immune competent cells (e.g. macrophages and dendritic cells) without triggering host cell death. The intra-macrophage environment plays an essential role in bacterial killing where engulfed bacteria are exposed to a hostile environment of low pH, high levels of proteolytic/lysosomal enzymes, high nitrosative and high oxidative stress, and the activation of a respiratory burst with generation of superoxide ions. Although a few stress response genes have been identified that likely support the paradigm ileal AIEC isolate LF82 to survive and replicate within the macrophage, the key molecular mechanisms involved in supporting Crohn’s disease (CD) mucosa-associated AIEC to resist killing by host mucosal macrophages within harsh environment of the phagolysosome still remains largely unclear. Here we aimed to compare the ability of a number of *E. coli* strains to survive and replicate inside macrophages, including a number of clinical isolates (from CD, colorectal cancer (CRC) and ulcerative colitis (UC) patients and other infective or non-inflamed sources), and this to toleration of growth in chemical-induced stress conditions mimicking the intra-phagolysosome environment. In addition, a focus was to further understand the molecular mechanisms responsible for acid tolerance of the paradigm CD isolates and examine their replication within macrophages defective in NF-κB pathway signalling. Finally, to also assess whether CD AIEC possess ability to alter host oxidative stress response gene expression in macrophages to support their survival/replication.

Both ileal and colonic CD isolates (AIEC) were found to possess ability to either survive and/or replicate within murine macrophages (i.e. J774-A1 cell-line and wild-type (WT) C57BL/6 bone marrow derived macrophages [BMDM]) and to tolerate all stress conditions mimicking those within the phagolysosome, e.g. low nutrient, high acid, high nitrosative, high oxidative stress including exposure to superoxide ions. Interestingly pathogenic *E. coli* isolates from urinary tract infection (UTI) and some healthy-mucosa associated *E. coli* strains behaved similarly. Crohn’s AIEC were unable to survive and replicate inside *Nfkb1*−/− and *Nfkb2*−/− BMDM, whilst they survived/replicated within WT and *c-Rel*−/− BMDM. Thus Crohn’s AIEC survival and replication appears dependent on host NFκB signalling within the
Conversely, all CRC and UC isolates tested and the majority of laboratory *E. coli* strains studied were unable to survive inside murine J774-A1 macrophage phagolysosomes and they were also intolerant to most stress conditions, in particular superoxidative stress. Colonic CD AIEC isolate HM605 showed higher initial levels of expression of acid response genes *gadA* and *gadB* that may support adaptation to the intra-macrophage phagolysosome niche. Adaptation to an intra-macrophage lifestyle appeared not to be through any ability to alter host macrophage oxidative stress response to infection as no differential changes were observed in the expression of 84 host genes related to oxidative stress to that seen with non-replicating laboratory *E. coli* strain.

Overall this study provides new insight into how CD mucosa-associated *E. coli* isolates resist killing by mucosal macrophages through adaptation to the acidic, high oxidative environment within the macrophage phagolysosome. The data may support future development of new therapeutic strategies that target the fundamental pathology of CD, in particular support a reduction in bacterial persistence/increased killing of intra-macrophage *E. coli* in CD patient mucosae.
Supporting papers


Scientific abstracts and presentations


## List of Abbreviations

### A

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>AAM</td>
<td>Alternatively activated macrophage</td>
</tr>
<tr>
<td>ADA</td>
<td>Adalimumab</td>
</tr>
<tr>
<td>Adi</td>
<td>Arginine decarboxylase antiporter</td>
</tr>
<tr>
<td>AIEC</td>
<td>Adherent, invasive <em>Escherichia coli</em></td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>APEC</td>
<td>Avian pathogenic <em>Escherichia coli</em></td>
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<tr>
<td>ATG16L1</td>
<td>Autophagy-related 16-like 1</td>
</tr>
<tr>
<td>AZA</td>
<td>Azathioprine</td>
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### B

<table>
<thead>
<tr>
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<th>Description</th>
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<tbody>
<tr>
<td>BM</td>
<td>Bone marrow</td>
</tr>
<tr>
<td>BMDM</td>
<td>Bone marrow-derived macrophage</td>
</tr>
<tr>
<td>BMMac</td>
<td>Bone marrow macrophages</td>
</tr>
<tr>
<td>β-arrs</td>
<td>β-arrestins</td>
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<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
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### C

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<tbody>
<tr>
<td>Caco-2</td>
<td>Human epithelial colorectal adenocarcinoma cells</td>
</tr>
<tr>
<td>CAM</td>
<td>Classically-activated macrophages</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CARD15</td>
<td>Caspase-recruitment domain 15</td>
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<tr>
<td>CD</td>
<td>Crohn’s disease</td>
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<td>Abbreviation</td>
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<td>--------------</td>
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<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
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<tr>
<td>CEACAMs</td>
<td>Carcinoembryonic antigen-related cell adhesion molecules</td>
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<tr>
<td>CFUs</td>
<td>Colony-forming units</td>
</tr>
<tr>
<td>CRC</td>
<td>Colorectal cancer</td>
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<tr>
<td>Cp</td>
<td>Crossing point</td>
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<td>DLG5</td>
<td>Disks large homolog 5</td>
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<td>DMEM</td>
<td>Dulbecco's Modified Eagle Medium</td>
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<td>DsbA</td>
<td>Disulphide isomerase</td>
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<td>EAEC</td>
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<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
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<td>Extra-intestinal pathogenic <em>Escherichia coli</em></td>
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<td>IFN-γ</td>
<td>Interferon gamma</td>
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<td>Immunoglobulin A</td>
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<td>Interleukin</td>
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<td>IL23R</td>
<td>Interleukin 23 receptor</td>
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<tr>
<td>InPEC</td>
<td>Intestinal pathogenic <em>E. coli</em></td>
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<td>IRGM</td>
<td>Immunity-related GTPase M</td>
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<td>L</td>
<td>Light chain 3-associated phagocytosis</td>
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<td>Light chain 3</td>
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<td>Lipopolysaccharide</td>
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<tr>
<td>LRRK2</td>
<td>Leucine-rich repeat serine, threonine protein kinase-2</td>
</tr>
<tr>
<td>M</td>
<td>Minimal microbial growth media</td>
</tr>
<tr>
<td>M9</td>
<td>Mycobacterium avium subspecies paratuberculosis</td>
</tr>
<tr>
<td>MAP</td>
<td>Macrophage Colony Stimulating Factor</td>
</tr>
<tr>
<td>M-CSF</td>
<td>Monocyte-derived macrophage</td>
</tr>
<tr>
<td>MDP</td>
<td>Muramyl dipeptide</td>
</tr>
<tr>
<td>MDR</td>
<td>Multidrug-resistant</td>
</tr>
<tr>
<td>MES</td>
<td>Morpholine ethanesulphonic acid</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>Magnesium chloride</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>MLN</td>
<td>Mesenteric lymph node</td>
</tr>
<tr>
<td>MOI</td>
<td>Multiplicity of infection</td>
</tr>
<tr>
<td>MV</td>
<td>Methyl viologen</td>
</tr>
<tr>
<td>Myd88</td>
<td>Myeloid differentiation primary response gene 88</td>
</tr>
<tr>
<td>N</td>
<td>Nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NaNO₂</td>
<td>Sodium nitrite</td>
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<tr>
<td>NCF4</td>
<td>Neutrophil cytosolic factor-4</td>
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<tr>
<td>NETs</td>
<td>Neutrophil extracellular traps</td>
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<tr>
<td>NF-κB</td>
<td>Nuclear factor kappa-light-chain-enhancer of activated B cells</td>
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<tr>
<td>NMEC</td>
<td>Neonatal meningitis-associated <em>E. coli</em></td>
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<tr>
<td>NOD2</td>
<td>Nucleotide-binding oligomerization domain-containing-2</td>
</tr>
<tr>
<td>NTC</td>
<td>No template control</td>
</tr>
<tr>
<td>O</td>
<td>Superoxide anion</td>
</tr>
<tr>
<td>O₂⁻</td>
<td>Superoxide anion</td>
</tr>
<tr>
<td>OCTN</td>
<td>Organic cation transporters</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
</tr>
<tr>
<td>OH</td>
<td>Hydroxyl radical</td>
</tr>
<tr>
<td>OMVs</td>
<td>Outer-membrane vesicles</td>
</tr>
<tr>
<td>P</td>
<td>Pathogen-associated molecular patterns</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>PBMCs</td>
<td>Peripheral blood mononuclear cells</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>PMNs</td>
<td>Polymorphonuclear neutrophils</td>
</tr>
<tr>
<td>PRRs</td>
<td>Pattern recognition receptors</td>
</tr>
<tr>
<td>PUMA</td>
<td>p53-upregulated modulator of apoptosis</td>
</tr>
<tr>
<td>PKS</td>
<td>Polyketide synthetase</td>
</tr>
<tr>
<td>Q</td>
<td>Quantitative reverse transcription polymerase chain reaction</td>
</tr>
<tr>
<td>R</td>
<td>Receptor-interacting serine threonine kinase-2</td>
</tr>
<tr>
<td>RNS</td>
<td>Reactive nitrogen species</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RpoS</td>
<td>RNA polymerase, sigma S</td>
</tr>
<tr>
<td>RPMI 1640</td>
<td>Roswell Park Memorial Institute 1640 medium</td>
</tr>
<tr>
<td>RSC</td>
<td>RNeasy Mini spin</td>
</tr>
<tr>
<td>S</td>
<td>Small interfering RNA</td>
</tr>
<tr>
<td>siRNA</td>
<td>Sequeostome 1-like receptors</td>
</tr>
<tr>
<td>SEPEC</td>
<td>Sepsis-causing enteropathogenic E. coli</td>
</tr>
<tr>
<td>SLRs</td>
<td>Single nucleotide polymorphisms</td>
</tr>
<tr>
<td>ST</td>
<td>Salmonella Typhimurium</td>
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<tr>
<td>T</td>
<td>Description</td>
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<td>-----</td>
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<tr>
<td>Th</td>
<td>T helper</td>
</tr>
<tr>
<td>TLRs</td>
<td>Toll-like receptors</td>
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<td>TLR4</td>
<td>Toll-like receptor 4</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumour necrosis factor</td>
</tr>
<tr>
<td>U</td>
<td>UC</td>
</tr>
<tr>
<td></td>
<td>Ulcerative colitis</td>
</tr>
<tr>
<td>UTI</td>
<td>Urinary tract infection</td>
</tr>
<tr>
<td>UPEC</td>
<td>Uropathogenic <em>Escherichia coli</em></td>
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### Abbreviations of amino acids

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<tr>
<th>Amino Acid</th>
<th>Abbreviation</th>
<th>One Letter</th>
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<tr>
<td>Alanine</td>
<td>Ala</td>
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<tr>
<td>Arginine</td>
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<td>Asparagine</td>
<td>Asn</td>
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<tr>
<td>Aspartate</td>
<td>Asp</td>
<td>D</td>
</tr>
<tr>
<td>Aspartate or Asparagine</td>
<td>Asx</td>
<td>B</td>
</tr>
<tr>
<td>Cysteine</td>
<td>Cys</td>
<td>C</td>
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<td>Glutamate</td>
<td>Glu</td>
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<td>Glutamine</td>
<td>Gln</td>
<td>Q</td>
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<tr>
<td>Glutamate or Glutamine</td>
<td>Glx</td>
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<tr>
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<td>Histidine</td>
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<td>Isoleucine</td>
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<td>Leucine</td>
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<td>Lysine</td>
<td>Lys</td>
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<tr>
<td>Methionine</td>
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<td>Phenylalanine</td>
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<td>Proline</td>
<td>Pro</td>
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<tr>
<td>Serine</td>
<td>Ser</td>
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<tr>
<td>Threonine</td>
<td>Thr</td>
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<td>Tryptophan</td>
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<tr>
<td>Tyrosine</td>
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<tr>
<td>Valine</td>
<td>Val</td>
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Chapter 1

Introduction
1.1 Definition and epidemiology of inflammatory bowel disease

Crohn’s disease (CD) is a chronic relapsing immunologically mediated inflammatory disorder, affecting the bowel and along with ulcerative colitis (UC), they form the two major groups of inflammatory bowel disease (IBD). Significant overlap in the clinical features of both CD and UC is evident, although the immune response in CD is different from that seen in UC. CD may affect any part of the gastrointestinal tract from mouth to anus, but commonly affects the terminal ileum at which the precise reasoning remains unexplained [1]. Crohn’s patients usually suffer from abdominal pain, diarrhoea, tenesmus and significant weight loss which may be associated with extra-intestinal manifestations such as skin rashes (e.g. erythema nodosum and pyoderma gangrenosum), eye inflammation (episcleritis and uveitis), venous thromboembolism, arthritis and renal stones [2, 3]. Meta-analysis also showed a positive association between IBD and the risk of stroke (7 studies for CD and 6 studies for UC) [4]; Extraintestinal manifestations of CD are illustrated in Figure 1.1. CD patients may have symptoms for many years before diagnosis, because the clinical manifestations of CD are more variable than UC [5, 6]. The diagnosis of CD is usually established with the imaging studies and endoscopic findings in a patient with a compatible clinical history.

The intestinal pathological findings in CD are characterised by transmural inflammation (inflammation in all layers from mucosa to serosa), deep mucosal ulcers, increased goblet cells, abscesses, fissures and granuloma formation; see Figure 1.1 [7]. These chronic inflammatory lesions are proposed to develop due to a disrupted intestinal barrier, Paneth cell dysfunction and a disturbed innate immune response, resulting in the accumulation antigen-presenting cells, such as dendritic cells and macrophages, lymphocytes and plasma cells within the intestinal mucosal layer [1, 8]. Pathological characteristics resemble the mucosal lesions and intestinal inflammation seen in response to classic enteric infection, with gut pathogen such as Shigella, Yersinia and Salmonella species (spp.) [9]. Whilst in UC, there is no inflammation beyond the submucosal level and inflammatory cells such as neutrophils are present in the lamina propria, with forming crypt abscesses and associated depletion of goblet cells (and mucins) from the epithelium is evident [10].
Males and females are equally affected in CD and has classically been described to have a bimodal incidence with the highest rates seen in adolescents and young adults and a second peak in later years[11, 12]. The CD concordance rate in monozygotic twins is estimated to be in the region of 20 to 50%, meanwhile in dizygotic twins, brought up in the same environment, it is below 10% [13]. The concordance rate of UC in monozygotic twins has been reported at around 16% and that in dizygotic twins, around 4% [14]. When it comes to the epidemiology, CD is more common in Europe and North America than observed in Africa and Asia [15]. For example, CD affects more than 115,000 people in the UK [16], and the frequency of onset and relapse in IBD showed seasonality in CD with a peak in July and August, but this has not been established for UC [17]. However, incidence rate of CD is rapidly increasing worldwide particularly in developing and developed nations adopting a western-style diet [15], as seen in Japan [18]. Likewise, those emigrating from poor and developing nations to the West, within a few years of moving are at increased risk of developing CD presumably due to a key change in their lifestyle and environment [19]. Animal studies in support of this include experiments in mice which have established that maternal high-fat diet (HFD) and resultant obesity promote the early onset of severe CD-like ileitis in genetically susceptible offspring [20]. CD is associated with very considerable morbidity, disrupting ability to work and study and family life, and also confers a small increase in mortality, with a standardised mortality ratio of 1.52 [21, 22]. Two meta-analyses also concluded that mortality in CD did not decrease over time, despite changes in patient management [21, 23]. Historically, nearly 80% of CD cases need surgery at some time during lifetime [24]. However, the use of immunosuppressants and biologics is associated with a reduction in risk of major surgery [25].
Figure 1.1 Crohn’s disease manifestations.

(A) The appearance of intestinal mucosa of a healthy individual and a Crohn’s patient. (B) The diagram illustrates extraintestinal manifestations of Crohn’s disease, adapted from http://www.physio-pedia.com/Crohn’s_Disease (accessed 08-12-2016).
1.2 Factors involved in Crohn’s disease pathogenesis:

1.2.1 Defective mucosal barrier function and alteration in the gut microbiota

The intestinal epithelium, as well as it established absorptive and digestive functions, is an efficient barrier against encroachment of the resident commensal microbiota and opportunistic enteric gut pathogens. There are a number of contributing factors involved in mucosal defence including the mucus layer, intestinal peristalsis, innate antibacterial factors such as lactoferrin and lysozyme, and hydrophobic antibacterial peptides produced by Paneth cells present at the base (crypts) of the small intestine. The intestinal mucosa also possesses specific immunological protection strongly facilitated by secretion of immunoglobulin A (IgA) [26].

Human gut microbiota plays an essential role in the shaping of the intestinal immune response in the healthy individuals [27]. Gut microbiome is established during the first 2 weeks of life and usually remains stable thereafter [28]. All human gut microbiome consists of around 1150 species, and each individual hosts approximately 160 species in which anaerobes of the Firmicutes and Bacteriodetes phyla are predominant [29].

In order to broaden insight into the pathogenesis of CD, multiple studies have been intensely carried out on CD intestinal microbiota over the last decade. Based on a number of previous human and animal experimental studies, the microbiota has been proposed to be involved in chronic inflammatory lesions formation particularly in two ways: first, a low-grade infection by a persistent pathogen, either traditional or opportunistic; and second, an imbalance between the beneficial commensals and the potentially harmful microbiota [30].

Results confirm a decrease in ‘protective’ bacterial phyla beneficial to gut health, including Firmicutes and Bacteriodetes (containing Gram-positive bacteria species) as compared to the microbiota of healthy controls, and the increase in abundance of Proteobacteria, including
potentially harmful Gram-negative intestinal bacteria, including *Escherichia coli* (*E. coli*) [31, 32]. *E. coli* which are known to be numerically dominant inhabitants of the healthy human gut microbiota and play an essential role for maintaining normal intestinal homeostasis and the stability of luminal microbiota via, for example involvement in synthesizing vitamin B and K as well as metabolizing bile acids [33]. However, there are virulent *E. coli* strains that are likely to cause a variety of intestinal and extra-intestinal diseases; this imbalance in the gut microbial population is referred to as 'dysbiosis'. Studies of faecal and gut mucosal-associated microbiota in patients with UC, demonstrated quantitative and qualitative changes in the composition, suggestive of dysbiosis [34-36].

Reduced diversity of other micro-organisms is also recently been described in IBD patients, including fungal microbiota (mycobiome) [37-39] and viruses (virome) [40, 41] and suggested to play a possible role in disease pathogenesis.

The correlations between CD and dysbiosis have been established to be more clearly marked in the mucosal biopsies (mucosa–associated bacteria populations) than bacterial communities from the intestinal lumen (faecal samples) [42-46]. In addition, the significant shift of normal gut microbial community is associated with intestinal inflammation in both experimental colitis and human IBD [47]. Even though CD pathogenesis is still poorly understood and the exact aetiology is still unknown, there is clear evidence suggestive that a number of lifestyle factors contribute to the dysbiosis of gut microbiota, including environmental triggers such as smoking [48, 49], ‘adolescent’ diet (notably a ‘westernised’ diet, high in fat and refined sugar, low in intake of fruit and vegetable fibre [50, 51]. For example, recent studies report that Western-style diet alters the microbiota composition within 1 day [52], and can result in increase in gut colonisation of a Crohn’s associated *E. coli* in transgenic CEABAC10 mice, expressing human CEACAMs, including CEACAM6, a receptor for CD-mucosa-associated ileal *E. coli* strains [53]. A result from a meta-analysis study indicated that the intake of dietary fibre, particularly fruit fibre, was significantly associated with a decreased risk of inflammatory bowel disease [54, 55]. It was also reported that supplementation of some types of dietary fibre may prolong remission and reduce the intestinal mucosa lesions during the course of the disease [56]. Avoidance of fibre was found to be associated with a greater risk of CD flare within a period of 6 months [57]. In addition, other studies, have revealed that
acutely ileitis promotes dysbiosis, especially increased mucosa association of *E. coli* in mice, and colonic inflammation (colitis) induced in rats can drive dysbiosis and lead to barrier disruption of the intestinal mucosa [58, 59].

Smoking has been proven to increase the risk of CD development and is associated with a higher rate of recurrence post-operation for CD patients [60]. It has also been proven to play a role in the pathogenesis of CD in children who are exposed to passive smoking, [61]. Smoking alters the gut microbial community (dysbiosis) and also results in dysfunction of mucosal macrophages to handle gut pathogens [62, 63]. Conversely for UC, the protective effects of smoking are well described with a reduction in the relapse rate among smokers with UC [64, 65].

Other key predisposing factors include genetic susceptibility (see section 1.2.2) and an inappropriate innate and adaptive immune host response have been proven as well to be implicated in increasing the susceptibility of the intestines to bacterial infection, chronic intestinal inflammation and consequently CD development (see Figure 1.2) [66]. A population-based cohort study from Denmark, demonstrated that individuals were at an increased risk of developing CD especially in those who had had previous gastrointestinal inflammation caused by *Salmonella* spp.[67].

Taken together, primary gut infections by specific bacterial pathogens might lead to dysbiosis of gut microbiota, and also to a defective intestinal mucosal barrier function which are implicated in CD aetiology. However, more efforts and further investigations are required to identify what the principal origin driving dysbiosis is, and also to understand the mechanisms of how whether the recent exposure to gastrointestinal pathogens or chronic carriers of these pathogens are implicated in the predisposition of CD [68]. The ‘Hygiene hypothesis’ suggests that those individuals having reduced microbial exposure during childhood might lend them to an immune hypersensitivity response later in their lives when challenged with bacteria, including those involved in CD pathogenesis [69]. The effect of sex hormones on IBD pathogenesis is still largely unclear. However, a key *in vivo* study showed that female mice
are less sensitive to dextran sodium sulphate (DSS)-induced colitis and this protection was proposed to be conferred by oestradiol [70].

1.2.2 Immunological and genetic susceptibility factors in bacterial recognition, autophagy and phagocyte function

The recent identification of genes associated with CD has been informative in improving our understanding of its pathogenesis, highlighting impairment of genetic components essential for innate immunity, intestinal barrier integrity and in microbial recognition and clearance[71] (see Figure 1.2). Following on from earlier work [72, 73], Genome-wide association studies (GWAS) have now identified 163 IBD risk loci, 30 of which are CD specific and 110 shared between UC and CD [74]. Many IBD loci are reported to be implicated in other immune-mediated disorders, including ankylosing spondylitis and psoriasis [74]. Key identified polymorphisms in the innate immune system of CD patients include genes that are linked to processes such as pathogen recognition [nucleotide-binding oligomerisation domain-containing-2 (NOD2)/Caspase-recruitment domain 15 (CARD15) and interleukin 23 receptor (IL23R)] and autophagy [immunity-related GTPase M (IRGM) and autophagy-related 16-like 1 (ATG16L1)], all relevant to killing of bacteria within macrophages [71-73].

CARD15 encoding the NOD2 receptor [75, 76]. Mutations in this gene probably account for about 15% of CD causation in the West although there are geographical variations with a lesser effect in northern European countries and no apparent impact on CD causation in Japan [77]. The NOD2/CARD15 protein is part of the innate immune system and is expressed in the cytoplasm of macrophages and Paneth cells [78]. CD-associated mutations in NOD2/CARD15 affect the leucine-rich domain recognising the bacterial cell wall peptidoglycan component, muramyl dipeptide (MDP), of both Gram-positive and Gram-negative bacteria. After recognition, NOD2 activates nuclear factor kappa B (NF-κB) and induces the production and release of proinflammatory cytokines. Crohn’s-associated NOD2/CARD15 mutations are considered to be loss of function mutations with evidence for reduced production of anti-bacterial defensins by Paneth cells and for a reduced IL-8
response to MDP by macrophages [79]. In association with NOD2/CARD15 mutations, polymorphism in genes SLC22A4 and SLC22A5, encoding the organic cation transporters OCTN1 and OCTN2 have also been identified with variants expressed in intestinal epithelial cells, T cells and macrophages[80]. In addition, a mutation in two haplotypes of DLG5, encoding scaffolding protein, has also been confirmed to be associated with NOD2/CARD15 mutations in CD patients [81].

**ATG16L1 and IRGM autophagy genes:** Two key genes associated with CD are the autophagy genes Autophagy-related 16-like 1 (ATG16L1) and immunity-related GTPase M (IRGM) [82-84]. Both encode proteins that play a key role in facilitating disposal of protein aggregates, DNA, lipids and damaged organelles but also in the mechanistic events by which macrophages degrade, kill and clear invading phagocytosed bacteria (a process also termed ‘xenophagy’), including pathogens such as Mycobacteria and Salmonella spp. [85-87].

**Other microbial handling/recognition genes:** Additional CD susceptibility loci relevant to aberrant microbial recognition and handling and/or phagocyte function include toll-like receptor 4 (TLR4), leucine-rich repeat serine, threonine protein kinase-2 (LRRK2), neutrophil cytosolic factor-4 (NCF4) and IL-23R. TLR4 is an apical cell-surface pathogen recognition receptor on intestinal epithelial cells, macrophages and dendritic cells, key in detection of lipopolysaccharide (LPS) presented on the outer-membrane surface of Gram negative bacteria, with polymorphism of TLR4 at D299G leading to hypo-responsiveness to LPS [88]. LRRK2 has been linked to CD through the association of a single nucleotide polymorphism on chromosome 12q12 [73] and in murine studies where LRRK2-deficiency resulted in increased inflammation and significantly poorer clinical outcomes following administration of dextran sodium sulphate to induce colitis [89]. A Meta-analysis study showed that the T1237C polymorphism of the TLR9 gene is implicated in the susceptibility of IBD [90].

The identification of NCF4 as a CD susceptibility gene is also important as it encodes the p40-phox subunit of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase crucial for reactive oxygen species (ROS) production by phagocytic cells in response to microbial infection [83]. Similar molecular defect in NADPH oxidase had also been established to be
associated in chronic granulomatous disease [91, 92]. Key studies show that altered neutrophil recruitment, along with an abnormal production of cytokines and reduced bacterial clearance, follow either acute trauma to the rectum and ileum [93] or subcutaneous injection of heat-killed *E. coli* in CD patients [94]. The latter of these studies suggests that macrophages may be involved in a key step of the observed immune dysfunction in CD. However, it is not yet clear whether this represents an inherent defect in macrophage function. Variants of the *IL-23R* gene have also been linked to CD [95, 96]. IL-23R is expressed by activated dendritic cells and macrophages, and IL-23 can induce production of pro-inflammatory cytokines that may contribute to intestinal inflammation [97]. CD is defined as a T helper 1 (Th1)-mediated disease where there is abundant interferon gamma (IFN-γ) producing lymphocytes found within the inflamed gut of CD patients [98], also associated with elevated levels of IL-12, inducing Th1 cell differentiation in humans [99]. Further studies in mice also reported the key involvement of loss of anti-inflammatory IL-10 in CD and UC pathogenesis [100, 101]. For example, *Il-10*−/−mice lacking IL-10 are observed to be colitis-free when were raised in a germ-free environment. However, when exposed to gut microbiota following transfer to specific pathogen-free conditions, a rapid development of chronic bacterial antigen-specific T-cell-mediated colitis resembling IBD was noticed, suggesting the importance of commensal intestinal bacteria in the pathogenesis of chronic, immune-mediated experimental colitis [102-104].

The role of vitamin D in several immune related diseases, including IBD, has been established [105]. For example in a mouse model (*Il-10*−/−mice), vitamin D deficiency increased susceptibility to dextran sodium sulphate (DSS)-induced colitis, whereas dietary supplementation with active 1,25(OH)2 vitamin D3 ameliorated symptoms [106]. Vitamin D deficiency also predisposes to adherent, invasive *E. coli* (AIEC)-induced barrier dysfunction and experimental colonic injury [107]. Additional evidence supporting this comes from the study of Lagishetty *et al.* where elevated levels of colonic bacteria (∼50-fold higher) were seen in colonic tissue of vitamin D deficient mice with DSS-induced colitis and reduced levels of anti-microbial peptides [108]. In addition, results of a cross-sectional study concluded an association between vitamin D deficiency/insufficiency and higher disease activity in IBD patients [109-111]. It is also reported that 70% of quiescent CD patients are deficient in vitamin D [112]. Vitamin D receptor (VDR) polymorphisms are also associated
with CD [113]. Treatment with vitamin D reduces the risk of clinical relapse in CD [114, 115].

Figure 1.2 A model for the development of Crohn’s disease.

Multiple predisposing factors are proposed to impair the intestinal mucosal barrier and promote alteration in the gut microbiota, resulting in an increase in mucosal recruitment by of Enterobacteriaceae, especially adherent, invasive \textit{E. coli}. Dysbiosis of gut microbiota may increase the susceptibility to infection or vice versa.
1.3 The role of specific bacteria in Crohn’s disease pathogenesis

Increased luminal concentrations of *Bacteroides* spp., *Eubacterium* spp., *Peptostreptococcus* spp., and *Coprococcus* spp. were reported in CD patients [116]. Key early studies from France [117], Netherlands [118], and Germany [119, 120] all reported an increase in mucosa-associated bacteria in CD. *Listeria* spp., and Streptococci, along with *E. coli* were also reported to play a role in the pathogenesis of CD [121].

There have been a number of distinctive studies that strongly favour the hypothesis that a specific bacterium plays a pivotal role in the initiation of chronic inflammation and development of CD. Early serological and culture studies suggested that *Mycobacterium avium* subspecies *paratuberculosis* (MAP), an obligate intracellular bacterium, causing a similar chronic inflammatory disease that primarily affect small intestine of cattle known as Johne’s disease, was more prevalent in CD patients [122-125]. A study by Ryan and colleagues [126] supports the idea of the presence of MAP DNA in granulomatous lesions of CD patients. MAP-reactive CD4 T cells have also been found in patients with CD [124, 127]. Even though, MAP has been hypothesised to be as contributing agent for CD pathogenesis, there is still great controversy, and absence of conclusive evidence, to fully supporting this hypothesis [128, 129]. However, a new study using better laboratory techniques proved the frequency detection of MAP in CD-patients which was significantly in greater proportion than in non-CD individuals [130].

Our own studies have suggested that microbial mannan (present in yeast cell wall and *Mycobacterium* species such as MAP) may be a key environmental factor to suppress macrophage killing of intracellular bacteria, including CD mucosa-associated *E. coli*, such as AIEC [131]. The shared susceptibility association of *NOD2* and *IL23R* polymorphisms seen in both CD and Mycobacterial disease (including leprosy) suggests MAP may yet be important in CD pathogenesis [132, 133].
Reduction in specific beneficial bacteria such as the Firmicute *Faecalibacterium prausnitzii* (*F. prau*), may also play an important role here. Low levels of *F. prau* are strongly associated with early disease recurrence after intestinal surgery in CD [43, 134]. This effect may be due to reduced production of bacterial anti-inflammatory molecules, with culture supernatants from *F. prau* shown to reduce the severity of colitis in animal models [43, 134].

Enteric *E. coli* bacterium is a member of the natural microbiota that is present in gut of normal healthy individuals, but also can be an opportunistic gut pathogen that when acquired causing significant morbidity and mortality worldwide [135]. Early serological studies described high antibody titres against mucosa-associated *E. coli* in the biopsies obtained from both paediatric and adult patients with CD [136, 137], and this was later supported by immunohistochemical studies demonstrating *E. coli* antigens within macrophages in CD tissue [121]. Many groups, including our own, have shown an increased number of mucosa-associated *E. coli* (including those with the AIEC phenotype) in CD, both in the ileum and in the colorectum [30, 117, 138-142]. We ourselves observed that aerobic culture of colonoscopic biopsies after removal of the mucus layer with dithiothreitol (DTT) is often sterile in the colon of control patients (irritable bowel syndrome (n = 13), sporadic polyposis (n = 4), piles (n = 3), diverticulitis (n = 2), pruritus ani (n = 1), and healthy (screening; n = 1), whereas the colon in CD and colon cancer contains increased bacterial numbers in this sub-mucus niche, more than 50% of which were *E. coli* [139], even though these organisms only account for less than 1% of the faecal microbiota [28]. Poor correlation between site of inflammation and presence of *E. coli* [142], and tendency to show that the same organisms can be identified from various sites within the same colon [139, 143] are compatible with the organisms having a causative role in the inflammation rather than merely colonising inflamed intestinal mucosa. Evidence for a primary pathogenic role is also given by their presence within granulomas [144], the histological hallmark of CD, by their ability to induce granuloma formation *in vitro* [145] and ability for similar *E. coli* to cause granulomatous colitis in Boxer dogs [146], and potentially in cats and swine too [147]. This *E. coli* pathovar associated with CD has been designated AIEC based on their ability to adhere to, and invade into, intestinal epithelial cell-lines (differentiated Caco-2 and undifferentiated I-407 cell-lines), induce release of pro-inflammatory cytokines, and possess an ability to survive and replicate within human peripheral blood monocyte-derived macrophages and within murine (J774-A1) macrophages [148, 149]. I-407 cell-line, of embryonic intestinal origin, is now
well documented to be contaminated with HeLa cells (originating from a cervical carcinoma), with other cell-lines derived from colorectal carcinomas, e.g. Caco-2 and HT-29, and the HEp-2 epithelial cell-line also documented as being HeLa cell contaminated [150]. Therefore, there is some significant doubt as to whether studies using some sources of these cell lines are indeed useful to understand mechanisms of CD pathogenesis (given their non-intestinal, transformed or cancerous origin) and/or their use as a model for measuring the ability of enteric bacteria to adhere to, and to invade into, intestinal epithelial cells.

ExPEC, belonging to the normal commensal gut microbiota of healthy individuals, are defined as facultative pathogens, having abilities to cause disease outside of the gut. Key examples include those strains from major subtypes such as Uropathogenic E. coli (UPEC), neonatal meningitis-associated E. coli (NMEC) and sepsis-causing E. coli (SEPEC) [151]. A study from Martinez et al., [152] showed only 4 out of 63 ExPEC isolates from different origins showed an AIEC phenotype suggesting little similarity between ExPEC and AIEC despite of the genetic similarities seen using multilocus sequence typing (MLST) [152], a technique used for typing of multiple loci of housekeeping genes [153].

According to the phylogenetic analysis, E. coli strains can be divided into four main phylogenetic groups (A, B1, B2 and D) [154] although eight phylo-groups are now recognized: A, B1, B2, C, D, E, F and the eighth Escherichia cryptic clade I [155]. Most commensal E. coli strains belong to group A, and virulent (ExPEC) strains have been shown to mainly belong to B2 and D [156]. Mucosa-associated E. coli that were detected in high numbers in the mucosal biopsies of patients with CD and those from patients with ulcerative colitis, another inflammatory bowel disease, belong to B2 and D phylogenetic groups [142]. Nowrouzian et al. also showed that E. coli strains found mainly in the faeces of healthy individuals belong to phylogroup A and B1, while E. coli from the microbiota of the colorectal mucosae of 13 Swedish schoolgirls sampled in the 1970s (all found to have asymptomatic bacteriuria in a school-screening program) belong to B2 and D [157]. Genomes of four CD mucosa-associated AIEC strains, belonging to B2 group have been sequenced and published [9, 158-160], and novel virulence factors, including those encoding a type-6 secretion system, were detected in their genomes [9]. A recent comparative genomic analysis of AIEC and non-AIEC strains however did not identify a molecular property
exclusive to the AIEC phenotype and recommended that a broader approach to the identification of the bacteria-host interactions and its role in CD pathogenesis [161].

1.4 Crohn’s disease associated *E. coli* - host intestinal mucosa interactions

Crohn’s *E. coli* phenotype AIEC colonise the intestinal epithelial cells via adhering to form biofilms over the intestinal mucosa and invade them via macropinocytosis-like process [162]. 17 out of 27 AIEC strains and only 9 out of 38 intestinal non-AIEC strains were biofilm producers [163]. *In vitro* studies have demonstrated the ‘paradigm’ ileal AIEC strain LF82 is able to translocate a number of human epithelial cell-lines, including HEP-2, intestine-407, Caco-2 and HCT-8 [164] other colonic AIEC strains, meanwhile, behaved similarly [30, 139]. Aphthous ulcers of the “dome” or follicle-associated epithelium (FAE), overlying Peyer’s patches in the distal ileum and lymphoid follicles of the colon are likely the initial mucosal lesions occurring in CD patients [165-167], and have been observed in patients using magnifying chromoendoscopy [168]. The FAE effectively forms the interface between the intestinal lymphoid system and the luminal environment. Specialized microfold (M) cells, accounting for about 5% of cells in the FAE, are optimized for antigen adherence and transport, and for immunological sampling of microorganisms [169].

Several invasive bacteria take advantage of the transcytotic characteristics of M cells to cross the gut mucosal barrier, including *Yersinia*, *Salmonella* and *Shigella* spp. [170-173]. It was suspected that the portal of mucosal entry of CD AIEC was also likely through M cells [174] and recent studies successfully modelling M cells *in vitro*, demonstrated that CD AIEC could indeed translocate through M cells (up to 20-fold compared with parent Caco-2 cells) and through isolated human ileal FAE [175]. Adhesion and subsequent translocation of CD AIEC across murine and human ileal Peyer’s patches, and across M cells *in vitro*, was observed to be dependent on possession of the *lpf* operon, encoding long polar fimbriae (Lpf) in CD AIEC [176, 177]. In a recent study, the genomes of 8 phylogenetically diverse AIEC strains were sequenced. AIEC were distributed across different phylogroups, with enrichment seen in genes encoding propanediol utilization, iron acquisition and long-polar fimbriae. These
traits correlated with the ability of AIEC to utilize fucose and iron, invade epithelial cells, translocate across M-cells, and persist within macrophages. It was also identified from analysis of the lpfA sequences that there were two variants, distinguished in part by the position of the lpf operon, lpfA141 and lpfA154. The lpfA141 operon of ileal E. coli encodes Lpf that exhibits high amino acid sequence similarity to enteropathogenic E. coli Lpf, whereas the lpfA154 operon encodes Lpf exhibiting high amino acid sequence similarity to Stg fimbriae from avian pathogenic E. coli. Prevalence of lpfA154 was observed in 71% of human ileal AIEC examined, and demonstrated to promote translocation of AIEC across M-cells, with deletion of lpfA154 from a murine AIEC strain CUMT8 effecting significantly reduced M-cell translocation, which is consistent with previous studies showing translocation across M cells in vitro and ex-vivo Peyer’s patches of the lpfA141-expressing ileal CD-mucosa associated AIEC LF82 [178, 179]. Isolates expressing lpf have been found to be more prevalent in colonic mucosae of CD patients than that of non-IBD controls [180].

Ex vivo studies also indicate a defective mucosal barrier to bacteria in the Peyer’s patches from CD patients [181, 182]. It is plausible therefore that increased bacterial load at M cells is important in the development of CD. A striking correlation also exists between the age-related incidence of CD and the number of Peyer’s patches in the small bowel, the latter peaking in late adolescence and then falling away [12]. CD ileal AIEC strains also typically produce type-1 pili (FimH) on their surface supporting adherence to ileal enterocytes via interaction with Carcinoembryonic antigen-related cell adhesion molecule 6 (CEACAM6) receptors known to be over expressed on the inflamed ileal (but not colonic) epithelium in CD [183]. Highly glycosylated CEACAMs have also been proposed as M cell microbial receptors [184]. It is plausible that one or more members of the CEACAM receptor family may play an important role in regulating endocytosis of CD mucosa-associated E. coli into host M cells. A recent study also reported that the glycoprotein 2 (GP2), specifically expressed on the apical plasma membrane of M cells among enterocytes, is recognized by FimH [185]. Mannose-derived FimH antagonists (such as biaryl or ‘two-ring’ mannosides) were reported to have promising therapeutic potential for UTI and CD, and also it was predicted that one or more FimH antagonists will be entering the clinic within the next two years [186].
A recent study, [187] also reported that the glycoprotein 2 (GP2), specifically expressed on the apical plasma membrane of M cells among enterocytes, [188] is recognized by FimH, the adhesin of type 1 pili on bacteria. By an intriguing coincidence it has also been found that the same GP2 protein is the epitope for the “anti-pancreatic” antibody found in CD sera [189]. In addition, CD mucosally-associated AIEC outer-membrane vesicles (OMVs) also show ability to interact with enterocyte endoplasmic reticulum stress response glycoprotein 96 receptor, increased in expression on the inflamed intestinal epithelium [190]. These OMVs, in association with flagellin, also possess significant ability to evoke pro-inflammatory cytokine release [149]. Colonic mucosa-associated AIEC strains expressing afimbrial adhesin afa operon, more commonly associated with diarrhoeagenic diffusely adherent E. coli (DAEC), have also been observed to be more prevalent in CD patients than in non-IBD controls. The presence of the afa operon correlates with diffuse adherence to, and invasion of intestinal epithelial cells [180]. A summary of Crohn’s AIEC host intestinal mucosal interactions is presented in **Figure 1.3**. CD mucosally-associated AIEC strains have also the ability to reduce the autophagy response inside intestinal epithelial cells by up-regulating levels of the microRNAs such as miR30C and miR130A [191]. Recently, an *in vitro* study reported that AIEC strain LF82 induces ROS production and mucin expression in intestinal epithelial T84 cells [192].
Ileal Crohn’s disease-associated (CD) *E. coli* colonise and translocate intestinal epithelial cells via binding of type 1 fimbrial (FimH) adhesive protein expressed on the surface of CD AIEC with host CEACAM-6 receptors over expressed on the apical surface of inflamed ileal tissue epithelial cells. Also interaction via FimH occurs with GP2 receptors expressed on the apical surface of Microfold (M) cells overlying Peyer’s patches. In addition, interactions have been described via outer membrane vesicles (OMV) binding to GP96 stress response protein expressed on the surface of inflamed ileal epithelial cells in CD patients. CD AIEC, after translocation, are taken up by, and survive/replicate within mucosal macrophages and dendritic cells (DC). Their residence here might then support formation of granulomata, characteristic of CD mucosae.
1.5 Neutrophils and their role in CD pathogenesis

Neutrophils are a type of polymorphonuclear leukocytes. They are professional, highly motile phagocytes involved in the first line of the innate immune defence against pathogenic microbes in healthy individuals and several lines of evidence indicate that they are also involved in chronic inflammatory conditions and adaptive immune responses [193, 194]. Neutrophils are typically the first leukocytes to be recruited to an inflammatory site and their key strategies to kill invaded pathogenic microorganisms are phagocytosis, release of soluble antimicrobials from their cytoplasm granules, including primary (azurophil), secondary (specific) and tertiary (gelatinase) granules, lysosomes [195], and generation of neutrophil extracellular traps (NETs) [196]. These killing strategies are activated via receptors that recognize bacterial peptides such as N-formyl-Met-Leu-Phe (f-MLP) or via pro-inflammatory mediators such as C5a and IL-8 [197, 198]. It is evident that neutrophils possess more rapid rates of phagocytosis and higher intensity of oxidative respiratory response than do macrophages [199].

In vitro, ileal AIEC LF82 has been shown survive and replicate inside human neutrophils, and also to induce interleukin-8 (IL-8) production from infected neutrophils. Up regulation of autophagy of infected-neutrophils enhanced intracellular killing of LF82 and limited the AIEC-induced inflammatory response. In other words, subversion of autophagy in LF82-infected neutrophils induces inflammation and cell death [200]. In vivo studies in CD patients on the other hand demonstrated a defect in neutrophil recruitment along, with an abnormal production of cytokines (including IL-8), following either acute trauma to the rectum and ileum [201], or subcutaneous injection of heat-killed E. coli (see Figure 1.4) [94]. Therefore, a defect of neutrophil chemotaxis has been suggested to be resultant of a reduced IL-8 secretion from macrophages [202]. However, ex vivo studies reported chemotaxis of CD neutrophils is normal [203, 204], and also our recent lab studies on MDM obtained from CD patients and healthy controls (HC) infected with colonic mucosa-associated AIEC HM605, E. coli K-12 or Staphylococcus aureus Oxford strain revealed that no significant differences were observed in killing of these bacteria between CD (active and quiescent) and HC, nor any differences in production pro-inflammatory cytokines TNF, IL-6 and IL-8) between groups. In addition, E. coli-infected MDM from CD patients and HC showed equivalent ability to
induce neutrophil chemotaxis relative to unaffected controls [205]. Another key study however suggested a defect in functionality and in signal transduction activation of peripheral neutrophils from quiescent CD patients and a decrease in their trans-epithelial migration in vitro in response to IL-8 compared to healthy controls [206].

Figure 1.4 Patients with Crohn’s disease (CD) exhibit reduced bacteria clearance of subcutaneously injected $^{32}$P-labelled heat-killed E. coli relative to healthy controls (HC) and patients with ulcerative colitis (UC).

1.6 Macrophages: Phagocytosis and Autophagy response mechanisms

Macrophages are professional phagocytes, playing an important role in innate immunity showing phagocytic activity against a wide range of pathogens, inducing synthesis and release of pro-inflammatory cytokines, although they are relatively less effective at bacterial killing than neutrophils [207-209]. Activated macrophages can crudely be classified into classically-activated macrophages (M₁-macrophages), which are immune effectors against pathogenic bacteria associated with a large amount production of lymphokines, and alternatively activated macrophages (M₂-macrophages), divided into four subgroups (2a, b, c and d) having a variety of functions, including immunity regulation, tissue repair and wound healing [210-212]. Monocytes and M₁ macrophages in the lamina propria are found to invade intestinal tissues directly and are involved in disrupting the intestinal epithelial barrier through deregulation of tight junction proteins and induction of epithelial cell apoptosis (i.e. leading to chronic intestinal inflammation) [213]. Macrophages in UC mainly act within the intestinal mucosa, while in CD it can also be found within the muscularis and the mesenteric fat tissue compartments [213].

Within macrophages, pathogenic bacteria are killed and degraded by either phagocytosis or autophagy initiated by two distinct mechanisms, although having similarities in the last stages where the phagosomes and autosomes merge with lysosomes and mature to phagolysosomes and autolysosomes. Phagocytosis is defined as a process at which extracellular particles such as pathogens are engulfed and surrounded by a double membrane to form internal vesicles termed phagosomes as presented in Figure 1.5. These phagosomes fuse with lysosomes to form mature phagolysosomes, in which their contents are degraded by ROS and proteolytic enzymes [209, 214]. ROS are chemically reactive molecules containing free oxygen radicals, including superoxide anions (O₂⁻), hydrogen peroxide (H₂O₂), and hydroxyl radicals (OH⁻) produced within the intracellular compartment under aerobic conditions through the activation of multicomponent NADPH oxidase. ROS are cytotoxic to foreign cells and are essential in microbicidal process of monocytes [215, 216]. Although macrophages and neutrophils are phagocytes, there are differences between them in how they perform phagocytosis and also in the final outcome of the phagocytotic process [217].
Figure 1.5 Bacteria phagocytosis process.

The phagocytosis process is initiated by the recognition of bacterium pathogen-associated molecular patterns (PAMPs) via membrane-bound pattern recognition receptors (PRRs) on phagocytes followed by bacteria engulfment into an intracellular vesicle called a phagosome. Phagosome-lysosome fusion then occurs to form a mature phagolysosome, followed by degradation of the bacterium. Degraded material is released by exocytosis.
Autophagy, or "self-eating", is a eukaryotic cellular process implicated in a range of physiological processes ranging from control of cell growth [218, 219], removal of old cellular structures and maintaining the intracellular nutritional homeostasis during starvation, and in contribution to type II programmed cell death [220]. Autophagy is also involved in inducing secretion of immune mediators [221], controlling adaptive immunity by inducing MHC class-II antigen presentation [222] and T-lymphocyte homeostasis [223], and in defence from cellular invasion by pathogenic bacteria, providing immune protection through targeted recognition and elimination of microbes such as Mycobacterium tuberculosis [85], group A Streptococcus and S. aureus [224]. Autophagy in response to pathogens is typically referred to as ‘xenophagy’. Autophagy is initiated in response to stresses such as hypoxia, nutrient starvation, endoplasmic reticulum stress and infection [225, 226]. Engulfed bacteria trigger xenophagy via a number of different mechanisms including induction of nutrient starvation (i.e. competing for nutrients) and stimulation of the innate immune receptors, including membrane bound Toll-like receptors (TLRs), cytoplasmic receptors such as ATG16L1 and NOD2 and sequestosome 1-like receptors (SLRs) [86, 219, 227]; as briefly summarised in Figure 1.6.
Figure 1.6 Intracellular elimination of invading microorganisms by autophagy.

Once a microorganism is engulfed by host cells, such as a macrophage, the autophagy machinery is activated by pathogens through one of the following mechanisms: 1, nutrient starvation; 2, stimulating innate immune receptors via membrane bound pathogen-recognition receptors (PRRs) and sequestosome1-like receptors (SLRs). Pathogens trigger Toll-like receptors (TLRs) leading to activation of TNF receptor-associated factor 6 (TRAF6) or NADPH oxidase 2/ reactive oxygen species. (NOX2/ROS) mediated autophagy or microtubule-associated protein 1A/1B-light chain 3 (LC3)-associated phagocytosis (LAP). Cytoplasmic PRRs, triggered by pathogens include nucleotide-binding oligomerization domain-containing protein 2 (NOD2) and autophagy-related protein 16-like 1. (ATG16L1) receptors). Lysosomes mature to become autolysosomes, where intraphagolysosome materials are then degraded by either oxygen-dependent (production of ROS) or oxygen-independent (production of granules containing proteolytic enzymes) at the final stage of the autophagy process. Source of Figure adapted from [219].
1.7 Defective autophagy and lack of clearance of Crohn’s disease mucosa-associated AIEC

ATG16L1 and IRGM function in autophagosome formation and evidence from recent studies supports a role for autophagy as an antimicrobial mechanism downstream of cell-surface TLR and cytosolic NOD-like receptor signalling. Activation of NOD2 by MDP induces autophagy in antigen-presenting cells (such as dendritic cells and macrophages) in a receptor-interacting serine/threonine kinase-2 (RIPK-2) dependent manner [228]. Knock-down of ATG16L1 and IRGM mRNA using siRNA approaches resulted in defective recognition and clearance of CD mucosa-associated E. coli within host epithelial cells and macrophages [229, 230]. However, deficiency in either gene did not interfere with the replication and survival ability of other non-pathogenic, environmental, commensal, or gastroenteritis-inducing E. coli, suggesting a specific role for autophagy in restraining Crohn’s AIEC. Similarly, expression of the CD variant ATG16L1*300A in intestinal Caco2 epithelial cells impairs their ability to capture internalized Salmonella spp. within autophagosomes [231] and is also associated with abnormalities in Paneth cell granule exocytosis [221], impaired production of antimicrobial α-defensins [232], and increased production of pro-inflammatory cytokines IL-1β and IL-18 by macrophages in response to LPS [233]. In addition, Lapaquette and colleagues established that impaired expression of ATG16L1, IRGM or NOD2 lead to an increase of intra-macrophage CD AIEC, and was associated with an increase secretion of both IL-6 and TNF, while induction of autophagy lead to a decrease in AIEC intramacrophage survival and replication and in pro-inflammatory cytokine production. It seems that any impairment of autophagy leads to a defect in bacterial clearance associated with increased intra-cellular bacteria replication within phagocytes (illustrated in Figure 1.7). Stimulating autophagic machinery in CD patients has therefore been postulated as a potential therapeutic approach to restrain the intra-macrophage CD AIEC replication and to reduce inflammation [234]. The anti-malarial drug chloroquine, for example, has been reported to enhance the late stage of autophagy, particularly fusion of autophagosomes with lysosomes [235], inhibiting intra-macrophage replication of Mycobacterium tuberculosis, and enhancing the anti-tuberculosis protectiveness of isoniazid and 25 OH-vitamin D3 [236]. However, stimulating autophagy with rapamycin, was reported to be ineffective particularly when intramacrophage replication of CD AIEC was well established [234].
Figure 1.7 Impairment of autophagy leads to a defect in bacterial clearance associated with increased intra-cellular bacteria replication within phagocytes.

1.8 Virulence factors supporting ability of Crohn’s disease mucosa-associated *E. coli* to survive within host macrophages

AIEC strains obtained from CD ileal and colonic biopsy tissue demonstrate ability to, survive and replicate within phagolysosomes of macrophages [8, 149]; see Figure 1.8. However, they are not unique in this ability as other pathogens are also known to survive and replicate within macrophages, including Mycobacteria, *Salmonella, Shigella, Legionella, Coxiella, Brucella* and *Listeria* species. Key adaptive defence mechanisms adopted by these pathogens support their resistance to killing within the low pH, low nutrient environment, high oxidative and nitrosative stress environment of the phagolysosome. Whilst *Shigella* and *Listeria* are able to escape from the mature phagolysosome, *Salmonella* spp. prevent fusion of phagosome with the lysosome, and *Mycobacterium tuberculosis* modify the intra-phagolysosome environment to support their survival respectively [238-241].

Some of the virulence factors supporting survival and replication of AIEC within macrophages have been identified using isogenic mutants of the “paradigm” ileal AIEC LF82. Genes identified include *htrA* (encoding high temperature stress protein), *dsbA* (encoding an oxidoreductase) and *hfq* (encoding a RNA chaperone important in mediating bacterial adaptation to chemical stress) [242-244]. However, HtrA and DsbA are fairly ubiquitous in *E. coli*, and it is likely that other unidentified factors are needed to support Crohn’s AIEC survival within the stressful conditions of the phagolysosome. One recently identified factor is GipA. GipA deletion impairs Crohn’s AIEC translocation across M cells and their ability to replicate inside macrophages; their colonization of PPs and dissemination to mesenteric lymph nodes in mice was also impaired. GipA deletion also reduced *lpfa* mRNA levels in Crohn’s AIEC. GipA expression was also found to be induced by bile salts, intraphagolysosome reactive oxygen species and acidic pH conditions. In addition, survival of Crohn’s AIEC-ΔgipA bacteria was reduced in medium containing either H$_2$O$_2$ or acidic pH [245].
Acid stress is the antimicrobial environment likely encountered by active enteric bacteria within the phagolysosome. *Salmonella* spp., *Shigella* spp. and *E. coli* have all been reported to possess a repertoire of low pH inducible systems that support resistance, tolerance and habituation during environmental acid stress. Likewise, Crohn’s AIEC certainly appear to be tolerant of the low pH intraphagolysosome environment [246]. *E. coli* is notable due to its possession of four known acid resistance systems. The first system requires sigma factor RpoS and the cyclic AMP (cAMP) receptor protein CRP, with RpoS functioning as a major environmental stress response regulator in both *E. coli* and *Salmonella* spp. [246].

Deletion of rpoS from a Crohn’s AIEC (strain O83:H1) has been observed to increase sensitivity of this clinical isolate to oxidative stress [247]. The second system requires extracellular glutamate. The components of glutamate-dependent acid response are two isoforms of glutamate decarboxylase encoded by *gadA* and *gadB*, and a glutamate-γ-aminobutyric acid antiporter encoded by *gadC* [248, 249]. Murine intestinal commensal *E. coli* have been observed to respond to chronic intestinal inflammation by up-regulating expression of stress response genes such as *gadA* and *gadB* [250]. Proven to be essential as regulators of *gad* stress response gene expression are *gadE* and *gadX* [251]. The third acid resistance system requires is arginine-dependent utilising of arginine decarboxylase (AdiA and AdiC) antiporter [246] and the fourth is lysine dependent, involving lysine decarboxylase [249]. In addition, *E. coli* also harbour specific mechanisms that enable them to resist high levels of reactive oxygen species (ROS) that form the oxidative and superoxidative to phagocytosed pathogens. These defensive resources have been found to be grouped into two regulated sets of genes designated as soxRS and oxyR regulons [252, 253]. *E. coli* SOS system is a network regulating the expression of more than 40 genes which is activated to rescue cells from severe DNA damage by physical and chemical factors, including ROS. Based on a knock-out study, *E. coli* SOS genes *dinf* was reported to protect against oxidative stress, although the exact nature of the DinF activity remains to be identified [254, 255]. Despite of all these fundamental pieces of knowledge on the virulence genes of Crohn’s-associated *E. coli* strains, genetic factors that are characteristic of AIEC phenotypes are still required to be studied.
Figure 1.8 Transmission electron micrograph of adherent, invasive *E. coli* within macrophages.

A: Crohn’s disease colonic mucosa-associated isolate HM605 surviving and replicating within vesicles of J774A1 murine macrophages; B: Double membrane around intra-macrophage vesicle indicates bacteria are contained within phagolysosomes (solid arrow). Images courtesy of Dr. Carol L. Roberts (University of Liverpool, United Kingdom).
1.9 Current therapeutic approaches for CD and the potential for future treatment via targeting intra-macrophage Crohn’s disease mucosa-associated AIEC

Superficial mucosal lesions in Crohn’s patients are likely to heal, while deep ulcers and fissures heal with more difficulty and may lead to the development of fibrotic strictures. When the lesions are extensive, CD becomes symptomatic associated with local complications such as dilatation, perforation, haemorrhage, abscess and fistula along with fibrosis and stricture formations [3]. Smoking cessation was found to be beneficial in patients with CD [256], which has been suggested to be as a result of the recovery of immune cell function. However, this has not been studied on CD patients [257]. The main goals of gastroenterologists, during treatment of CD patients, are first to induce remission and second to prevent relapse by either surgery or drugs. However, the ideal therapy that should reduce inflammation without inducing immunosuppression remains a challenge.

Current treatment approaches:

Patients with active CD are routinely treated with immune suppressing drugs such as corticosteroids (prednisolone), thiopurines (azathioprine, mercaptopurine) and anti-TNF antibody (infliximab and adalimumab) [258]. The use of 5-aminosalicylic acid (5-ASA) drugs, including Sulfasalazine and Mesalazine in CD is still controversial and studies evaluating their efficacy have produced mixed results [259-261]. According to a recent network meta-analysis study, the most effective therapies for induction and maintenance of remission of Crohn's disease are adalimumab (ADA) and infliximab+ azathioprine (AZA) [262]. It has been reported that biologic therapies had higher durability for induction and maintenance therapy than immunomodulators [263]. It is recently reported that ADA response in Crohn’s patients is genetically predisposed by a Single Nucleotide Polymorphism (SNPs) in CD-associated genes and found ATG16L1 as the most promising candidate gene for adalimumab response [264]. Using a polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) of 16S rRNA gene fragments and quantitative PCR, respectively in colonic mucosal biopsies from 15 CD patients compared with 4 healthy subjects, treatment with ADA was reported to induce short-term changes in the microbiota
with those phyla recovering after treatment including beneficial Firmicutes, and a decrease in the prevalence of Proteobacteria, such as *E. coli* [265].

Monoclonal anti-TNF antibodies used in CD have limitations including increased risk of side effects such as reactivation of hepatitis and granulomatous disease (tuberculosis), and bone marrow suppression, and long-term complications such as risks of malignancies [266, 267]. Since, TNF inhibitors are not useful for one third of all patients, other strategies have recently been developed such as monoclonal antibodies targeting the interleukin (IL)-6 receptors, which are expected to hold greater promise to be helpful in the future [268].

Surgical resection of an isolated inflamed ileal segment in adolescents with active CD is very effective, leading to a clinical improvement (symptom relief) and a reduction in medication requirements [269]. However, the recurrence of CD was observed approximately in 80% of cases after ileal resection surgery of the inflamed part and the recurrence was mostly occurred in ileal part which was proximal to the anastomosis with the large intestine, suggesting that the recurrence is more likely to be dependent on the proximity to the large bowel contents where the bacteria niches are abundant [270]. Identifying the risk factors for postoperative CD recurrence will be beneficial in order to assist the clinician in implementing more aggressive prophylactic treatment to sustain remission and to prevent recurrence.

Further meta-analysis of six randomised placebo-controlled clinical trials showed that the use of antibiotics, including ciprofloxacin, metronidazole and co-trimoxazole in these patients are highly likely to induce remission in patients with active CD, and patients who received antibacterial therapy were clinically improved compared with CD patients who received only placebo [271, 272]. Another clinical trial demonstrated CD endoscopic recurrence was at a high rate following 1 year surgery, but after CD patients received metronidazole or ornidazole antibiotics, the post-operative endoscopic recurrence was reduced [273]. However, a recent meta-analysis failed to provide any benefit for the use of antibiotics in maintaining remission period, and also in the treatment of active intraluminal and perianal disease [274].
Some probiotic microorganisms such as *E. coli* Nissle 1917 might have positive effects on maintaining remission of IBD, particularly UC [275, 276], and this probably occurs due to bacterial competition [277, 278], and they may be effective in maintaining remission period in postoperative prophylaxis [279]. However, a number of studies on children have shown antibiotic exposure in children, mainly at age $\leq 3$ months, is a significant risk factor for development of CD [280, 281], and a 4-year prospective study established that most patients with IBD, received antibiotic treatment demonstrate a more severe clinical course [282].

**Therapeutic targeting of intra-macrophage Crohn’s disease mucosa-associated AIEC:**

The hypothesis of the implication of AIEC in the pathogenesis of CD was supported by clinical and experimental data. Therefore, this bacterium is considered to be as a potential target in the treatment of Crohn’s patients using antibiotics [149]. It shows killing of intramacrophage *E. coli in vitro*. However, ileal CD AIEC has been observed to manifest resistance to a range of antibiotics, including ciprofloxacin, rifampicin, clarithromycin, tetracycline, and trimethoprim/sulfamethoxazole, plus 42% of ileal Crohn’s AIEC are categorised as Multidrug-resistant (MDR) [283].

A study investigating CD patients, having an active ileal lesion, with circulating antibodies directed against *E. coli* and *Pseudomonas fluorescens*, reported that these patients had a higher response rate to budesonide in combination with metronidazole and ciprofloxacin compared to those patients without circulating anti-bacterial antibodies [284]. The most appropriate target for use of antibiotics in CD patients is proposed as being those intramacrophage replicating CD AIEC. However, antibiotic treatment for active CD is still thought to be non-beneficial, although there is evidence supporting the use of antibiotics to prevent post-operative recurrence of CD [273, 285]. In addition, studies on both human and animal clinical isolates indicate that antimicrobial resistance is common in *E. coli* associated with CD and granulomatous colitis, and that resistance to antibiotics such as ciprofloxacin and enrofloxacin may correlate to poor clinical outcome of disease [283, 286, 287]. Administration of a triple antibiotic regimen has been recommended to avoid the antibiotic resistance which proved to reduce intra-macrophage survival of CD AIEC to 3% compared to untreated controls. Antibiotics that were able to enter macrophages, including azithromycin,
Ciprofloxacin, rifampicin, sulfamethoxazole, tetracycline and trimethoprim were all effective against CD AIEC surviving and replicating within the macrophage phagolysosome, while antibiotics that are unable to penetrate macrophages are considered to be ineffective [149]. However, due to drug to drug interactions/side effects that may occur between some class of antibiotics and azathioprine (an immunosuppressant regularly used to treat CD patients with active disease), other alternative strategies using agents to manipulate the intra-phagolysosome environment have been explored since CD AIEC are known to be dependent on an acidic environment for their survival [242]. This suggests that alkalinisation of the intra-phagolysosome would perhaps reduce Crohn’s AIEC survival within macrophages. For example, hydroxychloroquine, a weak base with the ability to increase phagolysosome pH, is reported to improve intra-macrophage killing of bacteria with an intra-phagolysosomal lifestyle such as Coxiella burnetii, causing Q-fever [288] and Tropheryma whippelii, causing Whipple's disease [289, 290]. In this context, recent research studies in our own lab revealed that hydroxychloroquine directly reduced survival and replication of intra-macrophage AIEC and enhanced the efficacy of antibiotics (such as Doxycycline and Ciprofloxacin) to kill intra-macrophage Crohn’s AIEC [205]. A recent study on the use of species-specific antibiotics termed colicins for treatment of CD-associated AIEC reported that the ability of colicins E1 and E9 to kill intra-phagolysosome AIEC in RAW264.7 macrophages, and no toxicity was observed toward macrophage cells, indicating the potential of colicins as therapeutics for the eradication of E. coli from the gastrointestinal tract of CD disease patients [291]. The corticosteroid dexamethasone has been found to promote phagocytosis and enhance killing of Staphylococcus aureus inside human monocytes/macrophages in vitro [292].

Vitamin D deficiency is common in approximately 70% of CD patients in the either active or quiescent phase of disease [112, 293]. A retrospective study where 3217 CD patients with a low requirement for surgery, showed higher levels of vitamin D at which 30ng/ml was considered as a cut off [294]. There was a reduction in the risk of relapse following patients with quiescent CD given vitamin D supplements [115]. Vitamin D (1, 25 OH2-vitamin D3) supplementation also enhanced killing process of intra-macrophage CD AIEC in both murine and human macrophage phagolysosomes [205]. However, there is no data yet, proving the effects of vitamin D on in vivo macrophage CD AIEC interactions. Several individual studies recently verified alterations in the composition of intestinal microbiota in CD patients and the
role of abnormal intestinal microbiota in CD pathogenesis. Faecal microbiota transplantation (FMT), which was successfully tested in recurrent Clostridium difficile infections [295], has been established as a promising therapeutic option for CD via modifying microbiota, restoring the balance of intestinal flora and reversing the inappropriate immune stimulation make intestinal ecosystem less suitable for intestinal E. coli colonization [296-298]. FMT has recently been reported to improve the quality of life in IBD patients [299]. The efficacy of exclusive enteral nutrition (EEN) for CD via (A) down regulation of inflammatory cytokines production, (B) reduction of intestinal permeability, and (C) modulating the intestinal microbiome have been also established [300-303]. Nutritional supplementation is essential for CD patients with evidence of malnutrition and it may help in maintaining remission [304]. Whether FMT, EEN and nutritional supplements alter macrophage function to support killing intra-macrophage Crohn’s AIEC is as yet undetermined.

1.10 Hypothesis and aims:

1.10.1 Hypothesis

Key additional molecular mechanisms are likely to be involved in supporting Crohn’s disease (CD) mucosa-associated AIEC to resist killing by host mucosal macrophages, and that these mechanism support their ability to survive and replicate within the low pH, low nutrient, high oxidative and nitrosative stress environment of the phagolysosome.
1.10.2 Aims

In this study, our main targets were to:

1. Assess and validate the ability of CD mucosally-associated *E. coli* to survive and replicate inside macrophages compared with other *E. coli* strains, including clinical isolates from colorectal cancer and ulcerative colitis patients.

2. To understand the role of host NF-κB pathway activation in supporting intra-macrophage replication of CD AIEC.

3. Investigate whether CD mucosally-associated *E. coli* can tolerate and grow in low pH, low nutrient, high nitrosative and high superoxidative stress conditions, mimicking the harsh environment within an active macrophage phagolysosome.

4. Identify bacterial molecular mechanisms underlying tolerance of CD AIEC surviving and replicating within the phagolysosome.

5. To assess whether CD AIEC possess ability to alter host oxidative stress response gene expression in macrophages.
Chapter 2

Materials and Methods
2.1 Bacterial strains and their culture conditions

2.1.1 Bacterial strains

The heterogeneous population of bacterial strains used throughout this study are:

1) **Ileal CD E. coli isolates**

Ileal Crohn’s disease (CD) *E. coli* strains LF10, LF11, LF13, LF82* and LF86, isolated from inflamed lesions of clinically active Crohn’s patients were kindly provided by the late Professor Arlette Darfeuille-Michaud (Pathogénie Bactérienne Intestinale, Laboratoire de Bactériologie, Université d’Auvergne, Clermont-Ferrand, France) [243]. An additional ileal CD strain, 541-15A*, was obtained from Professor Kenneth Simpson, Cornell University, College of Veterinary Medicine, Ithaca NY, USA) [178, 305].

2) **Colonic CD E. coli isolates**

Colonic CD strains were previously isolated from colonic biopsy tissue specimens of CD patients in the remission with either a history of active ileal inflammation, ileo-colonic disease or colonic inflammation alone [139]. The latter bacteria strains designated as HM were isolated from patients attending the Royal Liverpool University Hospital by Dr. Helen Martin; Gastroenterology Research Unit, University of Liverpool, Liverpool, UK [139].

<table>
<thead>
<tr>
<th>Table 2.1 Sources of colonic CD <em>E. coli</em> isolates.</th>
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<tr>
<td><strong>HM95</strong>, <strong>HM96</strong>, <strong>HM104</strong>, <strong>HM413</strong> and <strong>HM419</strong></td>
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<td><strong>HM427</strong></td>
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<td><strong>HM154</strong>, <strong>HM580</strong>, <strong>HM605</strong> and <strong>HM615</strong></td>
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3) **Ulcerative colitis** *E. coli* isolates

Ulcerative colitis *E. coli* strains HM233, HM250, HM378, HM394 and HM457* isolated from the inflamed colon tissues of clinically active UC patients and HM464 from colon tissues of UC patients during remission period.

4) **Colorectal cancer (CRC)** *E. coli* isolate

CRC *E. coli* strains HM229, HM230, HM244, HM358*, HM312, HM44*, and HM374, isolated from colon mucosa of patients with colon cancer [139].

**Note** - for all IBD (CD and UC) and CRC isolates detailed above, those indicated with an asterisk (*) indicates that they are adherent, invasive *E. coli* (AIEC) as per the definition defined by Darfeuille-Michaud A in 2001 [148], i.e. that they adhere to, and invade into, intestinal epithelial cell-lines (differentiated Caco-2 and undifferentiated I-407), induce release of pro-inflammatory cytokines, and possess an ability to survive and replicate within human and murine macrophages [148].

5) **Other *E. coli* clinical isolates**

*E. coli* strains ECOR40, ECOR48, ECOR50 and ECOR64 (ECOR48 and 64 from patients with acute cystitis, and ECOR40 and 50 with acute pyelonephritis) provided by the STEC Centre, Department of Microbiology and Molecular Genetics, Michigan State University (MSU), USA. *E. coli* strains SJH2, J96 and CP9 obtained from patients with urinary tract infection, termed Uropathogenic *Escherichia coli* (UPEC), were obtained from Professor Craig Winstanley (Department of Clinical Infection, Microbiology and Immunology, Institute of Global Health & Infection, University of Liverpool, UK) and Dr Alison O'Brien (Department of Microbiology and Immunology, Uniformed Services University of the Health Sciences, Bethesda MD USA) [306], *E. coli* strains obtained from healthy individuals ECOR1, ECOR35, healthy individuals with IBS [HM484 (AIEC) and HM488 (AIEC)] with sporadic polyposis [HM428 (AIEC), HM454 (AIEC) and HM456 (AIEC)] and with piles [HM463 (AIEC)], and from a healthy infant ECOR51.

Probiotic *E. coli* Nissle 1917 (EcN) was a kind gift to the department from Dr. C. Enders (Ardeypharm; Herdecke, Germany). *E. coli* K-12, i.e *E. coli* (Migula) Castellani and Chalmers ATCC® Number 10798, was obtained from the American Type Culture Collection (LGC Standards; Middlesex, UK) with *E. coli* XL-1Blue obtained from Agilent Technologies.
(Santa Clara, USA) and *E. coli* K-12 derivative (EPI300-T1) from Epicentre (Madison, USA).

6) **Other bacteria**

*Salmonella enterica* serovar Typhimurium (S. Typhimurium) strains LT2 and 4/74 were a kind gift to the department from Professor Craig Winstanley (Clinical Infection, Microbiology and Immunology, University of Liverpool, UK).

### 2.1.2 Bacterial storage and culture

All studied bacteria strains were stored in Protect® micro-preservation beads in a -80°C freezer, with routine working stocks kept at -20°C and grown on Luria Bertani (LB) agar plates for 24 h in the incubator at 37°C and 5% v/v CO₂ atmosphere. LB agar plates (pH 7) were made by mixing of 10g Bacto-tryptone, 5g Bacto-yeast extract, 5g NaCl, and 15g agar in a litre of distilled water and then autoclaving for sterilization, followed by pouring the sterile LB agar into sterile Petri dishes.
2.2 Murine macrophage J774-A1 cell-line culture

Obtained from the European Collection of Animal Cell Culture (ECACC catalogue number 91051511; Porton Down; United Kingdom), murine macrophage-like cell line J774-A1 (taken from ascites or reticulum cell sarcoma of a female mouse;) was maintained in Roswell Park Memorial Institute (RPMI) 1640 medium (supplied by Sigma-Aldrich, Poole, UK) supplemented with 10% v/v foetal calf serum (FCS) (Gibco; Paisley, Scotland), 100 U/ml penicillin (Sigma), 100 µg/ml streptomycin (Sigma), and 4mM L-glutamine (Sigma), within 75 cm² tissue culture flasks (Appleton Woods Limited; Birmingham, UK) in the incubator at 37°C in a humidified atmosphere of 95% v/v air and 5% v/v CO₂. J774-A1 cells were grown to 80% confluency and gently passaged using cell scrapers (Corning®; Chorges, France); see Figure 2.1. Macrophages were routinely passaged twice weekly and used in experiments at passage numbers between 8 and 20, with cells counted using a glass haemocytometer. In order to replenish the stock cultures to the cryobank, 80% confluent J774-A1 cells were aliquoted into cryo-vials in freezing media containing 90% v/v FCS and 10% v/v dimethyl sulphoxide (DMSO).

For experiments, J774A1 macrophages were routinely seeded to 24-well tissue culture plates at a density of 1x10⁵ cells per well (see section 2.7).
Figure 2.1 J774-A1 macrophage cell line at 80% confluency (48h post seeding; Magnification x20) cultured in RPMI medium.

The morphology of our macrophage cultures was representative of those images seen on-line http://www.pheculturecollections.org.uk/products/celllines/generalcell/detail.jsp?refId=91051511&collection=ecacc_gc (accessed 08-12-2016).
2.3 Generation of high yields of healthy bone marrow-derived macrophages (BMDM) from murine wild-type, Nf-kb and c-Rel deficient bone marrow (BM) cells

Femurs from mice as follows: Wild-type mouse C57BL/6 (n=2♂, n=2♀); mice deficient in NF-κB1 (Nfκb1−/−; n=1♂, n=3♀), mice deficient in NF-κB2 (Nfκb2−/−; n=2♂) and those deficient in c-Rel (c-Rel−/−; n=3♀) were kindly provided by Dr. Carrie Duckworth and Dr. Ahmed Elramli, Henry Wellcome Laboratories of Molecular & Cellular Gastroenterology, University of Liverpool, UK. Wild-type C57BL/6 mice supplied by Charles River (Margate, UK), and transgenic strains on a C57BL/6 background, including Nfκb1−/− and Nfκb2−/− mice [307, 308] and c-Rel−/− mice [309] were maintained at the University of Liverpool’s specific pathogen-free (SPF) Biomedical Services animal Unit. Bone marrow from Il-1r−/−, Il-18r−/− and Myd88−/− (on a C57Bl/6 background) were a kind gift from by Dr. Stuart Marshall-Clarke (Human Cell Biology & Anatomy, University of Liverpool, UK), with the Myd88−/− mice originally provided with the generous permission of Prof Shizuo Akira (Osaka University, Japan) [310].

High yields of healthy bone marrow-derived macrophages were obtained following adaptation of the method of Manzanero et al. [307, 311]. All femurs were first soaked in 70% v/v ethanol inside a Petri dish for disinfection. After 3 to 5 min, bones were rinsed off ethanol via placing them into a Petri dish containing sterile PBS, pH 7.3 (Life Technologies; Paisley, UK). Both bone ends were carefully trimmed with sterile scissors in order to expose bone marrow shaft and a needle was then inserted through a cut made at the top ends with sterile scissors, followed by flushing BM with 5 ml per bone of RPMI 1640 medium into a sterile tube. After centrifugation (250 g for 5 min), supernatants were removed and pellets of BM progenitor cells (at cell density ranging from 1.4 to 2.5 x 10^6 cells/ml) were resuspended in RPMI 1640 medium supplemented with 10% v/v foetal calf serum, 4mM L-glutamine. The steps of this procedure are briefly summarised in Figure 2.2. BM progenitor cells could then either stored in liquid nitrogen cell bank in freezing media (90% v/v FCS and 10% v/v dimethyl sulfoxide (DMSO) for future experimentation, or differentiated to adherent macrophages following addition of purified recombinant Mouse Macrophage Colony Stimulating Factor (rM-CSF) from PeproTech EC Ltd. (London, UK). For the latter, BM cells were placed into 12-well cell culture plates and volume adjusted up to 10 ml per dish.
with medium supplemented with rM-CSF, followed by incubation at 37°C, in a humidified incubator of 95% v/v air and 5% v/v CO₂ for 6 d. Using BM progenitor cells from C57Bl/6 and *Nfkβ2*-/- mice, initial concentrations of rM-CSF ranging from 50-100 ng/ml were examined for ability to effect differentiation of, and proliferation and survival of, macrophages (see Figure 2.3 and Figure 2.4). A concentration of 100 ng/ml rM-CSF showed greater activity and this level was selected therefore to differentiate BM progenitor cells from all other wild-type and knockout mouse strains (see Figure 2.5). After incubation, media containing the non-adherent, non-differentiated BM cells were discarded. Adherent differentiated macrophage cells (elongated in morphology) were washed twice with 5 ml sterile PBS, scraped and resuspended in a further 10 ml (5 ml per femur) medium supplemented with rM-CSF followed by seeding cells into 24-well plates (i.e. placing 1 ml of medium containing cells into each well) and then incubating for overnight prior to infection; see section 2.7 for detail of the intra-macrophage replication assay. For more information and details on the growth and differentiation of murine BM cells into macrophages, see [311].
Figure 2.2 The main steps of preparation of Bone Marrow (BM) cells.

(1) The femur is first disinfected and then the both ends of the femur are trimmed (2) BM is then flushed using a 30 gauge needle coupled to a one ml syringe into a 50mL Falcon tube. (3) After centrifugation (at 250 g for 5 min), BM cell pellets were resuspended in 10 ml RPMI 1640 medium supplemented with rM-CSF and (4) placed into a 10mL Petri dish for culture at 37°C, 95%/5% v/v air/CO2. Adapted from http://dx.doi.org/10.14440/jbm.2014.12 (accessed 08-12-2016). Permission sought from authors and publisher.
Figure 2.3 Frozen Bone marrow (BM) cells from a wild-type C57BL/6 mouse differentiate into macrophages.

Representative images at magnification x20 of a wild-type mouse (C57BL/6) BM cells differentiated into macrophages on day 3 (left hand panel) and day 6 (right hand panel).
**Figure 2.4** Comparative images illustrating morphological features of *Nf-κb2* knock-out bone marrow (BM) progenitor cells at different rM-CSF concentrations.

BM progenitor cells were cultured for 6 days in RMPI 1640 medium at rM-CSF concentration of 50 ng/ml (left hand panel) compared to those cultured in 100 ng/ml (right hand panel). The differentiation levels (conversion) of BM progenitor cells into macrophages are higher at r-MCSF concentration of 100 ng/ml.
Figure 2.5 Morphology of differentiated BM-derived macrophages from Nf-kb1 (A), Nf-kb2 (B) and c-Rel (C) knock-out mice at days 1, 2 and 6 post recombinant mouse colony stimulating factor (rM-CSF) treatment. (Panel A).

Representative images (Magnification x20) illustrate the morphology of BM progenitor cells from (A) Nf-kb1⁻/⁻, (B) Nf-kb2⁻/⁻ and (C) c-Rel⁻/⁻ mice following 1, 2 and 6 days of culture in RPMI 1640 medium supplemented with 100 ng/ml of rM-CSF. On day 1, BM progenitor cells are small in size with a defined cell-membrane, while adherent differentiated macrophages with branched and extended morphology are clearly visible from day 3 onwards.
Figure 2.5 (Continued): Morphology of differentiated BM-derived macrophages from *Nf-κb1* (A), *Nf-κb2* (B) and *c-Rel* (C) knock-out mice at days 1, 2 and 6 post recombinant mouse colony stimulating factor (rM-CSF) treatment. (Panel B)

Representative images (Magnification x20) illustrate the morphology of BM progenitor cells from (A) *Nf-κb1* (B) *Nf-κb2* (B) and *c-Rel* (C) knock-out mice following 1, 2 and 6 days of culture in RPMI 1640 medium supplemented with 100 ng/ml of rM-CSF. On day 1, BM progenitor cells are small in size with a defined cell-membrane, while adherent differentiated macrophages with branched and extended morphology are clearly visible from day 3 onwards.
Figure 2.5 (Continued): Morphology of differentiated BM-derived macrophages from \textit{Nf-\kappa b1} (A), \textit{Nf-\kappa b2} (B) and \textit{c-Rel} (C) knock-out mice at days 1, 2 and 6 post recombinant mouse colony stimulating factor (rM-CSF) treatment. (Panel C).

Representative images (Magnification x20) illustrate the morphology of BM progenitor cells from (A) \textit{Nf-\kappa b1}\textsuperscript{-/-} (B) \textit{Nf-\kappa b2}\textsuperscript{-/-} and (C) \textit{c-Rel}\textsuperscript{-/-} mice following 1, 2 and 6 days of culture in RPMI 1640 medium supplemented with 100 ng/ml of rM-CSF. On day 1, BM progenitor cells are small in size with a defined cell-membrane, while adherent differentiated macrophages with branched and extended morphology are clearly visible from day 3 onwards.
2.4 Generation of BMDM from a variety of genes knock out BM cells

Frozen BM cells from $\text{Il}^{-/-}$, $\text{Il18}^{-/-}$ and $\text{Myd88}^{-/-}$ mice underwent the same growth conditions and period of incubation as mentioned above to differentiate them to mature adherent macrophages (Figure 2.6). Myeloid differentiation primary response gene 88 (Myd88) is an adapter protein, used by almost all TLRs (except TLR3) to activate the transcription factor NF-κB [312].
Figure 2.6 Frozen BM cells from *Il-1r*−/−, *Il-18r*−/−, *Myd88*−/− mice well differentiate into macrophages.

Representative images (Magnification x20) illustrate the morphology of BM progenitor cells from (A) *Il-1r*−/− (B) *Il-18r*−/− and (C) *Myd88*−/− mice after 6 days of culture in RPMI 1640 medium supplemented with 100 ng/ml of rM-CSF. Differentiated macrophages with branched and extended morphology are clearly visible.
2.5 Verification of successful murine BM monocyte differentiation into mature macrophages

F4/80 antigen is a mature mouse cell surface glycoprotein expressed at high levels on various macrophages including: Kupffer cells, splenic red pulp macrophages, microglia, macrophages of the gut lamina propria, and Langerhans cells in the skin [313]. This antigen is also expressed on the macrophages within connective tissue, heart, kidney, and those within the reproductive and neuroendocrine systems. F4/80 antigen has been established as one of the most important antigens used to identify mature mouse macrophages (macrophage marker) [313]. Expression levels of F4/80 are frequently used to estimate the maturation of macrophage cells [314].

BM cells were first incubated for 6 days in Petri dishes. Cells were then transferred to 12 well cell cultures plates on to sterile 13mm glass cover-slips for 24h. Culture medium was then removed and replaced with 10% formalin. Following incubation for 30 min at room temperature, formalin was replaced with ice-cold 100% methanol and the culture plate was incubated for 5-10 min at a -20°C freezer. Methanol was removed and wells washed three times with sterile PBS. To block non-specific antibody binding, 10% w/v Bovine Serum Albumin (BSA) (Sigma) was applied to the cells for 1h at room temperature. The cells were then incubated overnight with primary antibody; F4/80/EMR1 antibody (CI-A3-1; catalogue number NB600-404; Novus Europe; Abingdon, United Kingdom) at dilution 1:100. The following day, the cells were again washed three times with PBS and the secondary antibody (anti-mouse Ig antibody; Vectashield Antifade Mounting Medium with DAPI; VECTOR Laboratories Ltd.; Peterborough, UK) as then applied to cells and the plate was incubated for an hour at room temperature. After washing with PBS, pre-prepared diaminobenzidine (DAB)-substrate solution [Two DAB tablets (Sigma) added to 5 ml distilled water] was applied to cells for 5-10 min at room temperature. Cells were then viewed after washing with sterile PBS. For more details regarding the F4/80 primary antibody please see the manufacturer’s datasheet; see http://www.novusbio.com/NB600-404 (accessed 08-12-2016).
2.6 Intramacrophage replication assay

Survival and replication abilities of CD *E. coli* strains, compared to other clinical *E. coli* isolates and to *S. Typhimurium* strains, within J774-A1 murine macrophage cell line were assessed by gentamicin protection assay, as previously described [131, 149, 205]. Replication assessment of ileal and colonic Crohn’s disease mucosa-associated *E. coli* strains LF82 and HM605 was also performed in wild-type bone marrow-derived macrophages (BMDM) and within macrophages derived from the BM of Nfκb1−/−, Nfκb2−/− and c-Rel−/− mice.

All macrophages tested were seeded onto two 24-well tissue culture plates at a density of 1x10⁵ cells per well, and incubated for 24 h at 37°C in atmosphere of 5% CO₂. Macrophage monolayers were infected in triplicate at multiplicity of infection (MOI) of 10 with a number of *E. coli* strains obtained from biopsies of CD, UC and CRC patients, those with UTI, from healthy individuals, and non-pathogenic laboratory *E. coli* strains. Before infection, these bacterial strains were resuspended in sterile PBS to the required optical density (OD) for each strain defining a bacteria cell count of 1 x 10⁹ bacteria/ml; see Figure 2.7. In order to allow macrophages to phagocytose bacteria, infected cells were incubated for 2 h. After incubation, the media were removed and infected macrophages were washed with sterile PBS. One millilitre of pre-warmed medium, containing gentamicin (20 µg/ml) in order to kill extracellular bacteria was added into each well. The first 24-well plate was then incubated for 1h, the second meanwhile for 3h to allow those resident intracellular bacteria to either replicate or be killed by the macrophage. After washing, cells with sterile PBS, 1 ml 1% v/v Triton X-100 (Sigma) in deionized water was added for 5 min to lyse cells. Ten-fold serial dilutions of the cell lysates were performed followed by plating of 50 µl of each, starting from 10² fold dilution of cell lysates down to 10⁴, on standard Luria broth (LB) agar plates (four plates per dilution step). After 24 h incubation at 37°C, bacterial colonies were enumerated as colony forming units (CFU). In order to measure the relative replication of bacteria strains, CFU at 6h post-infection were compared with CFU at 3h post-infection.
Figure 2.7 Bacteria growth calibration curve.

Representative bacterial growth curve to support *E. coli* enumeration; by comparison colony forming unit (CFU) counts per millilitre (CFU/ml) established by overnight culture on LB agar relative to optical density (OD$_{600nm}$) reading of the same bacterial suspension. Each value represents the mean ± standard error of the mean (SEM). This calibration curve was used to identify the required reading at OD$_{600nm}$, containing the target number of working stock bacteria (1$x10^9$ CFU/ml), $\equiv$ OD of 0.9 [n=3]. This curve is representative for the colonic mucosa-associated CD AIEC HM605.
2.7 Assessment of the phagocytic abilities of BMDM from knock-out strains of mice

Phagocytosis of NfkB1\textsuperscript{-/-}, NfkB2\textsuperscript{-/-}, c-Rel\textsuperscript{-/-}, Il-1r\textsuperscript{+} and Il-18r\textsuperscript{+} and Myd88\textsuperscript{-/-} BMDM were studied with an assay that detected internalization of Fluorescently-labelled heat-killed *Escherichia coli* K-12 strain BioParticles\textregistered, Alexa Fluor\textregistered 488 conjugate (Catalogue number E-13231; Life Technologies; Paisley, UK) at different time points (3, 6 and 24h post-infection) by fluorescence microscopy (Olympus bx51 microscope; images were taken by AQM software (Kinetic Imaging) and adjusted by AJ imaging software) C57BL/6 BMDM was used as a control. To maintain conditions in the standard intra-macrophage replication assays only, following addition of the K-12 BioParticles for 1h, a further 2h incubation in media containing gentamicin (20 µg/ml) was performed. Fluorescence of *E. coli* K-12 BioParticles conjugates bound to the macrophage cell surface was quenched with 0.4% w/v Trypan blue (Sigma) before imaging. For further information on Alexa Fluor\textregistered 488 conjugated *Escherichia coli* K-12 strain BioParticles\textregistered, visit http://ww.lifetechnologies.com/order/catalog/product/V6694 (accessed 08-12-2016).

2.8 Bacteria growth curve in standard nutrient media at differing pH

Overnight bacteria cultures were harvested and washed three times with sterile PBS, and then resuspended at an optical density (OD\textsubscript{600nm}) = 0.1 in RPMI 1640 medium without phenol red (Life Technologies). A 2 mL sample was placed into a cuvette tube and OD at wavelength 600\textsubscript{nm} was measured hourly using a GeneQuant Pro UV/VIS Spectrophotometer, DNA/RNA Calculator (Amersham BioSciences; Piscataway NJ, USA). This was performed using standard RPMI medium at pH 7 and at pH 5 (titration using 1M HCl and then 0.2µm sterile filtered).
2.9 Bacteria survival and growth in acidic nutrient-poor M9 medium

At early exponential growth phase (OD$_{600}$=0.1), bacteria were resuspended in a differing pH, ranging from 4 to 7, nutrient-poor (Minimal salts/Minimal microbial growth M9) medium (Life Technologies) supplemented with 100mM Bis-Tris (Sigma), 0.1% w/v Casamino Acids (MP Biomedicals; Loughborough Leicester, UK), 0.16% v/v glycerol (Sigma) and 10µM magnesium chloride (Sigma), each essential compounds for bacteria to grow. OD at wavelength 600$_{\text{nm}}$ of each bacterial suspension was subsequently measured hourly at time points 0 to 8h by spectrophotometer.

2.10 Low pH, high nitrosative and high oxidative stress tests on solid growth media

At OD$_{600}$=0.1, bacteria underwent ten-fold serial dilution steps. 20 µl from each dilution was placed, in triplicate, into five LB agar plates, containing one of the following stress agents: 100 mM 4-Morpholine ethanesulfonic acid (MES) (Sigma) pH 5.0, 100 mM MES pH 5.0 + 1 mM sodium nitrite (NaNO$_{2}$) (Sigma), 1 mM methyl viologen (MV) (Sigma) pH 7.0, 1 mM hydrogen peroxide (H$_2$O$_2$) (Sigma) pH 7.0, and a plain LB agar at pH 7.0 was used as a control. Plates were then subjected to the incubator for overnight incubation at 37°C (Summarised in Figure 2.8). This method as per [244].
Figure 2.8 A diagram summarising the main steps of performing bacteria stress tolerance tests.
2.11 RNA extraction and cDNA synthesis of intra-macrophage *E. coli* strains besides those grown in acidic M9 media

Using the RNeasy Mini Kit (Qiagen; Ltd, Crawley, UK), bacterial RNA was extracted from Crohn’s AIEC strain HM605, and laboratory *E. coli* strains (EPI300, XL-1Blue and K-12) cultured in acidic (pH 4.5) M9 media at 6h time point of their growth curve. In additional separate experiments, RNA from was isolated from AIEC HM605 and EPI300 inside J774-A1 macrophages post 3h and 6h infection.

Bacteria cells were lysed by adding 350 µl of RLT lysis buffer (Qiagen) to each bacterial pellet and mixed by pipetting up and down, followed by vortexing and centrifugation at full speed (Hawk 15/05 microcentrifuge, with rotor radius of 75 mm) in a 2ml Qia-shredder tube (Sigma) for 2 min. One volume of 70% v/v ethanol was added to the lysate and mixed by pipetting. A 700µl aliquot of each sample was then transferred into RNeasy Mini spin columns (RSC) placed in a 2 ml collection tube and centrifuged ≥8000 rpm (centrifuge as above) for 15 seconds. After discarding the flow-through, 700µl buffer RW1 was added to the RCS and samples again centrifuged for a further 15 seconds. The flow-through was discarded and 500µl buffer RPE was then added to the RCS followed again by 15 seconds centrifugation. After discarding flow-through, another 500 µl buffer RPE was again added to the RCS and centrifuged for 2 min, again at ≥8000 rpm. The RCS was placed in 1.5 collection tube and 45µl RNase-free water was directly added to centre of the RCS, and centrifuged at ≥8000 rpm for 1 min to elute the extracted RNA. A NanoDrop Lite Spectrophotometer (Thermo Fisher Scientific, USA) was used to quantify the eluted RNA (with concentrations routinely obtain ranging from 50 to 100 ng/µl). Complementary DNA (cDNA) at concentration ranging from 2-2.5 µg/µl was synthesised from the sample RNA using a Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics Ltd., Burgess Hill, UK) was initiated by adding the all components presented in the following Table 2.2. The reaction tube was then incubated at 25ºC in a bench heating block for 10 min, then at 55ºC for a further 30 min, followed by heating to 85ºC for 5 min to inactivate Transcriptor Reverse Transcriptase. The sample was then cooled on ice for 5 min. The cDNA was stored at -20ºC freezer for use in subsequent qRT-PCR experiments.
Table 2.2 Components for setting up a single reverse transcription reaction.

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anchord-oligo(dt)18 primer, 50pmol/µl</td>
<td>1 µl</td>
<td>2.5 µM</td>
</tr>
<tr>
<td>Transcriptor Reverse Transcriptase Reaction</td>
<td>4 µl</td>
<td>1 x</td>
</tr>
<tr>
<td>Buffer, 5x concentration</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Protector Rnase Inhibitor, 40 U/µl</td>
<td>0.5 µl</td>
<td>20 U</td>
</tr>
<tr>
<td>Deoxynucleotide Mix</td>
<td>2 µl</td>
<td>1 mM</td>
</tr>
<tr>
<td>Transcriptor Reverse Transcriptase, 20 U/µl</td>
<td>0.5 µl</td>
<td>10 U</td>
</tr>
<tr>
<td>Sample RNA</td>
<td>2 µl</td>
<td></td>
</tr>
<tr>
<td>Water, PCR-grade</td>
<td>10 µl</td>
<td></td>
</tr>
<tr>
<td><strong>Final volume</strong></td>
<td>20 µl</td>
<td></td>
</tr>
</tbody>
</table>
2.12 Quantitative real time polymerase chain reaction analysis (qRT-PCR) for assessment of bacteria stress genes supporting acid tolerance

Quantitative RT-PCR was performed in order to quantify the expression levels of stress response genes of the Glutamic acid decarboxylase operon (gad) system, used for tolerance to an acidic environment, by *E. coli* strains EPI300, K-12, XL-1Blue and CD AIEC HM605 grown in an acidic (pH 4.5) low nutrient (M9) growth medium and those within the phagolysosome of J774-A1 macrophages. After reverse transcription of extracted RNA from bacteria, 5 µl of the cDNA template was added into each well of the Light Cycler® 480 Multiwell plate, containing 15 µl of PCR mix (Roche Diagnostics Ltd., UK) prepared by mixing 10 µl Light Cycler® 480 Sybr Green I Master with 2 µl primers, listed in Table 2.3, which are real-time oligonucleotide primers designed by Eurogentec (Southampton, UK), in a final volume (3 µl) of water, PCR-grade. In each experiment, 5µl of water PCR-grade was added into PCR mix regarded as no template control (NTC). As a training test to fulfil our curiosity, a PCR mix was prepared by adding 2 µl of *uidA* primers into 2 µl Taqman probe (200nM), 10 µl Light cycler® 480 probe master mix and 2 µl water, PCR-grade and then 5 µl of each dilution from the fivefold serial dilutions (1:5) of *E. coli* K-12 cDNA templates were added to the PCR mix in the multiwell plate. Multiwell plates were sealed and then centrifuged at 1500 g (Sorvall Heraeus, Multifuge 3S-R, rotor radius 145 mm) for 2 min. Using the Light Cycler® 480 Instrument, multiwell plate was loaded and afterwards run with conditions as per the manufacturer’s instructions (Light Cycler® 480 Sybr Green I Master, Roche): 95 °C for 5 min then 45 cycles of 95 °C for 10 sec and 60 °C for 20 sec followed by 72°C for 30 seconds and then cooling step of 40 °C for 10 sec. The specificity of amplification was confirmed by melting curve analysis. All reactions were run in duplicate. Threshold cycle (Cp) for each well was automatically defined by the real time cycler software.
Table 2.3 Oligonucleotide primers used in this study to measure *gadA* and *gadB* expression by qPCR.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Oligonucleotide Sequence (5’ to 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>gadA-F</td>
<td>CCCCGTCAAGTTTATGG</td>
</tr>
<tr>
<td>gadA-R</td>
<td>CGCCGATGGTGTCCG</td>
</tr>
<tr>
<td>gadB-F</td>
<td>TGGTTCTTCGAGGCTGTA</td>
</tr>
<tr>
<td>gadB-R</td>
<td>TTGCCCAACGCCATT</td>
</tr>
</tbody>
</table>

Primer sequences taken from Smith *et al.*[315]
2.13 RT² Profiler PCR Array to quantify host macrophage oxidative stress response genes in response to *E. coli* infection

The Qiagen/SABiosciences RT² Profiler PCR Array (Catalogue Number 330231 PAMM-065ZA, Qiagen Ltd, Crawley, UK) was used to quantify the level of 84 oxidative stress genes expressed from J774-A1 macrophages post 6h infection with Crohn’s AIEC HM605, LF82 and laboratory *E. coli* strain EPI300 (see Table 2.4). RNA of these infected cells was extracted using RNeasy, Mini Kit (Qiagen), followed by cDNA synthesis using RT² First Strand Kit (Catalogue Number 330401; Qiagen). An aliquot (91 µl) of RNase-free water was then added into each sample cDNA. The cDNA concentration of all samples was ranging from 168 to 309 ng/µl, see Table 2.5.

RT² PCR components mix was prepared in a 5 ml tube as described in Table 2.6. Since the PCR Array plate already contains primers, 25 µl PCR components mix was then dispensed to each well of the RT2 Profiler PCR Array plate using an 8-channel pipette. The plate was carefully sealed and centrifuged for 1 min at 1500 g in a Sorvall Heraeus Multifuge 3S-R, rotor radius 145 mm) for 2 min. RT² Profiler PCR Array plate was placed in Light Cycler 480 and the PCR cycling program was set up according to Table 2.7. The Cp values for all wells were exported to Excel files and uploaded on [http://pcrdataanalysis.sabiosciences.com/pcr/arrayanalysis.php](http://pcrdataanalysis.sabiosciences.com/pcr/arrayanalysis.php) (accessed 08-12-2016) for analysis.
**Table 2.4 RT² PCR Oxidative stress profiler array gene list (Mouse).**

| Antioxidants | Glutathione Peroxidases (GPx): Gpx1, Gpx2, Gpx3, Gpx4, Gpx5, Gpx6, Gpx7, Gstk1, Gstp1.  
|             | Peroxiredoxins (TPx): Ehld1, Prdx1, Prdx2, Prdx3, Prdx4, Prdx5, Prdx6 (Aop2).  
|             | Other peroxidases: Apc, Cat, Csb, Duox1, Epx, Lpo, Mpo, Ptgs1 (COX1), Ptgs2 (COX2), Rag2, Serpinb1b, Tpo.  
|             | Other Antioxidants: Alb, Gsr, Sod1, Sod3, Srxn1, Txnrd1, Txnrd2, Txnrd3.  
| Reactive Oxygen Species (ROS) Metabolism | Superoxide Dismutases (SOD): Sod1, Sod2, Sod3.  
|             | Superoxide Metabolism Genes: Ccs, Cyba, Ncf1, Ncf2, Nos2 (iNOS), Nox1, Nox4, Noxa1, Noxa1, Recq4, Scd1, Ucp2.  
|             | Other Reactive Oxygen Species (ROS) Metabolism Genes: Aox1, Fmo2, Ifi19, Ii22.  
|             | Oxidative Stress Responsive Genes: Als2, Apoe, Cat, Ccl5 (RANTES), Csb, Duox1, Epx, Ercc2 (XPD), Ercc6, Pth1, Gclc,Gclm, Gpx1, Gpx2, Gpx3, Gpx4, G  
|             | Dx5, Gpx6, Gpx7, Gsr, Gss, Hmox1, Hspa1a (hsp70A1), Idh1, Krt1, Mpo, Nqo1, Park7,Prdx1, Prdx2, Prdx6 (Aop2), Pmp  
|             | , Psmb5, Sod1, Sqstm1, Tpo, Txn1, Txnip, Txnrd1, Txnrd2, Ucp3, Xpa.  
| Oxygen Transporters | Atr, Cygb, Dnm2, Fancc, Ifit172, Mb, Ngb, Slc38a1, Vim.  

Table 2.5 cDNA concentration of J774-A1 at 6h post infection.

<table>
<thead>
<tr>
<th>J774-A1macrophages</th>
<th>Uninfected</th>
<th>EPI300</th>
<th>HM605</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample 1</td>
<td>179 ng/µl</td>
<td>309 ng/µl</td>
<td>195 ng/µl</td>
</tr>
<tr>
<td>Sample 2</td>
<td>208 ng/µl</td>
<td>170 ng/µl</td>
<td>168 ng/µl</td>
</tr>
<tr>
<td>Sample 3</td>
<td>180 ng/µl</td>
<td>302 ng/µl</td>
<td>206 ng/µl</td>
</tr>
</tbody>
</table>

Table 2.6 RT² PCR component mix.

<table>
<thead>
<tr>
<th>Array Format</th>
<th>96-well</th>
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</thead>
<tbody>
<tr>
<td>RT2 SYBR Green Mastermix (2x)</td>
<td>1350 µl</td>
</tr>
<tr>
<td>cDNA reaction</td>
<td>102 µl</td>
</tr>
<tr>
<td>RNase free water</td>
<td>1248 µl</td>
</tr>
<tr>
<td>Total volume</td>
<td>2700 µl</td>
</tr>
</tbody>
</table>

Table 2.7 Cycling conditions for Roche Light Cycler 480.

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Cycles</th>
<th>Duration</th>
<th>Temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-incubation</td>
<td>1</td>
<td>10 min</td>
<td>95</td>
</tr>
<tr>
<td>Amplification</td>
<td>45</td>
<td>15 sec</td>
<td>95</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1 min</td>
<td>60</td>
</tr>
<tr>
<td>Melt Curve</td>
<td>1</td>
<td></td>
<td>95</td>
</tr>
<tr>
<td>Cooling</td>
<td>1</td>
<td>1 min</td>
<td>30</td>
</tr>
</tbody>
</table>
2.14 Data analysis

All data sets presented in this thesis are expressed as mean ± SEM unless otherwise stated, where N is the total number of individual experiments (or mice) and n is the number of sample replicates. Independent sample groups were assessed for normality by Shapiro-Wilk test. Equality of sample variances were analysed by Levene’s test. Following this, statistical comparisons of normally distributed data sets were performed by one way analysis of variance (ANOVA) followed by Dunnett’s post-hoc test to make pair-wise comparisons of treatment means to untreated/uninfected controls (StatsDirect version 2.6.2; Sale, United Kingdom). This included comparisons of survival and fold replications of CD E. coli strains compared to treatment with a non-replicating control E. coli EPI300 (i.e. for the intramacrophage replication assays), and also comparisons of the survival/growth of IBD and non-IBD E. coli strains under various chemical stress conditions compared to unstressed control conditions. Differences were considered significant when P<0.05. Where appropriate, non-parametric correlation coefficient (Spearman’s rank) analysis was used to assess for any association between % bacterial growth in stress conditions and ability to replicate inside murine macrophages.
Chapter 3
Investigating the ability of *E. coli* strains from various diseases to replicate inside murine macrophages
3.1 Introduction

Several studies examined gut microbiota in the inflamed and non-inflamed segments of CD and UC, and established a significant reduction in the biodiversity of faecal and mucosa-associated microbiome in IBD patients compared with those healthy controls [66], reported that a relative decrease of Firmicutes and Bacteriodetes phyla, and an increase of Proteobacteria, particularly the family Enterobacteriaceae (including species such as E. coli) in CD, while in UC, there is reduction in Clostridium spp. and an increase in E. coli [316], see Introduction, section 1.2.1. Mucus, coating the healthy intestine is discontinuous in the small intestine and continuous in the colorectum, consisting of two layers: the outer of which is a loosely adherent layer which contains/is inhabited by commensal bacteria representative of the faecal stream and an inner tightly adherent layer which is normally sterile, as is the sub-mucus niche, so as to protect the epithelium. Mucosa-associated E. coli (including the phenotype AIEC) have been found in increased number in the ileal and colonic mucosae (including the inner adherent mucus layer) of CD patients [30, 36, 317, 318] and to a lesser extent patients with UC [119, 142] and colorectal cancer (CRC) [139, 143]. AIEC phenotype has been found to represent 9.3%, 3.7% and 3.1% of E. coli population obtained from ileal, ileo-colonic and colonic biopsies of CD patients [319]. In addition, clear experimental evidence has been provided for the causal role of gut bacterial dysbiosis in chronic ileal inflammation development (Crohn's disease-like ileitis) [320].

A number of independent studies have reported that intra-mucosal E. coli or mucosa-associated E. coli isolated from CD patients possess invasive properties. For example, in vitro studies on paradigm CD AIEC strains from the ileum, LF82, and from the colon, HM605 [117, 138, 139] showed they possessed the ability to translocate and replicate within both murine and human monocyte-derived macrophages (HMDM) [8, 305, 321, 322]. Electron microscopy, in addition, validated the ability of Crohn’s strains LF82 and HM605 replication inside vacuoles/phagolysosome [8, 131, 305, 321]. Interestingly, AIEC strains were isolated from the mucosa of healthy subjects and found to be more abundant in the ileum than the colonic mucosal biopsies [30]. Supported by the fact that AIEC strains have been isolated from healthy individuals, they are probably better defined as pathobionts (disease-causing organisms that live as symbionts under circumstances of normal gut health) [138, 283].
Despite all the advances that have been made in the last decade, more knowledge on AIEC pathogenesis and that regarding AIEC-macrophage interactions are still required, including whether AIEC are involved in the aetiology of CD and/or the mechanisms/environmental triggers that determine their increased presence in high numbers the inflamed mucosae of CD patients. Understanding the mechanisms of AIEC-host macrophage interactions in the pathogenesis of CD may provide key insight into CD pathogenesis and enable to develop different therapeutic strategies, based on the killing/clearance of this CD mucosa-associated pathovar. Here, we aimed, as an initial step, to assess and compare the survival and replication abilities of a number of CD, UC and CRC mucosally-associated E. coli strains, pathogenic E. coli strains isolated from patients with urinary tract infection (UTI), non-pathogenic E. coli strains isolated from healthy individuals, and non-pathogenic laboratory E. coli strains within murine macrophages in vitro using a gentamicin protection assay (see Materials & Methods; section 2.6). This would likely shed light on the best paradigmatic E. coli strains representing each disease to be singled for further studies. Some of these studied E. coli strains isolated from CD, UC and CRC, meet the criteria of being designated as AIEC phenotype [148], including the following examples: CD (HM95, HM605 and HM615, and LF82), UC (HM457), and CRC (HM44 and HM358). For a full listing, see Materials & Methods, section 2.1.1. For all other E. coli strains, their origin/source is known, but their phenotype is not well established, i.e. they have not be examined to determine as to whether they can be designated as AIEC or non-AIEC. Crohn’s AIEC strains, during the later phase of murine macrophage infection, induce chronic activation of NF-κB, which correlates with increased TNF-α secretion [323]. AIEC replication within macrophages is suggested to be dependent on TNF-α secretion [8, 149, 324]. In addition, how intra-macrophage replicating Crohn’s AIEC strains HM605 and LF82 behave within murine BMDM from various Nfκb family member knockout mice, including the classical pathway Nfκb1−/− (p105→p50) and c-Rel−/− (p65) and alternative pathway Nfκb2−/− (p100→p52) was examined [325]. Phagocytosis of these BMDMs from mice with immune response-related gene deletions (e.g., Il-1r−/−, Il-18r−/− and the TLR adaptor Myd88−/−) was also examined.
3.2 Crohn’s disease mucosa-associated AIEC HM605 shows greater ability to survive and replicate inside macrophages compared to other E. coli

Determination of fold replication of bacteria within murine J774-A1 macrophages showed that the paradigm Crohn’s disease (CD) mucosa-associated ileal isolate LF82 significantly survived and replicated within macrophages (4.056 ± 1.16-fold [mean ± SD]) compared to a non-adherent, non-invasive laboratory strain E. coli K-12 derivative EPI300 (0.83 ± 0.42); P<0.05; n=3, ANOVA. Similarly, and to a greater extent, the two paradigm CD mucosa-associated colonic E. coli isolates HM615 (4.87 ± 1.06-fold; p<0.01) and HM605 (8.97 ± 0.67-fold increase; p<0.0001) also showed significant survival and replication within macrophages (see Figure 3.1).

Figure 3.1 Intramacrophage survival and replication of paradigm Crohn’s disease mucosa-associated AIEC.

Increased replication of Crohn’s disease mucosa-associated AIEC ileal isolate LF82, and two colonic isolates HM615 and HM605 compared with the reference non-pathogenic E. coli K-12 derivative strain EPI300 within J774-A1 murine macrophages. Results are expressed as the relative number of intracellular bacteria at 6h post infection relative to that obtained after 3h post infection (i.e. bacteria fold replication). Each value represents the mean ± standard error of the mean (SEM) of three independent experiments (N=3) with 3 replicates undertaken (n=3). Differences in survival and replication of each AIEC were statistically significant. *P ≤ 0.05, **P ≤ 0.01, and ****P ≤ 0.0001 respectively; ANOVA with Dunnett’s post-hoc test compared to the reference laboratory E. coli strain EPI300.
The gentamicin exclusion assay, using J774-A1 macrophages, was further expanded to compare the paradigm CD AIEC strains with other *E. coli* from various conditions (including additional *E. coli* strains from CD, mucosally-associated isolates from UC patients, CRC patients, UTI isolates and those from the non-inflamed intestine of healthy individuals.

Overall, Crohn’s disease (ileal and colonic) mucosa-associated *E. coli* strains showed significantly greater ability to survive and replicate within J774-A1 murine macrophages compared to *E. coli* isolates from healthy individuals and patients with UC, CRC and those with a UTI; see Figure 3.2. Comparison of intra-macrophage survival and replication inside J774-A1 macrophages of individual strains to that observed with CD AIEC HM605 (10.18 ± 1.82-fold [mean ± SD]; N=6, n=3) and non-pathogenic EPI300 (0.51 ± 0.16–fold; N=6, n=3) were as follows: CD colonic *E. coli* isolates HM95 (3.68 ± 0.54-fold) and HM413 (3.76 ± 0.27-fold) and ileal isolate LF86 (4.54 ± 0.43-fold) and LF82 (4.06 ± 1.16-fold) replicated significantly better than CD *E. coli* HM427 (1.07 ± 0.28-fold), HM96 (1.19 ± 0.20-fold) and HM104 (0.94 ± 0.15-fold); N=1-3, n=3; see Figure 3.2.

Non-pathogenic laboratory *E. coli* strains, including the reference strain EPI300 (see above), *E. coli* XL-1 Blue (0.89 ± 0.07-fold), and two *S. Typhimurium* strains (ST4/74 and ST LT2 [0.92 ± 0.06 and 0.88 ± 0.10-fold respectively], showed inability to resist intra-macrophage killing at 6h; Figure 3.2. Some isolates were just able to survive at 6h, not to replicate, inside macrophages, including *E. coli* K-12 strain (1.07 ± 0.05-fold), and isolates from healthy individuals ECOR51 (1.11 ± 0.18-fold) and HM488 (1.42 ± 0.13-fold). However, some healthy *E. coli* isolates were also seen to replicate effectively inside murine macrophages, such as ECOR35 (2.05 ± 0.33-fold) and ECOR1 (5.62 ± 1.28-fold). All UTI-associated *E. coli* strains showed ability to survive and replicate within J774-A1 macrophages, albeit to varying degrees, including significant replication by ECOR64 (7.15 ± 1.24-fold; P<0.001 ANOVA) and lower level (non-significant) replication by ECOR40 (2.47 ± 0.53), ECOR50 (2.24 ± 1.05) and ECOR48 (1.59 ± 0.52-fold). In contrast, all CRC mucosa-associated *E. coli* isolates HM44, HM312 and HM358 were killed by J774A1 macrophages at 6h. Similarly, some UC mucosa-associated *E. coli* isolates, including HM250 (0.67 ± 0.21-fold) and HM464 (0.50 ± 0.09-fold) were also effectively killed, although other UC mucosa-associated *E. coli* either just survived or minimally replicated within murine macrophages at 6h (HM394, 1.07 ± 0.04 -fold and HM233, 2.12 ± 0.50 –fold compared to EPI300).
Figure 3.2: Intra-macrophage replication of CD *E. coli* isolates compared with healthy, UC, CRC and UTI-associated *E. coli*.

In general, Crohn’s disease (ileal and colonic) mucosa-associated *E. coli* strains showed significantly greater ability to survive and replicate within J774-A1 murine macrophages compared with healthy, UC, CRC and UTI-associated *E. coli* strains. Non-pathogenic laboratory *E. coli* isolates and two Salmonella strains (ST4/74 and STLT2) showed little/no ability to replicate, within macrophages. Some healthy and UTI *E. coli* isolates were also seen to replicate effectively inside murine macrophage phagolysosomes. Results are expressed as the number of intracellular bacteria at 6h post infection relative to that obtained after 3h post infection. Each value represents the mean ± standard error of the mean (SEM) of three independent experiments (N=3) with triplicates undertaken (n=3), excepting for HM605 and EPI300 (N=6, n=3), and for ST4/74, STLT2, *E. coli* XL-1Blue, K-12, HM96, HM104, HM394 and HM488 in which each value represents one experiment run in triplicate (N=1, n=3); Differences in survival and replication of bacteria that were statistically significant are indicated as follows: *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001
3.3 Confirmation of generation of mature bone marrow-derived macrophages (BMDM) from cryopreserved mouse femur bone marrow (BM) cells by F4/80 glycoprotein expression analysis

In order to verify that the BM cells from wild-type C57Bl/6 mice and knock-out mice strains on C57Bl/6 background differentiated into mature macrophages after being cultured in media supplemented with rM-CSF (100 ng/mL) for 6 days, immunocytochemistry using anti-mouse F4/80 antibody was performed. The following representative images show the expression of F4/80 antigen on mature BMDM from wild-type and one of the knock out strains, Nfkb2−/− mice (Figure 3.3).

![Nfkb2−/− BMDM](image1)

![C57BL/6 BMDM](image2)

**Figure 3.3 Immunocytochemistry using anti-mouse F4/80 EMR1 antibody (C1-A3-1).**

Two representative images of adherent BM cells from Nfkb2−/− (A) and C57BL/6 (B) mice following 6d of differentiation in rM-CSF (100ng/mL) showing branched and extended cellular morphology and immunoreactivity with anti-mouse F4/80 antibody. Blue arrows point to sites of mature macrophage aggregation with high level F4/80 antigen expression. (Magnification x20) N=2 mice, n=2 replicates.
3.4 Crohn disease (CD) mucosa-associated AIEC HM605 and LF82 replicate inside C57Bl/6 BMDM

Intra-phagolysosome survival and replication of CD AIEC HM605 within wild-type C57Bl/6 BMDM at 6h post-infection was 4.47 ± 1.00-fold [mean ± SD] above that seen at 3h post infection; BM cells were obtained from a wild-type mouse C57BL/6 (n=2♂, n=2♀), N=3, n=2 replicates; see Figure 3.4A and Figure 3.4B. The paradigm ileal CD AIEC isolate LF82 also replicated within wild-type BMDM at a similar level (4.00 ± 0.75-fold), N=1, n=2 replicates; Figure 3.4A and Figure 3.4C. These results indicate these AIEC strains are able to survive and significantly replicate within wild-type C57Bl/6 BMDM differentiated from cryopreserved mouse femur bone marrow using rM-CSF (100 ng/mL, over 6 days).

3.5 Crohn’s disease AIEC strains HM605 and LF82 are unable to survive nor replicate within Nfκb1 and Nfκb2 deficient murine BMDM

Intra-macrophage replicating CD mucosa-associated AIEC strains HM605 and LF82 were both unable to replicate or to survive inside BMDM, derived from Nfκb1−/− (Figure 3.5A) or Nfκb2−/− mice (Figure 3.5B). Relative replication within BMDM from Nfκb1−/− was 0.47 ± 0.19-fold and 0.56 ± 0.28-fold respectively for HM605 (N=2, n=2) and LF82 (N=1, n=2); with relative replicate in Nfκb2−/− BMDM being 0.82 ± 0.06-fold and 0.25 ± 0.08-fold for HM605 (N=3, n=2) and LF82 (N=1, n=2) respectively.
Figure 3.4 Intra-macrophage replication of Crohn’s disease AIEC HM605 and LF82 in C57Bl/6 BMDM.

(A) CD mucosa-associated *E. coli* HM605 and LF82 showed ability to survive and replicate within wild-type C57BL/6 BMDMs as determined by gentamicin protection assay, 6h/3h post infection. Representative images of intra-macrophage HM605 and LF82 respectively at 3h and 6h post infection time for (B) HM605 and (C) LF82 as determined by overnight culture on LB agar and CFU enumeration; HM605; N=3, n=2 replicates, and LF82; N=1, n=2 replicates.
Figure 3.5 Crohn’s disease (CD) AIEC HM605 and LF82 show inability to survive and replicate within \( N\)f\( k\)b\( 1^-\) and \( N\)f\( k\)b\( 2^-\) BMDM.

As determined by gentamicin protection assay, at 6h/3h post infection, both CD mucosa-associated \textit{E. coli} HM605 and LF82 showed inability to survive and replicate within (A) \( N\)f\( k\)b\( 1^-\) BMDM [\( N=2, n=2\) replicates for HM605 and \( N=1, n=2\) replicates for LF82], and (B) \( N\)f\( k\)b\( 2^-\)BMDM [\( N=3, n=2\) replicates for HM605 and \( N=1, n=2\) replicates for LF82]. (C) Representative images of AIEC HM605 CFU grown overnight on LB agar plates obtained by lysis of \( N\)f\( k\)b\( 2^-\) BMDM at 3h and 6h post infection.
3.6 Crohn’s disease mucosa-associated AIEC strains HM605 and LF82 survive and replicate normally within c-Rel<sup>−/−</sup> BMDMs

Crohn’s AIEC strain HM605 survived and replicated within c-Rel<sup>−/−</sup> BMDMs (2.70 ± 0.62-fold; N=1, n=2) at levels similar to those seen for wild-type BMDM; see Figure 3.6A and Figure 3.6 B. For ileal CD mucosa-associated LF82, this isolate was seen to only survive (and not replicate) at 6h post infection within BMDM from c-Rel<sup>−/−</sup> mouse (1.10 ± 0.32-fold at 6h/3h; N=1, n=2) see Figure 3.6A and Figure 3.6C respectively. The ability to only survive (and not replicate) has been previously observed in some J774-A1 intramacrophage replication assays using the AIEC LF82.
Figure 3.6 HM605 and LF82 fold replication within c-Rel−/− BMDM.

(A) Bacteria fold replication of Crohn’s AIEC HM605 and LF82 within c-Rel−/− BMDM. (B) Representative examples of LB agar plates, containing bacteria colonies of AIEC HM605, and (C) AIEC LF82 obtained from intra c-Rel−/− BMDM at time points 3h and 6h post infection. N=1, n=2 replicates.
3.7 Other immune-related gene knockout BMDM infected with HM605 at MOI of 100 show better outcomes

Long-term cryopreserved BM (>10 years frozen in liquid N\textsubscript{2}) from immune-related gene knock-out animals showed high variability in ability to differentiate to BMDM. Whilst \textit{IL-1r}\textsuperscript{-/} \textit{IL-18r}\textsuperscript{-/} and \textit{Myd88}\textsuperscript{-/} BM progenitor cells differentiated well into macrophages when cultured within media supplemented with rM-CSF.

In gentamicin exclusion assay, the intracellular bacteria were typically counted at 10\textsuperscript{2} fold dilution of cell lysates infected at MOI of 10, but unfortunately no intracellular bacteria were obtained from, \textit{IL-1r}\textsuperscript{-/}, \textit{IL-18r}\textsuperscript{-/}, and \textit{Myd88}\textsuperscript{-/} BMDM cells in 10\textsuperscript{2} fold dilution plates (N=1, n=2 replicates). Therefore, in order to troubleshoot this issue, it was decided to infect BMDMs with CD AIEC HM605 and LF82 at both MOI of 10 and 100, and comparisons made (Figure 3.7). Representative images (Figure 3.7A and B) show that the intracellular bacterial numbers of the plates of the neat and 10\textsuperscript{1} fold dilutions of lysates of cells infected at MOI of 100 were higher than those infected at MOI of 10. Figure 3.7C and D show comparison between the numbers of intracellular bacteria in the plates of neat and 10\textsuperscript{1} fold dilutions of \textit{IL-1r}\textsuperscript{-/} cell lysates infected at MOI of 10 at 3h with those at 6h post infection. Intra-macrophage replication of both HM605 and LF82 were seen to be higher (~5 and ~1.5-fold respectively) in \textit{IL-1r}\textsuperscript{-/} murine BMDM (N=1, n=2). Other knockout BMDMs did not yield any conclusive datasets. Since all these BM cells were cryopreserved for many years (since 2002), it is more likely that the age of BM presented a challenge to effect adequate differentiation to mature/active macrophages and those that had differentiated showed little or no functional ability to examine intra-macrophage survival and replication.

Phagocytic function analysis of the various genes knock-out BMDM versus wild-type C57BL/6 BMDM cells showed no defect in ability to phagocytose and internalise fluorescein-labelled heat-killed \textit{E. coli} K-12 in \textit{Nfkb}\textsuperscript{-/} and \textit{Myd88}\textsuperscript{-/} BMDM cells (Figure 3.8). In contrast, phagocytosis was noticed to be impaired in \textit{IL-1r}\textsuperscript{-/} and \textit{IL-18r}\textsuperscript{-/} BMDM and these two cells were unable to degrade fluorescein-labelled heat-killed \textit{E. coli} K-12 (Figure 3.8).
Figure 3.7 Comparisons between cells infected at MOI of 10 and MOI of 100 (Panel A).

Bacteria inside C57BL/6 BMDM cells, 3h post infection with AIEC HM605 at MOI of 10 compared with at MOI of 100 (A) and with AIEC LF82 at MOI of 10 compared with at MOI of 100 (B). A comparison between the intra- *Il-1r*− BMDM numbers of HM605 (C) and LF82 (D) at 3h and 6h post infection. N=1, n=2 replicates.
Figure 3.7 (Continued): Comparisons between cells infected at MOI of 10 and MOI of 100 (Panel B).

Bacteria inside C57BL/6 BMDM cells, 3h post infection with AIEC HM605 at MOI of 10 compared with at MOI of 100 (A) and with AIEC LF82 at MOI of 10 compared with at MOI of 100 (B). A comparison between the intra- IL-1r−/− BMDM numbers of HM605 (C) and LF82 (D) at 3h and 6h post infection. N=1, n=2 replicates.
Figure 3.7 (Continued): Comparisons between cells infected at MOI of 10 and MOI of 100 (Panel C).

Bacteria inside C57BL/6 BMDM cells, 3h post infection with AIEC HM605 at MOI of 10 compared with at MOI of 100 (A) and with AIEC LF82 at MOI of 10 compared with at MOI of 100 (B). A comparison between the intra- II-1r−/− BMDM numbers of HM605 (C) and LF82 (D) at 3h and 6h post infection. N=1, n=2 replicate.
Figure 3.7 (Continued): Comparisons between cells infected at MOI of 10 and MOI of 100 (Panel D).

Bacteria inside C57BL/6 BMDM cells, 3h post infection with AIEC HM605 at MOI of 10 compared with at MOI of 100 (A) and with AIEC LF82 at MOI of 10 compared with at MOI of 100 (B). A comparison between the intra- \( II-1r^{-/-} \) BMDM numbers of HM605 (C) and LF82 (D) at 3h and 6h post infection. N=1, n=2 replicates.
Figure 3.8 Phagocytosis time course studies (Panel A).

Phagocytosis was intact in C57BL/6 BMDM (A) Nfkb2−/−(B), and Myd88−/−(E) BMDM cells. In contrast, phagocytosis was impaired in Il-1r−/− (C) and Il-18r−/− (D) BMDM respectively.
Figure 3.8 (Continued): Phagocytosis time course studies (Panel B).

Phagocytosis was intact in C57BL/6 BMDM (A) *Nfkb2<sup>−/−</sup>* (B), and *Myd88<sup>−/−</sup>* (E) BMDM cells. In contrast, phagocytosis was impaired in *Il-1r<sup>−/−</sup>*(C) and *Il-18r<sup>−/−</sup>*(D) BMDM respectively.
Figure 3.8 (Continued): Phagocytosis time course studies (Panel C).

Phagocytosis was intact in C57BL/6 BMDM (A) Nfkb2<sup>-/-</sup>(B), and Myd88<sup>-/-</sup>(E) BMDM cells. In contrast, phagocytosis was impaired in Il-1r<sup>-/-</sup> (C) and Il-18r<sup>-/-</sup> (D) BMDM respectively.
Figure 3.8 (Continued): Phagocytosis time course studies (Panel D).

Phagocytosis was intact in C57BL/6 BMDM (A) Nfkb2−/−(B), and Myd88−/−(E) BMDM cells. In contrast, phagocytosis was impaired in Il-1r−/−(C) and Il-18r−/−(D) BMDM respectively.
Figure 3.8 (Continued): Phagocytosis time course studies (Panel E).

Phagocytosis was intact in C57BL/6 BMDM (A) Nfkb2−/−(B), and Myd88−/−(E) BMDM cells. In contrast, phagocytosis was impaired in Il-1r−/− (C) and Il-18r−/− (D) BMDM respectively.
3.8 Summary of results

1. All Crohn’s ileal- and colonic-mucosa associated *E. coli* strains, excepting one isolate HM104, possessed ability to either survive and/or replicate within murine (J774-A1) macrophages. Interestingly, all UTI *E. coli* isolates and some healthy mucosa-associated *E. coli* strains behaved in a similar manner.

2. All CRC mucosa-associated *E. coli* strains tested, two out of four UC mucosa-associated isolates and the majority of laboratory *E. coli* strains (excepting *E. coli* K-12) were unable to survive inside J774-A1 macrophage phagolysosomes.

3. Wild-type C57BL/6 mouse BM cells and murine *Nfkb1*−/−, *Nfkb2*−/− and *c-Rel*−/− BM progenitor cells (fresh and 2-8 week frozen cells) were successfully differentiated into macrophages when cultured within media supplemented with rM-CSF. Mature BMDM macrophages generated were identified by cell morphology analysis and by their expression of F4/80 epitope using immunocytochemistry.

4. Crohn’s disease paradigm ileal and colonic AIEC strains LF82 and HM605, showed ability to survive and replicate within wild-type C57BL/6 BMDMs. Data suggests that both paradigm CD isolates were unable to survive and replicate inside *Nfkb1*−/− and *Nfkb2*−/− BMDMs, whilst they both survived and replicated within BMDM derived from *c-Rel*−/− mice. No defect in the phagocytosis was seen in *Nfkb*−/− family BMDMs.
3.9 Discussion

There is growing evidence supporting the hypothesis that Crohn’s disease (CD), a chronic-relapsing inflammatory bowel disease (IBD), is a result of innate immunodeficiency, such as a defect in bacteria handling and clearance, and impaired neutrophil chemotaxis [94, 326]. However, no abnormality in phagocytosis and respiratory burst function of monocyte-derived macrophages (MDM) obtained from CD patients has been described to date [327].

Our intra-macrophage replication assays, simultaneously comparing relative replication of various E. coli strains from 4 different diseases (CD, UC, CRC and patients with a UTI) along with from non-IBD control patients, is the first to our knowledge. Paradigm CD ileal and colonic AIEC (LF82 and HM605 respectively), were shown to possess ability to survive and replicate following engulfment by antigen-presenting immune cells such as macrophages and dendritic cells known to play critical roles in the induction of chronic inflammation (see Chapter 3, section 3.2) and this is in agreement with our own previous findings [131, 149, 179] and that observed by others [8, 229, 242, 243]. E. coli isolates from UTI patients along with some healthy-mucosa associated E. coli strains, obtained from healthy individuals also showed the ability to replicate within (J774-A1) macrophages. The latter result is consistent with findings of Subramanian et al. [149] in which mucosa-associated E. coli strains isolated from the non-inflamed intestine of non-IBD patients, diagnosed with sporadic benign polyp lesions or with IBS, were able to significantly replicate within J774-A1 macrophages (see reference [149]). Likewise, there is some evidence in the literature that UPEC strains isolated from patients with UTI can also survive and replicate within mouse macrophages, as well as replicating within urogenital epithelial cells [328, 329]. A previous study has shown that 7% of UPEC meet the criteria of the AIEC phenotype [30]. In contrast, all CRC mucosa-associated E. coli strains tested and the majority of laboratory E. coli strains (excepting E. coli K-12) were unable to survive inside J774-A1 macrophage phagolysosomes. E. coli K-12 was able to survive within murine macrophages over 6h and this result has also been previously observed by Subramanian and colleagues [149]. Two out of four UC mucosa-associated isolates and the majority of laboratory E. coli strains (excepting E. coli K-12) were unable to survive inside J774-A1 macrophage phagolysosomes. It has been documented that there is lower prevalence of E. coli-laden macrophages seen in UC [330], supporting earlier
A correlation between the colonic *E. coli* colonization density and the severity of ileal inflammation in Crohn’s patients has been demonstrated [157]. Pathogenic *E. coli*, being able to penetrate the intestinal epithelial barrier and resist macrophage killing process, have been found to be able to trigger a huge host inflammatory response [331]. Infected murine macrophages (J774-A1) harbouring Crohn’s disease AIEC strains within, including LF82 and 13I, found to secrete high levels of proinflammatory cytokine TNF [323, 324, 332]. AIEC LF82 and 13I strains appear to survive within murine macrophage phagolysosomes by suppressing acute NF-κB signal pathway activation during the initial phase of infection, a common strategy used by other pathogenic bacteria for their intra-cellular survival [323, 333, 334]. Persistence of intra-macrophage AIEC during the later phase of infection seems to be through induction of chronic activation of NF-κB, which correlates with increased TNF secretion from infected macrophages [323]. Exogenous addition of TNF was also reported to increase intra-macrophage replication of AIEC LF82 [324], with secreted levels correlating to number of intra-macrophage AIEC LF82 within infected monocyte-derived macrophages (MDM) [335]. Interestingly, AIEC (LF82)-infected MDM- from patients with quiescent CD appear to release significantly higher amounts of both IL-6 and TNF than those obtained from patients with active disease or those from healthy controls. although our own recent laboratory studies indicate that peripheral blood monocyte-derived macrophages obtained from healthy volunteers and Crohn’s patients produced roughly similar quantities of proinflammatory cytokines, including TNF, after infection with Crohn’s mucosa-associated AIEC HM605 [205].

Plasma TNF levels in Crohn’s patients with active disease are known to be significantly high [336]. Inhibition of proinflammatory cytokine production (including TNF) by triterpene ganoderic acid C1 (GAC1) treatment of either isolated peripheral blood mononuclear cells (PBMCs) or inflamed colonic biopsies taken from Crohn’s patients occurs via blockade of NF-κB activation [337, 338]. Gross impairment in the secretion of proinflammatory

studies where levels of intramucosal bacteria isolated from UC was found to be much lower than that seen in CD [139, 141, 330]. Here 2/3 colonic mucosa-associated isolates from UC mucosae were shown to possess ability to replicate within murine macrophages and further study on a greater number of clinical isolates is warranted.
cytokines TNF, IL-4, IL-5, IL-13, IL-15, and IFN-γ by macrophages from Crohn’s patients has been reported in response to infection with heat-killed \textit{E. coli} NCTC 10418 (HkEc) \cite{94, 339}, and recent published data from two different research groups showed that monocyte-derived macrophages (MDM) from Crohn’s patients stimulated with the same \textit{E. coli} strain released attenuated levels of TNF and interferon gamma (IFNγ) compared with those secreted from healthy control macrophages \cite{327, 340}. Moreover, THP-1 (human monocytic cell line) macrophages infected with the Crohn’s AIEC LF82 secrete large amounts of exosomes (i.e. extracellular vesicles that function in intercellular communication and have been implicated in host responses to intracellular pathogens) to induce a proinflammatory response \cite{341}.

NF-κB signalling can be attributed to actions of five family member protein subunits/protein subunit complexes, including NF-κB1, NF-κB2, RelA (p65), RelB and c-Rel, controlling DNA transcription and subsequent expression of proinflammatory cytokines to play a pivotal role in regulating immune response to infection \cite{342}. Results of our intramacrophage replication assays undertaken with Crohn’s AIEC strains within murine BMDM from various Nfκb family member knockout mice, indicated that neither ileal not colonic CD representative AIEC strains LF82 and HM605 survived within Nfκb1−/− (classical pathway p105→p50) nor alternative pathway Nfκb2−/− (p100→p52) BMDM. Both strains were able to survive and replicate within wild-type C57BL/6 and c-Rel−/− (p65) BMDM. Our phagocytosis function analysis data also showed that this engulfment process was not impaired in BMDM deficient in Nfκb family member proteins/protein subunits. Recent \textit{in vitro} studies on mice lacking the c-Rel subunit showed that they were more susceptible to colitis and colitis-associated cancer than wild-type mice, whilst Nfκb2−/− mice exhibited less severe colitis and an attenuated cytokine response in comparison to C57BL/6, Nfκb1−/− and c-Rel−/− mouse groups following DSS administration \cite{343}.

The fact that NF-κB pathway signalling is found to be chronically active in IBD (as well as in other inflammatory conditions, including gastritis) \cite{344} suggests that methods of inhibiting NF-κB signalling would likely have potential therapeutic application \cite{345}, including countering AIEC macrophage persistence seen in ileal and colonic Crohn’s disease mucosae.
Chapter 4

Studying the ability of Crohn’s *E. coli* strains to grow in conditions mimicking the harsh environment found inside vacuoles/phagolysosomes of the macrophage
4.1 Introduction

A number of strategies are adopted by differing pathogenic bacteria to avoid being killed inside the intra-cellular vacuoles/phagolysosomes of macrophages. These include a variety of adaptive responses, including avoidance of phagocytosis, inhibition of phagolysosome maturation and resistance of antimicrobial killing environment of mature phagolysosomes (i.e. low pH, low nutrient, high oxidative and nitrosative stress conditions) [346-348]. Classical pathogens known to survive and replicate within macrophages, including *Mycobacterium*, *Salmonella*, *Shigella*, *Brucella*, *Legionella* and *Listeria* spp. [224]. Key defence mechanisms adopted by these pathogens support their resistance to killing within the environment of the phagolysosome. *Shigella* and *Listeria* are able to escape from the mature phagolysosome, whilst *Salmonella* spp. can inhibit fusion of phagosome with the lysosome, and species such as *Mycobacterium tuberculosis* is able to modify the intra-phagolysosome environment [224]. *Salmonella* spp., *Shigella* spp. and *E. coli* have all been reported to possess a repertoire of low pH inducible systems that support resistance, tolerance and habituation during environmental acid stress likely encountered by active enteric bacteria within the phagolysosome. Other examples of pathogens showing adaptation to an intra-phagolysosomal life-style include *Coxiella burnetii* causing Q-fever [288] and *Tropheryma whipplei* causing Whipple's disease [289, 290].

Paradigm Crohn’s AIEC strains HM605 and LF82, and other CD mucosa-associated *E. coli*, have been shown to be present within mature phagolysosomes by gentamicin exclusion assay and microscopy, including transmission electron microscopy (TEM) [8, 131, 149]. Earlier studies have shown that ileal AIEC LF82 appears to be tolerant of a low pH intra-phagolysosome environment thus facilitating ability to replicate within the macrophage [349]. This persistence within macrophages supports giant cell formation and development of granulomata *in vitro* [350]. The presence of granulomas in mesenteric lymph nodes (MLN) has been found to be associated with postoperative recurrence in CD [351].
The mechanism of how these Crohn’s AIEC isolates resist the killing process and survive, even replicate, within macrophage phagolysosomes without inducing cell death is still poorly understood. Some key genes supporting AIEC survival and replication within macrophages have been identified using isogenic mutants of the ‘paradigm’ ileal AIEC LF82, including htrA, dsbA, hfq and gipA [241-245]. However, htrA and dsbA encoded stress response proteins HtrA and DsbA are fairly ubiquitous in E. coli, and it is likely that other unidentified factors are needed to support AIEC survival within the stressful conditions of the phagolysosome. Treatments that target the fundamental pathology of CD are still required and therefore studies understanding their ability to resist killing within mucosal macrophages are likely to improve current drugs used to maintain and treat this condition, and also support development of novel drug therapy that might be much more effective for patients with CD.

In this chapter, we aimed therefore to investigate the growth of intra-macrophage replicating Crohn’s mucosa-associated E. coli clinical isolates (including those with confirmed as AIEC), other IBD and cancer mucosa-associated E. coli, as well as pathogenic and non-pathogenic E. coli strains in chemical-induced stress conditions mimicking the intra-phagolysosome environment.

### 4.2 Growth of E. coli strains in a high nutrient environment

An initial examination of a collection of various E. coli strains isolated from healthy individuals and UTI patients (ECOR strains), in comparison to the colonic CD AIEC strain HM605 and the non-intra-macrophage replicating laboratory E. coli strain EPI300, grown in nutrient rich RPMI culture media showed little differences between strains at both pH 5 and 7 but that all showed significant reduction in growth at pH 5 (>50% less growth; HM605 54%, ECOR1 44%, ECOR35 56%, ECOR51 48%, ECOR40 49%, ECOR48 51%, ECOR64 52%, ECOR50 51%, and EPI300 43%; n=3) (see Figure 4.1)
Figure 4.1 *E. coli* growth patterns in high nutrient RPMI culture medium at differing environmental pH (pH 7 and pH 5).

Changing pH of high-nutrient media from 7 to 5 remarkably affected the bacteria growth. There was no significant differences seen in growth between strains. Each point represents the optical density mean of an experiment run in triplicate (n=3).
4.3 Crohn’s disease *E. coli* isolates are better able to grow in an acidic (pH 4.5), low nutrient environment

In order to assess their ability Crohn’s *E. coli* strains, including those of confirmed AIEC phenotype such as HM605 and LF82, to survive within the low nutrient, acidic environment inside the macrophage phagolysosome, strains were grown in low-nutrient medium (M9) at differing pH ranging from pH 4.0 to pH 7.0. The intra-phagolysosome pH of human-and murine derived macrophages after infection with bacteria such as *Mycobacterium tuberculosis* has been estimated in previous studies to be ~ pH 5 (pH 4.8 to 5.5) [352-354].

Four CD *E. coli* strains previously characterised as AIEC phenotype, LF82, HM605, HM615 and HM427 showed increased growth tolerance over 8h to an acidic, low nutrient M9 media, especially at pH 4.5, compared to that seen by non-AIEC laboratory *E. coli* strains K-12 EPI300 and XL1Blue, which demonstrated intolerance to low nutrient and low acid conditions over the same time frame (Figure 4.2). Two *S. Typhimurium* strains used showed better growth than laboratory *E. coli* strains down to pH 5 but remarkably could not tolerate low nutrient at pH 4.5 and below (Figure 4.2). In contrast, not all *E. coli* strains from CD patients (non AIEC types) were able to show significant growth at pH 4.5, suggesting that there are specific properties of adaption held by certain strains (see Figure 4.3). Other studied *E. coli* strains; i.e. those *E. coli* strains obtained from UC, cancer and UTI patients, and those from healthy individuals showed as similar inability, albeit with some variation, to grow at pH 4.5 as per the non-AIEC CD *E. coli* strains (see Figure 4.3). There was no remarkable growth in M9 minimal media of any bacteria studied at pH 4 (Figure 4.2). To support future studies using fluorophore expressing AIEC isolates, we compared the growth characteristics of colonic CD AIEC HM615 expressing enhanced green fluorescent protein (eGFP) which showed identical tolerance to growth in M9 minimal media at all differing pH as per the wild-type isolate (Figure 4.4).
Figure 4.2 Comparisons of the growth patterns of Crohn’s-associated *E. coli* strains and non-Crohn’s *E. coli* isolates in low-nutrient medium (M9) at differing pH from 7 to 4.

Four CD AIEC strains showed increased growth tolerance over 8 h to an acidic, low nutrient M9 media, at pH 4.5. All grew over 8h at pH 5, 6 and 7 and no significant differences among them were observed. Laboratory *E. coli* strains, XL-1 Blue, *S. Typhimurium* LT2 and 4/74 were unable to grow well at pH 4.5 and below. No bacteria studied were able to grow in M9 minimal media at pH 4; n=3.
Figure 4.3 The growth curves of a collection of *E. coli* strains isolated from a range of diseased individuals, besides two Crohn’s-associated *E. coli* strains, in a M9 medium at pH 4.5.

All bacteria’s growth lines increased regularly over time and no noticeable differences among them were observed. n=3.
Figure 4.4 A comparison of the growth curves of wild-type Crohn’s disease colonic mucosa-associated AIEC strain HM615 in differing pH M9 media with those of HM615 strain engineered to express enhanced Green fluorescent protein (eGFP).

eGFP expression has no adverse effect on the ability of this bacteria strain to grow in minimal media over a range of pH environments. n=3.
4.4 The majority of Crohn’s *E. coli* strains not only have better ability to tolerate low pH but also to tolerate high oxidative and nitrosative stress conditions typical of the phagolysosome environment

Bacterial strains were grown on plain solid LB agar plates at pH 7 (referred to as normal growth condition controls), and on LB agar plates providing environmental stress conditions, including (i) acid (low pH) stress, (ii) high nitrosative stress at pH 5, and (iii) oxidative and superoxidative stress, both at pH 7 (see **Methods section 2.10**). Unfortunately, it was not possible to grow bacterial strains on LB agar media at pH 4.5 and 4.0 because we found that warm LB agar media at each of these pH conditions resulted in inability of the agar to set/solidify in petri dishes. According to the results illustrated in **Figure 4.5**, the growth of Crohn’s *E. coli* strains (AIEC) HM605, HM615 and LF82, HM427 was not influenced by any of the chemical stress conditions that mimicked the harsh low pH, high nitrosative, and low and high oxidative stress environment within macrophage phagolysosome. All were able to tolerate growth on LB agar under these conditions at a similar level to that seen on LB agar at pH7 (**Figure 4.5**). Conversely, the growth of laboratory *E. coli* strains EPI300 and XL-1Blue were both significantly inhibited all of the stress conditions compared with growth seen on LB agar control plates at pH 7 (see **Figure 4.6**). Interestingly, *S. Typhimurium* strains LT2 and 4/74 and colonic cancer *E. coli* isolate HM358 showed the ability to tolerate the stress of low pH (pH 5), high nitrosative stress and mild oxidative stress conditions, but showed no tolerance under superoxidative stress condition where growth was remarkably impaired (see **Figure 4.6**). Surprisingly, non-pathogenic *E. coli* K-12 tolerated and grew in all stress challenge conditions (**Figure 4.6**). Again to support future *in vivo* intestinal mucosa oxidative stress response studies using fluorophore expressing AIEC isolates, we compared the growth characteristics of colonic CD AIEC HM615 expressing eGFP under the four stress environments compared to wild-type. Both wild type and the eGFP expressing isolate showed tolerance to all conditions tested (**Figure 4.7**) and this is consistent with the results observed for growth in minimal media at differing pH; **Figure 4.4**.
Figure 4.5 Crohn’s *E. coli* strains have better ability to tolerate low pH, high oxidative and nitrosative stress.

Crohn’s *E. coli* strains (AIEC); HM605 (A), LF82 (B), HM615 (C) and, and HM427 (D) in low pH, nitrosative, low oxidative, and superoxidative conditions. Bacteria growth in plain LB at pH 7 was used as a control. Bacteria CFUs were frequently counted at dilution factor $10^5$ and presented as relative growth response. N=4, n=3 replicates. Morpholine ethanesulphonic acid (MES) sodium nitrite (NaNO$_2$), methyl viologen (MV) and hydrogen peroxide (H$_2$O$_2$).
Figure 4.6 Laboratory *E. coli* strains, except K-12, and colonic cancer *E. coli* isolate HM358 and *S. Typhimurium* strains showed no tolerance under superoxidative stress condition.

Stress tolerance tests of Laboratory *E. coli* strains EPI300 (A), XL-1Blue (B) and ST strains 4/74 (C) and LT2 (D), Cancer AIEC strain HM358 (E) and *E. coli* strain K-12 (F) in low pH, nitrosative, oxidative, and superoxidative conditions. Bacteria growth in plain LB at pH 7 was used as a control. Bacteria CFUs were frequently counted at dilution factor 10^5 and presented as relative growth response. N=4, n=3 replicates. * P ≤0.05, ** P ≤ 0.01, **** P ≤ 0.0001, ANOVA with Dunnett’s post-hoc test. Morpholine ethanesulphonic acid (MES) sodium nitrite (NaNO₂), methyl viologen (MV) and hydrogen peroxide (H₂O₂).
Figure 4.7 A comparison of the growth of wild-type Crohn’s disease colonic mucosa-associated AIEC strain HM615 in stress conditions with those of HM615 strain engineered to express enhanced Green fluorescent protein (eGFP).

eGFP expression has no adverse effect on the ability of this bacteria strain to grow in low pH, nitrosative and low and high oxidative stress environments. N=4, n=3 replicates.
4.5 CD AIEC isolates survive the combined stress of low pH (pH 5) oxidative and superoxidative stress of the intra-macrophage phagolysosome

It is already known that bacteria within macrophage phagolysosomes face low pH environment, ranging from pH 4.8 to pH 5.5 [352-354] associated with other harsh conditions such as high nitrosative and oxidative/superoxidative stress. We therefore attempted to compare the survival and growth of some *E. coli* strains in an acidic environment (pH 5) in combination of either oxidative or superoxidative stress conditions to those bacteria grown under stress but under non-acid conditions (i.e. pH 7). Bacteria were also grown on standard LB agar plates at pH 7 and at low pH, pH 5 as controls. The results (see Figure 4.8; panels A, B, C and D) showed that Crohn’s *E. coli* strains had significantly greater ability to tolerate all oxidative and superoxidative stress conditions at both acidic and non-acidic environments. Likewise, similar results were seen for non-pathogenic *E. coli* K-12 (Figure 4.9; panel C). It was also noted that non-pathogenic and laboratory *E. coli* strains were seen to show greater growth under superoxidative stress conditions at pH 5 compared to superoxidative stress at pH 7 (see Figure 4.9; panels A and B), potentially suggesting that acidic pH affected negatively production of superoxide radicals from MV and perhaps to some degree H₂O₂ activities. Non-AIEC CRC strain HM358 was able to tolerate oxidative stress condition effected by H₂O₂ but not under superoxidative conditions at pH 7 (see Figure 4.8; panel D) and refer to Figure 4.6; panel E).
Figure 4.8: CD AIEC isolates survive the combined stress of low pH (pH 5) oxidative or superoxidative stress environments on solid LB a growth media, mimicking the environment of intra-macrophage phagolysosome.

(A-D) Comparisons of the growth of CD E. coli strains in LB media containing chemical stress conditions, either superoxidative (Left hand panel) or oxidative (right hand panel) at differing pH (pH 5 and pH 7) to that growth seen at differing pH but on plain LB media (control). N=4, n=3 replicates.
Figure 4.8 (continued): CD AIEC isolates survive the combined stress of low pH (pH 5) oxidative or superoxidative stress environments on solid LB a growth media, mimicking the environment of intra-macrophage phagolysosome.

(A-D) Comparisons of the growth of CD E. coli strains in LB media containing chemical stress conditions, either superoxidative (Left hand panel) or oxidative (right hand panel) at differing pH (pH 5 and pH 7) to that growth seen at differing pH but on plain LB media (control). N=4, n=3 replicates.
Figure 4.9: Laboratory *E. coli* strains and a CRC mucosa-associated *E. coli* show greater growth under superoxidative stress conditions at pH 5 compared to superoxidative stress at pH 7.

(A-D) Comparisons of the growth of three laboratory and one CRC *E. coli* strain in LB media containing chemical stress conditions, either superoxidative (Left hand panel) or oxidative (right hand panel) at differing pH (pH 5 and pH 7) to that growth seen at differing pH but on plain LB media (control). It is speculated that the acidic environment (at pH 5) negatively impacted on the production of superoxide radicals from MV and perhaps to some degree hydroxyl radicals from H$_2$O$_2$. N=4, n=3 replicates. * P $\leq$ 0.05, *** P $\leq$ 0.001, **** P $\leq$ 0.0001; ANOVA with Dunnett’s post-hoc test (using LB pH 7 as control).
Figure 4.9 (continued): Laboratory *E. coli* strains and a CRC mucosa-associated *E. coli* show greater growth under superoxidative stress conditions at pH 5 compared to superoxidative stress at pH 7.

(A-D) Comparisons of the growth of three laboratory and one CRC *E. coli* strain in LB media containing chemical stress conditions, either superoxidative (Left hand panel) or oxidative (right hand panel) at differing pH (pH 5 and pH 7) to that growth seen at differing pH but on plain LB media (control). It is speculated that the acidic environment (at pH 5) negatively impacted on the production of superoxide radicals from MV and perhaps to some degree hydroxyl radicals from H$_2$O$_2$. N=4, n=3 replicates. * P ≤ 0.05, *** P ≤ 0.001, **** P ≤ 0.0001; ANOVA with Dunnett’s post-hoc test (using LB pH 7 as control).
4.6 Correlation of the growth of various *E. coli* strains in superoxidative stress environment to ability to survive and replicate within macrophages

Given the interesting preliminary data from the solid LB agar bacteria stress tolerance tests, particularly in the MV-induced superoxidative stress environment, seen for a number of CD *E. coli* strains when compared to intolerant laboratory strains and one CRC isolate, we therefore decided to perform further studies on a wider range of *E. coli* strains obtained from Crohn’s disease, UC, CRC and UTI patients and those from non-inflamed disease controls and healthy individuals (see Appendix, Section 9.3 - supplementary stress tolerance data). This data is summarised in Figure 4.10, panel A, which includes the previous experimental datasets in section 4.5 and 4.6. Interestingly, other Crohn’s disease ileal mucosa-associated *E. coli* isolates LF10, LF11, LF13 and 541-15A, and colonic mucosa-associated isolates, HM96, and HM104, were unable to tolerate superoxidative stress conditions as seen by Crohn’s AIEC strains such as, for example, LF82 and HM605 (see Figure 4.5). Interestingly, other intra-macrophage replicating *E. coli* strains, obtained from healthy and UTI patients, were also observed to significantly tolerate (i.e. survive and replicate) in growth media containing MV. Other clinical isolates able to show survival on MV containing LB agar were *E. coli* K-12 and *E. coli* Nissle 1917 (EcN). On the other hand, all UC *E. coli* isolates studied, all CRC isolates (excepting for HM229) and all non-pathogenic laboratory *E. coli* showed inability to survive superoxidative conditions. Since, the majority of intra-macrophage replicating *E. coli* strains tolerated growing in the superoxidative stress environment, a correlation between fold replication of *E. coli* strains inside macrophages, previously shown in Figure 3.2, with their % growth observed in MV agar was performed (see Figure 4.10 panels B and C). Overall, although results indicate that there is variation in the data sets, Spearman's Rank Correlation Coefficient analysis showed that there is a strong link between the ability of bacteria to grow under superoxidative stress conditions and their ability to significantly replicate inside murine macrophage phagolysosomes. (rho = 0.74). This was supported by Principal component analysis (PCA) also indicating that a strong pattern exists between these measurable factors.
Figure 4.10: Comparison between the % growth of E. coli strains from different diseases in MV and their fold replication within murine macrophages. E. coli strains from different diseases. (Panel A)

(A) Survival and growth of a variety of E. coli strains in superoxidative stress condition and (B) comparison of their growth in MV with their fold replication inside macrophage phagolysosomes. Spearman’s rank correlation coefficient for this dataset is (rho =0.74. Principal component analysis (PCA) of % bacteria growth in MV and bacteria fold of replication (C).
Figure 4.10 (continued): Comparison between the % growth of *E. coli* strains from different diseases in MV and their fold replication within murine macrophages. *E. coli* strains from different diseases. (Panel B)

(A) Survival and growth of a variety of *E. coli* strains in superoxidative stress condition and (B) comparison of their growth in MV with their fold replication inside macrophage phagolysosomes. Spearman’s rank correlation coefficient for this dataset is (rho =0.74. Principal component analysis (PCA) of % bacteria growth in MV and bacteria fold of replication (C).
Figure 4.10 (continued): Comparison between the % growth of *E. coli* strains from different diseases in MV and their fold replication within murine macrophages. *E. coli* strains from different diseases. (Panel C)

(A) Survival and growth of a variety of *E. coli* strains in superoxidative stress condition and (B) comparison of their growth in MV with their fold replication inside macrophage phagolysosomes. Spearman’s rank correlation coefficient for this dataset is (rho =0.74. Principal component analysis (PCA) of % bacteria growth in MV and bacteria fold of replication (C).
4.7 Summary of results

1. Chemical stress conditions mimicking the intra-macrophage phagolysosome environment (low pH, low nutrient, high nitrosative stress, high oxidative/super-oxidative stress) were seen to have no adverse effects on the growth of Crohn’s disease mucosa-associated AIEC, and intramacrophage replicating UTI and healthy-mucosa associated *E. coli* isolates.

2. All laboratory, CRC and UC mucosally associated *E. coli* isolates (with only a few exceptions) were seen to be intolerant to stress conditions mimicking those within the phagolysosome, in particular superoxidative stress conditions.
4.8 Discussion

Within the macrophage phagolysosome, engulfed bacteria are killed and degraded in an acidic, high nitrosative, high oxidative, nutrient-limited stress environment to support subsequent antigen presentation [355]. Oxygen-dependent degradation in the phagolysosome relies on ROS and RNS, whereas oxygen-independent degradation depends on the release of proteolytic enzymes and antimicrobial peptides [356]. Tolerance of this acidic, nutrient-limiting environment inside intra-macrophage vacuoles has been found to be important for the survival and replication of key enteric bacteria such as Salmonella spp. [357, 358] and has also been suggested as important for ileal Crohn’s disease (CD) mucosa-associated adherent, invasive E. coli (AIEC) isolate LF82 [322].

The mechanism of how Crohn’s disease AIEC isolates resist the killing process and survive, even replicate, within macrophage phagolysosomes without inducing cell death is still poorly understood. Here we studied the ability of a collection of E. coli, including CD mucosa-associated E. coli isolates, to tolerate stressful growth conditions that would closely mimic the harsh environmental conditions typical of the macrophage intra-phagolysosome. Wild-type (and eGFP expressing) CD mucosa-associated isolates, including colonic and ileal strains (including LF82), showed tolerance to growth in nutrient minimal media at differing pH (including acidic conditions) and to all stress environments tested, including high oxidative, high superoxidative and high nitrosative conditions. This supports the previous study of AIEC LF82-containing phagosomes which when treated with alcalinising agents chloroquine and ammonium chloride, intracellular replication of this was inhibited [322]. It should be noted also, that alteration of phagolysosome pH in macrophages causes inhibition of acidic proteases which leads to a diminished proteolytic activity against intracellular pathogens [359].

Some key genes have been identified in AIEC LF82 as supporting intramacrophage replication, such as htrA and DsbA [242, 243] but these are fairly ubiquitous to all E. coli strains including colonic CD mucosa-associated E. coli [180]. A more recent paper has identified that gipA may be more importance in supporting Crohn’s disease AIEC to persist in
this phagolysosome niche [245]. The phagolysosomes stress environment (particularly acidic pH) may switch on expression of tolerance/virulence genes. Expression of *gipA* was induced by reactive oxygen and low pH treatment conditions [245].

Chemical stress conditions mimicking the intra-macrophage phagolysosome environment (low pH, low nutrient, high nitrosative stress, high oxidative/super- oxidative stress) were also seen to have no adverse effects on the growth of intramacrophage replicating UTI and healthy-mucosa associated *E. coli* isolates. Likewise, there is some evidence in the literature that UPEC strains isolated from patients with UTI can also survive and replicate within mouse macrophages, as well as replicating within urogenital epithelial cells [328, 329]. A previous study has shown that 7% of UPEC meet the criteria of the AIEC phenotype [30]. Having invaded bladder epithelial cells, internalized UPEC can clonally replicate into biofilm-like intracellular bacterial communities (IBCs) of thousands of bacteria while avoiding key host clearance mechanisms and that these bacteria tolerate oxidative stress within the IBCs [360].

Our data showed that all laboratory, CRC and UC mucosally-associated *E. coli* isolates (with only a few exceptions) were seen to be intolerant to stress conditions mimicking those harsh conditions encountered within the phagolysosome, in particular high superoxidative stress (as generated experimentally by MV). Two notable exceptions included non-pathogenic reference *E. coli* K-12 and Gram-negative EcN. Both strains tolerated all growth stress conditions (see Appendix, section 9.3). *E. coli* K-12 has previously been reported to tolerate 1.5 mM hydrogen peroxide (H₂O₂) levels over 24 hours of incubation in LB broth [149, 361] and this may perhaps support its noted ability to survive/persist (but not to replicate significantly) inside both murine J774-A1 macrophages and human peripheral blood monocyte-derived macrophages [149]. Gram-negative EcN, reported to be useful in maintenance of remission of UC [362], and its ability to tolerate growing within macrophages might perhaps support its anti-inflammatory activity in UC and counter increased numbers of mucosa-associated *E. coli* observed in UC some studies, albeit in lower numbers than that seen in CD mucosae. EcN and *E. coli* K-12 are genetically very similar strains, but differ markedly in their ability to activate the inflammasome where *E. coli* K-12 has a markedly greater ability than EcN [363].
Some of the mucosa-associated non-inflamed *E. coli* isolates and those ECOR healthy control *E. coli* isolates tested also showed ability to survive and/or replicate in our study and tolerate all stress conditions. The ability of commensal *Escherichia coli* to survive killing within macrophages (including murine commensal *E. coli* NC101, with an AIEC phenotype) has been previously documented, facilitated by the protective action of small heat-shock/chaperone proteins (e.g. IbpAB) specifically to prevent killing by macrophage-derived reactive oxygen species [364].

We had tried to further mimic the phagolysosome environment with a combination environment of both oxidative and superoxidative stress with low pH but it was also noted that non-pathogenic and laboratory *E. coli* strains (as per AIEC) were seen to show greater growth under superoxidative stress conditions at pH 5 compared to superoxidative stress at pH 7. We hypothesised that the acidic pH had negatively affected the production of superoxide radicals from MV and perhaps to some degree H$_2$O$_2$ oxidative activities. Our results were found to be consistent with findings of a published paper by van Dijk and colleagues [365] which demonstrated that MV generates superoxide radicals only at pH environments higher than 6 and not at pH 5 as tested in our studies [365]. Thus creating *in vitro* growth conditions to mimic multiple elements of the phagolysosome environment is a challenge for future studies, and may only be achievable in live isolated macrophages or *in vivo* with oxidative, nitrosative and pH sensitive molecular probes, and probes that measure proteolytic enzyme activities too.
Chapter 5

To characterise genes relevant to the ability of Crohn’s AIEC to tolerate the intraphagolysosome environment that would support their growth, survival and replication within macrophages.
5.1 Introduction

A number of key genes have been implicated as being important to support survival and replication of Crohn’s AIEC strains within macrophages including htrA, dsbA, hfq and gipA [190, 245, 366-368]. A screening study using a transposon mutant library constructed from AIEC LF82 identified both htrA and dsbA favouring LF82’s ability to resist macrophage killing and to replicate within macrophages [243, 367]. DsbA had already been reported to be necessary for virulence (including colonisation, adhesion, invasion and intra-macrophage survival) of pathogens such as S. Typhimurium [369], E. coli K-12 [370], Enteropathogenic E. coli [371] and Shigella flexneri [372]. Hfq protein was already shown to play a pivotal role in controlling the virulence of AIEC LF82, with hfq deletion found to affect both the bacteria stress tolerance and its motility, confirming the importance of this protein in LF82 virulence by RpoS and RpoE-independent mechanisms [244]. Previous studies had already established that Hfq is essential for bacteria virulence (motility and invasiveness) [373] of UPEC [374], Pseudomonas aeruginosa [375] and S. Typhimurium [376]. It was also reported that the absence of ibeA in the genome of AIEC strain NRG857c could result in inability of this strain to invade the mucosa, and to subsequently survive within macrophages of the inflamed murine intestine [377], which is consistent with the role of this invasion in other pathogenic E. coli strains, such as APEC [378]. In Crohn’s disease AIEC LF82, gipA appears important to support persistence in the phagolysosome niche with its expression induced by reactive oxygen and low pH [245]. Other virulence genes such as afaC (encoding afimbrial adhesin (Afa), pks, and lpf, which are not usually present in non-pathogenic E. coli, also been frequently found, in AIEC strains [179, 180], but none of these appear to be relevant in supporting survival and growth within macrophages.

It is however known that E. coli harbour specific mechanisms that enable them to resist high levels of reactive oxygen species (ROS) that form the oxidative and super-oxidative response to phagocytosed pathogens. These defensive resources may be grouped into two regulated gene sets, the soxRS and oxyR regulons [252, 253]. The soxRS and oxyR regulons orchestrate defence mechanisms of E. coli against oxidative stress via induction of transcription of a set of genes that increase resistance to oxidative stress [379, 380]. An additional study has also shown that overexpression of btuE, encoding glutathione peroxidase (GPXs) BtuE peroxidase
protein, causes a decrease in the amounts of oxidative damage and also a decrease in the levels of transcription of key stress-induced genes such as ibpA, soxS and katG [381].

Of significant note, *E. coli* may also possess acid environment tolerance response systems. Of the 4 known, the first system requires sigma factor RpoS and the cyclic AMP receptor protein CRP, with RpoS functioning as a major environmental stress response regulator in both *E. coli* and *Salmonella* spp. [246]. Deletion of RpoS from a CD AIEC (strain O83:H1) has been observed to increase sensitivity of this clinical isolate to oxidative stress [247]. The second acid resistance system requires is arginine-dependent utilising of arginine decarboxylase (AdiA and AdiC) antiporter [246]. The third system is lysine dependent, involving lysine decarboxylase [249]. The system requires extracellular glutamate. The components of glutamate-dependent acid response are two isoforms of glutamate decarboxylase encoded by gadA and gadB, and a glutamate-γ-aminobutyric acid (Gad) antiporter encoded by gadC [248, 249]. Various types of *E. coli* strains are already known to contain acid stress response genes of this system, including gadA and gadB which are up regulated in response to acidic environment changes ranging from pH 2.5 to 4.5 [246, 248, 382]. Of note, murine commensal *E. coli* have been observed to respond to chronic intestinal inflammation by up-regulating expression of gadA and gadB [250]. This altered expression of gadA and gadB in luminal commensal *E. coli* was shown to reduce bacterial survival and attenuate the colitis, which likely occurred due to decreased translocation of bacteria across intestinal epithelium and increase of their susceptibility to bacterial killing by host antimicrobial peptides (AMPs) [250].

We therefore aimed to measure the expression levels of acid stress genes gadA and gadB in 4 *E. coli* (CD AIEC colonic isolate HM605 or non-intra-macrophage replicating laboratory *E. coli* EPI300 in response to changes in M9 media under acidic conditions and also the expression levels of these two genes 3h and 6h post infection of J774-A1 macrophages. Also examined was the host oxidative stress responses that occurred when macrophages were infected with either CD AIEC colonic isolate HM605 or non-intra-macrophage replicating laboratory *E. coli* EPI300 (i.e. a strain susceptible to macrophage killing), using a Mouse Oxidative Stress RT² Profiler PCR Array (which profiles the expression of 84 genes related to oxidative stress; see *Methods section 2.13*).
5.2 Acid stress response genes *gadA* and *gadB* expression increases following a change of pH from 7 to 4.5 in M9 minimal nutrient media

Laboratory *E. coli* strains EPI300, XL-1Blue and *E. coli* K-12 showed some ability to tolerate acidic stress in M9 media at pH 4.5 following. Crohn’s disease AIEC HM605 growth in M9 demonstrated increased growth at 6h compared to 3h (see **OD growth curves, Figure 5.1**).

**Figure 5.1** A comparison of the studied *E. coli* strains (samples) growth in M9 minimal nutrient media under acidic stress conditions (pH 4.5) at 3h to 6h time points.

All strains were able to grow in this stress medium at pH 4.5 despite of the growth variations among them; N=2, n=3.
Bacteria were then taken for RNA extraction and amplified using qRT-PCR for gadA expression. *E. coli* strains EPI300, XL-Blue and K-12 all showed an increase shift in gene expression when grown at pH 4.5 compared to growth in media at pH 7 over 6h (see Figure 5.2; N=2, n=2). The expression of gadB was at similar levels to that seen for gadA in each of the 4 *E. coli* strains studied (see Figure 5.3; N=2, n=2). Shift in gadA gene expression (i.e. shift in Cp value, ΔCp ranged from 3.5 to 7.0 when grown at pH 4.5 compared to growth in media at pH 7 over 6h. For gadB, ΔCp ranged from 2.2 to 6.5; see Figure 5.4A and Figure 5.4B. The biggest shift in gad expression in response to acidic stress was seen with *E. coli* K-12 with a ΔCp for gadA of 7.00 ± 2.9 and gadB of 6.57 ± 1.78 (mean ± SEM) and confirms previous observations of tolerance to solid agar stress responses to acid (Chapter 4; Figure 4.6). Interestingly, both gadA and gadB expression was seen to be significantly high in the CD AIEC isolate HM605 grown in M9 media for 6h at pH 7 and as a consequence, following growth in M9 media at pH 4.5, this isolate showed much less of an increase shift in gad expression when grown at pH 4.5 (Figure 5.4A and Figure 5.4B; N=2, n=2).
Figure 5.2 Glutamate-γ-aminobutyric acid antiporter gadA expression levels of *E. coli* strains following growth in acidic stress conditions.

(A) Reverse transcription real-time qPCR for bacterial *gadA* expression in *E. coli* strains. Expression levels of *gadA* were observed to be increased by all four *E. coli* strains grown in M9 minimal nutrient media, over 6h, at pH 4.5 compared to pH 7, for (B) CD AIEC HM605, (C) K12, (D) EPI300 and (E) XL-1Blue. N=2, n=2 replicates. Low cycle number (Cp value) indicates higher expression levels. Negative template control is growth media without infection at pH 7 (black) and pH 4.5 (grey).
Figure 5.3 Glutamate-γ-aminobutyric acid antiporter gadB expression levels of E. coli strains following growth in acidic stress conditions.

(A) Reverse transcription real-time qPCR for bacterial gadB expression in E. coli strains. Expression levels of gadB were observed to be increased by all four E. coli strains grown in M9 minimal nutrient media, over 6h, at pH 4.5 compared to pH 7, for (B) CD AIEC HM605, (C) K12, (D) EPI300 and (E) XL-1Blue. N=2, n=2 replicates. Low cycle number (Cp value) indicates higher expression levels. Negative template control is growth media without infection at pH 7 (black) and pH 4.5 (grey).
Figure 5.4 (A) Shift in *gadA* and (B) *gadB* expression (ΔCp) from *E. coli* strains HM605, K-12, EPI300 and XL-1Blue when grown at pH 4.5 compared to growth in media at pH 7 over 6h.

Each value represents the mean ± standard error of the mean (SEM) of three independent experiments (N=2, n=2 replicate).
It was attempted to undertake qPCR analysis of *gad* expression in CD AIEC HM605 and all four *E. coli* strains (EPI300, XL-1Blue, K-12 and HM605) following a gentamicin protection assay of infected J774-A1 macrophages at 6h compared to 3h post-infection. **Figure 5.5 and Figure 5.6** shows Cp values of *gadA* and *gadB* expression which was detected at lower expression levels for all *E. coli* strains expressed from the *E. coli* strains inside murine macrophages studied showed similar but smaller shifts in *gad* expression at 6h within macrophages. (N=1, n=2).
Figure 5.5 Levels of gadA expressed from intra-macrophage E. coli strains.

(A) Amplification curves of gadA levels expressed from intra-macrophage E. coli strains (B) HM605, (C) K-12, (D) EPI300 and (E) XL-1Blue at 3h and 6h post-infection. These strains managed to express gadA and no significant differences were observed among them. N=1, n=2 replicates.
Figure 5.6 Levels of *gadB* expressed from intra-macrophage *E. coli* strains.

(A) Amplification curves of *gadA* levels expressed from intra-macrophage *E. coli* strains (B) HM605, (C) K12, (D) EPI300 and (E) XL-1Blue at 3h and 6h post-infection. These strains managed to express *gadB* and no significant differences were observed among them. N=1, n=2 replicates.
5.3 Mouse J774-A1 macrophage oxidative stress gene expression levels 6h post-infection with Crohn’s disease AIEC HM605

The expression of 84 genes related to oxidative stress, including peroxidases and reactive oxygen species following infection of J774-A1 murine macrophages for 6h with CD AIEC isolate HM605 or non-replicating strain *E. coli* K-12 derivative EPI300 were analysed using RT² Profiler Array. Scatter plot figures show the host oxidative stress response genes that show increased expression (in red), no change (in black) and lowered expression (in green) after infection with EPI300 (group 1; Figure 5.5) and Crohn’s disease AIEC HM605 (group 2; Figure 5.6) compared to uninfected controls. Ten genes were up-regulated and 8 down-regulated in response to EPI300 infection (Figure 5.5) and 9 up-regulated and 4 down-regulated following CD AIEC HM605 infection (Figure 5.6). Common up-regulated genes to both infections were *Cat* and *Ptgs2* (Peroxidase/cyclooxygenase), *Ccl5*, *Prdx5* and *Sqtsm1* (Oxidative Stress response), *Gpx3* (Glutathione Peroxidase), *Ncf1* and *Nos2* (Superoxide Metabolism) and *Sod2* (Superoxide Dismutases). Significant changes (>2 fold) seen in same 8 genes in response to EPI300 and HM605 infection (4 genes elevated and 4 decreased; see Figure 5.7). Superoxide stress response genes *Ncf1* and *Sod2* were all upregulated to similar levels at 6h (>2-fold) in both *E. coli* HM605 and K-12 derivative EPI300-infected macrophages compared to uninfected controls, suggesting that CD isolate HM605 does not alter macrophage oxidative stress response to infection to promote its own intra-phagolysosome growth (see Figure 5.7). For further detail of expression of those genes changed/unaltered by EPI300 and HM605 infection of J774A1 murine macrophages following RT² Profiler PCR Array analysis can be found in Chapter 9; see Appendix section 9.1 (Table 9.1) and section 9.2 (Table 9.2).
Figure 5.7 Scatter plot of host oxidative stress response genes following infection with non-pathogenic *E. coli* EPI300 compared to uninfected control.

Scatter plot figure illustrating host oxidative stress response genes that show increased expression (in red), no change (in black) and lowered expression (in green) after infection with *E. coli* EPI300 (group 1) compared to uninfected control. N=3, RT² Profiler PCR Array Data Analysis programme version 3.5. The id of the genes, their fold change and their position on the array plates can be found in Appendix 9, Table 9.2.
Figure 5.8 Scatter plot of host oxidative stress response genes following infection with Crohn’s mucosa-associated AIEC HM605 compared to uninfected control.

Scatter plot figure illustrating host oxidative stress response genes that show increased expression (in red), no change (in black) and lowered expression (in green) after infection with CD AIEC HM605 (group 2) compared to uninfected control. N=3, RT2 Profiler PCR Array Data Analysis programme version 3.5. The id of the genes, their fold change and their position on the array plates can be found in Appendix 9, Table 9.2.
Figure 5.9 Bar chart illustrating oxidative stress genes expression in J7774-A1 murine macrophages infected with E. coli EPI300 or HM605.

Bars indicate > 2-fold change (mean ± SEM) of key oxidative stress genes that were either over- or under-expressed (in J7774-A1 murine macrophages infected with E. coli EPI300 or HM605, each compared to uninfected controls. N=3.
5.4 Summary of results

1. Expression of bacteria acid stress response antiporter glutamate-γ-aminobutyric acid (Gad) genes gadA and gadB were observed to be much higher in the *E. coli* strains studied following 6h growth in minimal nutrient media at pH 4.5 (i.e. mimicking the environment inside murine macrophage phagolysosome), than that observed at pH 7.

2. Crohn’s disease mucosa-associated AIEC HM605 showed higher initial levels of expression of both gadA and gadB in low nutrient media at pH 7 and this elevated state of gad expression may support adaptation to an intra-macrophage phagolysosome survival/replication lifestyle (see Chapter 3; Figure 3.1 and Figure 3.2).

3. Other non-pathogenic *E. coli* strains that show some ability to tolerate acid stress, such as *E. coli* K-12 (see Chapter 4, Figure 4.6), were seen to show the greatest upregulation of gad gene expression in response to acid stress in low nutrient media.

4. The expression of gadB was observed to be at similar levels to that seen for gadA in each of the 4 *E. coli* strains studied (i.e. HM605, K-12, EPI300 and XL-1Blue), and again all strains showed an increase shift in expression of this particular gene when grown at pH 4.5 compared to growth in media at pH 7 over 6h.

5. Adaptation of Crohn’s disease AIEC HM605 to the phagolysosome niche appears not to be through its ability to alter host macrophage oxidative stress response to infection, as no differential changes were observed in the expression of 84 host genes related to oxidative stress to that response seen with EPI300, a non-intra-macrophage replicating laboratory *E. coli* strain.


5.5 Discussion

Having previously verified that Crohn’s disease AIEC strains possess greater ability to survive and replicate inside macrophages and that they may endure growth in chemical stress-induced conditions resembling that of the intra-macrophage phagolysosome environment, we subsequently examined for potential genes that might play a role in its virulence phenotype, using the AIEC strain, HM605 as a representative isolate. Gad system genes seemed likely good initial candidates, given that various types of E. coli are already known to contain this acid stress response system, which is upregulated in response to exposure to a low pH environment [382], and that two previous studies have shown that luminal commensal AIEC strain NC101 responds to chronic intestinal inflammation by upregulation of expression of these two acid stress response genes, gadA and gadB [250, 383]. We therefore initially looked for expression of these genes in CD E. coli strain HM605 compared to non-pathogenic and laboratory strains (i.e. EPI300, XL-1Blue and K-12). Higher initial basal levels of gad gene expression seen for the AIEC isolate HM605 meant that as a consequence this isolate showed much less of an increase shift in gad expression when grown in nutrient poor media at low (acidic) pH. One could speculate that E. coli with an AIEC phenotype may perhaps already be adapted with high level of gad expression to counter/respond rapidly to acid stress encountered when phagocytosed into the macrophage phagosome environment, and that they may use this acid stress tolerance system to not only facilitate their initial survival but also subsequently to extensively replicate rapidly within maturing macrophage vacuoles, i.e. when phagosomes fuses with lysosomes. Notably, the largest shift observed in gad gene expression in response to the experimentally-induced acidic stress conditions was for the non-pathogenic E. coli K-12 strain, suggesting this strain rapidly adapts to acid stress to facilitate its survival/tolerance of high acid growth conditions. This response was also seen in the earlier solid agar chemical stress growth assays described in Chapter 4 (see Figure 4.6). This gad system activation response may explain the observations in this study (Chapter 3; Figure 3.2) and that of others [149] that E. coli K-12 is also able to survive, albeit not replicate extensively, within murine macrophages over 6h study time.
Macrophages are well known to effectively generate reactive oxygen and nitrogen species, to facilitate intra-macrophage bacteria killing [384], and in order to defend themselves from the intra-phagolysosome toxic reactive oxygen species, E. coli strains trigger transcription of other stress-response regulators, such as OxyR, PerR and SoxR, which promote/enhance virulence [385, 386]. The RT2 Profiler PCR Array results presented here in this Chapter clearly illustrated that a number of key host macrophage oxidative stress genes were significantly upregulated in response to infection with AIEC HM605. This included; 1.) the neutrophil cytosolic factor 1 gene Ncf1 which encodes a cytosolic subunit protein of NADPH oxidase, p47 (phox) [387]; 2.) superoxide dismutase 2 mitochondrial gene Sod2, encoding a protein that binds superoxide by products of oxidative phosphorylation and then converts them to hydrogen peroxide and diatomic oxygen [388]; 3.) the p62/sequestosome 1 gene Sqstm1, encoding an autophagosome cargo protein that interacts with other proteins responsible for selective autophagy [389, 390]; and 4 ) Prdx5, encoding peroxiredoxin-5 protein [391]. These proteins are all involved in defending against superoxide/reactive oxygen species and control excessive inflammatory responses after macrophage activation [392], with peroxiredoxin 5 (PRX5) and manganese-containing superoxide dismutase (Sod2) being highly upregulated after TLR activation of macrophages during microbial infection [388, 393].

Oxidative stress genes that were significantly down-regulated in AIEC-infected macrophages, included the following; 1.) The glutamate-cysteine ligase gene Gclc, encoding a catalytic protein involved in the first step in the synthesis of glutathione important for protection against infection [394, 395]; 2.) The isocitrate dehydrogenase 1 gene Idh1, catalysing oxidative decarboxylation of citrate cytosolic NADP+-dependent ribozyme [396]; 3.) RecQ protein-like 4 gene Recql4, encoding the ATP-dependent DNA helicase Q4 [397], and 4.) The antioxidant prostaglandin-endoperoxide synthase 1/cyclooxygenase 1 gene Ptgs1, catalysing the conversion of arachidonic acid to prostaglandin [398]. Our data also indicate that no differential changes were observed in the expression of 84 host genes related to oxidative stress to that seen with the non-replicating laboratory E. coli strain EPI300 in terms of both up-regulation and down-regulation of the mouse oxidative stress genes post 6h infection. Overall, this result suggest that adaptation to the phagolysosome niche of Crohn’s disease AIEC, such as HM605, appears not to be through any ability to alter host macrophage oxidative stress response to infection.
Studies have identified presence of key virulence genes thought to associate to a ‘AIEC’ phenotype, i.e. being significantly higher among AIEC strains isolated from CD patients than non-IBD controls [180, 245, 399, 400]. Comparison of the complete genome of ileal CD AIEC E. coli NRG857c (O83:H1) had confirmed a phylogenetic linkage between AIEC and ExPECs [9]. A more recent comparative RNASeq analysis of AIEC strain LF82 compared to a non-invasive strain HS also showed enrichment for pdu operon genes [401], as did the study of Dogan et al. [178] and further analysis of the complete genomes for 13 AIEC and 11 non-invasive E. coli revealed that a number of CRISPR-associated Cas genes (encoding proteins that recognize foreign genetic material in plasmids and phages) may serve as AIEC-specific biomarkers [401]. One particular gene ibeA (encoding an invasion protein) also shown to be enriched in AIEC in the same study [401], plays a role in resistance of E. coli to hydrogen peroxide stress [402] and supporting survival of AIEC within macrophage [377]. However, a recent whole genome sequencing study of 41 B2 phylogroup E. coli strains, isolated from 19 patients with IBD (i.e. 14 with CD, 5 with UC), and 17 without IBD, interestingly showed no exclusive identifiable molecular features for the AIEC phenotype [161].
Chapter 6

Overall study outcomes
6.1 Overall summary of the key findings

The following list summarises the key findings on CD-\textit{E. coli} isolates that have been identified throughout working in the lab so far:

1. The majority of Crohn’s ileal- and colonic-mucosa associated \textit{E. coli} strains possess ability to either survive and/or replicate within murine (\textit{J774-A1}) macrophages. Notably, all pathogenic UTI \textit{E. coli} isolates examined and some healthy mucosa-associated \textit{E. coli} strains behaved in a similar manner. All CRC mucosa-associated \textit{E. coli} strains tested, two out of four UC mucosa-associated isolates and the majority of laboratory \textit{E. coli} strains were unable to survive inside \textit{J774-A1} macrophage phagolysosomes (see \textit{Chapter 3, Figure 3.2}).

2. Bone marrow progenitor cells from wild-type C57BL/6, \textit{Nfkb1}\textsuperscript{−/−}, \textit{Nfkb2}\textsuperscript{−/−} and \textit{c-Rel}\textsuperscript{−/−} mice were successfully differentiated into mature adherent macrophages when cultured in media supplemented with rM-CSF (see \textit{Chapter 2, section 2.3}). Paradigm Crohn’s ileal and colonic AIEC isolates LF82 and HM605 showed ability to survive and replicate within wild-type C57BL/6 bone-marrow derived macrophages (BMDM). They were, however, unable to survive and replicate inside \textit{Nfkb1}\textsuperscript{−/−} and \textit{Nfkb2}\textsuperscript{−/−} BMDM, whilst they both survived and replicated within \textit{c-Rel}\textsuperscript{−/−} BMDM. (see \textit{Chapter 3, section 3.4, 3.5 and 3.6}).

3. Chemical stress conditions mimicking the intra-macrophage phagolysosome environment were observed to have no adverse effects on the growth of Crohn’s disease mucosa-associated AIEC, and those intra-macrophage replicating UTI and healthy-mucosa-associated \textit{E. coli} isolates. All laboratory, CRC and UC mucosally-associated \textit{E. coli} isolates (with only a few exceptions) were seen to be intolerant to the same stress conditions, in particular superoxidative stress. (see \textit{Chapter 4, section 4.2 and 4.3}).
4. Expression of bacteria acid stress response antiporter glutamate-γ-aminobutyric acid genes *gadA* and *gadB* were observed to be much higher in the studied *E. coli* strains in minimal nutrient, low pH (acidic) growth conditions. CD AIEC HM605 showed higher base levels of expression of both *gadA* and *gadB* suggesting this may benefit survival in a highly acidic phagolysosome environment (see Chapter 5, section 5.2).

5. Adaptation to the phagolysosome niche of Crohn’s disease AIEC HM605 appears not to be any ability to alter host macrophage oxidative stress response to infection, as no differential changes were observed in the expression of 84 host genes related to oxidative stress to that seen with the non-replicating laboratory *E. coli* strain EPI300 (see Chapter 5, section 5.3).
Chapter 7

Discussion
7.1 Intra-macrophage survival and replication of Crohn’s disease mucosa-associated AIEC isolates inside the vacuolar environment of the mature phagolysosome

There is accumulating evidence supporting the importance of host–microbe interactions in the pathogenesis of CD and this has been consolidated by genome-wide association studies [326]. Delayed/aberrant clearance of bacteria from mucosal tissues of Crohn’s patients by macrophages, along with retention of undigested bacteria has been proposed to cause chronic granulomatous inflammation and stimulation of secondary adaptive immune response [403]. Th1-related cytokines (TNF, IFN-γ, and IL-12) and Th17-associated cytokines (IL-17A, IL-21, and IL-23) have been reported to markedly increase in the inflamed mucosa of Crohn’s patients and their increased levels are associated with the progression of CD [7, 404].

Several international research groups have identified the increased abundance of E. coli (including the proposed ‘pathovar’ AIEC) in both the ileal and colonic mucosa of active and newly diagnosed Crohn’s patients (both adult and paediatric) [30, 46, 117, 139, 305, 405]. These E. coli have frequently been observed to translocate the Crohn’s intestinal mucosa of patients to be found scattered within the lamina propria, submucosa, the muscle layers, and the perivascular areas of the subserosa [120]. Other studies have also reported increased E. coli abundance in active UC patients as well as in those with active CD [140, 142, 406-410]. These E. coli have been detected in the lamina propria of UC patients [120], but there is no evidence for their ability to translocate to mucosa layer [411, 412]. In addition, intra-macrophage E. coli were commonly found in lamina propria (LP) macrophages in mucosal biopsies from 71% CD, 11% UC patients, but from non-inflamed controls (0%) [330]. A Canadian group had also previously shown that E. coli strains from UC showed higher percent survival inside murine (RAW264.7) macrophages than those isolates from CD and healthy controls after 20h infection [410]. Similar intra-mucosal and intra-epithelial E. coli have also been found in increased numbers from patients with CRC [139, 143]. This is supported by recent studies, including quantitative profiling/detection by PCR [413, 414]. Some of these E. coli strains were shown to possess the characteristics of both DAEC and genotoxic AIEC [180],[413, 414]. Several studies have identified presence of key virulence genes thought to associate to a ‘AIEC’ phenotype, i.e. being significantly higher among
AIEC strains isolated from CD patients than non-IBD controls [180, 245, 399, 400]. Virulence factors supporting survival and replication within macrophages have been identified using isogenic mutants of the “paradigm” ileal AIEC LF82, including HtrA, DsbA, Hfq and GipA [242-245]. The AIEC phenotype has also been frequently observed amongst intestinal pathogenic E. coli (InPEC) strains from various animal species [415]. However, a recent whole genome sequencing study showed no identifiable molecular features for an exclusive AIEC phenotype [161].

In our study we have provided further evidence of the ability of Crohn’s disease mucosa-associated E. coli to replicate within murine macrophages and also shown that Crohn’s AIEC strains have better ability to survive and replicate than those isolates obtained from both CRC and UC patients. We have also shown that E. coli strains from other disease and non-inflamed conditions also share AIEC phenotypic properties (especially those obtained from UTI patients). AIEC replication within macrophages is suggested to be dependent on host TNF secretion [8, 149, 323, 324]. Crohn’s AIEC LF82 and 13I have been reported during macrophage infection to induce chronic activation/nuclear translocation of NF-κB, which correlates with increased TNF secretion and ongoing intracellular replication of these strains [323]. In this study, intra-macrophage Crohn’s AIEC strains HM605 and LF82 survival and replication were observed within murine BMDM from various Nfκb family member knockout mice, including the classical pathway, Nfkb1−/− (p105→p50) and c-Rel+/− (p65), and the alternative pathway, Nfkb2−/− (p100→p52). Phagocytosis of these AIEC was not impaired, but our data showed that both isolates showed little ability to replicate within Nfkb1−/− and Nfkb2−/− BMDM, while they managed to survive and replicate within c-Rel+/− BMDM, like that of WT BMDM. This supports the role of host Nfkb signal pathway activation in supporting intra-macrophage survival and persistence, although whether subsequent elevation TNF levels occur to support survival/replication was not studied here. However, previous data from our own lab show that peripheral blood monocyte-derived macrophages obtained from healthy volunteers and Crohn’s patients produced roughly similar quantities of proinflammatory cytokines, including TNF, after infection with Crohn’s mucosa-associated AIEC HM605 [205]. This contradicts other studies showing that exogenous addition of TNF increases numbers of intra-macrophage replicating AIEC LF82 [324, 335]. It would be interesting to examine the replication of these paradigm AIEC isolates replication within BMDM derived from knock-outs of TNF and TNF receptor family members [416, 417]. CD
AIEC LF82 isolate have also been reported to have the ability to delay apoptosis in the infected macrophages by a mechanism involving increase of S-nitrosylation and proteasomal degradation of Caspase-3 [418]. Interestingly, tumour necrosis factor-like weak inducer of apoptosis (sTWEAK) has been found to be elevated in Crohn’s patients, suggesting that TWEAK may play a role in the aetiology of CD [404].

Adaptation to an intracellular lifestyle is not uncommon in the pathogen world including Q-fever-associated Coxiella burnetii [288] and Whipple's disease-associated Tropheryma whippelii [289, 290] as key examples. Likewise, enteric pathogens Salmonella and Shigella have both been reported to possess a repertoire of inducible systems that support tolerance of the harsh phagolysosome environment [224]. Paradigm Crohn’s AIEC strain LF82, has also been shown to be present within mature phagolysosomes and appears to be tolerant of a low pH and high oxidative stress environment typical of the phagolysosome [322]. This persistence within macrophages supports giant cell formation and development of granulomata in vitro [145].

Our study data supports these earlier observations and extend this to a wider range of CD isolates. Here we demonstrated that following acid, oxidative, superoxidative and nitrosative stress, Crohn’s disease mucosa-associated E. coli strains possessing the AIEC phenotype were better able to tolerate growth in ‘killing’ conditions inside the macrophage phagolysosome. Interestingly, other studies on ExPEC strains has also demonstrated the ability to survive/adapt to/tolerate the intracellular bactericidal mechanisms within peripheral blood-derived human neutrophils [419].

Other important mechanisms may also be important for intracellular survival of CD mucosa-associated E. coli at the level of the epithelial interface. Hypoxia inducible transcription factor (HIF)-1α protein, encoded by HIF1A gene, plays an essential role in cellular and systemic responses to hypoxia [420]. There is strong evidence showing that CD AIEC strains promote gastrointestinal inflammatory disorders via activation of HIF-dependent responses leading to an over expression of HIF-1α protein in inflamed ileal epithelium of Crohn’s patients [421] and also HIF-1α was found to mediate CEACAM6 expression and regulate the xenophagy process of CD AIEC within intestinal epithelial cells [422].
Identifying the other stress response genes favouring Crohn’s AIEC survival and replication ability within the macrophage phagolysosome was a key target in this study. The glutamate-dependent acid response system, particularly two isoforms of glutamate decarboxylase encoded by gadA and gadB [248, 249], was a good target given that a number of E. coli are already known to contain acid stress response genes of this system [246, 248, 382], and is likely the most effective system in protecting E. coli strains from low pH compared with other acid resistance mechanisms [423-425]. Here we showed that expression of the acid stress response antiporter glutamate-γ-aminobutyric acid system genes gadA and gadB from bacteria grown in low nutrient media (pH 4.5) mimicking the active phagocytic-vacuole environment, were observed to be much higher in all E. coli strains than that seen at pH 7. Crohn’s AIEC HM605 showed higher initial levels of expression of both gadA and gadB in low nutrient media at pH 7, which might suggest that elevated levels at initial entry to the phagolysosome confer an advantageous position in adaptation for survival/replication within this niche. AIEC adaptation to the phagolysosome niche appears not to be through ability to alter host macrophage oxidative stress response to infection as no differential changes were observed in the expression of 84 host genes related to oxidative stress to that seen by with non-replicating laboratory E. coli strain EPI300. Murine commensal E. coli have been observed to respond to chronic intestinal inflammation by up-regulating expression of gadA and gadB [250] and in the same study, it was hypothesised that these strains might upregulate these acid response genes during inflammation to enhance their survival and virulence. Conversely, they showed that upregulation of acid tolerance pathways limited commensal E. coli survival and colitogenic potential [250]. Notably though, the largest shift observed in gad gene expression to the experimentally-induced acidic stress conditions in our study was for the non-pathogenic E. coli K-12 strain, and this certainly supported its survival/tolerance of high acid growth conditions. The higher initial gad gene levels observed here in human CD mucosa-associated E. coli may also suggest that AIEC differ considerably to commensal strains.
7.2 Host macrophage–Crohn’s disease mucosa-associated AIEC interactions: Opening up new therapeutic strategies

Understanding the Crohn’s disease (CD) mucosa-associated E. coli-host macrophage interaction may perhaps provide key insight into CD pathogenesis and support the development of new therapeutic strategies that target the fundamental pathology of CD and support development of novel drugs that might be much more effective for patients with CD. The main focus of the studies of this thesis, i.e. to investigate how CD mucosa-associated AIEC isolates resist killing process of mucosal macrophages through adaptation to the environment within the macrophage phagolysosomes, would clearly lend towards this goal.

Our data has clearly validated the ability of Crohn’s ileal- and colonic-mucosa associated AIEC isolates to survive and replicate within mucosal macrophages, while the majority of ulcerative colitis (UC) and colorectal cancer (CRC) mucosa-associated E. coli isolates were unable to replicate. Crohn’s AIEC survival and replication appear also to be dependent on host NFκB signalling and possibly subsequent host TNF secretion from the macrophage. Blockade of the NFκB signaling pathway, both classical and alternative pathways, has become a potential target for pharmacological intervention [426]. Various anti-inflammatory agents including glucocorticoids, non-steroid anti-inflammatory drugs (NSAIDs), and immunosuppressants (some commonly used to treat CD patients) strongly inhibit NF-κB activation by mechanisms that are not fully understood [426] but may support a reduction in bacterial persistence/increase in killing of intra-macrophage E. coli as part of their therapeutic action to get CD patients into remission and maintain them there. Clearly, future agents that might specifically target NFκB activation within antigen-presenting cells harbouring persistence AIEC may have greater therapeutic potential here.

These CD E. coli isolates are also clearly tolerant of the harsh chemical environment within the phagolysosome suggesting innate or acquired adaptation to an intracellular lifestyle/persistence. Therefore, understanding the factors that support intra-macrophage E. coli (mainly AIEC) persistence needs to reach beyond conventional immune response manipulation; i.e. approaches that decrease the intra-macrophage bacterial load. Such
approaches are being undertaken, including use of a) vitamin D supplementation to enhance AIEC intra-macrophage killing [205], supported by Vitamin D supplementation trail data to prevent relapse in Crohn’s [115]; b) antibiotics to target AIEC [149] and use of phagolysosome environment modifying agents that change the niche to support killing of intra-vacuolar persisting *E. coli* [205]. The latter study has led to a trial of combination Antibiotics and Hydroxychloroquine (APRiCOT Trial) [https://clinicaltrials.gov/ct2/show/NCT01783106].
Chapter 8

References
8.1 References


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Chapter 9

Appendices
9.1 Appendix

Table 9.1 Fold-change values of all 82 stress related gene, expressed from mouse J774-A1 macrophage after infection with either *E. coli* EPI300 or HM605, each compared to uninfected control, using the Qiagen/SABiosciences RT² Profiler PCR Array. Increased expression (in red), no change (in black) and lowered expression (in green) (N=3).

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<th>Fold Change</th>
<th>95% CI</th>
<th>Comments**</th>
<th>Fold Change</th>
<th>95% CI</th>
<th>Comments**</th>
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**Footnote:**

* Fold change values greater than 1 indicate a positive- or an up-regulation. Fold change values less than 1 indicate a negative or down-regulation.
**Comments:**

(Comment A): This gene’s average threshold cycle is relatively high (> 30) in either the control or the test sample, and is reasonably low in the other sample (< 30). These data mean that the gene’s expression is relatively low in one sample and reasonably detected in the other sample suggesting that the actual fold-change value is at least as large as the calculated and reported fold-change result. This fold-change result may also have greater variations if p value > 0.05; therefore, it is important to have a sufficient number of biological replicates to validate the result for this gene.

(Comment B): This gene’s average threshold cycle is relatively high (> 30), meaning that its relative expression level is low, in both control and test samples, and the p-value for the fold-change is either unavailable or relatively high (p > 0.05). This fold-change result may also have greater variations; therefore, it is important to have a sufficient number of biological replicates to validate the result for this gene.

(Comment C): This gene’s average threshold cycle is either not determined or greater than the defined cut-off value (default 35), in both samples meaning that its expression was undetected, making this fold-change result erroneous and un-interpretable.

The following SABiosciences/Qiagen web link is the array gene list studied:

### Table 9.2 Oxidative stress genes that were either over- or under -expressed (> 2-fold change) in J7774-A1 murine macrophages infected with *E. coli* EPI300 or HM605, each compared to uninfected controls. Increased expression (in red), no change (in black) and lowered expression (in green) (N=3).

**Note:** only the genes which are checked are exported into this file

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**Footnote:**

Fold Regulation represents fold change results in a biologically meaningful way. Fold change values greater than 1 indicate a positive- or an up-regulation, and the fold regulation is equal to the fold change. Fold change values less than 1 indicate a negative or down-regulation, and the fold regulation is the negative inverse of the fold change.
9.3 Appendix - Supplementary Stress tolerance data

Figure 9.1 Non-pathogenic clinical isolate Nissle 1917 (EcN) tolerated stress conditions. (Included as part of the data set, regarding tolerance to MV, in Chapter 4, Figure 4.10).

Bacteria growth in plain LB at pH 7 was used as a control. Bacteria CFUs were frequently counted at dilution factor $10^5$ and presented as relative growth response. Each value represents the mean ± standard error of the mean (SEM) of three independent experiments (N=4, n=3 replicate). * P ≤0.05, ** P ≤ 0.01, **** P ≤ 0.0001, ANOVA with Dunnett’s post-hoc test.
Figure 9.2 (A-I) *E. coli* isolates from healthy individuals were able to tolerate all stress conditions (Included as part of the data set, regarding tolerance to MV, in Chapter 4, Figure 4.10). Each value represents the mean ± standard error of the mean (SEM) of three independent experiments (N=4, n=3 replicate).
Figure 9.3 (A-L) CD E. coli isolates, excepting LF86 and HM413 were intolerant to the superoxidative stress condition (included as part of the data set, regarding tolerance to MV, in Chapter 4, Figure 4.10). Each value represents the mean ± standard error of the mean (SEM) of three independent experiments (N=4, n=3 replicate). * P ≤0.05, ** P ≤ 0.01, **** P ≤ 0.0001, ANOVA with Dunnett’s post-hoc test.
Figure 9.4 (A-G) *E. coli* isolates from UTI patients were tolerant to all stress conditions (Included as part of the data set, regarding tolerance to MV, in Chapter 4, Figure 4.10). Each value represents the mean ± standard error of the mean (SEM) of three independent experiments (N=4, n=3 replicate).
Figure 9.5 (A-F) *E. coli* isolates, excepting HM229 were unable to tolerate the superoxidative stress conditions (included as part of the data set, regarding tolerance to MV, in Chapter 4, Figure 4.10). Each value represents the mean ± standard error of the mean (SEM) of three independent experiments (N=4, n=3 replicate). * P ≤0.05, ** P ≤ 0.01, **** P ≤ 0.0001, ANOVA with Dunnett’s post-hoc test.
Figure 9.6 (A-F) *E. coli* isolates from UC patients were intolerant to the superoxidative stress condition (Included as part of the data set, regarding tolerance to MV, in Chapter 4, Figure 4.10). Each value represents the mean ± standard error of the mean (SEM) of three independent experiments (N=4, n=3 replicate). * P ≤0.05, ** P ≤ 0.01, **** P ≤ 0.0001, ANOVA with Dunnett’s post-hoc test.