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TOPIC HIGHLIGHT

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Escherichia coli-host macrophage interactions in the pathogenesis of inflammatory bowel disease

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Abstract

Multiple studies have demonstrated alterations in the intestinal microbial community (termed the microbiome) in Crohn's disease (CD) and several lines of evidence suggest these changes may have a significant role in disease pathogenesis. In active and quiescent disease, both the faecal and mucosa-associated microbiome are discordant with matched controls with reduced biodiversity, changes in dominant organisms and increased temporal variation described. Mucosa-associated adherent, invasive *Escherichia coli* (*E. coli*) (AIEC), pro-inflammatory and resistant to killing by mucosal macrophages, appear to be particularly important. AIEC possess several virulence factors which may confer pathogenic potential in CD. Type-1 pili (FimH) allow adherence to intestinal cells *via* cell-surface carcinoembryonic antigen-related cell adhesion molecules and possession of long polar fimbrae promotes translocation across the intestinal mucosa *via* microfold (M)-cells of the follicle-associated epithelium. Resistance to stress genes (*htrA*, *dsbA* and *hfq*) and tolerance of an acidic pH may contribute to survival within the phagolysosomal environment. Here we review the current understanding of the role of mucosa-associated *E. coli* in Crohn's pathogenesis, the role of the innate immune system, factors which may contribute to prolonged bacterial survival and therapeutic strategies to target intracellular *E. coli*.

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Key words: Crohn's disease; Inflammatory bowel disease; *Escherichia coli*; Intra-macrophage survival and replication; Phagolysosome; Autophagy

Core tip: There is significant evidence implicating adherent, invasive mucosa-associated *Escherichia coli* (AIEC) in the pathogenesis of Crohn's disease. AIEC translocate M-cells of Peyer's patches and lymphoid follicles of the colon, and then to survive and replicate within underlying mucosal macrophages. How Crohn's AIEC resist killing and adapt to the environment within the phagolysosme to survive and grow within macrophages is still poorly understood. Here we review the current understanding of the role of AIEC in Crohn's pathogenesis, the role of the innate immune system, factors which may contribute to prolonged bacterial survival and therapeutic strategies to target intracellular AIEC.

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INTRODUCTION

Crohn's disease (CD) is a chronic relapsing inflammatory bowel disease (IBD) of multifactorial aetiology, affecting any part of the gastrointestinal tract from mouth to anus. Patients typically suffer from abdominal pain, diarrhoea and weight loss which may be associated with extra-intestinal manifestations including erythema nodosum, iritis and arthritis. The intestinal pathological findings are characterised by transmural inflammation, deep mucosal ulcers, abscesses, fissures and granuloma formation[1]. 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 of antigen-presenting cells (such as dendritic cells and macrophages), lymphocytes and plasma cells within the intestinal mucosal layer[1,2]. Pathological characteristics resemble the mucosal lesions and intestinal inflammation elicited by known enteric gut pathogens such as *Shigella* and *Salmonella* spp[3].

CD is classically described to have a bimodal incidence with the highest rates seen in adolescents and young adults and a second peak in later years, although this has recently been questioned[4]. It is associated with a small increase in mortality (standardised mortality ratio 1.52) but very considerable morbidity, disrupting work, study and family life[5]. Historically approximately 80% of cases needed surgery at some time[6] but the use of immunosuppressants and biologics has increased and is associated with a reduced 5 years risk of major surgery[7]. The condition is more common in Europe and North America[8]. However, incidence is rapidly increasing worldwide particularly in developed nations adopting a western style diet, as seen in Japan[9]. 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[10].

The gut microbiota plays an essential role in the shaping of the intestinal immune response in healthy individuals[11]. There is now very strong evidence that both a reduction in the numbers of beneficial bacteria and increases in numbers of harmful bacteria living naturally in the gut are present in CD[12] although it is less clear which of these changes might be causative and which might be a consequence of inflammation. Several independent groups have consistently shown changes in both the faecal and mucosa-associated microbiome in Crohn's patients and unaffected relatives[13-15], an imbalance referred to as "dysbiosis" (Figure 1). Changes are typified by reduced biodiversity and alterations in the dominant organisms, specifically reduction in beneficial firmicutes and increase in numbers of proteobacteria [including *Escherichia coli* (*E. coli*)][14,16,17].

There is also clear evidence to suggest that a number of lifestyle factors contribute to the dysbiosis of gut microbiota observed in CD (see Figure 1). This includes key environmental triggers such as smoking[18], with cessation abrogating the observed dysbiosis[19]. Also a key risk factor in CD is a intake of a "westernised" diet, high in fat and sugar, low in fruit and vegetable fibre[20]. In a mouse model with a humanised microbiota, a switch to a high fat, high sugar diet altered the microbiome within 1 d[21]. A similar diet has also been observed to increase numbers of Proteobacteria, such as *Bilophila wadsworthia*[22] and mucosally adherent, invasive *E. coli* (AIEC)[23].

GENETIC SUSCEPTIBILITIES IN BACTERIAL RECOGNITION, AUTOPHAGY AND PHAGOCYTE-SPECIFIC GENES IN CD

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[24] (see Figure 1). Following on from earlier work[25,26], Genome-wide association studies have now identified 163 IBD risk loci, 30 of which are CD specific and 110 shared between ulcerative colitis and Crohn's[27]. Identified polymorphisms in the innate immune system of Crohn's patients include genes that are linked to processes such as pathogen recognition [nucleotide-binding oligomerization domain-containing-2 (*NOD2*)/Crohn's-associated gene identified was 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[24-26].

The first *CARD15* encoding the NOD2 receptor[28,29]. Mutations in this gene probably account for about 15% of Crohn's 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[30]. The NOD2/CARD15 protein is part of the innate immune system and is expressed in the cytoplasm of macrophages and Paneth cells[31]. 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 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[32]. In association with *NOD2/CARD15* mutations, polymorphism in genes *SLC22A4* and *SLC22A5*,

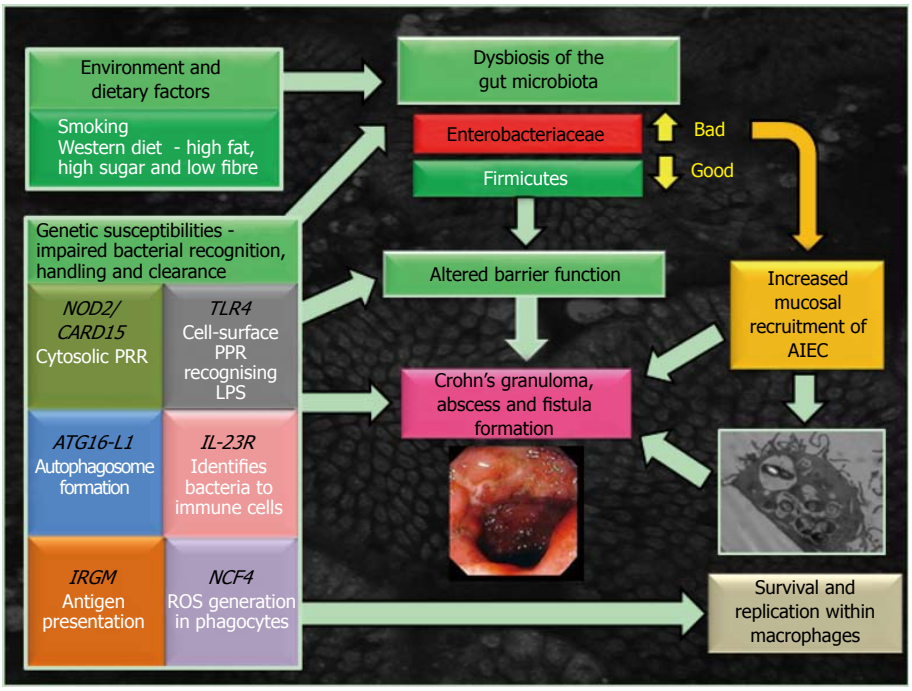

Figure 1 Model for the development of Crohn's disease. AIEC: Adherent, invasive *Escherichia coli*; ATG16L1: Autophagy-related 16-like 1; CARD15/NOD2: Caspase-recruitment domain 15/nucleotide-binding oligomerization domain-containing-2 receptor; IL-23R: Interleukin-23 receptor; IRGM: Immunity-related GTPase M; LPS: Lipopolysaccharide; *NCF4*: Neutrophil cytosolic factor-4 gene; PRR: Pathogen recognition receptor; ROS: Reactive oxygen species; TLR4: Toll-like receptor 4.

encoding the organic cation transporters OCTN1 and OCTN2 have also been identified with variants expressed in intestinal epithelial cells, T cells and macrophages[33]. In addition, a mutation in two haplotypes of *DLG5*, encoding scaffolding protein, has also been confirmed to be associated with *NOD2/CARD15* mutations in Crohn's patients[34].

Two other key genes associated with Crohn's are *ATG16L1* and *IRGM*[35-37]. Both encode proteins that play a key role in autophagy, a cellular process facilitate not only disposal of protein aggregates, DNA, lipids and damaged organelles but also an integral step in the mechanism by which macrophages degrade, kill and clear invading phagocytosed bacteria (a process also termed xenophagy), including Mycobacteria and Salmonellae[38-40].

Additional Crohn's 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[41]. LRRK2 has been linked to CD through the association of a single-nucleotide polymorphism on chromosome 12q12[26] 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[42]. The identification of *NCF4* as a Crohn's susceptibility gene is also important[36]. *NCF4* 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, with molecular defects in NADPH oxidase already established to result in chronic granulomatous disease[43]. 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[44], or subcutaneous injection of heat-killed *E. coli* in Crohn's patients[45]; (see Figure 2). Whilst these studies suggest macrophages may be involved in a key step of the observed immune dysfunction in CD, 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 Crohn's[46]. IL-23R is expressed by activated dendritic cells and macrophages, and IL-23 can induce production of inflammatory cytokines that may contribute to intestinal inflammation[47].

SPECIFIC BACTERIA IN THE PATHOGENESIS OF CD

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 chronic intestinal inflammatory disease

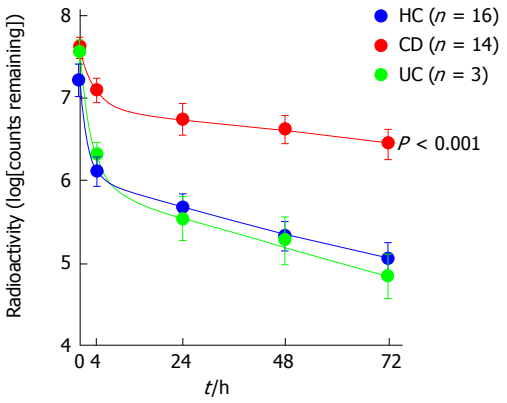

Figure 2 Patients with Crohn's disease exhibit reduced bacterial clearance of subcutaneously injected ^{32}P-labelled heat-killed *Escherichia coli* relative to healthy controls and patients with ulcerative colitis. Reproduced with permission. © 2009 Rockefeller University Press. Originally published in *Journal of Experimental Medicine* 206: 1883-1897[45]. CD: Crohn's disease; HC: Healthy controls; UC: Ulcerative colitis.

in cattle (Johne's disease), was more prevalent in Crohn's patients[48,49]. A study by Ryan and colleagues[50] also confirmed the presence of MAP DNA in granulomatous lesions of CD patients. MAP-reactive CD4 T cells have also been found in patients with Crohn's[51]. Even though, MAP has been hypothesised to be as contributing agent for Crohn's pathogenesis, there is still great controversy, and absence of conclusive evidence, to fully supporting this hypothesis[52]. Our own studies have suggested perhaps that microbial mannan (present in yeast cell walls and Mycobacterium species such as MAP) may be a key environmental factor to suppress macrophage killing of intracellular bacteria[53]. The shared susceptibility association of *NOD2* and *IL-23R* polymorphisms seen in both CD and Mycobacterial disease suggests MAP may yet be important in CD pathogenesis[54].

Faecalibacterium prausnitzii may also be important with low levels strongly associated with early disease recurrence after intestinal surgery[55]. This effect may be due to bacterial production of anti-inflammatory molecules with culture supernatant shown to reduce the severity of colitis in an animal model.

The finding of increased mucosa-associated *E. coli* in the sub-mucus niche or within the mucosa itself has proved particularly consistent in CD[12]. Early serological studies described high antibody titres against *E. coli* in Crohn's patients compared to unaffected controls[56] and this was later supported by immunohistochemical studies demonstrating *E. coli* antigens within macrophages in CD tissue[57]. Many groups, including our own, have shown an increase in mucosa-associated *E. coli* in CD, both in the ileum and in the colorectum[58-64]. We ourselves observed that aerobic culture of colonoscopic biopsies after removal of the mucus layer with dithiothreitol is often sterile in control colons whereas the colon in CD and colon cancer contains increased bacterial numbers in this sub-mucus niche, more than half of which are *E. coli*[60], even though these organisms account for less than 1% of the faecal microbiota[65]. Poor correlation between site of inflammation and presence of *E. coli*[63] and tendency to show that the same organisms can be identified from various sites within the same colon[60,66] are compatible with the organisms having a causative role in the inflammation rather than merely colonising inflamed mucosa. Evidence for a primary pathogenic role is also given by their presence within granulomas[67], the histological hallmark of CD, by their ability to induce granuloma formation *in vitro*[68] and ability for similar *E. coli* to cause granulomatous colitis in dogs[69], and potentially in cats and swine too[70].

These *E. coli* pathovars associated with CD have been designated AIEC based on their ability to adhere to, and invade into, intestinal epithelial cell-lines, induce release of pro-inflammatory cytokines, and possess an ability to survive and replicate with intestinal macrophages[71]. Phylogenetic analysis shows that most mucosa-associated *E. coli* isolated from the tissue of Crohn's patients belong to groups B2 and D[65] as per extra-intestinal isolates, whereas most commensal *E. coli* strains would belong to group A[72].

CROHN'S AIEC-HOST INTESTINAL MUCOSA INTERACTIONS

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 Crohn's patients[73-75], and have been observed in patients using magnifying chromoendoscopy[76]. 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[77]. Several invasive bacteria take advantage of the transcytotic characteristics of M cells to use them to cross the gut, including *Yersinia*, *Salmonella* and *Shigella* spp[78-80]. It was suspected that the portal of mucosal entry of AIEC was also likely through M cells[81] and own recent studies successfully modelling M cells *in vitro*, demonstrated that Crohn's AIEC could indeed translocate through M cells (up to 20-fold compared with parent Caco2 cells) and through isolated human ileal FAE[82]. Adhesion and subsequent translocation of AIEC across murine and human 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 AIEC[83]. Isolates expressing *lpf* have been found to be more prevalent in Crohn's mucosae than that of non-IBD controls[84]. *Ex vivo* studies also indicate a defective mucosal barrier to bacteria in the Peyer's patches from Crohn's patients[85,86]. It is plausible therefore that increased bacterial load at M cells is important in the development of Crohn's. 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

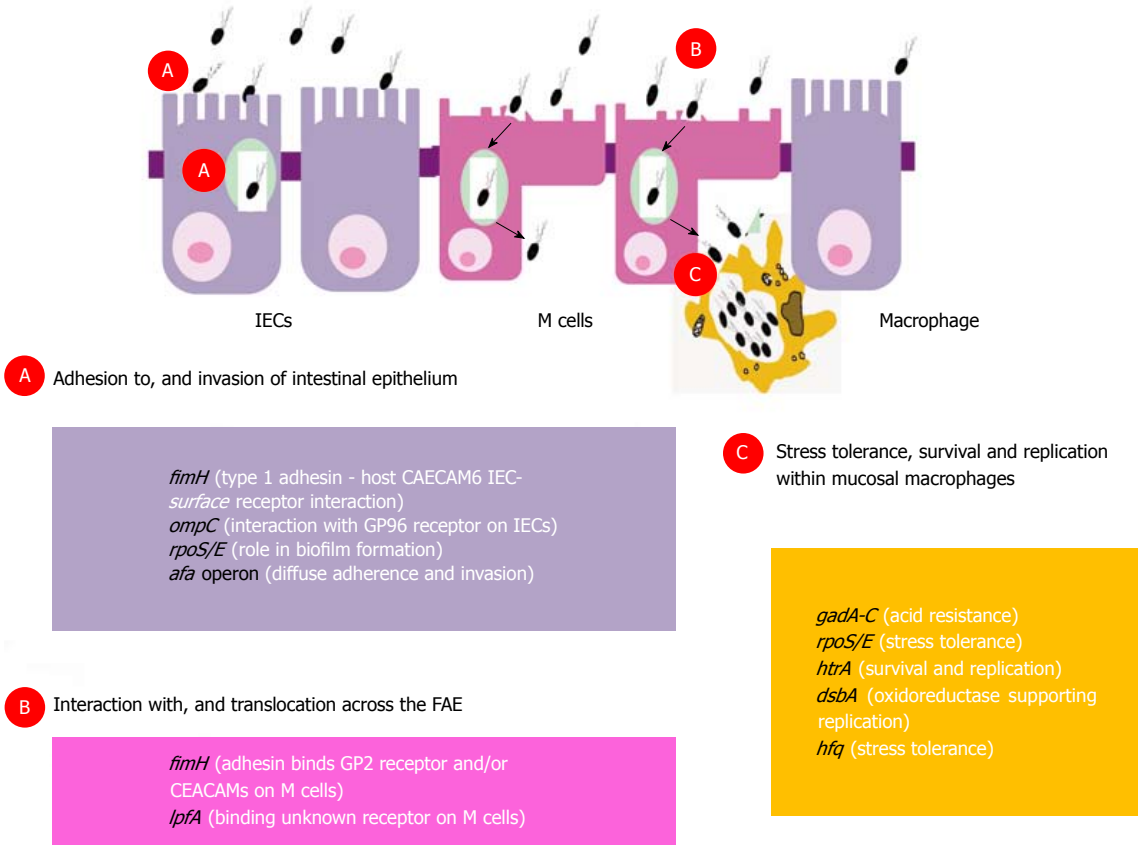

Figure 3 Crohn's mucosally associated adherent, invasive *Escherichia coli* host mucosa interactions: genotype-phenotype relationships. A: Adhesion to, and invasion of intestinal epithelium; B: Mucosal entry across the follicle-associated epithelium; C: Tolerance to stress, habituation and replication within mucosal macrophages. *afa*: Operon encoding afimbrial adhesin; CEACAM: Carcinoembryonic antigen-related cell adhesion molecule; *dsbA*: Gene encoding bacterial disulfide oxidoreductase; *fimH*: Gene encoding bacterial type-1 fimbrial adhesin; *gadA-C*: Glutamate-dependent acid resistance genes; GP2: Glycoprotein 2 receptor; GP96: Endoplasmic reticulum stress response glycoprotein 96; *hfq*: Gene encoding RNA-binding host factor essential for replication of the bacteriophage Qβ; *htrA*: Gene encoding high temperature stress protein A; IECs: Intestinal epithelial cells; *lpfA*: Gene encoding long polar fimbriae adhesin; M cells: Microfold cells; *ompC*: Gene encoding outer-membrane vesicle protein C; *rpoS/E*: Genes encoding stress tolerance sigma factors.

falling away[87].

Ileal AIEC isolates also typically express 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 Crohn's[88]. Highly glycosylated CEACAMs have also been proposed as M cell microbial receptors[89]. 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[90]. By an intriguing coincidence it has also recently been found that the same GP2 protein is the epitope for the "anti-pancreatic" antibody found in CD sera[91]. In addition, Crohn's AIEC outer-membrane vesicles (OMV), also show ability to interact with enterocyte endoplasmic reticulum stress response glycoprotein 96 receptor, increased in expression on the inflamed intestinal epithelium[92]. These OMVs, in association with flagellin, also possess significant ability to evoke pro-inflammatory cytokine release[93]. Colonic mucosally associated AIEC isolates expressing afimbrial adhesin *afa* operon, more commonly associated with diarrhoeagenic diffusely adherent *E. coli*, have also been observed to be more prevalent in CD patients than in non-IBD controls[84]. The presence of the *afa* operon correlates with diffuse adherence to, and invasion of intestinal epithelial cells[84].

A summary of Crohn's AIEC genotype relevant to host intestinal mucosa interactions is summarised in Figure 3.

VIRULENCE FACTORS SUPPORTING CROHN'S AIEC SURVIVAL AND REPLICATION WITHIN HOST MACROPHAGES

AIEC isolated from Crohn's ileal and colonic biopsy tissue demonstrate ability to survive and replicate within phagolysosomes of host macrophages[94,95]; see Figure 4. However, they are not unique in this ability as other pathogens are also known to survive and replicate within macrophages, including Mycobacteria, *Salmonella, Shigella, Coxiella, Brucella, Legionella* and *Listeria* species. Key de-

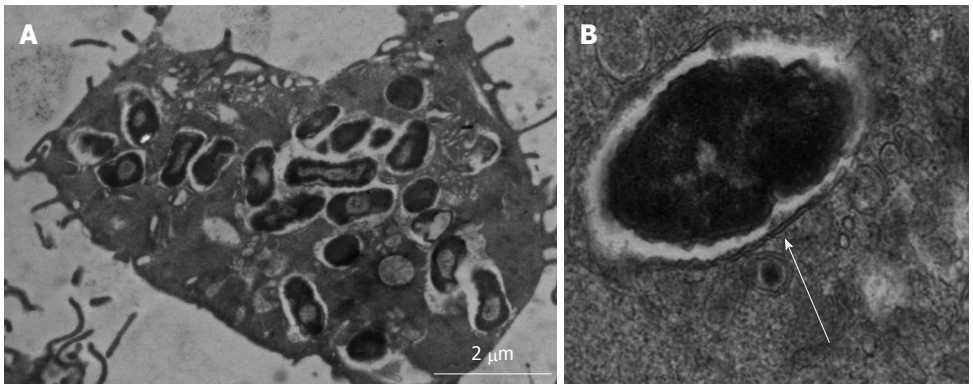

Figure 4 Transmission electron micrograph of adherent, invasive *Escherichia coli* within macrophages[1]. A: Crohn's disease colonic mucosa-associated isolate HM605 surviving and replicating within vesicles of J774-A1 murine macrophages; B: Double membrane around intra-macrophage vesicle indicates bacteria are contained within phagolysosomes (arrow). [1]Images courtesy of Dr. Carol L Roberts (University of Liverpool, United Kingdom).

fence 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. For example, *Shigella* and *Listeria* are able to escape from the mature phagolysosome, Salmonellae can inhibit fusion of phagosome with the lysosome, whilst *Mycobacterium tuberculosis* is able to modify the intra-phagolysosome environment[96]. Key genes supporting AIEC survival and replication within macrophages have been identified (see Figure 3) using isogenic mutants of the "paradigm" ileal AIEC LF82, including *htrA* (encoding high temperature stress protein), *dsbA* (encoding an oxidoreductase) and *hfq* (encoding a RNA chaperone important in mediating bacterial adaptation to chemical stress)[97-99]. However, 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.

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, AIEC certainly appear to be tolerant of the low pH intra-phagolysosome environment[97]. *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 receptor protein CRP, with RpoS functioning as a major environmental stress response regulator in both *E. coli* and Salmonellae[100]. Deletion of RpoS from a Crohn's AIEC (strain O83:H1) has been observed to increase sensitivity of this clinical isolate to oxidative stress[101]. 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*[102,103]. Murine AIEC have been observed to respond to chronic intestinal inflammation by up-regulating expression of *gadA* and *gadB*[104]. The third acid resistance system requires is arginine-dependent utilising of arginine decarboxylase (AdiA and AdiC) antiporter[100] and the fourth is lysine dependent, involving lysine decarboxylase[103]. In addition, *E. coli* also harbour specific mechanisms that enable them to resist high levels of ROS that form the oxidative and super-oxidative response to phagocytosed pathogens. These defensive resources have recently been found to be grouped particularly into two regulated sets of genes *soxRS* and *oxy*R regulons[105,106].

DEFECTIVE AUTOPHAGY AND LACK OF CLEARANCE OF AIEC

ATG16L1 and IRGM function in autophagosome formation and evidence from our own studies supports a role for autophagy as an antimicrobial mechanism downstream of toll-like receptor and 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 dependent manner[107]. Knock-down of *ATG16L1* and *IRGM* using siRNA approaches results in defective recognition and clearance of Crohn's mucosa-associated *E. coli* within host epithelial cells and macrophages[108]. 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 AIEC. Similarly, expression of the Crohn's variant ATG16L1*300A in intestinal Caco2 epithelial cells impairs their ability to capture internalized *Salmonella* spp. within autophagosomes[109] and is also associated with abnormalities in Paneth cell granule exocytosis[110], impaired production of antimicrobial α-defensins[111], and increased production of pro-inflammatory cytokines IL-1β and IL-18 by macrophages in response to LPS[112].

STRATEGIES TO TARGET INTRA-MACROPHAGE AIEC IN CD

If AIEC have a primary pathogenic role then it follows

that targeted treatment should lead to clinical benefit. This hypothesis is supported by studies in Boxer dogs which develop a granulomatous colitis following infection with an AIEC strain[69], with subsequent clinical resolution following treatment with the 4-quinolone antibiotics, enrofloxacin[113]. However bacterial antibiotic resistance is common both in animal and human studies and is associated with poor clinical outcome[114]. Trials of antibiotics in the treatment of active CD have been disappointing to date with good evidence only for their use in the prevention of post-operative disease recurrence[115,116]. A large metanalysis recently failed to show any clear benefit for their use in maintenance of remission or in the treatment of active luminal or peri-anal disease[117]. In some trials, early open label studies were positive only for later randomised trials to fail to show clear benefit[118,119], which may, in part, be due to the development of antibiotic resistance. *In vitro,* quinolone-based antibiotics regimens to target intra-macrophage Crohn's AIEC isolates are effective[95] but again single antibiotic use likely increases the risk of drug resistance, a problem highlighted by a recent study in which multidrug resistance was seen in 61.5% of Crohn's AIEC isolates[120]. Triple antibiotic regimens are superior to ciprofloxacin mono-therapy and reduce intra-macrophage AIEC survival to 3% relative to untreated controls[95]. Unfortunately significant drug-drug interactions occur with some antibiotics and azathioprine which have limited the use of triple combinations to date. Consequently, alternative strategies are being explored including using adjuvant agents to manipulate the phagolysosomal environment to support microbial phagocytosis.

A more promising strategy may be to alter phagolysosomal pH to aid bacterial killing within macrophages. It has already been shown that AIEC are dependent on an acidic environment for survival[97] and that alkalinisation leads to reduced survival. Hydroxychloroquine, a weak base able to increase phagolysosomal pH, is known to improve killing of bacteria where intra-macrophage survival plays a key step in disease pathogenesis[119]. For example, *Coxiella burnetii* the agent of Q fever, maintains an intracellular lifestyle through adaptation to survival at an acidic pH[121,122]. *Coxiella* survival was significantly reduced *in vitro* by hydroxychloroquine treatment and this benefit translated into clinical response in a randomised trial[123,124]. Hydroxychloroquine in combination with antibiotics, is also now standard therapy for treatment of Whipple's disease, where replication of *Tropheryma whipplei* within tissue macrophages is a central part of the pathogenesis[125]. Similarly, our own recent studies have shown that dose-dependent enhancement of macrophage killing of Crohn's AIEC can be seen with hydroxychloroquine treatment and synergy with standard antibiotics is also observed[126].

Vitamin D supplementation also enhances killing of intracellular AIEC in both murine and human macrophages[127]. This may be due to enhancement of the respiratory burst but effects are likely to be multimodal with influences on several intracellular pathways. Cellular production of the antimicrobial peptides, such as cathelicidin antimicrobial peptide (CAMP) and β2 defensin, follows stimulation of toll-like receptors in the presence of vitamin D and conversely, vitamin D deficiency leads to impaired macrophage function due to defective defensin production[128]. This has significance in CD, where muramyl dipeptide stimulation in the presence of vitamin D leads to increased *CAMP* expression. Futhermore, vitamin D stimulates NOD2 expression and leads to downstream β2 defensin production[129]. Vitamin D deficiency is common in CD with up to 70% of patients affected, even in quiescent disease[130,131]. This now appears to have clinical consequence with several studies demonstrating a correlation between serum levels and disease behaviour. In a large prospective cohort study with nearly 1.5 m patient years of follow up, a validated method for predicting vitamin D levels was used to compare the incidence of CD in the lowest quartile relative to the highest quartile, finding the highest risk associated with the lowest Vitamin D levels[132]. This correlation is not limited to the relative disease risk and recent studies now show a clear correlation between disease behaviour and serum concentrations. CD activity, defined both by CDAI and CRP level, has been shown to be inversely correlated with Vitamin D levels, with greatest activity seen in those with the lowest levels[133]. Furthermore, in a retrospective study of 3217 patients, a lower likelihood of requiring surgery for Crohn's was seen with higher vitamin D levels, when using a cut off of 30 ng/mL[134]. Given these findings we might therefore expect a clinical effect from Vitamin D supplementation. This question was addressed in a randomised double-blind placebo-controlled trial in which a trend was seen towards lower relapse rates in patients treated with 1200 U/d of Vitamin D, although this did not quite reach significance[135]. However a significant reduction in risk of requiring surgery was seen for deficient patients who normalised their vitamin D levels with supplementation[134]. Overall these data suggest a clinical role for vitamin D supplementation in CD although further clinical trials are required. Whilst no data yet exists for the effect of vitamin D on AIEC-macrophage interactions *in vivo*, it appears that supplementation may hold promise as a clinical strategy for targeting Crohn's mucosa-associated *E. coli*.

Smoking has long been associated with disease activity and leads to greater treatment requirements, more stricturing disease, more peri-anal disease and shorter disease free survival[135,136]. These affects are likely to be multimodal in origin with effects seen on macrophage function, gut microbiota and vitamin D levels[137-139]. Interventional studies clearly show benefit from smoking cessation[140] and that this is an achievable therapeutic aim[141]. There are some data to support a hypothesis that this may in part be due to recovery of immune cell function but to date this has not been systematically studied in CD[142].

CONCLUSION

Based on the findings of a diversity of individual studies,

there has been accumulating evidence proving the implication of bacteria such as AIEC in the pathogenesis of CD, a chronic-relapsing IBD. AIEC have been shown to translocate M cells of Peyer's patches and lymphoid follicles of the colon, and then to survive and replicate within underlying mucosal macrophages and dendritic cells. However, the mechanism of how Crohn's AIEC resist killing process and adapt to the environment within the phagolysosme to survive and grow within macrophages without inducing cell death is still poorly understood. There is no doubt that further investigation is warranted to characterise and identify the key virulence factors relevant to AIEC phenotype, supporting current and novel, targeted treatments for future clinical benefit.

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