Characterisation of *Salmonella enterica* Typhimurium iron uptake mutants in human dendritic cells

Thesis submitted for the degree of

Master of Philosophy

at the University of Leicester

By

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October 2009
Dedicated to my parents in acknowledgement of a lifetime of support and to Dan, Eva and Isaac for their love, patience and encouragement
Abstract

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Julie Wilkinson

Dendritic cells are antigen presenting cells which play an important role in the initiation of immune responses. This study analysed plastic adherence methods of differentiating human DCs from precursors in peripheral blood. It was determined that this is an effective method of producing DCs however if levels of GM-CSF and IL-4 are lowered differentiation appears to be slowed.

FoxA is an iron-regulated outer membrane protein of Salmonella enterica Typhimurium required for the utilisation of ferrioxamines in iron uptake. Using an in vitro assay a mutant foxA strain was recovered from cultured human DCs at a higher percentage than its wild type parental strain or mutant strains carrying complementing plasmids; confirming foxA has a role in the interaction of S. enterica Typhimurium and human DCs

The outer membrane proteins FepA and IroN are receptors for the siderophore enterobactin and DHBS which can also use the receptor Cir. Using strains with mutations preventing the utilisation of enterobactin and DHBS as well as in the production of the siderophore salmochelin, it was shown that only the inability to utilise DHBS (fepA iroN cir) affects recovery of S. enterica Typhimurium from within human dendritic cells.

The cell surface marker expression of dendritic cells exposed to; the foxA mutant, the fepA iroN cir triple mutant and their respective parental strain were compared to mature dendritic cells. Results showed that infection by S. enterica Typhimurium causes maturation of dendritic cells. Furthermore no differences were seen between the mutants and their parental strains suggesting these mutations do not affect dendritic cell maturation.

Collectively this data indicates that FoxA and DHBS utilisation play a role during Salmonella infection of human dendritic cells however they do not affect their maturation.
Acknowledgement

This thesis is the result of work carried out over 6 years and has involved guidance and support by numerous people. First, I would like to thank my supervisors Colin Hewitt and Peter Williams for the opportunity to undertake the work described in the thesis and for their constant guidance and support.

I would also like to thank Richard Haigh for his help and advice with all things genetic, and Julian Ketley for his comments and advice as well as the undergraduate students who assisted with some of the work; Suzanne, Mandip and Nikki. Equally, I would like to thank all those people who selflessly (if sometimes grudgingly) donated blood; Kris Ridley, Oliver Bridle, Richard Haigh, Jenny Foxon, Rob Mason, Miranda Johnson and Alex Woodacre. Also thanks for their help and friendship go to Julie Turner, Gemma Balderson and Emma Dave. I would like to thank Jenny Foxon for her help and advice concerning tissue culture techniques; Lynne Howells and Louise Fox for their assistance with flow cytometry; and Renata Walewska and Colin Hewitt (again) for their phlebotomy skills.

On a personal note I offer endless thanks to my parents and to Teresa Wilkinson for invaluable babysitting, as well as all my family and friends for their support. I am also indebted to my husband, Dan, whose constant support and encouragement have helped me in so many ways, and to my angels Eva and Isaac for keeping me focused and sane.
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CHAPTER ONE

Introduction

1.1 General Introduction

*Salmonella* are a genus of Gram negative, non-spore forming and rod-shaped predominantly motile enterobacteria. They are chemoorganotrophs and facultative anaerobes found worldwide in animals and humans as well as in non-living habitats. Members of this genus are responsible for causing diseases in both animals and humans including typhoid fever, salmonellosis and paratyphoid fever.

Dendritic cells (DCs) are cells of the mammalian immune system involved in antigen presentation. They are found in most organs of the body in small numbers, as well as in the blood and the skin. Upon exposure to antigen these cells undergo a process of maturation causing migration to lymphoid tissue where they interact with T and B cells resulting in initiation of an adaptive immune response.

DCs play an important role during bacterial infection and the development of any resulting immune response however, our understanding of the exact nature of their interaction with *Salmonella* is limited. This study aims to investigate the interactions of a number of *Salmonella* strains with mutations in their iron uptake systems with human monocyte derived DCs.

1.1.1 *Salmonella* nomenclature

*Salmonella* nomenclature is complex and many attempts have been made to produce uniformity to enable accurate communication between scientists, health officials and the public. In 1966, Kaufmann and White produced a scheme, which is still widely used today, based on antigenic variations of two components: O (lipopolysaccharide [LPS] antigens) and H (flagellar antigens). This scheme defined
serotypes as if they were separate species resulting in the common designations *Salmonella typhimurium*, *S. typhi*, *S. enteritidis*, etc.

More recently, a new system has been developed using DNA sequence comparisons and multilocus enzyme electrophoresis, which offers a more accurate reflection of the relatedness of the serovars (Reeves *et al*, 1989, Boyd *et al*, 1996). Table 1.1 shows how this scheme divides the genus *Salmonella* into two species, *S. enterica* and *S. bongori*, and the further subdivision of the *S. enterica* into six subspecies. Although it is now known that *S. bongori* is a separate species from *S. enterica* it was originally designated as *S. enterica* subspecies V and in many instances is still referred to as Subspecies V for convenience and simplicity.

The majority of clinical isolates, approximately 99%, are assigned to subspecies I (Aleksic *et al*, 1996), including the serovars Typhimurium, Typhi and Enteritidis, which are considered to be major threats to public health. This bias is likely to be a result of serovars of subspecies I being almost exclusively associated with warm-blooded animals, such as domestic animals, livestock and farm produce. Man is much more likely to have sustained contact with these than with cold-blooded animals such as reptiles and amphibians with which the other subspecies and *S. bongori* are associated (Bäumler *et al*, 1997).
Table 1.1. Current numbers of *Salmonella* serovars in each species and subspecies

(Grimont and Weile, 2007)

<table>
<thead>
<tr>
<th><em>Salmonella</em> species and subspecies</th>
<th>Number of serotypes in subspecies</th>
</tr>
</thead>
<tbody>
<tr>
<td>I - <em>S. enterica</em> subsp. <em>enterica</em></td>
<td>1531</td>
</tr>
<tr>
<td>II – <em>S. enterica</em> subsp. <em>salamae</em></td>
<td>505</td>
</tr>
<tr>
<td>IIIa – <em>S. enterica</em> subsp. <em>arizonae</em></td>
<td>99</td>
</tr>
<tr>
<td>IIIb – <em>S. enterica</em> subsp. <em>diarizonae</em></td>
<td>336</td>
</tr>
<tr>
<td>IV – <em>S. enterica</em> subsp. <em>houtenae</em></td>
<td>73</td>
</tr>
<tr>
<td>VI – <em>S. enterica</em> subsp. <em>indica</em></td>
<td>13</td>
</tr>
<tr>
<td>V – <em>S. bongori</em></td>
<td>22</td>
</tr>
<tr>
<td>Total</td>
<td>2579</td>
</tr>
</tbody>
</table>
1.1.2 Diseases caused by serovars of the genus *Salmonella*

A wide spectrum of disease ranging from mild self-limiting gastroenteritis to life threatening systemic enteric fever can result from infection by a serovar of the genus *Salmonella*. The clinical outcome of such infections depends greatly on the infecting serovar, the host species and the immunological status of the host.

Serovars of *Salmonella* are referred to as host-adapted or non-host adapted with respect to a specific host. *S. enterica* Typhi is adapted to the human host causing typhoid fever but is normally unable to infect other host species. *S. enterica* Typhimurium causes a similar typhoid–like infection in mice but in other host species, such as humans and cattle, infections result in non-typhoid symptoms that rarely become systemic. However, in immuno-compromised individuals, non-host adapted as well as host-adapted species can cause systemic infections which may also lead to other infections such as meningitis (Swe Swe *et al*, 2008) and osteomyelitis (Afzal *et al*, 2003). The main clinical presentations of salmonellosis are described below.

**1.1.2.1 Salmonella gastroenteritis**

The most common cause of *Salmonella* gastroenteritis is the consumption of food of animal origin contaminated with *Salmonella* serotypes. Theoretically, all *Salmonella* serovars can cause salmonellosis in humans although the most commonly isolated are the *S. enterica* serotypes Typhimurium and Enteritidis (CDC *Salmonella* surveillance data, 2006).

Symptoms begin 12-72 hours after the ingestion of contaminated food and include abdominal pain, chills, mild fever, vomiting and diarrhoea. The disease is usually self-limiting with the infection localised to the ileum, colon and mesenteric lymph nodes, and a full recovery generally occurs after 3-14 days without any treatment. In severe cases, hospital treatment may be required but this is typically
limited to fluid replacement, with antibiotic use being restricted to those at risk of developing complications, such as immunocompromised individuals. Death from Salmonella gastroenteritis can be a result of dehydration or complications such as bacteraemia or the spread of infection to other areas of the body causing problems such as endocarditis, pneumonia or meningitis.

Salmonella are the second most commonly reported cause of foodborne infection in the US and Europe after Campylobacter. There are approximately 30,000 cases reported annually in the US although the actual incidence is likely to be much higher, an estimated 14 million cases, as many mild cases go unreported (CDC Salmonella surveillance, 2006).

1.1.2.2 Septicaemia

Septicaemia is the presence of large numbers of bacteria in the bloodstream and is the least common disease associated with Salmonella infection of man. Salmonella septicaemia is most commonly associated with the S. enterica serotypes Cholerae suis and Dublin (Jones et al, 2008); however, infections by any serovar can cause septicaemia in individuals with a weakened immune system, such as the very young, elderly or those immunologically compromised.

The incubation period leading to septicaemia is highly variable but is generally longer than that described for gastroenteritis. Septicaemia differs from gastroenteritis in that there are few gastrointestinal symptoms, with diarrhoea being recorded in only about one third of patients (Fang and Fierer, 1991). Septicaemia is characterised by the rapid dissemination of bacteria throughout the blood and lymphatic system, causing the sudden onset of fever followed by a spiking temperature. This distinguishes the disease from typhoid fever in which a continuous fever is observed.

Salmonella septicaemia often causes conditions such as pneumonia,
osteomyelitis, meningitis and endocarditis. These complications can result in a very high mortality rate; Salmonella endocarditis is highly destructive with a fatality rate of 70% (Cohen et al, 1987) and treated infantile Salmonella meningitis results in unfavourable outcomes in 48% of cases with 11% of these being fatalities (Chang et al, 2004).

1.1.2.3 Typhoid fever

Typhoid fever is a serious systemic infection caused by strains of S. enterica Typhi, although a similar disease can be seen upon infection by serovar Paratyphi. Bacteria are transmitted by ingestion of food or drink contaminated by the faeces or urine of infected people. Symptoms begin to appear after an incubation period of 7-21 days and include high fever, lethargy, abdominal pain, vomiting, rose-coloured rash and headaches. A second stage occurs and if untreated the mortality rate is relatively high with 15% of cases resulting in death due to complications (Edelman and Levine, 1986). The most common complications are septicaemia, intestinal perforation and bleeding but others such as pneumonia, myocarditis, meningitis and osteomyelitis can occur (Butler et al, 1991). Typhoid fever is treated with antibiotics, such as ceftriaxone or ciprofloxacin and their use has been responsible for a decrease in the mortality rate to approximately 1% compared to 10-30% if untreated.

In 10% of cases, patients will have a relapse after one to two weeks remission but in 3-5% of cases those infected become carriers of the pathogen, showing no symptoms, but shedding bacteria in their faeces. As infection is by the faecal-oral route carriers present the possibility of epidemics in areas of poor hygiene and sanitation. The historical account of ‘Typhoid Mary’, an immigrant in New York in the early 1900s who unwittingly infected 22 customers through her cakes and pies, exemplifies this point.
The incidence of typhoid fever is decreasing worldwide but there are still an estimated 12-33 million cases reported annually (Huang and DuPont, 2005) that are associated with 216,000–600,000 deaths (Steele, 2008). The vast majority of these cases occur in developing countries whilst cases in the developed world usually occur in individuals returning from abroad (Edelman and Levine, 1986).

1.2 *Salmonella* Pathogenesis

1.2.1 Infection by *Salmonella*

All pathogenic bacteria have to overcome or evade a wealth of defences produced by the host organism for a period long enough to cause a reaction that manifests as a disease. Opportunistic pathogens are able to cause such diseases only in the event of a weakness in these defences, whereas pathogens have evolved methods to overcome intact defences. In the case of *Salmonella* and other gastrointestinal pathogens, before the bacteria even arrive at their site of infection numerous host defences must be circumvented. The majority of studies of *Salmonella* infections have concentrated on *S. enterica* Typhimurium infections of mice, and these are what are described below, unless otherwise stated.

1.2.1.1 Initial entry and survival

Upon arrival in the stomach, bacteria have already survived the antibacterial agents present in the saliva including secretory IgA, lactoferrin, lysozyme and peroxidase (Karolewska *et al.*, 2008). A number of studies have highlighted the importance of stomach acidity as a defence against infection. Increasing the production of gastric acid in mice using histamine resulted in a 5.4 fold decrease in numbers of *S. enterica* Typhimurium surviving passage through the stomach (Tennant, 2008). However, it appears *S. enterica* Typhimurium possesses an acid tolerance response (ATR) which allows adaptation to survival at lower pH by minimising the damage
caused by these acidic environments (Gahan et al., 1999). There appear to be two inducible forms of ATR (Bang et al., 2000), that are seen in stationary phase cells (stat ATR) and in exponentially growing cells (log ATR). Using insertional mutagenesis of a number of global regulators has revealed that ompR mutants are defective in stat ATR but not log ATR (Bang et al., 2000), whereas mutations in rpoS (Hall et al., 1996) and fur (ferric uptake regulator) (Lee et al., 1995) effect only log ATR. A number of other loci have also been shown to be involved in ATR including part of the two component regulator PhoP/PhoQ (Bearson et al., 1998) and atp (Mg²⁺-dependent ATPase) (Foster, 1991). Single point mutations in fur or atp (Portillo et al., 1993) cause increased acid sensitivity resulting in attenuated virulence in mice and a mutation in rpoS has been identified as the cause of attenuated virulence of strain LT2 (Fang et al., 1992). These three genes are pleiotropic in nature so their exact role in ATR is difficult to ascertain.

A number of acid sensitive mutants have also been identified using a lethal screening mechanism; atrB, atrC, atrD, atrF and atrG, (Foster and Bearson, 1994). Some of the proteins were induced at pH 5.8 and some at pH 4.5 suggesting a two stage system and many were also involved in other metabolic functions (atrD and atrF mutations affected iron metabolism). These results support the concept of overlapping acid protection systems in S. enterica Typhimurium. However, the effect these determinants have on survival in the host stomach is still being investigated, although assays using swine stomach contents showed decreased survival of S. enterica Typhimurium phoP mutants and zero recovery of rpoS and fur mutants after 20 minutes exposure (Bearson et al., 2006).

1.2.1.2 Colonisation of the intestine

Salmonella entering the intestine need to resist specific antibodies, such as those directed against the O antigen of LPS (lipopolysaccharide - the primary targets of
adaptive mucosal immunity to Gram-negative enteric pathogens). The host’s gut flora also competes for resources, such as iron, as well as more specific antibacterial activities such as that described for the commensal bacteria *Lactobacillus acidophilus* LA1, which are effective *in vitro* and *in vivo* against Gram negative and positive pathogens, possibly due to a secreted unknown antimicrobial substance (Bernet-Garnard *et al*, 1997).

Bile is also present in the lumen of the intestine and is composed primarily of bile salts, which raise the pH of the intestines compared to the stomach and also degrade and disperse lipids, and as such are potently antimicrobial. Serovars Typhi and Typhimurium, however, have a particularly high resistance to bile salts with bactericidal levels being much higher than the concentrations found within the intestinal lumen (Prouty *et al*, 2004). This high resistance is dependent on loci regulated by the PhoP/Q regulatory system (Miller *et al*, 1989; van Velkinburgh and Gunn, 1999) and involves the AcrB-TolC efflux system, a system involved in the extrusion of a number of other cytotoxic substances (Buckley *et al*, 2006).

A number of factors are involved in bacterial colonisation of the intestine, including survival of peristaltic movement and the establishment of contact between the bacteria and the epithelial cells of the gut that allows invasion. The expression of LPS is important in the colonisation of the intestine, providing an increased ability of bacteria to penetrate the mucosal layer compared to LPS mutant strains (Nevola *et al*, 1987). Indeed the ability of serovar Typhimurium to colonise the mouse intestine decreases as the structure of LPS becomes more defective (Nevola *et al*, 1985).

Fimbriae are proteinaceous, hair-like appendages that mediate attachment to host cells. Members of the *Enterobacteriaceae* produce fimbriae and their importance to the pathogenesis of a number of enteric pathogens has been well documented; *IngA*, a
structural gene encoding a pilus of enterotoxigenic *E. coli* (ETEC) is conserved among diarrhoea causing strains (Giron *et al*, 1994) and TcpA fimbriae of *Vibrio cholerae* are required for colonisation of the intestine (Hall *et al*, 1988). Comparatively less is known about the role of fimbriae in *Salmonella* pathogenesis. Originally, three types of enterobacterial fimbriae were described and designated Type 1, 2 or 3 based upon their interaction with erythrocytes. Type 1 fimbriae are widespread among *Salmonella* serovars and it is this type that has been most extensively studied; type 1 fimbriae are important for the attachment of serovar Typhimurium to enterocytes and promote intestinal colonisation of swine *in vivo* (Althouse *et al*, 2003).

Other classes of fimbriae have been identified; type IV fimbriae, plasmid encoded fimbriae (*pef*) and long polar fimbriae (*lpf*). Type IV fimbriae are filamentous polar organelles whose biosynthesis in *Pseudomonas aeruginosa* involves 40 genes (Alm and Mattick, 1997). They are divided into two sub-groups based on their subunit structure, with Type IVb being exclusively identified on bacteria, including *Salmonella enterica* Typhi, that colonise the human intestine (Zhang *et al*, 2000). This suggests a role not only in the pathogenesis of *Salmonella* but also in human host specificity. Long polar fimbriae were initially identified in serovar Typhimurium and have been subsequently shown to be involved in adhesion to murine Peyer’s patches (Bäumler *et al*, 1996). Plasmid encoded fimbriae have been identified in serovars Typhimurium and Enteritidis and mutations in *pef* results in reduced numbers of bacteria associated with the intestinal villous (Bäumler *et al*, 1996).

Analysis of genomic fimbrial gene sequences revealed that serovar Typhi contains 14 putative fimbrial operons (Humphries *et al*, 2001). Many of these operons, however, are present in non-typhoidal strains of *Salmonella* so a simple correlation between ability to infect a host and a single fimbrial operon is unlikely, although it
should be noted that Typhi does have a unique combination of fimbrial operons that is highly conserved among strains (Townsend et al, 2001). There is evidence, however, that fimbriae have some involvement in Salmonella adaption to their hosts as in Typhimurium bcf (bovine colonisation fimbriae) has been identified as the fimbrial operon required for colonisation of bovine, but not murine Peyer’s patches (Tsolis et al, 1999). S. enterica Typhimurium has 13 operons for fimbriae, stj operon is only detected in this serovar suggesting serovar specific virulence characteristics (Akkoc et al, 2009)

1.2.1.3 Invasion of epithelial cells of gastrointestinal tract

A number of in vitro and in vivo methods have been used to study the interactions between S. enterica serovars and epithelial cells. Human cell lines including HeLa (Gianella et al, 1973) and HEP-2 (Douce et al, 1991) cells have been used in tissue culture, as well as Caco-2 cells, which are also capable of differentiating into a polarised epithelial layer (Finlay and Falkow, 1990). In vivo studies have used intestinal loops, mainly in rabbits and mice, to study the epithelial invasion of host cells (Boyen et al, 2006) and interaction with the epithelium of Peyer’s patches (Jensen et al, 1998).

During infection of mice S. enterica Typhimurium enter the host through the M cells of the follicle-associated Peyer’s patches of the small intestine (Jepson and Clark, 2001), an area lacking in goblet cells that is covered with a thinner layer of protective mucus than elsewhere in the gut. The M cells’ primary function is the sampling and internalisation of luminal antigens and their subsequent presentation to underlying lymphoid tissue of the Peyer’s patches. S. enterica Typhimurium preferentially associate with M cells causing morphological changes in these cells including membrane ruffling and destruction of microvilli (Daniels et al, 1996). In infections
with some other serovars, the microvilli are seen to regenerate but Typhimurium infection eventually results in the death of murine M cells. Although Typhimurium preferentially invade M cells, bacteria have also been isolated from non-phagocytic enterocytes and appear to be taken up by host dendritic cells (DCs) (Kiama et al, 2006) that penetrate tight junctions and project their dendrites into the lumen of the gut (Rescigno et al, 2001). *Salmonella* in the M cells enter the follicular dome situated beneath the follicle-associated epithelium which contains a population of macrophages, DCs and lymphocytes. Systemic infection results when these bacteria disseminate *via* the lymphatic system to infect the mononuclear phagocytic system, including the liver and spleen (Carter and Collins, 1974). There is good evidence that bacterial growth in these organs occurs intracellularly within phagocytes (Dunlap et al, 1992; Richter-Dahlfors et al, 1997) resulting in hepato- and splenomegaly followed by re-entry into the blood and secondary bacteraemia. In mice, death caused by Typhimurium is due to a pro-inflammatory cytokine response to Lipid A of LPS resulting in inflammatory damage of the liver and spleen (Khan et al, 1998). In infections of other hosts, such as humans, Typhimurium establishes a localised infection which is unable to proceed beyond the gut-associated lymphoid tissue.

1.2.1.4 Diarrhoeagenesis

Diarrhoea is characterised by the frequent passing of loose stools and results in the rapid loss of water from the body as well as salts, electrolytes and other nutrients. Acute diarrhoea is a common cause of death in the developing world and is the second most common cause of infant death worldwide. Diarrhoea in humans as a result of *Salmonella* infection is difficult to study using the mouse model as mice never exhibit diarrhoea during salmonellosis caused by serovar Typhimurium.

Zhang et al (2003) used bovine Typhimurium infection to determine that this
serovar causes inflammatory diarrhoea in cows. Gastroenteritis causes a massive inflammatory response as a result of bacterial proteins secreted by the Inv/Spa type III secretion system into host cells. These proteins include SipA, SopA, SopB, SopD and SopE2 which are required to elicit infiltration of neutrophils presumably inducing the production of chemokines in ileal tissue. This results in an inflammatory response, an increase in vascular permeability and mucosal oedema. This is in contrast to other studies which suggest that diarrhoea occurs as a result of a secretory mechanism involving stimulation of adenylate cyclase (Giannella et al, 1975).

A number of determinants, including an ability to invade cells, appear to be responsible for Salmonella-induced diarrhoea. Avirulent strains were unable to cause diarrhoea in studies using rabbit ileal loops whereas virulent invasive strains could (Amin et al, 1994). The involvement in gastroenteric virulence of an enterotoxin (Stn) isolated from S. enterica Typhimurium, which has similarities to E. coli labile toxins and cholera toxin, appears to be complex as both virulent and avirulent strains of Typhimurium make the enterotoxin (Wallis et al, 1986).

1.2.2 Virulence determinants

1.2.2.1 Salmonella pathogenicity island I (SPI1)

Salmonella pathogenicity island I (SPI1) is a 40 kb region of DNA acquired sometime prior to the speciation of Salmonella and is currently present in all lineages of S. enterica and bongori, regardless of the normal host species infected by a particular serovar (Ochman et al, 1996). Molecular genetic studies have shown that SPI1 is an important virulence determinant involved in the intestinal phase of infections that result in both enteric fevers and inflammatory gastroenteritis (Darwin and Miller, 1999). The most significant role of SPI1 during infection is the production of a type III secretion system (TTSS) that transports effector proteins towards or directly into host cells where
they interact with host proteins causing effects that are associated with disease (Hueck, 1998). It appears that, although the SPI1 encoded TTSS system is common to all *Salmonella* serovars, each serovar encodes different effectors that are appropriate to their host populations (Zhou and Galen, 2001).

The virulence phenotypes associated with the SPI1 TTSS system are not constitutively expressed but subject to transcriptional regulation in response to environmental and physiological conditions. Signals resulting from conditions such as high osmolarity and extreme pH (Bajaj *et al*., 1996) converge on expression of *hilA*, which directly regulates transcription of TTSS apparatus genes (Ellermeier and Slauch, 2007). HilD, HilC and RtsA constitute a feed forward loop that controls expression of HilA and RtsA also induces expression of *dsbA* (Ellermeier and Slauch, 2008). DsbA is required for both SPI1 and SPI2 TTSS to translocate effector proteins into the cytosol of cells (Ellermeier and Slauch, 2004).

The TTSS apparatus forms a needle complex that spans the inner and outer membrane of the bacterial wall. The proteins, including SpaO, P, Q, R, S, InvA, InvC and OrgB, that make up the core export apparatus facilitate the sec-independent export of distal components of the needle complex as well as the effectors. When this complex contacts a host cell, it undergoes a rearrangement that introduces part of the apparatus, termed the translocon (SipBCD), into the host cell membrane. Effector proteins that subvert cellular processes to favour bacterial colonisation can then be introduced directly into the cytoplasm of the host cell. Many of these effectors are encoded elsewhere on the chromosome, although SPI1 itself also encodes a number, including SipA, SptP and AvrA, that have important roles in the invasion of host cells. SipA has actin regulating activities which appear to play a role in membrane ruffling (Perrett and Jepson, 2009) whereas AvrA has a role in stabilising host cell permeability (Liao *et al*., 2009).
SPI1 also encodes a number of chaperone proteins, which facilitate efficient secretion and translocation of specific effector proteins. InvE controls translocation by regulating the functions of Sip protein translocases (Kubori and Galan, 2002) and SicA partitions SipB and SipC preventing their premature association and degradation (Tucker and Galan, 2000). Expression of a number of virulence genes located on SPI1 are under the control of the global regulator, FNR. Furthermore, S. enterica Typhimurium fnr mutants have been shown to be non-motile, lack flagellar, shows attenuation in mice and cannot survive in macrophages (Fink et al., 2007).

1.2.2.2 Other Salmonella pathogenicity islands

A number of other Salmonella pathogenicity islands have been identified in S. enterica Typhimurium including another that encodes a TTSS, SPI2. This system translocates effector proteins that modulate host cell functions enabling avoidance of antimicrobial activities and which promote intracellular proliferation. A number of these effector proteins are also involved in inhibition of antigen presentation, SifA, SspH2, SlrP, PipB2, and SopD2 (Halici et al., 2008). SifA also co-operates with the SPI1-encoded effector SipA to promote replication within the customised Salmonella-containing vacuoles of the host cells (Brawn et al., 2007). SPI2 plays a role in disrupting the intestinal ecosystem of the murine gastrointestinal tract (Barman et al., 2008) and in suppression of inducible nitric oxide synthase in activated macrophages (Das et al, 2009).

Other Salmonella pathogenicity islands include; SPI4 that encodes a type I secretion system and a protein SiiE, a non-fimbrial adhesin involved in adhesion of S. enterica Typhimurium to in vitro polarised epithelial cells (Gerlach et al., 2007); and SPI5 that encodes SopB an effector that is secreted via the SPI1 TTSS system and mediates invasion of cultured cells (Erhbar et al., 2002). However, exactly how all these
effectors are regulated and their entire roles in pathogenesis are not yet fully understood.

1.2.2.3 Virulence plasmid genes

A large number of Salmonella serovars possess large virulence plasmids (50-100 kb), which are required for full virulence (Rychlik et al., 2006), although interestingly the human host-adapted serovar Typhi does not carry any such plasmids. Using transposon insertion mutagenesis, the region of the plasmid required for the virulence phenotypes was identified to encode a number of genes, spvRABCD (Gulig et al., 1992). Expression of these genes are upregulated upon invasion of both macrophages and epithelial cells (Hautefort et al., 2008). This region is highly conserved among serovars of Subspecies I, and is also found in some isolates from Subspecies II, IIIa, IV and V, although, in contrast, the spv genes in these are located on the chromosome (Libby et al., 2002). SpvR positively regulates transcription of the genes in the spv operon, which is also regulated by RpoS, a global regulator of genes expressed during stress and starvation (Kovarz et al., 1995; Wilson and Gulig, 1998). The SpvB protein is primarily responsible for the spv virulence phenotype and has been identified as an ADP ribosyltransferase that modifies actin and destabilises the cytoskeleton of the infected cell (Lesnick et al., 2001). The operon is regulated by the iron status of the cell (Spink et al., 1994). Depending on the serovar, the plasmid also encodes for a number of other virulence associated genes (Table 1.2).

A number of serovars also carry high molecular weight plasmids responsible for antibiotic resistance. The serovar Typhimurium resistance genes cat (chloramphenicol), strAB (streptomycin) and tetA, tetB and tetC (tetracycline) are found on such plasmids (Llanes et al., 1999; Guerra et al., 2002).
Table 1.2. Virulence factors encoded by different *Salmonella* serovar specific plasmids.

<table>
<thead>
<tr>
<th></th>
<th>Typhimurium</th>
<th>Enteritidis</th>
<th>Colerae-suis</th>
<th>Gallinarum</th>
<th>Dublin</th>
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</thead>
<tbody>
<tr>
<td><em>spv</em></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>Rck</em></td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td><em>Pef</em></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>srgA</em></td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>?</td>
</tr>
<tr>
<td><em>mig-5</em></td>
<td>+</td>
<td>+</td>
<td>+</td>
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</table>

1.2.3 The mouse typhoid model

*S. enterica* Typhimurium infection of mice causes an infection similar to typhoid fever caused in humans by serovar Typhi. As a result, this mouse model is often used to identify and characterise virulence mechanisms important for pathogenesis of human typhoid fever. Although both serovars possess SP1, SP2 and SP3 they do not possess identical virulence genes.

The plasmid-encoded *spv* operon involved in the growth of serovar Typhimurium in the murine liver and spleen is not present in the Typhi genome (Woodward, 1989). Although both serovars share a number of operons responsible for the production of fimbriae, including type 1 fimbriae (*fim*), aggregative fimbriae (*agf*) and *Salmonella* fimbriae (*saf*), Typhi has a unique combination of operons.

Serovar Typhi also possesses the *viaB* locus which encodes a capsule, not present in serovar Typhimurium, which has a role in bacterial resistance to complement (Hashimoto *et al*, 1991). A further difference between the mouse model and typhoid fever is that mechanisms leading to death are different. Murine deaths from Typhimurium infection are caused by tissue damage in the liver and spleen, whereas human death from typhoid fever is usually due to perforation of the small intestine (Butler *et al*, 1985).

These differences have not prevented the widespread use of the mouse model for the development of typhoid fever vaccines. Mutations in genes affecting virulence that were identified in serovar Typhimurium using the mouse model and have subsequently been used to attenuate homologous genes in Typhi include *phoPQ* (Hohmann *et al*, 1996), *aroC aroD htrA* (Lowe *et al*, 1999).

There are at present two vaccines against typhoid fever currently recommended by the World Health Organisation, the live oral vaccine Ty21a and the parenteral
subunit Vi-antigen vaccine. Both vaccines confer protection to 50-80% of recipients. The oral vaccine is based on Typhi strain Ty21a which, although it is known to contain a galE mutation (McKenna et al, 1995) and does not possess the Vi-antigen, is otherwise genetically uncharacterised. This, along with the fact that four separate doses are required for maximum efficacy, has resulted in much investigation into single dose oral vaccines.

The Vi-antigen vaccine is administered by intramuscular injection and is based on purified Vi-antigen, a linear homopolymer of galacturonic acid that is purified from the bacteria. Field trials of the vaccine conferred 75% and 64% protection after 20 months of active surveillance, declining to 55% at 3 years after vaccination. Conjugating this vaccine with recombinant Pseudomonas aeruginosa exotoxin A as the protein carrier increased protection to 91% for 27 months (Kossaczka et al, 1999). However this has not been commercially developed due to complexities in production.

1.3 Iron and Infection

1.3.1. Iron in biological systems

All organisms, with the exception of lactobacilli, require iron for growth and replication. In iron limited conditions bacteria show a reduction in growth and in the production of iron-containing proteins as well as the inhibition of DNA replication and cell division (Vinella et al, 2005). These observations are explained by the requirement for iron of many proteins involved in essential metabolic pathways and reactions. The proteins that have a requirement for iron are diverse, a result of the two stable valencies of iron and the wide range of oxidation and reduction potentials (Byers, 1987). Iron-requiring proteins include a number involved in oxygen metabolism (Halliwell and Gutteridge, 1985), such as superoxide dismutase, catalase, hydrogenase, ferridoxin and the cytochromes, as well as the enzymes ribonucleotide reductase, RNA polymerase III
and various amino acid dioxygenases and hydroxylases.

Iron is not freely available to plants and microbes despite its abundance in the earth’s crust as it forms insoluble ferric hydroxides at both neutral and alkaline pH. Inside host organisms, iron is even more limited as it is maintained at low levels due to its central role in Haber-Weiss-Fenton reactions.

\[
\begin{align*}
\cdot O_2^- + Fe^{3+} &= O_2 + Fe^{2+} \\
2O_2 + 2H^+ &= H_2O_2 + O_2 \\
H_2O_2 + Fe^{2+} &= Fe^{2+} + OH^- + OH^-
\end{align*}
\]

These reactions produce potentially harmful hydroxide free radicals, which react with most biological molecules but are particularly associated with disruption of cell membranes and DNA breakage (Kehrer, 2000).

1.3.2 Iron in the vertebrate host

Higher organisms have evolved mechanisms for sequestering free iron, which involves the formation of complexes with iron binding proteins. These mechanisms prevent the participation of iron in the potentially damaging Haber-Weiss-Fenton reactions whilst ensuring there is sufficient available to meet the needs of metabolising cells.

The majority of iron in the human body is bound with haem proteins, predominantly haemoglobin (around 66%), where it assists with oxygen transport. Iron released from lysed erythrocytes is recycled by binding to haptoglobin, which is then removed from circulation by the mononuclear phagocytic system (Wassell, 2000). Iron is stored within cells primarily by binding to the protein shell of ferritin, where ferrous iron is converted into the ferric state by oxidation. This is a reversible reaction that allows ferritin to act as a buffer (Orino and Watanabe, 2008), protecting the cell from high concentrations of free iron whilst also meeting the cell’s changing requirements for
Transferrin and lactoferrin are two related glycoproteins with an extremely high affinity for iron that maintain low levels of iron in the serum and at mucosal surfaces respectively. Transferrin has an important role in the transport and recycling of iron; after binding iron it circulates in the blood until it identifies a transferrin receptor on the surface of a host cell. Transferrin is drawn into the cell in a vesicle and iron is then released when the contents of that vesicle become acidic (Nunez et al., 1990). The receptor and transferrin are then recycled back to the outside of the cell.

Lactoferrin does not appear to have a role in recycling or transport of iron but instead contributes to innate immunity at mucosal surfaces (Legrand et al., 2008) where it is found in secretions such as milk, tears, saliva, hepatic bile, gastrointestinal fluid, seminal fluid, nasal and cervical mucus (Ward and Conneely, 2004). Lactoferrin binds two atoms of iron, effectively scavenging any free iron and preventing microbial access to iron in mucosal environments. Another feature that contributes to lactoferrins’ antimicrobial role is its ability to function below pH4.5, a condition that prevails at sites of sepsis (Weinberg, 1984). This is in contrast to transferrin which loses its affinity for iron at pH<5.

1.3.3 Bacterial iron acquisition strategies

Iron is required by most bacteria for optimum growth, but since available iron concentrations are extremely low, not only in both aqueous and terrestrial environments but also within the host organism, bacterial pathogens have evolved a number of mechanisms for iron acquisition. In iron limited conditions bacteria can acquire iron by reduction of ferric iron, by utilisation of host iron-binding compounds or by production of siderophores. Some bacteria utilise all these strategies but most rely on a particular subset that they may have evolved because of the niche the particular organism
occupies. Alternatively, some bacteria have evolved to become completely independent of the need for iron.

1.3.3.1 Iron independent bacteria

Lactobacilli are Gram-positive, non-pathogenic bacteria and some of the members of this species have evolved to be completely independent of the need for iron (Pandey et al, 1994). These bacteria are able to grow equally well in iron free culture media as in media supplemented with iron. Several studies have shown that lactobacilli have replaced iron-requiring co-factors with unique enzyme co-factors; for example, manganese co-factored RNA polymerase and cobalt co-factored ribonucleotide reductase (Blakley et al, 1964).

The ability to survive in iron-free conditions allows lactobacilli to exploit unique environmental niches but limits their ability to compete with iron-dependent bacteria in iron-rich environments. A good example occurs in breastfed infants; human milk is rich in the iron-binding protein lactoferrin, which prevents growth of iron-dependent toxigenic bacteria and allows colonisation of the infant gut by non-pathogenic lactobacilli. Unfortunately, as iron is added to the infants’ diet iron-dependent bacteria then displace the non-pathogenic lactobacilli (Weinberg, 1994).

1.3.3.2 Reduction of ferric iron

A number of bacteria are capable of mobilising iron by using ferric reductases to reduce the metal from Fe$^{3+}$ to Fe$^{2+}$. Ferrous iron is soluble and readily available to bacteria as well as having a much lower affinity for host iron-binding compounds. *Listeria monocytogenes* produces an extracellular ferric reductase (Barchini and Cowart, 1996) and *Campylobacter jejuni* uses a ferric reductase co-factored with riboflavin (Crossley et al, 2007) to mobilise iron from a number of iron complexes including transferrin.
1.3.3.3 Utilisation of host iron-binding compounds

Haem and haemoglobin contain around two thirds of the total iron in the human body but as it is contained within erythrocytes it is not readily available to pathogens. A number of pathogens have evolved methods of removing iron bound to these molecules; *E. coli* (Suits *et al.*, 2009) and *Yersinia* species (Schneider and Paoli, 2005) can use trace amounts of serum haem as a source of iron. *Neisseria* species produce two surface receptors involved in haem iron acquisition, HmbR which mediates iron acquisition from haem and haemoglobin (Perkins-Balding *et al.*, 2003) and HpuA/HpuB which acquires iron from haemoglobin alone (Rohde *et al.*, 2002) or from haemoglobin complexed with haptoglobin (Rohde and Dyer, 2004). A number of pathogens increase the availability of haem and haemoglobin in the blood by producing toxins that lyse erythrocytes. *Vibrio cholerae* produces a haemolysin (Zhang and Austin, 2005) and invasive strains of *E. coli* produce α-haemolysin (Linggood and Ingram, 1982), both in response to iron restriction.

*Neisseria* species have also evolved outer membrane receptors that directly bind to iron associated with transferrin and lactoferrin. The development of these iron-scavenging methods is probably due to the inability of these bacteria to produce any known siderophores. The proteins TbpA and TbpB bind specifically to human transferrin (Agarwal *et al.*, 2005; DeRocco *et al.*, 2007), removing and internalising the iron. The receptor for lactoferrin is LbpA (Biswas and Sparling, 1995), although a second protein which has yet to be fully characterised, LbpB (Biswas *et al.*, 1999) appears also to be involved. Although a number of other bacteria also remove iron from host iron binding protein in this way, serovars of the genus *Salmonella* do not appear to possess this ability.
1.3.3.4 Production of siderophores

Siderophores are low molecular weight, high affinity iron-binding compounds secreted by most bacteria under iron-restricted conditions. Generally, they are catecholates or hydroxamates (Fig. 1.1) although a number of other compounds, such as citrate for *E. coli*, have been found to have siderophore-like properties (Wagegg and Braun, 1981). Endogenous siderophores are synthesised in the cytoplasm *via* pathways separate from those involved in primary metabolism. Siderophore molecules in the extracellular environment scavenge free iron and in some cases are able to displace it from host iron-binding proteins.

Many bacteria are also able to scavenge iron containing siderophores produced by other bacteria in the extracellular environment by producing outer membrane receptors and transport systems. *E. coli* produces a number of iron regulated outer membrane receptors (IROMPs) including FepA (Hollifield *et al.*, 1978) and IutA (Carbonetti and Williams, 1984) that are receptors for the endogenous siderophores enterobactin and aerobactin respectively. *E. coli*, however, can also take up siderophores produced by other species using the IROMPs FhuA (Coulton *et al.*, 1986), a receptor for ferrichrome synthesised by *Penicillium* spp and *Ustilago sphaerogena*, and FhuE (Hantke, 1983) the receptor for coprogen synthesised by *Penicillium* and *Neurospora* spp.

Transport of the ferric-siderophore complexes by Gram-negative bacteria into the cytoplasm is against a concentration gradient and involves outer membrane proteins and iron transport proteins (Fig. 1.2). The energy transduction protein TonB and proton motive force overcome the lack of obvious energy at the outer membrane (Braun *et al.*, 1991). TonB has three functional domains, a hydrophobic amino terminus anchored in the cytoplasmic membrane, a rigid central domain that spans the periplasm and a
Fig. 1.1. Examples of siderophores. A, enterobactin and B, vibriobactin are examples of catecholate type siderophores. C, coprogen and D, aerobactin are examples of the hydroxamate type.
Fig. 1.2. **General model of ferrisiderophore complexes.** The ferric-siderophore complex binds specifically to cognate iron-regulated outer membrane protein (IROMP) initiating transport. The protein receptor interacts with TonB protein, which interacts with ExbB and ExbD proteins resulting in conformational changes allowing transport across the outer membrane. Periplasmic binding protein dependent ABC transporter complexes mediate transport across the inner membrane.
hydrophobic carboxy terminus, which interacts with IROMPS (Traub et al., 1993). These IROMPs, along with all other TonB dependent transporter proteins, exhibit a conserved pentapeptide sequence at the amino terminus called the TonB box (Brewer et al., 1990) which mediates contact with TonB protein. Although TonB is clearly the energy transducer, two other proteins ExbB and ExbD are required for the transport of ferric-siderophore complexes (Fischer et al., 1989). In E. coli, these proteins are localised in the cytoplasmic membrane and mutations in either produce the same phenotype, more rapid degradation of TonB and a reduction in TonB dependent functions (Ahmer et al., 1995). However, their full importance in TonB dependent transport is difficult to determine, partly because TolQR proteins involved in another energy transducing system can partially compensate for ExbB and ExbD mutations (Braun and Herrmann, 1993).

ABC transporter complexes consisting of periplasmic binding proteins, ATP binding proteins and one or more hydrophobic cytoplasmic membrane proteins transport ferric-siderophore complexes across the cytoplasmic membrane. All E. coli hydroxamate siderophores in the periplasm are transported by a system composed of FhuD, a periplasmic binding protein, FhuC, which contains an ATP binding domain, and FhuB, a highly hydrophobic membrane component (Fecker and Braun, 1983). Ferric enterobactin is transported by the FepBCDG system (Pierce and Earhart, 1986) and ferric citrate by the FecBCDE system (Staudenmaier et al., 1989).

### 1.3.4 Iron and genetic regulation

The ability of bacteria to sense and adjust to their environment by altering their expression of virulence genes is essential for effective pathogenesis. A number of environmental signals, including pH, osmolarity and temperature, regulate gene expression (McIver et al., 1995; Mikulskis et al., 1994). A considerable number of
bacterial virulence genes, however, are regulated in response to low levels of iron as found in the mammalian host (Litwin and Calderwood, 1993). Siderophore and haemolysin production are regulated by iron concentration as well as a number of other virulence determinants. A number of pathogenic bacteria produce toxins in response to iron deficiency; *Shigella dysenteriae* produces shiga toxin (Svinarich and Palchaudhuri, 1992) and *Corynebacterium diphtheriae* produces diphtheria toxin (Pope, 1932). Production of such toxins can have serious effects on the infected host ranging from digestive upsets to myocarditis and can even result in death.

In *E. coli* and other Gram-negative species including *Yersinia*, *Salmonella*, *Vibrio*, *Campylobacter* and *Neisseria*, the Fur (ferric uptake regulation) protein controls regulation of genes in response to changes in iron concentration (Bagg and Neilands, 1985). The *E. coli* Fur protein is 17 kDa and acts as a transcriptional repressor of iron-regulated promoters by a process involving Fe$^{2+}$-dependent DNA binding. In iron-rich conditions, Fur binds the divalent ion and acquires a configuration which is able to bind to specific operator sequences thus preventing transcription of functional genes (de Lorenzo et al, 1986). Fur-Fe$^{2+}$ binds at sites known as Fur boxes, which all consist of a similar structure, a 19 bp A-T rich DNA sequence (Escholar et al, 1999). In contrast, in iron-limited conditions Fe$^{2+}$ is displaced, allowing RNA polymerase access to promoters and resulting in the expression of genes required for siderophore biosynthesis and other iron-regulated genes.

A number of studies using techniques such as two-dimensional SDS PAGE have revealed that the synthesis of large numbers of proteins is affected by iron availability. Foster and Hall (1992) discovered that, in *S. enterica* Typhimurium, of 34 Fur-regulated proteins, 15 appear to be positively regulated, some in the absence of iron. A mutation in the H90 residue of the *S. enterica* Typhimurium *fur* gene results in an inability to
sense iron but maintains the role of Fur as a mediator of the ATR, a function which appears to be positively regulated (Hall and Foster, 1996). A number of positively regulated genes have also been suggested in *E. coli*, including those involved in catabolism of succinate (Hantke, 1987). Expression of ftnB and dps are Fur-regulated and are induced under conditions of Fe limitation, producing the ferritons FtnB and Dps which have a role in protection from oxidative stress (Velayudhan et al, 2007).

**1.3.5 Iron acquisition and virulence**

The role of iron acquisition in the virulence of pathogenic bacteria was initially suggested by observations of infected host organisms with lowered or elevated levels of iron as a result of underlying infections or due to the administration of iron containing compounds. These observations implicated iron as an important factor in the infections of a number of bacteria including *E. coli*, *S. enterica* Typhimurium, *Proteus mirabilis* and *Yersinia* species (Payne, 1988).

Diseases such as malaria and sickle cell anaemia result in the lysis of erythrocytes and the shedding of haemoglobin into the serum which results in an increase in the incidence of *Salmonella* infection (Bronzan et al, 2007; Onwubalili, 1983). Other conditions associated with iron overload in humans, such as E-β-thalassaemia and transfusional iron overload of severely anaemic patients, result in susceptibility to infection by both Gram-negative and Gram-positive bacteria (Cunningham et al, 2004; Vento et al, 2006). Increases in virulence because of iron overload are confirmed by the addition of exogenous iron; inoculation of mice with *S. enterica* Typhimurium alongside ferric citrate resulted in increased bacterial growth in tissues (Sawatzki et al, 1983).

*S. enterica* Typhi mutants defective in enterobactin synthesis or transport exhibit decreased growth in HeLa cell monolayers and attenuation of virulence in mice.
demonstrating this system’s role in pathogenesis (Furman et al, 1994). The aerobactin system for scavenging iron, encoded on the ColV plasmid, enhances the virulence of strains of *E. coli* in a mouse model of infection (Williams et al, 1979). Pathogenic strains cured of the ColV plasmid have their virulence restored by cloned genetic determinants of the aerobactin system (Roberts et al, 1989). The siderophore yersiniabactin (Haag et al, 1993) and its TonB dependent outer membrane protein FyuA (Rakin et al, 1994) are required for the full virulence of *Yersinia enterocolitica* in mice.

**1.3.6 Salmonella and iron acquisition**

*Salmonella* species encounter iron-limited environments within their host organisms during infection of deeper tissues. Consequently, as with many other pathogenic bacterial species, they have evolved a number of iron acquisition systems. These include endogenously produced siderophore systems, specialised transport systems for exogenously produced siderophores as well as systems not requiring siderophores.

Ferrous iron is soluble and so is able to diffuse through porins into the periplasmic space; although in *S. enterica* Cholerasuis an outer membrane protein Omb has also been identified as being a ferrous binding protein (Su et al, 2009). In *Salmonella* transport across the inner membrane is mediated by FeoB protein encoded by the *feoAB* operon. Mutations in *feoB* did not, however, cause attenuation of *S. enterica* Typhimurium virulence after oral or intraperitoneal infection of mice (Tsolis et al, 1996). It has been recently reported (Jeon et al, 2008) that RstA promoted expression of FeoB enhances Fur activity of *S. enterica* in iron replete conditions. RstA, a *Salmonella* response regulator, binds directly to the *feoA* promoter, activating expression of the *feoAB* operon resulting in an increase in the uptake of ferrous iron into the bacterial cell and thus increased levels of Fur-Fe$^{2+}$. Consequently, FeoB through the
activation of RstA is involved in the hyper-repression of Fur regulated genes.

The principle siderophore produced by more than 99% of isolates of *Salmonella* serovars is enterobactin, a phenolate molecule, which is a cyclic triester of 2,3-dihydroxy-N-benzoyl serine (DHBS) (Pollack and Neilands, 1970). An intermediate of enterobactin biosynthesis, 2,3-dihydroxybenzoic acid (DHBA) (Wilkins *et al*., 1970), as well as enterobactin breakdown products, such as linear dimer and trimer forms of DHBS (Rabsch *et al*., 1996) can also be used to supply iron for growth. A number of IROMPS are involved in the uptake of enterobactin and its metabolites; FepA transports enterobactin and 2,3-dihydroxybenzoylserine, IroN promotes uptake of enterobactin, 2,3-dihydroxybenzoylserine, and an additional siderophore, salmochelin, and Cir is a receptor for 2,3-dihydroxybenzoylserine. Studies suggest that the uptake of enterobactin is not required for the virulence of *S. enterica* Typhimurium in mouse and chicken models of infection but an inability to uptake 2,3-dihydroxybenzoylserine results in significant attenuation in mice infections (Rabsch *et al*., 2003).

The siderophore salmochelin is a C-glucosylated enterobactin whose production is dependent on the synthesis of enterobactin and the *iroBCDEN* gene cluster. The ABC transporter IroC exports salmochelin as well as enterobactin, and the ABC transporter FepD as well as IroN are involved in the uptake of ferric-salmochelin. Mutation of *iroN* does not appear to cause attenuation in mouse and chicken models (Rabsch *et al*., 2003) but *iroC* mutants exhibit reduced virulence during systemic mouse infections (Crouch *et al*., 2008). Salmochelin production is interpreted as a bacterial evasion mechanism against the mammalian enterobactin binding protein NGAL-lipocalin and some salmochelin producing strains also produce micosins which may inhibit growth of competitors for catecholate siderophores (Muller *et al*., 2009).

The siderophore aerobactin and its corresponding IROMP IutA are produced by
some serovars of *Salmonella* with the highest proportion of these being found in serovars of Subspecies IIIa and IIIb. Aerobactin production by Subspecies I is almost exclusively restricted to isolates from nosocomial infections, particularly in children’s wards, with the most common serovars being Typhimurium, Wien, Infantis and Haifa (Kingsley *et al*, 1996). Interestingly, host-adapted serovars such as Typhi for man, Choleraesuis for pigs and Dublin for cattle produce no aerobactin and only low levels of enterobactin. Aerobactin production appears to have a role in systemic infections by serovars that are not usually associated with these types of infection in man and this may be, in part, because aerobactin is highly effective at removing iron from transferrin (Ford *et al*, 1988).

*S. enterica* Typhimurium can also transport a number of siderophores that are produced by other organisms (Table 1.3). As discussed previously, the uptake of these ferric-siderophores is dependent on specific IROMPS and transport systems. In *Salmonella*, transport of all ferric-siderophores is TonB-dependent with the exception of DHBA and ferrioxamines B and E (Kingsley *et al*, 1999). It has also been reported (Reissbrodt *et al*, 1997) that under conditions of iron limitation *S. enterica* Typhimurium excretes high levels of α-keto acids (predominantly pyruvic acid) which can function in iron uptake. However, *Salmonella* are unable to utilise haem as an iron source and unlike *E. coli* are devoid of a ferric citrate transport system (Wagegg and Braun, 1981).

A system involved in the transport of ferric-siderophores complexes across the inner membrane has been identified in *S. enterica* Typhimurium. The *sitABCD* operon located on SPI1 (Zhou *et al*, 1999) shows homology with a number of other iron transporter systems including the *Yersinia pestis yfuABC* system (Gong *et al*, 2001), *Haemophilus influenzae hitABC* system (Sanders *et al*, 1994) and *Neisseria*
Table 1.3. Siderophores utilised by *Salmonella*

<table>
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<tr>
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<th>ENDOGENOUS</th>
<th>EXOGENOUS</th>
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<tr>
<td><strong>CATECHOLATE TYPE</strong></td>
<td>Enterobactin</td>
<td>Myxochelins (from myxobacteria)</td>
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<tr>
<td></td>
<td>Salmochelin</td>
<td>Amonabactins (from <em>Aeromonas</em> sp.)</td>
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<tr>
<td></td>
<td>2,3–dihydroxybenzoic acid</td>
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</tr>
<tr>
<td></td>
<td>Linear 2,3-dihydroxybenzoylserine</td>
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<td><strong>HYDROXAMATE TYPE</strong></td>
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<td>Ferrichrome (from fungi)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ferrichrosin (from fungi)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Coprogen (from fungi)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ferrioxamine B (e.g. from <em>Streptomyces pylosus</em>)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ferrioxamine E (e.g. from <em>Erwinia herbicola</em>)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ferrioxamine G (e.g. from <em>Hafnia alvei</em>)</td>
</tr>
<tr>
<td><strong>α- KETO AND α-HYDOXYACIDS</strong></td>
<td>Primary metabolites of <em>Salmonella</em> and other microorganisms, and of cells of host animals</td>
<td></td>
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</tbody>
</table>
gonorrhoeae fbpABC system (Anderson et al, 2004). The sitA gene encodes a putative periplasmic binding protein, sitB encodes an ATP-binding protein, and sitC and sitD encode two putative permeases. The locus is induced under iron-limited conditions and is regulated by MntR and Fur (Ikeda et al, 2005). The transport system encoded by sitABCD is an important transporter of both manganese and ferrous ions that are required for survival of S. enterica Typhimurium in macrophages in vitro (Boyer et al, 2002); it also appears to have a role in systemic infection of mice (Zaharik et al, 2004).

It is not clear what the primary source of iron is during infection despite extensive study of Salmonella uptake mechanisms in vitro. Mutations in single genes of iron uptake systems generally have a minimal effect on virulence or no effect at all, a result of the existence of several redundant systems for the uptake of this essential nutrient.

1.3.7 Ferrioxamine and Salmonella

Ferrioxamines are hydroxamate siderophores first isolated and characterised from Streptomyces species (Bickel et al, 1960) and are produced by a number of enterobacterial species (Matzanke et al, 1991; Reissbrodt et al, 1990) including Erwinia herbicola (ferrioxamine E) and Hafnia alvei (ferrioxamine G). A number of other members of the Enterobacteriaceae, although unable to produce ferrioxamines themselves, are able to utilise iron from these siderophores. E. coli uses FhuE, an outer membrane receptor for coprogen, to mediate low level ferrioxamine B transport (Sauer et al, 1990) and Yersinia enterocolitica uses a corresponding receptor FoxA to efficiently take up ferrioxamine B (Bäumler and Hankte, 1992).

The plant pathogen E. herbicola is never found in the vertebrate gut and H. alvei only rarely during infections. The fact that members of the Enterobacteriaceae can synthesise ferrioxamines does give rise to the possibility that gut commensals may export these siderophores into the intestinal contents where they could be utilised by
pathogenic bacteria. The early stages of salmonellosis occur in the intestine, where bacteria adhere to and invade host cells of mucosal epithelium. Iron is limited at the epithelium-lumen interface due to the presence of lactoferrin in mucus. A number of Salmonella serovars are able to acquire iron complexed with ferrioxamines B, E and G (Kingsley et al., 1999) and therefore a role for ferrioxamine-mediated iron transport in Salmonella pathogenesis is possible.

As previously discussed, a number of systems are involved in the transport of ferric-siderophores across the outer membrane, periplasmic space and inner membrane, and in release of the iron from the siderophore. The transport of ferrioxamines has been characterised in a number of organisms and found to require similar components. The transport of ferrioxamine B by Yersinia enterocolitica and Erwinia amylovora has been described and the genes encoding the outer membrane receptors designated foxA (Bäumler and Hantke, 1992) and foxR (Kachadoorian et al., 1996) respectively. S. enterica Typhimurium is able to utilise the closely related but distinct molecules B, E and G (Fig. 1.3) and although transport across the outer membrane is generally highly specific, all three siderophores have been found to use the receptor encoded by the Salmonella foxA gene (Kingsley et al., 1999). The product of this gene shows 45% identical amino acid sequence with Yersinia enterocolitica FoxA (Tsolis et al., 1996). The transport of ferrioxamines B and E is TonB independent, so that these siderophores, but not ferrioxamine G, are transported even in the absence of a functional TonB protein, albeit at lower efficiency than in the parental strains. However, all three ferrioxamines are dependent, like other hydroxamate siderophores, upon the FhuBCD inner membrane permease complex for transport.

Distribution of the foxA gene in the genus Salmonella is restricted to subspecies I, II and IIIb and absent from subspecies IIIa, IV and VI as well as S. bongori. Kingsley
**Fig. 1.3. Ferrioxamine molecular structures.** The diagram shows the base structure with the table showing the side groups which distinguish the different molecules (from Essen *et al.*, 2007).
et al (1999) observed that *S. enterica* Typhimurium *foxA* mutants are attenuated in mice inoculated by either the intravenous or intragastric route. Mice inoculated by the intragastric route with *foxA* mutant strain BK102 showed an LD$_{50}$ of $>10^9$ CFU compared to the LD$_{50}$ of $10^5$ CFU when inoculated with the parental strain. Furthermore, surviving mice inoculated with a dose of $5 \times 10^8$ of the parental strain all survived showing that the *foxA* mutant was protective. When the intravenous route was used to inoculate mice, a similar attenuation was seen with the mutant *foxA* strain. This attenuation cannot simply be explained by the inability of the mutant strains to utilise ferrioxamines as a source of iron, as no evidence currently exists for gut commensal secretion of ferrioxamines, nor is ferrioxamine present in the blood. Makki (2003) was able to clone the *foxA* gene and develop a complemented strain (CSL2MK) providing further information about the relationship between *foxA* and virulence. This study demonstrated that *in vitro* the *foxA* mutant was less able to kill dendritic cells compared to the parental and complemented strains. However, an *in vivo* study using a mouse model produced contrasting results to those of Kingsley *et al* (1999) with the mutant behaving similarly to the parental and complemented strains with respect to systemic infection and the death of mice.

Ferroxamine mediated iron uptake and the ferrioxamine receptor protein gene *foxA* appear to have a role in infection by *S. enterica* Typhimurium and potentially other serovars, although the exact nature of this role and its importance in virulence has yet to be fully determined.

1.4 Dendritic cells (DCs)

1.4.1 Origin and molecular biology of DCs

DCs with different phenotypes and functions have been identified in different tissues indicating the existence of heterogeneous populations of DCs. It appears that
DCs can be generated along distinct developmental pathways from different haematopoietic lineages (Young and Steinman, 1996). Progenitor cells are lineage restricted causing segregation of cells into lymphoid or myeloid lineages and it appears that DCs can be derived from either (Fig. 1.4).

1.4.2 Phenotypic characterisation

DCs reside in most tissues of the body and can be classified by their location. This method of classification, however, does not take into account the function, morphology or lineage of the DCs. DCs are CD45+ leukocytes that express high levels of MHC II molecules and with an absence of markers associated with other leukocyte lineages, CD3 (T cells), CD15 (neutrophils), CD14 (monocytes), CD20 (B cells). Levels of expression of other CD markers can be used to further define these lineage-negative (lin-) populations, but exactly pinpointing any subset is complicated by the fact that DCs change their phenotype in response to stimuli and so do not carry stable markers. DCs initially exhibit an immature phenotype with low expression of MHC class II molecules (Romani et al, 1989). These cells are poor stimulators of T cells but are very effective at the uptake and processing of antigens, resulting in their maturation. Maturation, which is signified by the high expression of MHC class II and CD80/CD86 on the surface, can be induced by infectious agents such as bacteria and viruses or their products LPS and lipoteichoic acid (Granucci et al, 1999). Mature DCs are capable of stimulating T cells to initiate a primary immune response (Mehta-Damani et al, 1994).

Conventional DCs that reside in secondary lymphoid organs can be identified based on a high expression of the integrin CD11c and MHC-II whereas DCs in peripheral tissues, including Langerhans cells and interstitial DCs, have a lower expression of CD11c (Kissenpfennig et al, 2005; Leon et al, 2007). DCs resident in the lymphoid organs of mice are often divided into three phenotypic subsets: CD8α+,
**Fig. 1.4. Model of DC haematopoiesis:** Haematopoietic stem cells (HSC) differentiate into multipotential progenitor cells (MP) which can give rise to megakaryocytes (Meg), erythrocytes (E), granulocytes (G), monocytes (M), lymphocytes (L) and cells of the DC system. Distinct molecular events signal developing haematopoietic cells to control formation of different DC populations (Lotze and Thomson, 2001).
CD8α-CD4+ and CD8α-CD4-. (Henri et al, 2001). In addition, a fourth subset lacking expression of CD8α, CD4 and CD11b has been described in the Peyer’s patches (PP) of the gut (Iwasaki et al, 2000).

DCs in the gut-associated lymphoid tissue have some unique characteristics including the induction of expression of α4β7 and CCR9, gut-homing receptors, on T cells allowing T effector cells to home to the gut (Mora et al, 2003). A subset of gut DCs expressing CD103 produces the vitamin A metabolite retinoic acid (Johansson-Lindbom et al, 2005; Iwata et al, 2004) which is required for gut tropism and is also involved in class switching to IgA of B cells (Mora et al, 2006).

1.4.3 DCs and Salmonella

DCs have an important role in the pathogenicity of Salmonella as evidence shows that S. enterica Typhimurium localises mainly in the DCs of the Peyer’s patches in primary infections of mice (Hohmann et al, 1978).

Recently two novel subpopulations of DCs have been identified in the intestine; CX3CR1+ and CCR6+ DCs (Salazar-Gonzalez et al, 2006). CX3CR1+ DCs are located in the lamina propria and subepithelial dome of Peyer’s patches and sample the antigen content of the gut lumen by extension of protrusions (Neiss et al, 2005). Mice deficient in CX3CR1 lack DC protrusions across epithelial barriers and are more susceptible to Salmonella infections. In response to infection, CCR6+ DCs migrate to the follicle-associated epithelium of the Peyer’s patches (Rescigno et al, 2006) where they phagocytose bacteria and present bacterial antigen to CD4+ T cells, initiating the adaptive immune response. These DCs appear to be vital in initiating anti-Salmonella-specific T cell responses. DCs therefore fulfil more than one role during the host defence against Salmonella (Grassl and Finlay, 2008).
*S. enterica* Typhimurium are targeted for internalisation by murine DCs as a result of type 1 fimbrial adhesion FimH interaction with DCs. However, *sipB* is not required as it is for invasion of mammalian epithelial cells (Guo *et al.*, 2007). The majority of *S. enterica* Typhimurium that invade cells reside in a membrane-bound compartment known as the *Salmonella* containing vacuole (SCV). The bacteria re-model this compartment using over 30 virulence effectors secreted into the host cell cytosol by TTSSs encoded by SPI1 or SPI2 (Bakowski *et al.*, 2008). The SPI1 encoded effector SopB is important during the early stages of infection and in the maturation of the SCV (Mallo *et al.*, 2008). SopB also activates myosin II, which is involved in the positioning of the SCV near to the cell nucleus, a process necessary for maximal growth in macrophages (WasylNka *et al.*, 2008). *Salmonella* with mutations in *sopB* exhibit a reduced cytotoxic effect on DCs compared to the parental strain (Link *et al.*, 2006) suggesting a role for SCV development during invasion of DCs. However, these SVCs show differences to those found in macrophages and other cells studied as Typhimurium reside in murine DCs in compartments lacking lysosomal membrane glycoproteins (LGPs) and do not require the PhoP/PhoQ response regulator for intracellular survival (Garcia-del Portillo *et al.*, 2000).

*S. enterica* Typhimurium within murine DCs survive within an SCV whose maturation is dependent on a functional SPI2 (Jantsch *et al.*, 2003). Entry and intracellular survival within DCs does not require macrophage-specific virulence factors (Niedergang *et al.*, 2000), suggesting that different survival mechanisms have evolved for different cell types. DCs also blunt acidification of phagosomes by Rab27a-dependent recruitment of NOX2, reducing antigen proteolysis allowing more efficient processing (Round and Mazmanian, 2009).

Internalisation of *Salmonella* causes DCs to begin synthesis of a number of
chemokines and cytokines required for the survival of the host cell. These include IL-12, which is crucial in inducing IFN-γ-mediated responses for the clearance of intracellular bacteria (Wick, 2003, Siegemund et al, 2009), and TNF-α, which appears to have a role in indirectly activating DC maturation during oral challenge of mice by S. enterica Typhimurium (Sundquist and Wick, 2005). Human DCs pulsed with live or heat inactivated S. enterica Typhi increase expression of CD80 and CD83 and production of IL-12 and IFNγ (Salerno-Goncalves and Sztein, 2009). Indeed the amplification of cytokine production following infection appears to be the cause of DC maturation resulting in the modification of cell surface markers. These include modifications required for antigen presentation and the activation of CD4+ T cells, such as the upregulation of the costimulatory molecules B7.2 and CD40 and the translocation of MHC molecules at the cell surface. Another result of Salmonella internalisation is an increase in the stability of both MHC class I and class II peptide complexes, although it has been reported (Lapaque et al, 2009) that S. enterica Typhimurium can effect expression of DC surface expression of HLA-DR by a post-translational polyubiquitination. Increasing the half-life of these MHC peptide complexes means that DCs have a greater chance of encountering antigen-specific T cells in the lymph nodes. However, systemic S. enterica Typhimurium alters production of FilC, an antigen recognised by CD4+ T cells by compartmentalising it within DCs; furthermore changes in gene expression limit DC maturation and cytokine production (Alaniz et al, 2006).

SPI2 encodes a TTSS, which translocates effector proteins that interfere with the ability of murine DCs to stimulate antigen-dependent T cell proliferation (Kuhle and Hensel, 2004). It has been proposed that this interference is a result of effector proteins altering the loading of antigen-derived peptides onto MHC II complexes. The proteins
identified as having a strong effect on T cell proliferation are SifA, SspH2, SlrP, SopD2 and PipB2, all of which are equally required, suggesting non-redundant roles of these effectors (Halici et al, 2008). However, the molecular mechanisms by which *Salmonella* interferes with DC functions have yet to be fully ascertained.

**1.4.4 DCs and the development of vaccines**

An important area of vaccine development is the method of antigen delivery to the immune system. Targeting of the antigen can affect the type and effectiveness of the immune response, local or systemic, cellular or humoral, T<sub>H1</sub> or T<sub>H2</sub>. The immunomodulatory function of DCs has prompted many researchers to work on utilising these cells for the development of prophylactic vaccines as well as using them in therapeutic vaccines.

A great number of studies have concentrated on the development of DC therapies against neoplastic diseases. It has been shown that the immune system is capable of recognising and rejecting autologous tumour cells (Krikorian et al, 1980; Ralfkiaer et al, 1987; Ruiter, 1982); however, this natural defence is ineffective in many cases. Therefore, the goal of DC based immunotherapy of cancer is the delivery of tumour associated antigens (TAA) into DC populations in such a way as to promote effective induction of cytotoxic T lymphocyte responses against tumour cells bearing the target antigen(s). Such responses have been generated *in vitro* with DCs loaded with; prostrate cancer TAAs including the prostate cancer-associated protein trp-p8 (Kiessling *et al*, 2003) and PMSA (Horiguchi *et al*, 2002); cervical cancer TAAs HPV16E7 and HPV18E7 (Nonn *et al*, 2003); and the breast cancer TAA HER2/neu (Roses *et al*, 2007).

Immunisation against bacterial diseases has also been investigated with respect to DCs. Immunization of mice with DCs loaded with α-galactosyl ceramide alongside
listeriolysin (the main cytotoxic T lymphocyte epitope of *Listeria monocytogenes*) elicited specific cytotoxic T lymphocyte activity and protected against infection by the bacteria (Enomoto et al, 2007). Treatment of mice with Jaws II cells (murine DC line) pulsed with whole cell sonicates of *Helicobacter pylori* resulted in a 2 log reduction in the number of colonising bacteria. With respect to *Salmonella*, similar studies have yet to be carried out; however, *S. enterica* Typhimurium has been used in the development of anti-tumour vaccines by the pulsing of DCs with this bacterium in combination with heat shock proteins from 4T1 tumours. DCs primed with this proinflammatory bacterial stimulus and tumour-associated antigens induced a protective anti-tumour immune response in a murine model (Shilling et al, 2007).

1.6 Aims of this Project

To further our understanding of the genes involved in iron uptake systems during human infection, recovery of these strains from cultured human DCs was investigated. Ferric-ferrioaxmine uptake is dependent on the outer membrane protein encoded by *foxA* and although ferrioaxamines are unlikely to be present in a mammalian host previous experiments (Kingsley et al, 1999) have shown *S. enterica* Typhimurium *foxA* mutants to be attenuated in the mouse model. Similarly, a strain with mutations in genes encoding the outer membrane proteins, FepA, IroN and Cir also showed attenuation (Rabsch et al, 2003). The effects on virulence of mutations in genes involved in salmochelin production (*iroB*) and transport (*iroC*) were also investigated.

The following chapters describe in detail the production and classification of cultured DCs from human peripheral blood (chapter 3) and attempts to clone the *foxA* gene and produce a construct capable of complementing the *foxA*– strain SL2MK (chapter 4). Gentamicin killing assays involving the co-culturing of DCs with *S. enterica* Typhimurium strains are described in chapter 4; these aimed to assess the
involvement of the *foxA, fepA, iroN, iroBC* and *iroBC* genes in internalisation and possible subsequent killing of these cells.

These studies showed that the *foxA* mutant was recovered from DCs in higher numbers than the wild type parental strain or the complemented strains, suggesting an attenuated ability to kill or harm DCs. Furthermore, the inability to transport iron complexed with enterobactin or salmochelin or to produce salmochelin had no significant effect on recovery of strains from DCs. However, the inability to transport iron complexed with 2, 3-DHBS results in lower numbers being initially recovered from DCs compared to the wild type parental strain, but after continued exposure this inability resulted in recovery of higher numbers than the parental strain.

In chapter 5 a number of techniques are described that had the aim of confirming the cause of the differences in number of bacteria recovered from DCs in chapter 4. Attempts were made to transfer GFP containing plasmids into the strains; however, only the *foxA* mutant could be transformed but this did allow for comparison of this strain to its parental strain. Results showed that the increased recovery of *S. enterica* Typhimurium from DCs as a result of the mutation in *foxA* cannot be directly correlated to an increase in numbers of bacteria entering DCs. Finally, also in chapter 5, the effect of the mutations on maturation of DCs was investigated. The maturation of DCs exposed to the mutants and wild type strains were compared using the analysis of CD marker expression by flow cytometry. These results showed that exposure to *S. enterica* Typhimurium causes maturation of DCs and that the mutations in *foxA, fepA, iroBC, cir* and *iroN* have no significant effect on this maturation.
CHAPTER TWO

Materials and Methods

2.1. Bacterial Strains

2.1.1 *Salmonella enterica* Typhimurium

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<tr>
<th>Designation</th>
<th>Relevant Characteristics</th>
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<td>SL1344/nr</td>
<td>SL1344, Nal$^f$</td>
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<td>CSL2MK</td>
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### 2.1.2 *Escherichia coli*

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<td>XL1 Blue</td>
<td>endA1 gyrA96(nalR) thi-1 recA1 relA1 lac glnV44 F' [::Tn10 proAB+ lacIq Δ(lacZ)M15] hsdR17(rK- mK+), Nalr, TetR</td>
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### 2.2 Plasmid vectors and recombinant plasmids

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<thead>
<tr>
<th>Plasmid</th>
<th>Characteristics</th>
<th>Source/Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pWSRM</td>
<td>pWKS30, foxA+</td>
<td>Makki, 2003</td>
</tr>
<tr>
<td>pRHX2</td>
<td>pWKS30, foxA+</td>
<td>This study</td>
</tr>
<tr>
<td>pGFP</td>
<td>pUC ori, lacZ</td>
<td>Wang and Hazelrigg, 1994</td>
</tr>
<tr>
<td>pTR1-ompE</td>
<td>PTRI-pMEK91-5 DHa5E, ompE, lacZ</td>
<td>Rao, 2005 (unpublished work)</td>
</tr>
</tbody>
</table>
Fig. 2.1. Plasmid maps of pGFP (Map 1) and pTR1 + EcoR1 of pMEK (Map 2). *S. enterica* Typhimurium SL1344 pGFP was kindly provided by P. Everest (Glasgow Veterinary School) and *E. coli* JM109 pTR1-ompE plasmid expressing GFP was a gift from T. Rao (University of Leicester).
2.3 Media

Luria Bertani broth (LB) consisted of 1% tryptone (Oxoid), 0.5% yeast extract (Oxoid), and 0.5% NaCl, pH 7.0.

Luria Bertani agar (LUA) consisted of LB with 1.5% agar (Difco).

Nutrient broth (NB) and nutrient agar (NA) consisted of 25 g of nutrient broth No.2 powder (Oxoid) added to 1 litre of d.H2O. 1% agar No.1 was added to this base mixture prior to autoclaving to produce solid medium.

SOC medium consisted of 20 g of bacto-tryptone, 5 g bacto-yeast and 0.5 g of NaCl added to 980 ml of d.H2O. This was mixed and then autoclaved before cooling to room temperature and 20 ml of sterile 1 M glucose added.

Egg white agar medium (EWA): 2 × nutrient agar was prepared by adding the following to 100 ml of d.H2O: 2.56 g of nutrient broth (Difco), 0.6 g of yeast extract (Oxoid), 1.8 g of Na2HPO4.12H2O and 2.2 g of agar (Oxoid). This was then autoclaved and cooled in a water bath to 50°C. Egg white was separated from the yolk of fresh hens’ eggs aseptically and liquefied by addition of 0.71 M NaHCO3 and thorough mixing. For each plate, 9 ml of egg white at 37°C was mixed with 11 ml of 2 × nutrient agar.

Minimal Medium: 400 ml of M9CA salts were prepared as per the table below. The solution was then adjusted to pH 7.4 and autoclaved.

<table>
<thead>
<tr>
<th>Quantity</th>
<th>Chemical formula</th>
<th>Chemical name</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.4 g</td>
<td>Na2HPO4.2H2O</td>
<td>Di-sodium hydrogen orthophosphate dihydrate</td>
</tr>
<tr>
<td>1.2 g</td>
<td>KH2PO4</td>
<td>Potassium dihydrogen orthophosphate</td>
</tr>
<tr>
<td>0.2 g</td>
<td>NaCl</td>
<td>Sodium chloride</td>
</tr>
<tr>
<td>0.4 g</td>
<td>NH4Cl</td>
<td>Ammonium chloride</td>
</tr>
<tr>
<td>to 400 ml</td>
<td>dH2O</td>
<td>Distilled water</td>
</tr>
</tbody>
</table>
All the following were filtered (with the exception of glycerol) and added to the M9CA salts to produce minimal medium.

<table>
<thead>
<tr>
<th>Quantity</th>
<th>Chemical formula</th>
<th>Chemical name</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 mM</td>
<td>MgSO$_4$</td>
<td>Magnesium sulphate</td>
</tr>
<tr>
<td>0.2 M</td>
<td>C$<em>6$H$</em>{12}$O$_6$</td>
<td>Glucose</td>
</tr>
<tr>
<td>0.1 mM</td>
<td>CaCl$_2$</td>
<td>Calcium chloride</td>
</tr>
<tr>
<td>1 mM</td>
<td>C$<em>{12}$H$</em>{17}$N$_4$O$_5$</td>
<td>Thiamine</td>
</tr>
<tr>
<td>8 mM</td>
<td>C$_3$H$_8$O$_3$</td>
<td>Glycerol</td>
</tr>
<tr>
<td>0.8 g</td>
<td></td>
<td>Casamino acids</td>
</tr>
</tbody>
</table>

2.4 Medium supplements

<table>
<thead>
<tr>
<th>Supplement</th>
<th>Solvent</th>
<th>Stock concentration</th>
<th>Final concentration in medium</th>
<th>Storage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ampicillin</td>
<td>d.H$_2$O</td>
<td>100 mgm$^{-1}$</td>
<td>100 μgm$^{-1}$</td>
<td>4°C</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>d.H$_2$O</td>
<td>50 mgm$^{-1}$</td>
<td>50 μgm$^{-1}$</td>
<td>4°C</td>
</tr>
<tr>
<td>Kanamycin</td>
<td>d.H$_2$O</td>
<td>50 mgm$^{-1}$</td>
<td>25 μgm$^{-1}$</td>
<td>4°C</td>
</tr>
<tr>
<td>Nalidixic Acid</td>
<td>d.H$_2$O</td>
<td>50 mgm$^{-1}$</td>
<td>50 μgm$^{-1}$</td>
<td>4°C</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>50% d.H$_2$O: 50% EtOH (v/v)</td>
<td>12.5 mgm$^{-1}$</td>
<td>20 μgm$^{-1}$</td>
<td>4°C</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>d.H$_2$O</td>
<td>50 mgm$^{-1}$</td>
<td>150 μgm$^{-1}$</td>
<td>4°C</td>
</tr>
<tr>
<td>α,α´-dipyridyl</td>
<td>d.H$_2$O</td>
<td>25 mM</td>
<td>Variable</td>
<td>RT</td>
</tr>
<tr>
<td>X-GAL</td>
<td>DMSO</td>
<td>20 mgm$^{-1}$</td>
<td>25 μgm$^{-1}$</td>
<td>4°C</td>
</tr>
<tr>
<td>IPTG</td>
<td>d.H$_2$O</td>
<td>20 mgm$^{-1}$</td>
<td>25 μgm$^{-1}$</td>
<td>-20°C</td>
</tr>
</tbody>
</table>
2.5 Culture of bacterial strains

Bacterial strains were cultured overnight at 37°C with shaking in LB or overnight at 37°C on LUA plates. Where appropriate, antibiotics were added to media at concentrations indicated in Section 2.4.

2.6 Storage of bacterial strains

Overnight cultures of bacteria grown at 37°C with shaking in LB were mixed 1:1 with a solution of 50% glycerol and stored frozen at –80°C. Strains were recovered by scraping a small amount of glycerol stock onto a sterile loop and streaked onto LUA containing appropriate antibiotics.

2.7 Miscellaneous buffers and solutions

20 × phosphate buffered saline was prepared from NaCl (2.74 M), KCl (0.54 M), KH2PO4 (0.03 M) and Na2HPO4.12H2O, adjusted to pH7.4 with 2 M HCl before autoclaving.

40 × TAE buffer was prepared from Tris (1.6 M) and EDTA (40 mM) with pH adjusted to 7.7 using glacial acetic acid.

10 × MOPS buffer was prepared from 41.8 g of 3-\{N-morpholino\} propanesulphonic acid (MOPS), 6.8 g of sodium acetate, 20 ml of 0.5 M EDTA dissolved in 1,000 ml of H2O and stored in the dark at 4°C.

2.8 Agarose gels

Agarose gels were prepared by melting 0.8 to 1.5g agarose in 100 ml of 1 × TAE buffer; when cooled to 50°C, 500 ngml⁻¹ of ethidium bromide were added.

2.9 Tissue culture media and supplements

RPMI 1640 (Gibco): without phenol red and without GlutaMAX™.

Lymphoprep (Axis-Shield): sodium diatrizoate based density gradient medium.
FBS (Sigma-Aldrich): foetal bovine serum, heat inactivated, sterile-filtered, cell culture tested.

D-PBS (Sigma-Aldrich): Dulbecco’s phosphate buffered saline, without calcium or magnesium.

HBSS (Sigma Aldrich): Hank’s balanced salt solution, without calcium or magnesium.

<table>
<thead>
<tr>
<th>Supplement</th>
<th>Stock concentration</th>
<th>Final concentration in medium</th>
<th>Storage</th>
</tr>
</thead>
<tbody>
<tr>
<td>GlutaMAX™</td>
<td>200 mM</td>
<td>1%</td>
<td>-20°C</td>
</tr>
<tr>
<td>Heparin ammonium salt from porcine mucosa</td>
<td>140 units/mg</td>
<td>1 uml⁻¹</td>
<td>4°C</td>
</tr>
<tr>
<td>Bovine serum albumin (BSA)</td>
<td>10 mg/ml⁻¹</td>
<td>0.1 mg/ml⁻¹</td>
<td>-20°C</td>
</tr>
<tr>
<td>Etoposide</td>
<td>DMSO: 30 mg/ml⁻¹</td>
<td>50 µg/ml⁻¹</td>
<td>-20°C</td>
</tr>
</tbody>
</table>

2.10 Cytokines and cell stimulating agents

The following cell stimulating agents were used in this study:

GM-CSF (granulocyte/macrophage colony stimulating factor) at a concentration of 100 ng/ml⁻¹ (normal) or 25 ng/ml⁻¹ (reduced).

IL-4 (interleukin-4) at a concentration of 50 ng/ml⁻¹ (normal) or 5 ng/ml⁻¹ (reduced).

M-CSF (macrophage colony stimulating factor) at a concentration of 25 ng/ml⁻¹.

LPS from E. coli serotype O55:B5 at a concentration of 1 µg/ml⁻¹.

2.11 Antibodies

The following FITC-conjugated mouse anti-human monoclonal antibodies were used in this study:

IgG1 (clone 11711), IgG2a (clone 20102), CD3 (clone UCHT-1, isotype IgG1), CD14 (clone 134620, isotype IgG1), CD1a (clone HI149, isotype IgG1), CD16 (clone 3G8,
isotype IgG1), CD19 (clone 4G7-2E3, isotype IgG1), CD80 (clone 37711, isotype IgG1), CD86 (clone 37301, isotype IgG1), HLA-DR (clone L243, isotype IgG2a) and HLA-DQ (clone TU169, isotype IgG2a).

Table 2.1. CD antigens used in this study to investigate DC differentiation and response to bacterial infection.

<table>
<thead>
<tr>
<th>Marker</th>
<th>Other names</th>
<th>Cellular expression</th>
<th>Function</th>
<th>Expression on DC</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD1a</td>
<td>Leu6</td>
<td>Thymocytes, Monocytes, B cells, DCs</td>
<td>Antigen presenting cell molecule important in presentation of glycolipids and lipopeptide antigens</td>
<td>++</td>
</tr>
<tr>
<td>CD3</td>
<td>OKT3</td>
<td>Thymocytes, Peripheral T cells</td>
<td>Required for TCR cell surface expression and signal transduction</td>
<td>-</td>
</tr>
<tr>
<td>CD14</td>
<td>LPS receptor</td>
<td>Monocytes, Macrophages, B cells</td>
<td>Pattern recognition receptor</td>
<td>+</td>
</tr>
<tr>
<td>CD16</td>
<td>Fc receptors (FcγRIIIa and FcγRIIIb)</td>
<td>NK cells, monocytes and macrophages</td>
<td>Bind to the Fc portion of IgG antibodies.</td>
<td>-</td>
</tr>
<tr>
<td>CD19</td>
<td>-</td>
<td>Pre B cells, B cells</td>
<td>Regulates B cell development, activation and differentiation</td>
<td>-</td>
</tr>
<tr>
<td>CD80</td>
<td>B7-1</td>
<td>Activated B cells, T cells, MΦ and DC</td>
<td>APC costimulatory molecule</td>
<td>+++</td>
</tr>
<tr>
<td>CD86</td>
<td>B7-2</td>
<td>Memory B cells, Activated T cells, DCs</td>
<td>APC costimulatory molecule</td>
<td>+++</td>
</tr>
<tr>
<td>HLA-DR</td>
<td>-</td>
<td>APC</td>
<td>αβ heterodimer of the MHC Class II type</td>
<td>+++</td>
</tr>
<tr>
<td>HLA-DQ</td>
<td>-</td>
<td>APC</td>
<td>αβ heterodimer of the MHC Class II type</td>
<td>+++</td>
</tr>
</tbody>
</table>

- = no detectable expression  
+ = low expression  
++ = medium expression  
+++ = high expression
2.12 DC investigations

2.12.1 DC precursor enrichment and differentiation of adherent cells from peripheral blood monocytes

100 ml of heparinised human peripheral blood were diluted 1:1 with HBSS (without Ca^{2+} or Mg^{2+}). Diluted blood was layered onto Lymphoprep® and centrifuged at 22°C, 800 × g for 30 min. Cells at the interface between Lymphoprep and medium were removed and washed with RPMI 1640 (10% FCS, 1% glutamine) and centrifuged for 10 min at 1200 × g. Cells were resuspended at 10⁵ cells per ml in RPMI (10% FCS, 1% GlutaMAX™). 2 ml of cell suspension per well were distributed into each well of 12 well plates and incubated at 37°C, 5% CO₂, 95% humidity for 2 hours.

After incubation, non adherent cells were discarded and the wells washed. Adherent cells were washed and 2 ml of RPMI (10% FCS, 1% glutamine) containing GM-CSF (100 ng/ml) and IL-4 (50 ng/ml) were added to each well. Plates were then incubated at 37°C, 5% CO₂, 95% humidity for 7 days. Every two days, 500 μl of the supernatant were discarded from each well and replaced with 500 μl of fresh RPMI (10% FCS, 1% glutamine) containing GM-CSF (100 ng/ml) and IL-4 (50 ng/ml). After the 7 day incubation period, cells were washed with HBSS and dissociated from the bottom of the wells using Cell Dissociation Fluid (Sigma). Cells were washed and resuspended in RPMI (without phenol red, containing 10% FCS and 1% GlutaMAX™).

2.12.2 Antibody staining of DC surface markers

DCs were resuspended in RPMI (without phenol red, containing 10% FCS and 1% GlutaMAX™) at 10⁶ cells per ml. 100 μl of the cell suspension were then placed into each of the wells of a 96 well plate and centrifuged for 4 min at 1200 × g. The wells were washed with PBS, centrifuged and the supernatant discarded to produce dry pellets. Pellets were resuspended in 20 μl of the FITC labelled antibodies per well.
Plates were incubated in the dark for 30 min at 4°C, centrifuged and the supernatant discarded. Cells were washed in PBS and then:

EITHER: Resuspended in 100 µl of PBS for immediate analysis by flow cytometry.

OR: fixed with 3% formaldehyde at 4°C for 20 min. Cells were then washed and resuspended 100 µl of PBS and stored at 4°C until ready for analysis.

Cells were stained as described above using FITC (fluorescein isothiocyanate) -labelled mouse anti-human monoclonal antibodies against the following cell surface molecules: CD1a CD80, CD86, HLA-DR and HLA-DQ. CD14 was used to indicate a macrophage type phenotype, and CD19 and CD3 to indicate the level of contaminating B and T cells respectively. IgG1 and IgG2a were used as negative controls.

2.12.3 Maturation of DCs upon exposure to LPS

After 7 days of culture, LPS from E. coli (serotype O55:B5) was added to DCs at a concentration of 1 µgml⁻¹ and cells cultured for 3 days at 37°C, 5% CO₂, 95% humidity. The phenotype of the cells was then determined by flow cytometry using the antibodies listed in Section 2.11.

2.12.4 Differentiation to DC with reduced levels of cytokines

Adherent cells from the same donor, prepared by the 2 hour adherence method, were differentiated using two separate concentrations of cytokines.

1. Standard concentrations – GM-CSF at 100 µgml⁻¹ and IL-4 at 50 µgml⁻¹ (DCs)
2. Reduced concentrations - GM-CSF at 25 µgml⁻¹ and IL-4 at 5 µgml⁻¹ (DClow)

To compare phenotypes the cells produced were then stained using mouse anti-human monoclonal antibodies as described in section 2.11. Cells produced at both concentrations of cytokines were also exposed to LPS using the method described in Section 2.12.3.
2.12.5 Phenotypic analysis of DC<sub>low</sub>, adherent cells and macrophages

Three different cell populations were prepared, DC<sub>low</sub> cells produced using reduced concentrations of GM-CSF and IL-4, adherent cells cultured without cytokines and macrophages cultured in the presence of macrophage colony stimulating factor (M-CSF) at a concentration of 25 ng/ml<sup>-1</sup>. The phenotypes of the cells were then determined by flow cytometry using the antibodies listed in Section 2.12 along with FITC labelled antibody specific for CD16.

2.13 DNA manipulations

2.13.1 Preparation of plasmid DNA

Plasmid preparations were carried out using QIAprep kits. Briefly, a 5 ml overnight culture of <i>S. enterica</i> Typhimurium harbouring the required plasmid was grown in LB containing suitable antibiotics. Cells were harvested by centrifugation at 800 × g for 10 min at 4ºC, the supernatant was discarded and the pellet was resuspended in 250 µl of P1 buffer containing RNase. 250 µl of P2 buffer were added and the tube was gently inverted to lyse the cells. 250 µl of N3 buffer were added and the tubes inverted to precipitate proteins, cell debris and high molecular weight DNA. The tube was centrifuged for 10 min at 10,000 × g to produce a dense white pellet. The pellet was discarded and the supernatant was removed and loaded onto a QIAprep column. The column was centrifuged for 1 min and the flow through discarded before 500 µl of PB buffer were added to the column, which was centrifuged for 1 min and the flow through discarded. 750 µl of PE buffer were added and the column centrifuged for 1 min; the flow through was discarded and the column was centrifuged again for 1 min to remove residual PE. The column was placed into a 1.5 ml microcentrifuge tube to elute the DNA; 50 µl of EB buffer were added to the centre of the column, left to stand for 1
min and then centrifuged for 1 min. 10 µl of the resulting solution was then run on an agarose gel.

2.13.2 Agarose Gel Electrophoresis

6 × Orange G loading buffer was added to DNA samples and samples were loaded onto agarose gels. Gels were submerged in 1 × TAE buffer and electrophoresis was carried out at constant voltage of 5 V per cm.

2.13.3 Isolation of DNA from agarose gels

To recover DNA from agarose gels a GENECLEAN kit was used. Gels were run using 1 × TAE and stained with ethidium bromide before being viewed using a UV transilluminator. A sterile razor blade was used to excise the required band. The agarose fragment was weighed and 3 × volume of NaI was added before being incubated for 5-10 min at 55°C until dissolved. The glass milk suspension was vortexed until it was resuspended and then 5 µl were added to the dissolved agarose solution. The tube was mixed, inverted and vortexed, and placed on ice for 5 min, mixing every 2 min. The tube was then spun for 5 sec in a microfuge before the supernatant was removed. The pellet was washed with cold NEW wash (50% ethanol in 20mM Tris Cl (pH 7.6 or 8.0), 1mM EDTA and 0.1M NaCl) three times before the supernatant was again removed. The pellet was air dried for 15 min and then 12 µl of TE were added. This solution was incubated at 55°C for 3 min before it was centrifuged for 30 sec and the supernatant, containing the DNA, removed into a clean tube.

2.13.4 Restriction digests

Plasmid DNA was digested in a reaction mixture comprising 2 µl of 10 × restriction buffer, 2 µl of BSA, 1 µl of eluted plasmid DNA, 0.5 µl of restriction enzyme(s) and sterile H₂O to make up the volume to 20 µl. The contents were mixed, briefly vortexed and then incubated at 37°C for 1-4 hours. 5 µl of the digest were
mixed with 5 µl of loading dye and run on a 1 × TAE gel alongside a 10 kb marker ladder and undigested plasmid.

2.13.5 Isolation of chromosomal DNA from bacteria

Bacterial chromosomal DNA was prepared using an adaptation of the procedure described by Chen and Kuo (1993). 1.5 ml of an overnight bacterial culture was centrifuged at 10,000 × g for 5 min and the supernatant discarded. The pellet was resuspended by vigorous pipetting in 200 ml of lysis buffer comprising 40 mM Tris-acetate, pH 7.8, 20 mM sodium acetate, 0.1 mM EDTA and 1% (w/v) SDS. Cell debris and cell proteins were precipitated by the addition of 0.3 M NaCl, which on mixing produced a viscous suspension. This mixture was centrifuged for 10 min at 10,000 × g (4°C) and the supernatant removed to a fresh tube. An equal volume of phenol/chloroform equilibrated with Tris-HCl pH 8.0 was added and the mixture was gently inverted to produce a milky solution. The layers were separated by centrifugation for 3 min at 10,000 × g and the aqueous fraction transferred to a fresh tube and similarly extracted with chloroform:isoamyl alcohol (24:1). DNA was precipitated with 95% ethanol and washed twice in 70% ethanol, vacuum dried and dissolved in 20 µl of sterile water.

2.13.6 Isolation of the S. enterica Typhimurium foxA gene using polymerase chain reaction (PCR)

Primers were designed for short PCR, with the forward primer located within the foxA gene producing a fragment of 540 bp. Full length PCR produced the entire foxA gene in a 2.4 kb fragment and was used to clone foxA from the bacterial chromosome and to confirm the presence of the foxA clone within plasmid DNA.
Table 2.2. Primers used in PCR

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
<th>Restriction site</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forward 1 (F1)</td>
<td>AACGGGCCC CGCCAGAG</td>
<td>ApaI</td>
</tr>
<tr>
<td>Forward 3 (F3)</td>
<td>TAATCAGACCAATATCGCGACC</td>
<td>N/A</td>
</tr>
<tr>
<td>Reverse 2 (R2)</td>
<td>AGCGAATTCAAACGCCCATGAC</td>
<td>EcoRI</td>
</tr>
</tbody>
</table>

The optimum conditions for the PCR reactions were determined by testing the two pairs of primers in various combinations of the following factors:

- 5 and 7 min extension time
- 50 and 55°C annealing temperatures
- 30 and 35 cycles
- 4, 6 and 8 mM MgCl₂.

**Fig. 2.2. PCR conditions** – both types of PCR used 6 mM MgCl₂:

<table>
<thead>
<tr>
<th>foxA PCR from chromosomal DNA (F1 and R2) – full length</th>
<th>foxA PCR from plasmid DNA (F3 and R2) - short</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Incubate at 95°C for 5 minutes</td>
<td>1. Incubate at 94°C for 5 minutes</td>
</tr>
<tr>
<td>2. Incubate at 95°C for 30 seconds</td>
<td>2. Incubate at 94°C for 30 seconds</td>
</tr>
<tr>
<td>3. Incubate at 50°C for 30 seconds</td>
<td>3. Incubate at 50°C for 30 seconds</td>
</tr>
<tr>
<td>4. Incubate at 72°C for 7 minutes</td>
<td>4. Incubate at 72°C for 2 minutes</td>
</tr>
</tbody>
</table>

A 50 µl master mix (enough for four 10 µl reactions) contained the following components; the template was either chromosomal DNA (from SL1344/nr) or cloned foxA plasmid DNA from bacterial transformants (to confirm presence of the foxA insert).
<table>
<thead>
<tr>
<th>Template</th>
<th>F1/ F3</th>
<th>R2</th>
<th>10 x reaction buffer</th>
<th>dNTP</th>
<th>MgCl₂</th>
<th>H₂O</th>
<th>Taq polymerase</th>
</tr>
</thead>
<tbody>
<tr>
<td>20 µg/ml⁻¹</td>
<td>1 µM</td>
<td>1 µM</td>
<td>1 x</td>
<td>200 µM</td>
<td>6 mM</td>
<td>up to 50 µl</td>
<td>25 U/ml</td>
</tr>
</tbody>
</table>

2.13.7 Purification of PCR products using QIAGEN PCR clean-up kit

PCR products were purified using the commercially available QIAGEN PCR clean-up kit. The method supplied with the kit was used; briefly 200 µl of the sample were mixed with 1 ml of Buffer PB, transferred to a spin column and centrifuged at maximum speed for 1 min. The flow through was discarded. 600 µl of Buffer PE were added, the column was re-spun and the flow through was again discarded. The column was centrifuged again to ensure the removal of any residual buffer before the DNA was eluted with 50 µl of water into a fresh tube.

2.13.8 Ligation reactions

The plasmids and PCR products were purified as described and cut with restriction enzymes (section 2.13.4). Following digestion, the samples were purified before ligation reactions were carried out. 3 µg/ml⁻¹ of insert (or water for the control), 1 µg/ml⁻¹ of vector, 2 µl of 10 x ligase buffer (50 mM Tris-HCl, 10 mM MgCl₂, 1 mM ATP, 10 mM dithiothreitol, pH 7.5 at 25°C) and 0.1 units of ligase and distilled water to a total volume of 20 µl, were mixed and incubated at room temperature overnight.

2.13.9 Precipitation of ligation mix for electroporation

One tenth volume of 3 M sodium acetate, 2.5 volumes of ethanol and 10 ng/µl⁻¹ of yeast carrier tRNA were added to the overnight ligation incubation. This solution was mixed by vortexing, left on ice for 10 min and then centrifuged for 10 min. The
pellet was washed with 400 µl of 70% ethanol, dried under vacuum and re-dissolved in 10 µl of distilled water.

2.13.10 Preparation of *E. coli* and *S. enterica* Typhimurium cells for electroporation

100 ml of LB were inoculated with 1 ml of an overnight culture of the required bacterial strain and incubated at 37°C on a shaker at 150 rpm for two hours until the optical density at 600 nm was approximately 0.6. This culture was then centrifuged at 1500 × g for 10 min and the supernatant discarded. The cells were washed 6-8 times with an ice-cold solution of 1x 3-N-morpholinopropanesulfonic acid (MOPS) buffer using 10 min centrifugations at 800 × g to remove any salt and then resuspended in 200 µl of MOPS and stored on ice.

2.13.11 Transformation of *E. coli* and *S. enterica* Typhimurium by electroporation

50 µl of electrocompetent *E. coli* cells were mixed with 5 ng of precipitated ligation mix or plasmid and transferred into electroporation cuvettes. The cells were electroporated at 1.5 kV, with a resistance of 1000 Ω and a capacitance of 25 µF. *E. coli* were recovered by immediately resuspending the bacteria in 1 ml of SOC medium, followed by incubation at 37°C for 45 min. 100 µl samples were spread onto agar plates (20 ml of LA, 100 µgml⁻¹ of ampicillin, 25 µgml⁻¹ of XGAL and 25 µgml⁻¹ of IPTG) and incubated at 37°C overnight. Recombinants were identified by blue/white selection. 40 µl of competent *S. enterica* Typhimurium cells were mixed with 5-10 ng of plasmid DNA prepared using a QIAprep Miniprep kit and electroporated using settings of 2.5 kV, 200 Ω and 25 µF. Electroporated cells were allowed to recover by immediate suspension in 1 ml of LB at 37°C, and incubated for 1 hour at 37°C. Cell suspensions were then plated onto selective media and incubated at 37°C overnight.
2.13.12 Post-transformation selection

Plasmid DNA from the transformants was digested using the enzyme HindIII using the method described in Section 2.13.5. This restriction site would only be present in pWSK30 if there was no insert present and therefore should enrich for clones. DNA from the digest was purified by mini-prep procedure before being transformed into DH5α by electroporation.

2.13.13 Chemical transformation

A starter culture of the required *E. coli* strain was prepared as previously described. The cells were harvested and resuspended in 1 ml of ice-cold 100 mM MgCl$_2$ and immediately centrifuged. They were then resuspended in 1 ml of ice-cold 100 mM CaCl$_2$ and put on ice for 30 minutes. The solution was centrifuged, the supernatant discarded and the competent cells resuspended in 500 µl of 100 mM CaCl$_2$. 10 ng of DNA was added to 100 µl of competent cells and put on ice for 5 minutes. The samples were subjected to a 90 second heat shock (37°C) and then immediately recovered with 1 ml SOC medium and incubated at 37°C for 45 minutes before being plated with appropriate antibiotics.

2.13.14 Ferrioxamine diffusion plate bioassay

Diffusion plate bioassays on egg white medium were used to assess growth stimulation of enterobactin producing strains. Egg albumin imposes iron limitation upon a medium due to the presence of ovotransferrin; it also inhibits the scavenging of iron by enterobactin allowing observation of growth around a disc loaded with other specific siderophores. 2-3 colonies of the test strain, grown overnight in LB containing appropriate antibiotics, were resuspended in 100 µl of d.H$_2$O containing α,α´-dipyridyl at a concentration of 200 µM. This inoculum was then added to the egg white agar which was equilibrated to 50°C and mixed very gently by inversion. The seeded agar
was then poured into Petri dishes and allowed to set before being inverted in an incubator at 37°C to dry the plates. 6 mm filter paper discs were soaked in a solution of ferrioxamine B (1 µg/disc), dried at room temperature and placed on the centre of the seeded plates. The plates were incubated at 37°C overnight and then inspected for the presence of growth, indicated by a halo around the filter paper discs.

2.14 *Salmonella enterica* Typhimurium growth curves

Overnight cultures were prepared for each bacterial strain in LB containing necessary antibiotics. 10 µl of each culture were transferred to either 40 ml of LB or 40 ml of minimal medium. Cultures were incubated at 37°C with 100 µl samples being removed at regular time points for the next 24 hours. Samples were diluted 10 fold to 10^6 cells per ml (10^9 for samples taken after 12 hours). These were plated out onto selective media, incubated at 37°C overnight. Colonies were then counted.

2.15 DCs and bacterial interactions

2.15.1 Gentamicin assays

Overnight cultures of bacteria were washed with PBS and 2 × 10^6 cells per ml were resuspended in RPMI containing 150 µgml⁻¹ of gentamicin. At 20 min time points for the next 3 hours samples were taken and plated on selective media. After overnight incubation at 37°C colonies were counted and used to determine the effectiveness of the gentamicin treatment.

2.15.2 Exposure of DCs to *Salmonella enterica* Typhimurium strains (gentamicin killing assays)

DCs were counted, washed and resuspended in PBS at concentration of 10^5 cells per well of a 96 well plate. Overnight cultures of bacteria were diluted 50 fold in LB and grown at 37°C to an OD_{600} of 0.8, which approximates to 10^9 bacteria per ml. Bacteria were washed and resuspended in PBS (~10^9 bacteria per ml). 100 µl of
bacterial suspensions were added to wells containing DCs (10^5 per ml) and the plates were centrifuged for 4 min at 600 × g. Plates were incubated for 2 hours or 24 hours at 37°C, 5% CO₂ and 95% humidity and then centrifuged at 800 × g for 4 min. The supernatant was discarded and the cells washed in 200 μl of PBS. DCs were resuspended in PBS containing 150 μgml⁻¹ of gentamicin (freshly prepared) and incubated for 2 hours at 37°C, 5% CO₂ and 95% humidity. The DCs were washed and lysed in 100 μl of 0.05% Triton X-100 and then incubated at room temperature for 15 min. The lysates were centrifuged and washed before being resuspended in 100 μl of PBS. For each strain being investigated dilutions of lysates were made tenfold to 10⁻⁶; these and an undiluted sample were plated onto LB plates containing the required antibiotics. Plates were incubated at 37°C overnight and the colonies counted.

2.15.3 DC maturation upon exposure to Salmonella enterica Typhimurium

DCs were exposed for 48 hours to the following strains of Salmonella enterica Typhimurium: SL1344/nr, SL2MK, CSL2MK, C2, ATCC 14028 and WR1728. Unexposed DCs were used as a resting DC control and cells exposed to LPS as a mature DC control. The phenotype of the cells was then determined by flow cytometry using the antibodies specific for CD1a, CD14, CD86, HLA-DR and HLA-DQ listed in Section 2.11.
CHAPTER THREE

Preparation and phenotyping of DCs cultured from human peripheral blood

3.1 Introduction

This study aims to investigate the interactions of human dendritic cells with various strains of the intestinal pathogen *Salmonella enterica* serovar Typhimurium. To date relatively few studies have been carried out using human intestinal DCs mainly due to the difficulty in obtaining sufficient numbers of cells. Studies using mice and rats have provided more information about intestinal DCs. Iwasaki and Kelsall (2001) isolated three distinct subsets of DCs within murine Peyer’s patches (PP), CD11b⁺ (myeloid), CD8α⁺ (lymphoid) and CD11b⁻ / CD8⁻ (double negative (DN) DC). These types of DC were shown to be localised to different areas within the PP and to secrete different cytokines upon exposure to T cells or microbial stimulus. Myeloid DCs were more capable of producing a Th2 differentiation of naïve T cells and lymphoid and DN DCs were more capable of producing Th1 differentiation. During infection by intracellular bacteria such as *Salmonella*, the first cells to become infected are those in the subepithelial dome (SED), the myeloid and DN DCs (Hopkins and Kraehenbuhl, 1997). These cells then migrate to the interfollicular region (IFR) or the mesenteric lymph nodes (MLN) where the SED DN DCs directly prime Th1 responses and the SED myeloid DCs present antigen to resident lymphoid or DN DCs and cross prime T cells.

Myeloid-derived DCs (CD11b⁺) are found throughout the murine lamina propria (Neiss et al, 2005). These cells are able to actively sample gut bacteria through the formation of transepithelial dendrites by a mechanism that is dependent on the expression of the chemokine receptor CX3CR1 (Fotin and Ricciardi-Castagnoli, 2005).
Therefore it appears that a number of different DC subsets have a role during bacterial infection of mice.

There is evidence that human myeloid (CD11b⁺ CD8α⁻) and lymphoid (CD11b⁻ CD8α⁺) DCs also exist, as their precursors can be isolated from human blood and differentiated into immature myeloid or lymphoid DCs by culture with GM-CSF and IL-4 or interleukin 3 (IL-3) respectively. Human DCs isolated from the lamina propria have been confirmed to be derived from myeloid precursors (Howe et al, 2009) but have not been further characterised. Increased numbers of DCs were found in the PP of patients with Crohn’s disease compared with normal subjects (Salim et al, 2009). However, little study has been carried out to confirm whether the DN DCs (CD11b⁻ CD8α⁻) described in mice are also present in humans.

DCs are difficult to detect in vivo as there is no single monoclonal antibody that can be used to recognise all DCs and exclusively DCs. Furthermore, DCs constitute only a small proportion of cells in non-immune tissues and their isolation is fraught with difficulties because most techniques involve mincing the tissue, enzymatic digestion and differential adherence followed by positive or negative selection of the DCs. These processes result in a low yield of cells, which may be selective for subpopulations of DCs and which may induce changes in the isolated DCs that are not associated with in vivo events. As a result, detailed analyses similar to those carried out in mice have not been carried out with human intestinal DCs.

The majority of studies investigating human DC have relied upon cultured DCs. There are a number of methods designed to culture DCs; however they produce cells with varied characteristics and therefore great consideration is required before deciding upon the most suitable method for a particular experiment. For example, the phenotype and function of DCs can be affected by the precursor cells from which they are derived,
the differentiation factors used in culture and the anatomical location from which the DC precursors were recovered. DCs have been cultured from a number of human tissues and organs, including skin (Lenz et al., 1993), lymph nodes and tonsils (Grouard et al., 1997), foetal thymus (Res et al., 1999) and the liver, via chimaeric organ culture in the thymus of mice with severe combined immunodeficiency (SCID) (Plum et al., 1999). All of these methods are dependent on availability of organs and therefore present as many problems as directly culturing cells from the human intestine. The more readily available tissues bone marrow and cord blood can also be used to produce dendritic cells. CD34+ cells in the bone marrow can be positively selected using paramagnetic beads and then cultured with GM-CSF and tumour necrosis factor α (TNFα) to induce differentiation into DCs (Young et al., 1995). Myeloid, lymphoid and Langerhan's dendritic cells can also be differentiated from CD34+ cells in cord blood by culture with GM-CSF, TNFα and stem cell factor (SCF) for 5 days, followed by cell sorting to obtain CD1a+ CD14− cells (from which Langerhans cells are derived) and CD1a− CD14+ cells (from which DCs of a myeloid type are derived) (Caux et al., 1996). CD34/CD11c negative selection can also be used to purify cells that differentiate into lymphoid DCs (Sorg et al., 1999).

The most commonly used and best studied precursors of cultured human DCs are peripheral blood monocytes. Whole blood is obtained by phlebotomy and then separated by one step density gradient centrifugation (Romani et al., 1996; Bender et al., 1996) to isolate peripheral blood mononuclear cells (PBMC). Monocytes are enriched by adherence to plastic and the adherent cells are cultured with GM-CSF to prolong cellular survival and differentiate the cells towards larger, more granular cells, and IL-4 to inhibit macrophage development and induce DC growth (Sallusto and Lanzavecchia, 1994). Others have cultured adherent cells with the haemopoietic growth factor FMS-
like tyrosine kinase–3 ligand (Flt-3L) and IL-4 with or without the addition of TNFα (Miller et al, 2003; Baca-Estrada et al, 2002). After about 7 days CD1a+ CD14– CD83low cells were produced that were able to stimulate an allogeneic mixed leukocyte reaction (MLR). Culture of monocytes with IL-4 alone has also been reported to produce myeloid DCs, although the population produced appears to have a different phenotype (CD1alow CD83+) from those grown with additional cytokines (Roy et al, 2004). Functionally, no matter which method is used, DC cultured from monocytes will undergo maturation upon exposure to LPS for 2-3 days.

The majority of previous studies investigating interactions between DCs and salmonellae have used murine DCs from various tissues. The majority of studies with human DCs have tended to use monocyte-derived DCs (Salucci et al, 2008; Pietila et al, 2007; Kiama et al, 2006). Following the precedent of these studies and because DCs produced from monocytes appear to be representative of the PP DCs, this method of culturing DCs will be used for this study. Unpublished works by Makki (2003) and Thomas (2002) investigated interactions between human DCs and S. enterica Typhimurium and S. enterica Typhi, respectively. In both cases the enrichment of DC precursors by adherence for 2 hours followed by differentiation with GM-CSF and IL-4 was used to produce DCs from PBMCs. This chapter describes experiments to confirm that this method produces monocyte derived DCs (as determined by changes in CD marker expression) that are capable of maturation upon exposure to LPS. An alternative monocyte differentiation method, in which the concentration of cytokines is reduced compared with the method described above (Zou et al, 2000) was also assessed to determine what effect this alternative protocol has on the phenotype and maturation of the DCs produced.
3.2 Results

DCs have been cultured by a variety of methods as described in Section 3.1 and different methods can produce DCs representative of different DC subsets. The following experiments are aimed at confirming the phenotype of DCs produced from monocytic DC precursors enriched from peripheral blood and cultured with GM-CSF and IL-4. As different subsets have been shown to respond to stimuli in different ways it is important to know the subset of cultured DCs and their response to PAMPs (in this case LPS) before their use in assays.

3.2.1 DC precursor enrichment and differentiation

Monocytic DC precursors were enriched by adherence for 2 hours followed by culture with GM-CSF and IL-4 as described in Section 2.12.1. These cells were analysed by flow cytometry alongside cells cultured without the addition of cytokines to confirm that the method produced monocyte derived DCs. On flow cytometry forward and side scatter plots, adherent cells cultured in the absence of GM-CSF and IL-4 had a less granular morphology and were smaller compared with the larger, more granular cells that had been cultured in the presence of GM-CSF and IL-4 (Fig. 3.1). Differences can also be seen between the cell surface marker phenotypes of the two cell populations (Fig. 3.2)

Cells cultured in the absence of GM-CSF and IL-4 have low expression of the cell surface molecules CD1a, CD80, CD86, HLA-DQ and HLA-DR and high expression of CD14. Cells cultured in the presence of GM-CSF and IL-4 upregulate their expression of CD1a (from 42% to 66%), CD80 (from 9% to 27%), CD86 (from 29% to 45%), HLA-DQ (from 40% to 60%), and HLA-DR (from 31% to 82%) and down-regulate expression of CD14 (from 53% to 24%) (Fig. 3.3a). The mean fluorescence intensity (MFI) of CD14 decreased (from 134 to 76) after exposure to the
cytokines whereas MFI increased for CD1a (from 55 to 236), CD80 (from 43 to 123), CD86 (from 32 to 241), HLA-DQ (from 88 to 168), and HLA-DR (from 143 to 1472) (Fig. 3.3b). Experiments were repeated four times and the means were used to calculate the changes in expression; standard deviations were also calculated to provide an estimate of variance. The results confirm that cells with a DC phenotype (Zhou et al., 2001; Makki, 2003; Kiama et al., 2006) can be isolated from peripheral blood using this method.
Fig. 3.1. Representative forward (FSC-H) and side (SSC-H) scatter plot of DC precursors enriched by 2 hours adherence without (a) and with (b) exposure to GM-CSF and IL-4. Adherent DC precursors were cultured for 7 days in the absence or presence of GM-CSF and IL-4. In the absence of cytokines (a) the majority (91%) of cells were characteristic of either monocyte-type (medium size, some granules – region RO) or lymphocytes (small, no granules, below and to the left of RO). In the presence of cytokines (b) the largest proportions of cells were found to be large and granular (51%).
PBMCs adherent at 2 hr cultured without cytokines

PBMCs adherent at 2 hr cultured with cytokines

IgG1 isotype control

IgG1 isotype control

IgG2a control

IgG2a control

CD3

CD3

CD19

CD19
PBMCs adherent at 2 hr cultured without cytokines

PBMCs adherent at 2 hr cultured with cytokines

CD14

CD1a

CD80

CD86
Fig. 3.2. Comparison of phenotypes of adherent DC precursors cultured in the absence and presence of IL-4 and GM-CSF. Adherent DC precursors were isolated and cultured for 7 days in either the absence (green) or presence (red) of GM-CSF and IL-4 after which the phenotypes of the cell populations were determined by flow cytometry. The markers were set using the isotype controls IgG1 and IgG2a so that less than 1% of cells were positive. The background staining seen with the controls is due to the presence of high levels of Fc receptors on these cells.
Fig. 3.3. Percentage (a) and MFI (b) of gated adherent DC precursors expressing cell surface molecules from cell population cultured in the absence (green) and presence (red) of GM-CSF and IL-4. Adherent DC precursors were isolated and cultured for 7 days in the presence (red) or absence (green) of GM-CSF and IL-4, after which the phenotypes of the cell populations were determined by flow cytometry. Cells cultured in the presence of the cytokines exhibit an increase in expression of CD1a (+22%), CD80 (+18%), CD86 (+17%), HLA-DR (+22%) and HLA-DQ (+51%) and a decrease in expression of CD14 (-28%) and an increase in the MFI of: CD1a (+181), CD80 (+80), CD86 (+209), HLA-DQ (+79), a large increase in MFI of HLA-DR (+1329) and a decrease in MFI of CD14 (-58) compared to cells cultured without cytokines. Both sets of cells show low expression of CD3 (a T cell marker) and CD19 (a B cell marker).
3.2.2 Maturation of DC upon exposure to LPS

Immature DCs are effective at antigen uptake and processing but are poor stimulators of T cell responses. Maturation results in the migration of DCs to lymphoid tissue, upregulation of costimulatory molecules and the subsequent activation of the immune system and as such is a crucial function of DCs. Maturation can be induced \textit{in vitro} by exposure of DCs to pathogen associated molecular patterns (PAMPs) associated with infectious organisms including LPS or lipoteichoic acid. Changes in cell surface marker expression are used to identify mature DCs, which include increases in expression of MHC class II molecules, CD80 and CD86 and a slight decrease in CD1a. A number of other changes are also seen such as alterations in the production of cytokines and chemokines.

In the following experiments DC were exposed for 2 days to \textit{E. coli} LPS (Section 2.12.3) to confirm that cells produced by this method undergo maturation. Cell surface marker expression on DCs before and after exposure to LPS was analysed using flow cytometry. DCs analysed before and after exposure to LPS were found to have similar forward and side scatter profiles (Figures 3.4). Further analysis of the larger, more granular cells showed that exposure to LPS results in changes in the expression of cell surface markers (Fig. 3.5).

Compared with unstimulated DCs, those exposed to LPS upregulated their expression of CD80 (from 28\% to 58\%), CD86 (from 46\% to 73\%) and HLA-DR (from 86\% to 93\%); there was a large increase in HLA-DQ expression (from 46\% to 91\%), a slight decrease in CD1a expression (from 56\% to 47\%) and CD14 expression remained essentially unchanged (less than 1\%) (Fig. 3.6a). The MFI of CD1a decreased slightly (from 181 to 160) after exposure to LPS whereas MFI increased slightly for CD14 (from 80 to 111) and significantly for CD80 (from 76 to 440), CD86 (from 127 to 468),
HLA-DQ (from 208 to 680), and HLA-DR (from 732 to 1381) (Fig. 3.6b). Experiments were repeated five times and the means of these were used to calculate the changes in expression; standard deviations were also calculated to provide an estimate of variance.

These experiments confirm that DCs isolated from precursors in adherent PBMC and differentiated with the cytokine, GM-CSF and IL-4 mature upon exposure to LPS in a manner consistent with that reported in the literature (Granucci et al, 1999; Sallusto et al, 1995; Rescigno et al, 1998).
Fig. 3.4. Representative forward (FSC-H) and side (SSC-H) scatter plot of DCs cultured without (a) and with (b) LPS. DCs were cultured for 2 days in the absence of LPS (a) and upon flow cytometric analysis the proportion of cells found to be large and granular was 49%. DCs were cultured for 2 days with *E. coli* LPS (b) and upon flow cytometric analysis, the largest proportion of cells was found to be large and granular (57%).
Unstimulated DC (no LPS)  

Stimulated DC (+LPS)

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IgG1 isotype control

IgG1 isotype control

IgG2a isotype control

IgG2a isotype control

CD3

CD3

CD19

CD19
Unstimulated DC (no LPS)  

Stimulated DC (+LPS)
Fig. 3.5. Comparison of phenotypes of gated DC populations before and after stimulation with LPS. DCs were cultured in the presence (blue) or absence (red) of LPS. The phenotypes of the cells were determined by flow cytometry. The markers were set using the isotype controls IgG1 and IgG2a so that less than 1% of cells were positive. The background staining seen with the controls is due to the presence of high levels of Fc receptors on these cells.
Fig. 3.6. Percentage (a) and MFI (b) of gated cells expressing cell surface molecules on cell populations either left unstimulated or stimulated by LPS. DCs were cultured in the presence (blue) or absence (red) of LPS for 2 days. The phenotypes of the gated cell populations were then determined by flow cytometry. In comparison to unstimulated cells, exposure to LPS results in a slight increase in HLA-DR (+7%) expression and large increases in expression of CD80 (+30%), CD86 (+27%) and HLA-DQ (+45%). There is a slight decrease in CD1a expression (-9%). Results for MFI showed there was a slight increase for CD14 (+21) and large increases for CD80 (+516), CD86 (+341), HLA-DR (+649) and HLA-DQ (+472). There is a slight decrease for CD1a (-21). Both sets of cells show low expression of CD3 (T cell marker) and CD19 (B cell marker).
3.2.3 Differentiation of DCs at reduced cytokine concentrations

The previous experiments confirmed that cells with a DC phenotype can be produced by culture of adherent DC precursors from PBMC with GM-CSF and IL-4. The concentrations of cytokines used in these experiments (GM-CSF at 100 ng ml\(^{-1}\) and IL-4 at 50 ng ml\(^{-1}\)) are costly and therefore the possibility of reducing the concentration of cytokines whilst producing cells of the same phenotype and function was examined. Differentiation of adherent cells with GM-CSF at 25 ng ml\(^{-1}\) and IL-4 at 5 ng ml\(^{-1}\) has been reported to produce cells with the same phenotype as those produced with normal concentrations of cytokines (Zou et al., 2000). To assess this method, adherent DC precursors were differentiated using cytokines at either normal or reduced concentrations of cytokines followed by analysis of cell surface marker expression by flow cytometry. Cells grown under both sets of conditions were also exposed to LPS for 2 days to determine whether those cells differentiated with reduced concentrations of cytokines underwent the same maturation associated phenotypic changes as DCs produced at normal cytokine levels.

In flow cytometry, forward and side scatter distributions of cells differentiated using normal and reduced levels of cytokines appeared to be similar (Fig. 3.7). Further analysis of cell surface markers, however, showed that the gate containing the larger, more granular cells under each set of conditions showed a different pattern of expression (Fig. 3.8).

If the DCs differentiated by the two methods were identical, their phenotypes would be expected to be similar. However, the following differences were seen between DCs differentiated in low concentrations of cytokines (DC\(_{\text{low}}\)) and DCs differentiated in normal concentrations of cytokines; expression of CD14 was higher in DC\(_{\text{low}}\) than DCs (28% compared to 18%) and expression of CD1a (38% on DC\(_{\text{low}}\), 64%
on DCs), HLA-DQ (41% compared to 51%) and HLA-DR (47% compared to 92%) was lower. Expression of the markers CD80 (32 % on DC$_{low}$, 28% on DCs) and CD86 (40% DC$_{low}$, 43% on DCs) was similar on both cell types (Fig. 3.9a). Differences were also seen between MFI of cell surface markers (Fig. 3.9b); this was slightly higher on DC$_{low}$ compared to DCs for CD80 (239 compared to 206), CD86 (294 compared 232) and HLA-DQ (309 compared to 280) but lower on DC$_{low}$ compared to DCs for CD1a (359 compared to 667) and HLA-DR (850 compared to 1467). Results for CD14 were similar on DC$_{low}$ (112) and DCs (118). Experiments were repeated four times and the means of the values obtained used to calculate the changes in expression, standard deviations were also calculated to provide an estimate of variance.

If the DCs produced by the two methods were identical, the phenotypic changes in response to LPS may also be expected to be the same, but again, this was not the case (Fig. 3.10). Upon exposure to LPS DC$_{low}$ upregulated expression of CD14 (from 28% to 37%) but for DCs exposure to LPS resulted in no change in CD14 expression. DC$_{low}$ also upregulated expression of CD1a (from 38% to 52% after exposure to LPS) whereas DCs down-regulated CD1a expression (from 48% to 64% after exposure to LPS). Expression of CD80 remained similar after exposure to LPS for DC$_{low}$ (32% before and 37% after) whereas DCs up-regulated expression (from 28% before to 72% after). There was upregulation of CD86 after exposure to LPS for both cell types, DC$_{low}$ (from 40% to 59%) and DCs (from 43% to 77%) as well as upregulation of HLA- DQ (DC$_{low}$ from 41% to 70% and DCs from 59% to 94%) and upregulation of HLA-DR (DC$_{low}$ from 47% to 67% and DCs from 93% to 96%) (Fig. 3.11a).

The MFI also showed differences between the regulation of cell surface marker expression on DCs and DC$_{low}$ upon exposure to LPS (Fig. 3.11b). CD14 MFI increased with exposure to LPS for DC$_{low}$ (from 112 to 426) but decreased for DCs (from 118 to
81). CD1a MFI increased upon exposure for DC\textsubscript{low} (from 359 to 620) but a slight decrease was seen upon exposure for DCs (from 667 to 589). For DC\textsubscript{low} MFI increased upon exposure to LPS for CD80 (from 239 to 280), CD86 (from 294 to 592) and HLA-DQ (from 309 to 624). Increases in expression upon exposure to LPS were also seen for DCs but these changes were more significant; CD80 (from 206 to 893), CD86 (from 233 to 1981) and HLA-DQ (from 280 to 1665). There was a slight decrease in HL-DR expression for both DC\textsubscript{low} (from 851 to 629) and DCs (from 1467 to 1304). Experiments were repeated four times and the mean of these values was used to calculate the changes in expression; standard deviations were also calculated to provide an estimate of variance.

These results show that the DC\textsubscript{low} differentiated using the reduced cytokine levels do not exhibit the same cell surface phenotype or mature in the same way upon exposure to LPS as cells differentiated using normal levels of cytokines.
Fig. 3.7. Representative forward (FSC-H) and side (SSC-H) scatter plot of DCs and DC$_{low}$. DCs were differentiated for 7 days at cytokine concentrations of GM-CSF at 100 ngml$^{-1}$ and IL-4 at 50 ngml$^{-1}$ to produce DCs or GM-CSF at 25 ngml$^{-1}$ and IL-4 at 5 ngml$^{-1}$ to produce DC$_{low}$. Upon flow cytometric analysis, 32% of cells differentiated using normal cytokine concentrations (a) and 31% of cells differentiated using low cytokine levels (b) were found to be large and granular.
IgG1 isotype control

IgG2a control

DC

DC\textsubscript{low}

CD3

CD3

CD19

CD19
DC

CD14

DC_{low}

CD14

CD1a

CD80

CD86

CD1a

CD80

CD86
**Fig. 3.8. Comparison of phenotypes of DCs and DC\textsubscript{low}.** The phenotypes of DCs (red) and DC\textsubscript{low} (yellow) were determined by flow cytometry. The markers were set using the isotype controls IgG1 and IgG2a so that less than 1% of cells were positive. The background staining seen with the controls is due to the presence of high levels of Fc receptors on these cells.
Fig. 3.9. Percentage (a) and MFI (b) of cells expressing cell surface molecules on gated cell populations of DC and DC\textsubscript{low}. Adherent cells were exposed to normal levels of GM-CSF (100 ng ml\textsuperscript{-1}) and IL-4 (50 ng ml\textsuperscript{-1}), DC (red) or reduced levels of GM-CSF (25ngml\textsuperscript{-1}) and IL-4 (5ngml\textsuperscript{-1}), DC\textsubscript{low} (yellow) for 7 days. Cell surface marker expression on the resulting cells was determined by flow cytometry. The results shown are the means of four experiments and indicate that DC\textsubscript{low} are not identical to DC with respect to the expression of cell surface markers.
Stimulated DC (+ LPS)  
Stimulated DC_{low} (+ LPS)

IgG1 isotype control

IgG2a isotype control

CD3

CD19
Stimulated DC (+ LPS)

Stimulated DC$_{low}$ (+ LPS)
Fig. 3.10. Comparison of phenotypes of DCs and DC_{low} exposed for 2 days to LPS.

DCs (blue) and DC_{low} (pink) were exposed to LPS for 2 days followed by phenotypic analysis by flow cytometry. The markers were set using the isotype controls IgG1 and IgG2a so that less than 1% of cells were positive. The background staining seen with the controls is due to the presence of high levels of Fc receptors on these cells.
Fig. 3.11. Percentage (a) and MFI (b) of cells expressing cell surface molecules on gated cell populations of DC and DC$_{low}$, exposed for 2 days to LPS. DC (blue) and DC$_{low}$ (pink) were exposed to LPS for 2 days followed by phenotypic analysis by flow cytometry. The results shown are the means of four experiments and indicate that DC and DC$_{low}$ have different phenotypes upon exposure to LPS.
3.2.4 Further analysis of DC_{low}

The previous study determined that DC_{low} neither exhibit the same phenotype as DCs nor undergo maturation upon exposure to LPS. Two methods were designed to further investigate and attempt to identify these cells.

3.2.4.1 Comparison of DC_{low} with adherent cells and in vitro cultured macrophages

Experimentally, PBMCs can be used to produce macrophages after differentiation with M-CSF or GM-CSF (Komuro et al., 2001); therefore by reducing the concentrations of GM-CSF and IL-4 used to differentiate adherent cells into DCs, it is possible that the DC_{low} cells may have more in common with macrophages than DCs. The concentrations of cytokines used to differentiate DC_{low} could also have been so low that adherent cells were unable to differentiate into either DCs or macrophages and so DC_{low} could have more in common with cultured adherent cells. M-CSF at a concentration of 25 ngml^{-1} was used to differentiate adherent cells to produce macrophages and their cell surface phenotype analysed by flow cytometry to confirm they exhibited a macrophage phenotype. These cells alongside adherent cells (produced by incubation without any cytokines) were compared to DC_{low} using flow cytometry to analyse expression of cell surface markers. This analysis showed differences in the patterns of expression of cell surface markers between the three cell types (Fig. 3.12).

Compared with in vitro cultured macrophages, DC_{low} show a significant difference in the pattern of cell surface marker expression (Fig. 3.13a). DC_{low} have a higher expression of CD1a (35%) than macrophages (14%). Expression of CD80 is slightly higher for DC_{low} (26%) compared to macrophages (18%). However, expression is lower for DC_{low} than macrophages for CD14 (21% compared to 75%), CD16 (8% compared to 84%), CD86 (33% compared to 67%), HLA-DQ (43% compared to 93%) and HLA-DR (34% compared to 94%). The MFIs of each marker also showed
differences in patterns of expression (Fig. 3.13b). The MFI of CD1a was similar for DC\textsubscript{low} (68) and macrophages (61). The MFI of CD80 was higher for DC\textsubscript{low} (183) compared to that of macrophages (94). However, the MFI was lower for DC\textsubscript{low} compared to macrophages for CD14 (56 compared to 389), CD16 (54 compared to 335), CD86 (282 compared to 564) and HL-DQ (221 compared to 792). MFI of HLA-DR was significantly lower for DC\textsubscript{low} (165) compared to macrophages (2101).

DC\textsubscript{low} do show some similarities to adherent cells in the pattern of cell surface marker expression (Fig. 3.14a). The levels of expression of CD80 (26% for DC\textsubscript{low} and 22% for adherent cells) and HLA-DR (34% for DC\textsubscript{low} and 32% for adherent cells) are very similar and expression of CD16 is very low for both cell types (8% for DC\textsubscript{low} and 16% for AC). However, there are also notable differences. DC\textsubscript{low} have higher expression than adherent cells of the markers CD1a (35% compared to 23%), CD86 (33% compared to 18%) and HLA-DQ (43% compared to 35%). The most significant difference is seen between of the levels of expression of CD14 with expression being lower for DC\textsubscript{low} (21%) than for adherent cells (64%).

The MFI of each marker was also different for the DC\textsubscript{low} and adherent cells (Fig. 3.14b). The MFI was similar for both cell types for the markers CD1a (68 for DC\textsubscript{low} and 62 for adherent cells) and CD16 (54 for DC\textsubscript{low} and 42 for adherent cells). However, the MFI was higher for DC\textsubscript{low} than for adherent cells for the markers CD80 (183 compared 123), CD86 (282 compared to 51) and HLA-DQ (221 compared to 104). The MFI was lower for DC\textsubscript{low} than for adherent cells in the case of the markers CD14 (56 compared to 177) and HLA-DR (165 compared to 267). All experiments were repeated four times and the means of these was used to calculate the changes in expression. Standard deviations were also calculated to provide an estimate of variance. DC\textsubscript{low} do
not exhibit patterns of cell surface marker expression that are similar to either that shown by *in vitro* cultured macrophages or by undifferentiated adherent cells.
IgG1 isotype control

IgG2a Isotype control
CD14

DC_{low}

Adherent cells

Macrophage

CD1a

DC_{low}

Adherent cells

Macrophage
CD86

HLA-DQ
Fig. 3.12. Comparison of phenotypes of adherent cells from adherent cells, DC\textsubscript{low} and macrophages. The phenotypes of DC\textsubscript{low} (yellow), macrophages (grey) and adherent cells (green) were determined by flow cytometry. The markers were set using the isotype controls IgG1 and IgG2a so that less than 1% of cells were positive. The background staining seen with the controls is due to the presence of high levels of Fc receptors on these cells.
Fig. 3.13. Percentage (a) and MFI (b) of cells expressing cell surface molecules on gated cell populations of DC<sub>low</sub> and macrophages. The phenotypes of DC<sub>low</sub> (yellow) and macrophages (grey) were determined by flow cytometry. The results shown are the means of four experiments and show the phenotypes of DC<sub>low</sub> and <i>in vitro</i> cultured macrophages are different. Both cell populations expressed low levels of CD3 and CD19 (results not shown).
Fig. 3.14 Percentage (a) and MFI (b) of cells expressing cell surface molecules on gated cell populations of DC$_{low}$ and adherent cells. The phenotype of adherent cells (green) and DC$_{low}$ (yellow) were determined by flow cytometry. The results shown are the means of four experiments and show the phenotypes of DC$_{low}$ and adherent cells are different. Both cell populations expressed low levels of CD3 and CD19 (results not shown).
3.2.4.2 Comparison of DC_{low} to adherent cells during differentiation into DCs

Previous experiments in this chapter have concluded that DC_{low} have differentiated from adherent cells but not into DCs or in vitro cultured macrophages. Therefore, to determine whether the reduced concentration of cytokines delays the differentiation of DCs and thus explains the DC_{low} phenotype, a time course was carried out to compare cell surface marker expression on seven day differentiated DC_{low} with adherent cells undergoing differentiation into DCs. Adherent DC precursors were exposed to normal levels of cytokines (GM-CSF – 100 ngml^{-1} and IL-4 – 50 ngml^{-1}) and the expression of cell surface markers was analysed by flow cytometry on each day of their 7 day differentiation into DCs. DC_{low} were differentiated using reduced concentrations of cytokines and incubated for 7 days before their cell surface marker expression was also analysed by flow cytometry. The phenotype of DC_{low} (on day 7) was then compared to the phenotype of adherent cells on each day of their differentiation into DCs.

Comparison of the cell surface phenotypes of DCs and DC_{low} cells shows that there are similarities in the patterns of expression of cell surface markers on each day of the differentiation of DCs with day 7 DC_{low}. Results are the means of three experiment and standard deviations were also calculated to determine variance. Upon comparison of DCs during each day of their differentiation (Fig. 3.15 – Fig. 3.21) the percentages of cells expressing cell surface markers were most similar between Day 6 DCs and Day 7 DC_{low} as follows (DCs compared with DC_{low}); CD14 (19% compared with 23%), CD1a (32% compared with 36%), CD80 (29% compared with 31%), CD86 (32% compared with 39%), HLA-DQ (51% compared with 43%) and HLA-DR (56% compared with 48%). The MFI of markers were again most similar between Day 6 DCs and Day 7 DC_{low} as follows (DCs compared with DC_{low}); CD14 (63 compared with 79), CD1a
(213 compared with 111), CD80 (123 compared with 184), CD86 (355 compared with 170), HLA-DQ (467 compared with 336) and HLA-DR (309 compared with 465).

Paired t tests with a significance threshold of 0.05 were used to analyse both these sets of results and it was found that the differences between Day 6 DCs and Day 7 DC\textsubscript{low} with respect to percentage positive and MFI were not statistically significant. From these results we can conclude that DC\textsubscript{low} have the same phenotype as Day 6 DCs and therefore that the reduction in cytokines appears to delay differentiation. Further investigation would be required to fully assess whether increasing the incubation time would allow differentiation into functional DCs. However, having identified the DC\textsubscript{low} and confirmed that functional DCs can be successfully produced using the normal levels of cytokines; further experiments were considered beyond the scope of this study.
DAY ONE DC vs DAY SEVEN DC\textsubscript{low}

Fig. 3.15. Comparison of percentages of Day 1 DCs (green) and Day 7 DC\textsubscript{low} (yellow) expressing cell surface markers (a) and their mean fluorescence intensity (b)
DAY TWO DC vs DAY SEVEN DC\textsubscript{low}

Fig. 3.16. Comparison of percentages of Day 2 DCs (turquoise) and Day 7 DC\textsubscript{low} (yellow) expressing cell surface markers (a) and their mean fluorescence intensity (b).
Fig. 3.17. Comparison of percentages of Day 3 DCs (blue) and Day 7 DC\textsubscript{low} (yellow) expressing cell surface markers (a) and their mean fluorescence intensity (b)
Fig. 3.18. Comparison of percentages of Day 4 DCs (pink) and Day 7 DC_{low} (yellow) expressing cell surface markers (a) and their mean fluorescence intensity (b).
Fig. 3.19. Comparison of percentages of Day 5 DCs (orange) and Day 7 DC_{low} (yellow) expressing cell surface markers (a) and their mean fluorescence intensity (b)
Fig. 3.2. Comparison of percentages of Day 6 DCs (purple) and Day 7 DC\textsubscript{low} (yellow) expressing cell surface markers (a) and their mean fluorescence intensity (b)
Fig. 3.21. Comparison of percentages of Day 7 DCs (red) and Day 7 DC_{low} (yellow) expressing cell surface markers (a) and their mean fluorescence intensity (b)
3.3 Discussion

This chapter aimed to determine whether plastic adherence could be used to isolate DC precursors from peripheral blood and then differentiated into DCs. Different methods were used to generate cells, which were compared with those in the literature to determine which population most closely approximates to the cells that are normally called in vitro generated DC.

The use of plastic adherence of PBMC followed by exposure of DC precursors to IL-4 and GM-CSF causes significant changes in the expression of cell surface markers, changes which, as described in the literature (Sallusto and Lanzavecchia, 1994), confirm differentiation into cells with a DC phenotype. However, a number of other cell surface markers have been identified that can now be used in the identification and phenotyping of both in vivo and in vitro cultured DCs. CD11b has been identified on DCs in the murine lamina propria (Neiss et al, 2005) and two subsets of DCs found in the murine spleen can be identified by their ability to express CD8α (Martinez del Hoyo et al, 2002). CD123 can be used to differentiate between DC subsets in the blood (Jun et al, 2009) and DC-SIGN has been found to be an important DC marker involved in their interaction with T cells (Caminschi et al, 2001), although it is also expressed on CD68+ macrophages (van Lent et al, 2003). None of these markers can be used alone to identify DCs as they are all expressed on other cell types of the immune system and some have only been reported on murine DCs, but they could be useful in future studies to further characterise DCs produced by the method described in this study. This information would be useful for comparison of these DCs to those identified in vivo.

Exposure of DCs to LPS resulted in a change in marker expression that indicated the development of a mature DC phenotype. Comparison of cell surface marker
expression of the immature DCs and mature DCs also illustrated the usefulness of using both percentages of cells positive and MFI to analyse flow cytometry data. The percentage of cells positive results showed only slight changes in expression suggesting that the number of cells expressing the markers does not markedly increase. However, analysing the results using the MFI showed marked differences in expression of some markers before and after exposure to LPS, suggesting dramatic increases in the expression on the positive cells.

This chapter also examined a second method of producing DCs from DC precursors in peripheral blood, where the concentrations of cytokines GM-CSF and IL-4 used were decreased. This method was used by Zou et al (2000) to produce cells with a DC phenotype which also matured upon exposure to LPS. Unfortunately, using this method the cells produced in this study (DC_{low}) were not identical to those produced with the higher concentrations of cytokines (DCs). The phenotype of DC_{low} appears not to be the result of differentiation into an alternative DC subset as upon exposure to LPS DC_{low} did not display the changes of expression indicative of DC maturation.

In an attempt to both identify DC_{low} and to determine the reason for the differences between them and the DCs produced by Zou et al (2000) a number of further experiments were carried out. Comparisons with adherent DC precursors and cultured macrophages by flow cytometry determined that DC_{low} were not comparable to either. Therefore the reduction in cytokines has not prevented differentiation or changed it to result in the development of macrophages.

The analysis of the DC differentiation time course provided some interesting information not only about DC_{low} but also about normal DC differentiation. It appears that DC_{low} are comparable to DCs on day 6 of their normal differentiation, suggesting that reducing the cytokine concentration has slowed differentiation, although further
experiments would be required to confirm this and also to determine whether continued
culture could result in cells with a phenotype comparable to DCs. This result does not
help determine the cause of the differences between DC$_{low}$ and DC produced by Zou et
al (2000), particularly as they produced DCs after 6 days culture at the reduced cytokine
concentrations instead of the 7 days used to produce DCs and DC$_{low}$ in this study. Their
shorter culture time should have produced cells similar to our DCs on day 4 or 5 of
differentiation.

The time course also showed that upon exposure to GM-CSF and IL-4 the
expression of surface markers on DC precursors changes steadily during the culture
period until the levels associated with DCs are reached on day 7. There was an
exception to this though, as expression of CD86 remained relatively low until day 6. A
number of studies have cultured DC precursors with cytokines for only 6 days (Pickl et
al, 1996; Zou et al, 2000; Woszczek et al, 2008) instead of the 7 days used here. This
study however shows that during differentiation day 6 DCs, which are comparable to
DC$_{low}$, do not show the same phenotype as day 7 DCs. Therefore further investigation
is required into the differentiation of DCs, including the stage at which they are able to
mature upon exposure to LPS; such studies were, however, considered to be beyond the
scope of this study.
CHAPTER FOUR

Recovery of Salmonella enterica Typhimurium iron uptake mutants from monocyte-derived DCs

4.1 Introduction

All bacteria, with the exception of lactobacilli, require iron for growth and replication; however, within the biological fluids of vertebrates most iron is bound by lactoferrin and transferrin. To establish infection, pathogenic bacteria such as Salmonella enterica Typhimurium need mechanisms that enable them to use these various forms of complexed iron. One of the major mechanisms by which pathogenic bacteria obtain iron from their host organism is by the production of siderophores and their cognate outer membrane receptors.

The iron uptake mechanisms utilised by Salmonella have been extensively studied in vitro but it is still not clear what the primary source of iron is during infection. Generally, mutations in a single gene involved in regulating iron uptake has a minimal effect on virulence due to the existence of redundant systems for the uptake of this essential nutrient. However, studies involving mutations in certain specific iron regulated outer membrane proteins have suggested attenuation of virulence in mouse models of S. enterica Typhimurium infection.

Kingsley et al (1999) found that S. enterica Typhimurium mutants with a non-polar frameshift mutation in the foxA gene were unable to utilize ferrioxamines B, E or G, confirming that this gene encodes the outer membrane receptor for these siderophores. A number of animal models were also used to investigate the virulence of
strains carrying the mutation in \textit{foxA} and it was found that they exhibited a significantly reduced ability to colonize rabbit ileal loops.

A possible reason put forward for these results was that \textit{foxA} might have an essential role other than the uptake of ferrioxamines. Expression of \textit{foxA} is derepressed under iron-limiting conditions (Tsolis \textit{et al}, 1995) and therefore it is likely that high levels are present on the bacterial surface under conditions such as those found in blood serum and interstitial medium as a result of the presence of transferrin. This potential additional role of \textit{foxA} under these conditions, however, was not investigated.

Kingsley \textit{et al} (1999) were not able to complement the frameshift mutation in assays of virulence and therefore there also remained the possibility that indirect effects of the mutation rather than the mutation itself were the cause of the attenuation of virulence. Makki (2003) attempted to address this problem by complementation of the \textit{foxA} mutation. In these experiments, the \textit{foxA} mutant strain SL2MK was used; this strain contained a kanamycin resistance cassette inserted into the \textit{foxA} gene of SL1344/nr to allow positive selection of \textit{foxA} mutant bacteria. At this point, the genome sequence flanking \textit{foxA} was not available, and a number of cloning strategies had to be attempted before the \textit{foxA} gene was successfully cloned and used to complement the \textit{foxA} mutation in SL2MK.

The \textit{foxA} mutant and complemented strains produced by Makki (2003) were used in mouse model assays and contrary to the work by Kingsley (1999) showed that the virulence of \textit{foxA} mutants is not attenuated in mice. These different outcomes raise a number of questions. If the attenuation seen previously (Kingsley \textit{et al}, 1999) is due to the mutation in \textit{foxA} why did the mutant strain from Makki (2003) not show the same attenuation? Was the attenuation seen previously a result of secondary spontaneous
mutations in other genes? Further investigations would be required to answer these questions.

Makki (2003) also aimed to assess the role of the foxA gene in the internalisation and subsequent killing of DC. The study compared the ability of the mutant foxA strain (SL2MK) to survive within cultured DC compared with the foxA+ parental strain (SL1344/nr) and a derivative of the foxA mutant strain SL2MK complemented by the foxA+ plasmid (CSL2MK). All three strains were internalised by DCs, but the mutant foxA strain (SL2MK) was recovered in higher numbers than the parental or complemented strains. These results could be explained by an attenuated ability of the foxA mutant to kill the DC, although no further investigation to address this point was carried out during that study.

Rabsch et al (2003) had shown that siderophores and their outer membrane receptors may play a role in the virulence of S. enterica Typhimurium. Transposon insertion mutation of genes involved in the uptake of a number of siderophores (Fig. 4.1) produced strains with the phenotypes and genotypes described in Table 4.1.

Intragastric infection of mice showed that a strain with mutations in fepA, iroN and cir exhibited attenuated virulence compared to the parental strain, suggesting that the ability to utilise 2, 3-DHBS is important for virulence. However, mutations in fepA and iroN had no effect on virulence suggesting that the ability to utilise enterobactin and salmochelin as siderophores was not required for full virulence. Furthermore mutations in genes involved in salmochelin production (iroB) and transport (iroC) also did not affect the virulence of S. enterica Typhimurium in the mouse model.

This chapter describes experiments to determine the interactions of DCs with the foxA mutant (SL2MK), the parental strain SL1344 and the foxA+ complemented strain CSL2MK, as well as the mutants WR1726 (fepA), WR1727 (fepA iroN), WR1728 (fepA
*iroN cir* and WR1729 (*iroB iroC*) and their parental strain ATCC 14028. Initially experiments were carried out to optimise the gentamicin-killing assay used by Makki (2003) for all strains of *S. enterica* Typhimurium being used in these studies.

The clone used by Makki (2003) to complement the *foxA* mutation in strain CSL2MK contained a significant amount of up- and downstream sequence. As the genome sequence of *S. enterica* Typhimurium is now available, this chapter describes experiments to clone the *foxA* sequence without flanking sequence with the aim of clarifying whether the complementation reported by Makki (2003) was related to polar effects on neighbouring genes or was caused by the mutation of *foxA*. The growth rates of all strains were determined and compared to ensure that differences noted in future experiments were not caused by the mutations affecting their ability to replicate. Gentamicin killing assays were then carried out with DCs using the *foxA* strains and the IROMP and siderophore mutants (Table 4.1) with the aim of ascertaining whether the mutations attenuated their ability to kill DCs. This is of particular importance when considering the role that DC play in antigen capture and presentation and the possibility of developing vaccines from strains that are strongly attenuated.
Fig. 4.1. Salmonella siderophores and their cognate iron-regulated outer membrane proteins (IROMPs). FepA or IroN can transport ferric-enterobactin across the outer membrane. FepA, IroN and Cir can transport ferric-DHBS and IroN transports salmochelin. IroB and IroC are involved in the production of salmochelin and its transport out of the cell.
Table 4.1. Genotypes and phenotypes of *S. enterica* Typhimurium strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genes mutated</th>
<th>Fe-Siderophore uptake affected</th>
<th>Virulence affected</th>
<th>Source / Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATCC 14028</td>
<td>None – parental strain</td>
<td>None</td>
<td>N/A</td>
<td>Rabsch <em>et al</em>., 2003</td>
</tr>
<tr>
<td>WR1726</td>
<td><em>fepA</em></td>
<td>None</td>
<td>No*</td>
<td>Rabsch <em>et al</em>., 2003</td>
</tr>
<tr>
<td>WR1727</td>
<td><em>fepA iroN</em></td>
<td>Enterobactin Salmochelin</td>
<td>No*</td>
<td>Rabsch <em>et al</em>., 2003</td>
</tr>
<tr>
<td>WR1728</td>
<td><em>fepA iroN cir</em></td>
<td>Enterobactin Salmochelin DHBS</td>
<td>Yes*</td>
<td>Rabsch <em>et al</em>., 2003</td>
</tr>
<tr>
<td>WR1729</td>
<td><em>fepA iroN iroBC</em></td>
<td>Enterobactin Salmochelin (+production)</td>
<td>No*</td>
<td>Rabsch <em>et al</em>., 2003</td>
</tr>
<tr>
<td>SL1344/nr</td>
<td>None – parental strain</td>
<td>None</td>
<td>N/A</td>
<td>Makki, 2003</td>
</tr>
<tr>
<td>SL2MK</td>
<td><em>foxA</em></td>
<td>Ferrioxamines B, E and G</td>
<td>No*^{(2)}</td>
<td>Makki <em>et al</em>., 2003</td>
</tr>
<tr>
<td>CSL2MK</td>
<td><em>foxA</em>, complemented by pWRSM</td>
<td>None</td>
<td>No*^{(4)}</td>
<td>Makki <em>et al</em>., 2003</td>
</tr>
<tr>
<td>C2</td>
<td><em>foxA</em>, complemented by pRXH2</td>
<td>None</td>
<td>Unknown</td>
<td>This study</td>
</tr>
</tbody>
</table>

* compared to parental strain in *in vivo* experiments in mice and rabbits
*^{2} compared to parental strain in *in vivo* experiments in mice
*^{3} recovered from cultured DCs in higher numbers than the parental strain
*^{4} recovered from cultured DCs in similar numbers to the parental strain
4.2 Results

The effects of mutations in *foxA* have been reported to cause attenuated virulence in mice models of *S. enterica* Typhimurium infection and increased recovery from human monocyte derived DCs (Kinsley et al., 1999; Makki, 2003). A similar attenuation in virulence was seen in mouse models of infection by a strain containing mutations in *fepA* *iroN* *cir* resulting in the inability to utilise enterobactin, salmochelin and 2, 3-DHBS as siderophores. No work however, was carried out with this strain and human DCs. The following experiments aim to confirm whether the mutation in *foxA* and the triple mutations *fepA* *iroN* *cir* do cause an increase in recovery of *S. enterica* Typhimurium from human DCs.

4.2.1 Analysis of gentamicin killing assays for use as a method of investigating *S. enterica* Typhimurium infection of DC

Makki (2003) used a gentamicin killing assay to assess intracellular survival of *S. enterica* Typhimurium phagocytosed by DCs. Gentamicin is an aminoglycoside antibiotic that binds the 30S subunit of the bacterial ribosome preventing protein synthesis. It is highly effective against many types of Gram-negative bacteria including *Salmonella* but has no known influence on the function of DCs and has been used in many studies involving bacterial infections of DCs (Banks *et al.*, 2007; Adkins *et al.*, 2007; Skjolaas *et al.*, 2006). It is therefore an ideal antibiotic to use to kill extracellular bacteria before DCs are lysed to measure intracellular bacteria. To confirm this method and determine the effectiveness of gentamicin killing at a concentration of 150 µgml⁻¹, a time course was conducted (detailed in Section 2.15.1) with all bacterial strains.

The effects of gentamicin exposure on the parental strain SL1344/nr, SL2MK (*foxA*⁻) and CL2MK (SL2MK complemented with *foxA*) were compared. All three strains showed a similar pattern of a rapid decrease in viable bacteria over time and they
all failed to produce any colonies after 100 min exposure to 150 µgml⁻¹ gentamicin (Fig. 4.2a). The gentamicin resistance of the strains produced by Rabsch *et al* (2003), namely ATCC 14028 (parental strain), WR1726 (*fepA*), WR1727 (*fepA iroN*), WR1728 (*fepA iroN cir*) and WR1729 (*iroB iroC*), were also examined. Again all strains showed similar rapid decreases in viable bacteria over time with all bacteria being killed by 100 min (Fig. 4.2b). These results confirm that the concentration of gentamicin used in the gentamicin killing assay (150 µgml⁻¹) is sufficient to kill all the bacterial strains used within the 2 hours. This method therefore is suitable for use in future studies of intracellular bacterial survival within DCs.
Fig. 4.2. Survival of *Salmonella enterica* Typhimurium strains upon exposure to gentamicin. Overnight cultures of bacteria were suspended in RPMI containing 150 μgml⁻¹ gentamicin and samples were taken for counting every 20 min. The results shown are the means of three experiments and standard deviations are also shown. At the 100 min time point no colonies were produced from samples taken from any of the strains.
4.2.2 Production of complemented foxA mutant

The genome sequence of a number of strains of S. enterica Typhimurium were available at the start of the present study; but were not available when CSL2MK, the foxA complemented foxA mutant, was produced by Makki (2003). As previously discussed, the non availability of foxA flanking sequence had caused difficulties in cloning the foxA gene to prepare a construct that could be used to complement the foxA mutation in SL2MK. The construct used in CSL2MK included a fragment (designated SI-Kan) that contained over 4.4 kb of sequence flanking the 2.1 kb foxA gene (Fig. 4.3). This flanking sequence included a number of genes: one encoding STM0363, a putative transcriptional regulator; yahN encoding a putative RhtC-like transport protein; yahO encoding a putative periplasmic protein; and prpR encoding a prp operon regulator.

Therefore the aim of the following experiments was to re-clone foxA excluding the flanking sequences and create a new complemented strain which could be used in gentamicin killing assays to characterise the role of foxA during infection of DC. This chapter describes the strategies used to produce and test a complemented strain.

4.2.2.1 PCR and cloning of foxA

Chromosomal DNA for PCR analysis was prepared on a small scale from S. enterica Typhimurium strain SL1344/nr. The primers used for the PCR reactions are shown in Table 2.2. The forward primer F1 begins upstream of foxA and contains the promoter that is regulated by iron, the reverse primer was designated R2.

The PCR reaction was optimized and the PCR products were purified and analysed by electrophoresis on 1% agarose gel to ensure that a band of 2.4 kb was present (Fig. 4.4). The PCR product was ligated into pWKS30 and recombinants identified by blue/white selection. Individual white colonies grown from the transformants were tested for the presence of foxA by PCR.
Fig. 4.3. Diagrammatic representation of genes included in SI-Kan fragment used in the construct to complement the SL2MK foxA mutation in CSL2MK (Makki, 2003). Also shown are the positions of the primers designed in this study for PCR from chromosomal DNA to produce the foxA fragment (F1 and R2 – full PCR) and (F3 and R2 – partial PCR).
Because none of the colonies tested by PCR contained the $foxA$ gene, plasmids from the remaining colonies were digested with $Hind$III to enrich for clones containing the $foxA$ insert (section 2.13.13). The DNA was then chemically transformed (Section 2.13.14) into DH5$\alpha$ as well as a second $E. coli$ strain XL1 Blue in case the failure to transform into DH5$\alpha$ was due to incompatibility with the pWKS30 containing $foxA$. A number of white colonies were produced on each of the following plates;

**Table 4.2. Plates containing positive colonies analysed by PCR.**

<table>
<thead>
<tr>
<th>Plate</th>
<th>Enzymes cut with;</th>
<th>Transformed into;</th>
</tr>
</thead>
<tbody>
<tr>
<td>N1</td>
<td>$ApaI$ and $EcoR1$</td>
<td>DH5$\alpha$</td>
</tr>
<tr>
<td>R1</td>
<td>$ApaI$ and $EcoR1$</td>
<td>XL1Blue</td>
</tr>
<tr>
<td>N2</td>
<td>$ApaI$ $EcoR1$ and $HindIII$</td>
<td>DH5$\alpha$</td>
</tr>
<tr>
<td>R2</td>
<td>$ApaI$ $EcoR1$ and $HindIII$</td>
<td>XL1Blue</td>
</tr>
</tbody>
</table>

Colonies were collected from all the plates, the DNA isolated and used as the template for PCR. Using the short PCR cycle only colonies from plate N1 produced a band on agarose gel. DNA from these samples was then used in a confirmatory PCR reaction using the full-length cycle and the primers F1 and R2 to ensure the whole $foxA$ gene was present. Fig. 4.5 shows the results of the PCR reactions confirming that the clones 2, 4, 9, 11, 19 and 20 carry the $foxA^+$ construct, pRXH2.

**4.2.2.2 Transfer of pRHX2 into S. enterica Typhimurium strain SL2MK**

To test for complementation, pRHX2 from the six $foxA$ positive clones was transformed into the $foxA$ mutant strain $S. enterica$ Typhimurium strain SL2MK. Plasmid DNA was isolated from colonies and analysed using full length PCR, to ensure the entire $foxA$ gene was present. The $foxA$ mutants transformed with pRXH2 were designated C2, C4, C9, C11, C19 and C20.
4.2.2.3 Growth stimulation of *S. enterica* Typhimurium strains by ferrioxamine

Wild type, *foxA* mutant and *foxA* mutant transformed with pRXH2 *S. enterica* Typhimurium were tested in diffusion plate bioassays to determine their ability to acquire ferrioxamine B as a sole source of iron (Section 2.13.14). There was no growth of the *foxA* mutant strain (SL2MK) around ferrioxamine loaded filter paper discs although the wild type strain (SL1344/nr) and the original complemented mutant (CSL2MK) were able to grow in haloes around the ferrioxamine loaded filter paper discs. Strains C2, C4, C9, C11, C19 and C20 carrying pRXH2 showed clear haloes of growth around the siderophore-loaded discs (Table 4.3). Strains C2 and C9 showed the strongest growth around the ferrioxamine loaded disk when compared to the growth of the wild type. These results confirm that a functional *foxA* gene has been cloned in pRXH2 which complemented the *foxA* mutation in strain SL2MK. The strain that will be used later in these studies is designated as C2.
Fig. 4.4. **PCR of foxA from SL1344/nr.** PCR was carried out on chromosomal DNA prepared from SL1344/nr using primers for foxA. Lane 1 is the positive control and Lane 3 the negative control. Lane 5 contains the PCR sample and shows a band of the correct size (2.4 kb).

Fig. 4.5 **PCR products of mini-preps of transformants.** Mini-preps from colonies on plate N1 were tested by short PCR (Lanes 9-14) and the 6 clones that produced positive PCR products (clones 2, 4, 9, 11, 19 and 20) were re-tested using full length foxA PCR (Lanes 2-7). All 6 produced correct size bands on agarose gel confirming that they contained the foxA gene.
Table 4.3. Growth stimulation of *S. enterica* Typhimurium SL2MK strains complemented by pRXH2 or pWRSM, their parental strain and the mutant strain SL2MK. Growth stimulation was assessed using the egg white medium (EWM) diffusion plate assay. Ferrioxamine B was loaded onto sterile filter paper discs (6 mm diameter) and placed on the surface of seeded EWM agar supplemented with 200 µM dipyridyl. Diameters of growth halos are the means of 3 experiments.

<table>
<thead>
<tr>
<th>Strain</th>
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<th>Responses to Ferrioxamine (mean diameter of halo (mm))</th>
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</thead>
<tbody>
<tr>
<td>SL1344/nr</td>
<td>SL1344, nal^R</td>
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</tr>
<tr>
<td>SL2MK</td>
<td>SL1344/nr, <em>foxA</em></td>
<td>No growth</td>
</tr>
<tr>
<td>CSL2MK</td>
<td>SL2MK, pWRSM</td>
<td>19</td>
</tr>
<tr>
<td>C2</td>
<td>SL2MK, pRXH2</td>
<td>19</td>
</tr>
<tr>
<td>C4</td>
<td>SL2MK, pRXH2</td>
<td>10</td>
</tr>
<tr>
<td>C9</td>
<td>SL2MK, pRXH2</td>
<td>15</td>
</tr>
<tr>
<td>C11</td>
<td>SL2MK, pRXH2</td>
<td>12</td>
</tr>
<tr>
<td>C19</td>
<td>SL2MK, pRXH2</td>
<td>16</td>
</tr>
<tr>
<td>C20</td>
<td>SL2MK, pRXH2</td>
<td>No growth</td>
</tr>
</tbody>
</table>
4.2.3 Growth rates of *S. enterica* Typhimurium iron uptake mutants

The strains under investigation in this study (Table 4.1) carry mutations in one or more different genes that might cause differences in growth rates. The gentamicin killing assays to be used in future experiments compare numbers of bacteria recovered after a fixed period of time and therefore it is important to determine whether there are any significant differences in growth rates to ensure the numbers of bacteria recovered from within DCs can be confidently associated with the mutations in iron regulation genes. Growth curves were determined for all strains in LB and M9CA media.

LB is an iron replete culture medium providing optimal growth conditions for *S. enterica* Typhimurium strains. M9CA is an iron restricted medium and was used to determine growth rates under iron poor conditions.

Culture samples taken at different time points were diluted and plated onto agar allowing colonies to be counted after overnight incubation. Viable counts were performed three times and the average used to produce growth curves. From these curves and using the formula shown below the growth rate and the doubling time for each stain were calculated (Herbert *et al*, 1956).

\[
\text{No. of generations (g) = } \frac{\log_{10}(N_t/N_0)}{\log_{10}2}
\]

\[
\text{Growth rate = } g/t_1
\]

Where: \(N_t\) is the number of bacteria at time \(t\), \(N_0\) is the number of starting bacteria, and \(t_1\) is the elapsed time.

Growth rates were first examined for the strains; SL1344/nr (wild type), SL2MK (foxA), CSL2MK (SL2MK complemented by foxA\(^+\) plasmid pWRSM) and C2 (SL2MK complemented by foxA\(^+\) plasmid pRXH2). The growth curves produced (Fig. 4.7) show that in both LB and M9CA medium all four strains exhibit a similar pattern of growth,
although in M9CA the lag phase is longer at approximately 9 hours compared with 3 hours in LB.

The doubling times and growth rates (Fig. 4.6) for the four strains also appeared to be similar to each other under both sets of conditions. In LB medium differences in doubling times between wild type strain (SL1344/nr) and the other strains were all less than 1 min. In M9CA medium the differences in doubling times between SL1344/nr and the other strains were all less than 0.5 min. This suggests that the mutations in foxA and their complementation have no significant effect on growth of *S. enterica* Typhimurium.

When grown in LB medium (iron replete) all the strains showed a slight increase in doubling time compared to when grown in M9CA (iron poor) (Fig. 4.7); SL1344/nr (+12%), SL2MK (+11%), CSL2MK (+10%) and C2 (+17%) and a decrease in growth rate; SL1344/nr (-3.7%), SL2MK (-5.4%) and CSL2MK (-5.6%), except C2, which remained the same. These differences are not considered statistically significant using a paired t test with 95% confidence; therefore growth of these strains is not significantly affected by the differences in the composition of LB and M9CA.
Fig. 4.6 *Salmonella enterica* Typhimurium growth curves. Growth curves of strains grown under iron replete (A) and iron poor (B) conditions. Results shown are the mean of three experiments and show that the four strains (SL1344/nr (wild type), SL2MK (*foxA*), CSL2MK (SL2MK, pWRSM) and C2 (SL2MK, pRXH2) have similar growth characteristics to each other under both sets of conditions. The lag time is longer for all strains under iron poor conditions at 9 hours compared to 3 hours under iron replete conditions.
Fig. 4.7 Doubling time and growth rates of Salmonella enterica Typhimurium. The results were calculated using growth curves produced from the means of four experiments and show that all four strains exhibit similar doubling times and growth rates to each other under both iron replete and iron poor conditions. Under iron poor conditions however, all strains show an increase in doubling time and a decrease in growth rate, with the exception of C2 which showed the same growth rate under both conditions.
The growth rates of the strains from the studies by Rabsch et al (2003), ATCC 14028 (parental strain), WR1726 (fep$^A$), WR1727 (fep$^A$ iro$^N$), WR1728 (fep$^A$ iro$^N$ cir$^C$) and WR7129 (fep$^A$, iro$^N$, iro$^{BC}$) were also analysed. The growth curves show that all five strains showed very similar growth dynamics under both sets of conditions (Fig. 4.8) although again a difference was seen in the length of the lag phase, around 9 hours under iron poor conditions compared with under 3 hours for iron replete medium.

The doubling times and growth rates (Fig. 4.9) for the five strains also appeared to be similar under both sets of conditions. In LB medium differences in doubling times between wild type strain (ATCC 14028) and the other strains were all less than 1 min. In M9CA medium the differences in doubling times between ATCC 14028 and the other strains were all less than 1 min.

When grown in LB medium (iron replete) all the strains showed a slight increase in doubling time compared to when grown in M9CA (iron poor) (Fig. 4.9); ATCC 14028 (+6.2%), WR1726 (+4.7%), WR1727 (+8.4%) and WR1728 (+2.5%) except WR1729, which showed a slight decrease (-4.2%). Growth rates in iron poor conditions decreased compared to iron replete for all strains; ATCC 14028 (-17%), WR1726 (-9.7%), WR1727 (-7.8%), WR1728 (-13%) and WR1729 (-19%). These differences are not considered statistically significant using a paired t test with 95% confidence therefore growth of these strains is not significantly effected by the differences in the composition of the LB and M9CA.

These results confirm that there are no significant differences in the growth characteristics of parental strains and those carrying the mutations and therefore in future experiments any differences seen between numbers of bacteria recovered can be concluded to be a result of other factors.
**Fig. 4.8 Salmonella enterica Typhimurium growth curves.** Growth curves of strains grown under iron replete (A) and iron poor (B) conditions. Results shown are the mean of three experiments and show that the five strains ATCC 14028, WR1726 (fep\(^{-}\)), WR1727(fep\(^{-}\), iroN\(^{-}\)), WR1728 (fep\(^{-}\), iroN\(^{-}\), cir\(^{-}\)) and WR1729 (fep\(^{-}\), iroN\(^{-}\), iroBC\(^{-}\)) have similar growth to each other under both sets of conditions.
**Fig. 4.9** Doubling time and growth rates of *Salmonella enterica* Typhimurium. The results were calculated using growth curves produced from the means of four experiments and show that all four strains exhibit similar doubling times and growth rates to each other under both iron replete and iron poor conditions. Under iron poor conditions however, all strains show an increase in doubling time, except WR1729, and a decrease in growth rate.
4.2.4 Recovery of *S. enterica* Typhimurium from DC

To determine whether mutations in iron uptake genes affect recovery of *S. enterica* Typhimurium from human DC, gentamicin killing assays were carried out as described in Chapter 2 (Section 2.15.2). DCs were infected with *S. enterica* Typhimurium for 2 or 24 hours before bacteria not internalised were killed with 150 μg/ml of gentamicin. DCs were then lysed and bacteria present in the lysate were diluted, plated and incubated overnight to determine a viable count.

Initially, recovery of the strains SL1344/nr, SL2MK (*foxA*), CSL2MK (SL2MK pWRSM) and C2 (SL2MK pRXH2) from DC was analysed to determine the effects of the mutation in *foxA* upon survival in DC. The results after 2 hours infection (Fig. 4.10) show that the mutant strain is indeed recovered in higher numbers than the parental and complemented strains. Compared with SL1344/nr, the percentage of bacteria recovered from DC was as follows: SL2MK (*foxA*) (239%), CSL2MK (SL2MK pWRSM) (85%) and C2 (SL2MK pRXH2) (94%).

A similar pattern is seen after 24 hours infection (Fig. 4.11) although for all strains the percentage of bacteria recovered is significantly lower than at 2 hours; this may be the result of either the bacteria being killed by the DC or the DCs being killed by the bacteria. Compared to SL1344/nr the percentage of bacteria recovered at 24 hours was as follows: SL2MK (*foxA*) (179%), CSL2MK (SL2MK pWRSM) (84%) and C2 (SL2MK pRXH2) (103%). Paired t tests with a significance threshold of 0.05 were used to compare recovery of the parental strain with the *foxA* mutant and complemented strains. At both time points the difference in the percentages of SL2MK (*foxA*) compared to SL1344/nr recovered from DCs is statistically significant but the differences between SL1334/nr and the complemented strains CSL2MK and C2 are not.
These results confirm that the mutation in foxA does affect the intracellular survival of S. enterica Typhimurium in DCs.

The recovery from DCs of the iron uptake mutants developed by Rabsch et al (2003), WR1726 (fepA), WR1727 (fepA iroN), WR1728 (fepA iroN cir) and WR1729 (fepA iroN iroBC) and the parental strain ATCC 14028, was also analysed using gentamicin killing assays. After 2 hours infection (Fig. 4.12) there are differences in recovery between the strains when compared to the parental strain ATCC 14028; the percentages recovered were WR1726 (fepA) (92%), WR1727 (fepA iroN) (88%), WR1728 (fepA iroN cir) (53%) and WR1729 (fepA iroN iroBC) (97%). Although all strains carrying mutations were recovered from DCs at lower percentages than the parental strain, upon analysis of results using a paired t test, only the difference between ATCC 14028 and WR1728 (fepA iroN cir) was statistically significant.

After 24 hours infection, again a significantly lower percentage of bacteria were recovered for all strains compared with the parental strain (Fig. 4.13). However, a different pattern of recovery was seen with WR1728 being recovered at a significantly higher percentage whereas at 2 hours it was recovered at a lower percentage. Compared to the parental strain ATCC 14028, the percentage of bacteria recovered was as follows: WR126 (fepA), (91%), WR1727 (fepA iroN) (91%), WR1728 (fepA iroN cir) (163%) and WR1729 (fepA iroN iroBC) (92%). Again paired t tests were carried out with a threshold of significance of 0.05 and the only significant difference was between ATCC 14028 and WR1728 (fepA iroN cir). These results show that the inability to utilise iron complexed with DHBS does appear to have an effect as shown by the data from WR1728 (fepA iroN cir). Furthermore, it appears that this inability has different effects on S. enterica Typhimurium recovery from DCs after 2 hours infection compared to that seen after 24 hours infection.
Fig. 4.10 Recovery of Salmonella strains from DC after 2 hour infection. DCs were infected for 2 hours with the following strains of *S. enterica* Typhimurium: SL1344/nr, SL2MK, CSL2MK and C2. After infection for 2 hr DCs were lysed and the surviving intracellular bacteria quantified after plating. Recovery of each mutant strain is expressed as a percentage of the parental strain (SL1344/nr) recovered. The results are the means of six experiments and show that the *foxA* mutant strain, SL2MK, was recovered at a significantly higher percentage than the parental strain and the complemented strains CSL2MK and C2.
Fig. 4.11 Recovery of Salmonella strains from DC after 24 hour infection. DCs were infected for 24 hours with the following strains of *S. enterica* Typhimurium: SL1344/nr, SL2MK, CSL2MK and C2. After incubation DCs were lysed and the surviving intracellular bacteria quantified after plating. Recovery of each mutant strain is expressed as a percentage of the parental strain (SL1344/nr) recovered. The results are the means of six experiments and show that the *foxA* mutant strain, SL2MK, was recovered at a significantly higher percentage than the parental strain and the complemented strains CSL2MK and C2.
Fig. 4.12 Percentage of bacteria recovered from DC after 2 hour exposure. DCs were infected for 2 hours with the following strains of *S. enterica* Typhimurium: ATCC 14028, WR1726, WR1727, WR1728 and WR1729. After infection for 2 hr DCs were lysed and the surviving intracellular bacteria quantified after plating. Recovery is expressed as a percentage of the recovered wild type strain (ATCC 14028). The results are the means of five experiments and show that the strain WR1728 (*fepA*-, *iroN*-,*cir*) was recovered at a significantly lower percentage than the parental strain and the other mutant strains.
Fig. 4.13 Percentage of bacteria recovered from DC after 24 hour exposure. DCs were infected for 24 hours with the following strains of *S. enterica* Typhimurium: ATCC 14028, WR1726, WR1727, WR1728 and WR1729. After infection for 24 hr DCs were lysed and the surviving intracellular bacteria quantified after plating. Recovery is expressed as a percentage of the recovered wild type strain (ATCC 14028). The results are the means of six experiments and show that WR1728 (*fepA*, *iroN*, *cir*), was recovered at a significantly higher percentage than the wild type and the other mutant strains.

<table>
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<tr>
<th>COMPARISON OF STRAINS</th>
<th>p VALUE</th>
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<tbody>
<tr>
<td>WT: WR1726 (<em>fepA</em>)</td>
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</tr>
<tr>
<td>WT: WR1727 (<em>fepA</em>- <em>iroN</em>)</td>
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</tr>
<tr>
<td>WT: WR1728 (<em>fepA</em>- <em>iroN</em>- <em>cir</em>)</td>
<td>0.0005</td>
</tr>
<tr>
<td>WT: WR1729 (<em>fepA</em>- <em>iroN</em>- <em>iroBC</em>)</td>
<td>0.0635</td>
</tr>
</tbody>
</table>
4.3 Discussion

The gentamicin killing assay for determining recovery of bacteria from within DCs was assessed. Gentamicin at 150 µgml\(^{-1}\) was found to have an effective bactericidal effect on all strains under investigation after 100 min (Table 4.1). This result confirms that the 2 hour incubation used in gentamicin killing assays is sufficient to kill extracellular *S. enterica* Typhimurium. It is however, worth noting that the effects of gentamicin on bacteria were also shown not to be instant with the numbers of viable bacteria decreasing gradually during the first 100 min exposure. The interaction between DCs and *Salmonella* is not static; studies have reported that *Salmonella* can rapidly kill murine and human DCs (van der Velden, 2003; Sundquist and Wick, 2008), and so it is possible that extracellular bacteria which had been released by lysed DCs towards the end of the 2 hour incubation with gentamicin would not be killed by the gentamicin. However, it was considered that although it is not possible to totally rule out some extracellular bacteria surviving, this number is likely to be negligible compared to the numbers recovered from within the DCs.

Before the recovery of *S. enterica* Typhimurium strains from DCs could be carried out using the gentamicin assay, production of a new complemented *foxA* strain was required. The cloned *foxA* fragment (7.3 kb) used to produce the complemented strain used in the study by Makki (2003), CSL2MK, contained considerable flanking sequence and therefore allowed the possibility of complementation of polar effects on neighbouring genes. The *foxA* gene was cloned using PCR from the wild type, SL1344/\( \text{nr} \) and ligated into pWKS30 before transformation into *S. enterica* Typhimurium strain SL2MK (\( \text{foxA}^- \)). The presence of *foxA* was confirmed by PCR and diffusion plate bioassays. Results confirmed that the successful production of a strain containing a cloned *foxA* fragment which complemented the mutation in SL2MK but
which did not contain any flanking genes had been achieved. C2 (SL2MK, pRXH2) could be used in future experiments to confirm differences in the interaction of SL2MK and the wild type strain, SL1344/nr with DCs were the result of the mutation in foxA.

Makki (2003) reported that a mutation in foxA resulted in increased recovery of S. enterica Typhimurium from within human cultured DCs. The simplest explanation for these differences is that the mutation results in a change in the growth rate of the strain. The growth rates of all the strains used in this study (Table 4.1) were determined using growth curves produced from 24 hour cultures in both iron replete and iron-limited conditions. No significant differences were recorded in growth rates between the mutants and their wild type strains in either iron-poor or iron-replete conditions. This is likely to be a result of the redundant nature of Salmonella iron uptake mechanisms (Zhou et al, 1999) compensating for the mutations. Also they suggest that any secondary roles FoxA, and the protein products of the other genes mutated, may also have no effect on the growth rates of the strains.

The results from gentamicin killing assays carried out after exposure of DCs to strains S. enterica Typhimurium wild type (SL1344/nr), foxA mutant (SL2MK) and the complemented mutants (CSL2MK and C2) for 2 hours and 24 hours confirm that the mutation in foxA is the cause of increased recovery of S. enterica Typhimurium from DCs. All strains were recovered in lower numbers after 24 hours than after 2 hours. This could be a result of a number of factors. It could be due to increased DC death as a result of Salmonella infection, as reported by van der Velden et al (2003) and by Sundquist and Wick (2008), as well as in studies with other bacteria including the Yersinia enterocolitica induced death of murine DCs (Grobner et al, 2006). Alternatively, it could be due to an increase in the death of Salmonella as a result of killing by DCs. A number of studies have reported killing of ingested bacteria by DCs;
Schoppet et al (2000) reported 95% killing of ingested E. coli and 75% killing of S. enterica serovar Typhimurium by DCs after 6 hours of incubation. Nagl et al (2002) also reported killing of bacteria but at a greatly reduced efficiency, 50 to 70% reduction in the number of E. coli CFU and a 50% reduction in the number of Staphylococcus aureus CFU. Ferrioxamines are siderophores produced by bacteria; examples include Erwinia herbicola (Berner et al, 1998) which produces ferrioxamine E and Streptomyces antibioticum which produces ferrioxamine B and E (Yang and Leong, 1988) and are utilised by many other bacteria by the use of IROMPs specific for their uptake. However, as ferrioxamines are not present in cultured DCs, the increase in recovery of S. enterica Typhimurium with a mutation in foxA cannot be a result of the inability to utilise these siderophores. It appears that FoxA may have an unknown role in addition to its role in ferrioxamine uptake.

Rabsch et al (2000) reported that a triple mutation resulting in the inability to utilise DHBS as a siderophore caused attenuated virulence upon intragastric or oral infection of mice compared to the wild type strain. However, mutations resulting in the inability to utilise enterobactin or salmochelin or to produce salmochelin showed virulence similar to the wild type strain. The strains from the studies by Rabsch et al (2003), ATCC 14028 (parental strain), WR1726 (fepA'), WR1727 (fepA' iroN'), WR1728 (fepA' iroN' cir') and WR7129 (fepA, iroN, iroBC') were all recovered from DCs in lower numbers at 24 hours compared to at 2 hours, confirming that the duration of infection affects survival of DCs or Salmonella or both, as discussed previously. After 2 hours, only the fepA iroN cir' mutant WR1728 was recovered from DCs at a percentage statistically different to the wild type. Contrary to the result obtained with the foxA mutant though, WR1728 was recovered at a lower percentage than the wild type. The triple mutation in WR1728 results in the inability to utilise enterobactin,
salmochelin and DHBS as siderophores. However, as the other mutants were recovered at levels similar to the wild type it appears that it is only the ability to utilise DHBS that effects recovery from DCs. DHBS is a breakdown product of enterobactin, a siderophore utilised by a wide range of bacteria, including *E. coli* (Pettis and McIntosh, 1987) and *Klebsiella pneumoniae* (Koczura and Kaznowski, 2003). Therefore, the inability of the *fepA iroN cir* mutant to utilise DHBS as a siderophore to take up iron during exposure to DCs could be the cause of the decrease in recovery from within DCs. It is also possible that, like FoxA, Cir also has a role beyond that of siderophore uptake.

Interestingly, after 24 hours the *fepA iroN cir* mutant was recovered from DCs at a percentage significantly different to the wild type, but this time a higher percentage were recovered. Are the difference caused by an inability to utilise DHBS initially affecting uptake into DCs (*fepA* mutant strains of serovar Typhi were reported as having a reduced ability to invade HeLa cells, Furman et al, 1994) but an attenuated ability to kill DCs once within the cells?

**Further investigation is required to** determine the roles of FoxA, DHBS uptake and Cir during *S. enterica* Typhimurium during infection of DCs. Makki (2003) suggested the *foxA* mutation caused an attenuated ability to kill DCs, although no work to confirm this was carried out. It has been reported that DCs kill ingested bacteria (*Schoppet et al., 2000; Nagl et al., 2002*), so it is also possible that the mutations protect *S. enterica* Typhimurium from killing by DCs. These aspects of the interactions of DCs and *Salmonella* would have to be investigated before the cause of the differences seen in recovery of the *foxA* and *fepA iroN cir* mutants and their respective parental strains could be confirmed.
CHAPTER FIVE

Uptake of *Salmonella enterica* Typhimurium iron uptake mutants into human DCs and their effect on DC maturation

5.1 Introduction

The gene products required for invasion of non-phagocytic cells by *Salmonella* are delivered by a TTSS and are encoded on SPI1 and SPI2. The SPI1 encoded proteins SipG and SipA promote membrane ruffling (Zhou *et al.*, 1999) and SopE, SopE2 and SopB are involved in the rearrangement of actin (Galen and Zhou, 2000). The SPI2 encoded proteins SseG and SifA promote survival and replication in the SCV (Deiwick *et al.*, 2006).

*Salmonella* can enter macrophages by either SPI1-dependent invasion (Galan, 1996) or phagocytosis (Alpache-Aranda, 1994). Irrespective of the process of internalisation *Salmonella* survive and replicate within SCVs, in a process dependent on the two component PhoP/PhoQ system. The morphology of the SCV and *Salmonella* intracellular gene expression are both determined by the method of internalisation (Drechtrah *et al.*, 2005).

*Salmonella* have also been shown to enter and survive within DCs (Marriott *et al.*, 1999), a process significantly enhanced by the Type 1 fimbrial adhesin FimH but does not require sipB, a gene essential for *Salmonella* mediated invasion of mammalian epithelial cells (Guo *et al.*, 2007). In contrast to macrophages and non-phagocytic cells (Garcia-del Portillo *et al.*, 1995; Oh *et al.*, 1996; Rathman *et al.*, 1997), trafficking of *S. enterica* Typhimurium within DCs is not associated with targeting to host vacuoles.
containing lysosomal membrane glycoproteins (LGPs). Furthermore, survival of a \textit{S. enterica} Typhimurium \textit{phoP} mutant strain in DCs was comparable to that of the wild type (Garcia-del Portillo \textit{et al}, 2000). The formation and characteristics of the SCV in DC appears to be different to that of other cells.

\textit{Salmonella} within DCs have been reported to cause host cell death; Sundquist and Wick (2008) reported that in \textit{S. enterica} Typhimurium-infected mice TNF mediates death of CD8α⁺ DCs but not CD11c\textsuperscript{int}CD11b⁺ inflammatory DCs. \textit{Salmonella} can also directly induce rapid DC death (within hours) by a mechanism dependent on \textit{sipB} and the SPI encoded TTSS system and host derived caspase-1 (van der Velden \textit{et al}, 2003). This cytotoxicity occurred in CD11c⁺ DCs from murine bone marrow, murine splenic DCs and human DCs; these findings are contrary to other reports of \textit{Salmonella} surviving in DCs for much longer periods. Pietila \textit{et al} reported that \textit{S. enterica} Typhimurium strain ATCC 14028 could be recovered from within DCs 3 days after exposure and Svensson \textit{et al} (2000) reported no change in bacterial recovery from murine bone marrow derived DCs after 48 hours exposure to \textit{S. enterica} Typhimurium.

The ability of DCs to regulate immunity is dependent on maturation and is characterised by a decreased capacity to capture antigen, increased surface expression of MHC and costimulatory molecules (CD80 and CD86), the secretion of chemokines CCL22, CCL17 and CCL18 (Lin \textit{et al}, 1998), cytokines and proteases, and the surface expression of adhesion molecules and chemokine receptors CXCR4 and CCR7 (Sallusto \textit{et al}, 1996).

Dendritic cells have been shown to mature upon infection by \textit{Salmonella}; murine splenic DCs underwent maturation upon exposure to \textit{S. enterica} Typhimurium (Yrlid \textit{et al}, 2001) as did monocyte derived DCs upon exposure to a range of strains of \textit{S. enterica} Typhimurium (Dreher \textit{et al}, 2001). In both these studies up-regulation of
MHC and costimulatory molecules was used to determine DC maturation. It has also been reported that the pathogenicity factors of *S. enterica* Typhimurium can affect the response of human monocyte derived DCs. Wild type *S. enterica* Typhimurium induced maturation but a non-flagellated strain (a *fliC* mutant), induced up-regulation of HLA-DR but increases in expression of CD80 and CD83 were not so significant (Rimoldi *et al*, 2005).

In the study by Makki (2003) it was suggested that the increased recovery of the *foxA* mutant from within DCs compared with wild type was a result of an attenuated ability to kill DCs. However, the entry of *Salmonella* into DCs is not fully understood and therefore it is also possible that the *foxA* mutation could have resulted in an increase in the numbers of *Salmonella* entering or surviving within DCs. The situation with WR1728 (*fepA* *iroN* *cir*) is more complex as the mutation reduced the number of bacteria recovered at 2 hours +exposure and increased it at 24 hours, compared to the parental strain (section 4.2.4).

This chapter describes experiments designed to explain the differences in recovery from DCs of the strains described in chapter 4 (Table 4.1). Green fluorescent protein (GFP)-labeled bacteria have been used in studies to quantify *E. coli* infected macrophages (Gille *et al*, 2006), as well as in studies involving *Salmonella* infections of M cells (Jang *et al*, 2004) and murine liver DCs (Johansson and Wick, 2004). Attempts were made to label the strains of bacteria with GFP using pGFP, followed by an assessment of uptake into DCs by flow cytometry. The effects of *foxA* and *fepA* *iroN* *cir* triple mutants on DC maturation were also analysed by flow cytometry.
5.2 Results

*S. enterica* Typhimurium strains with mutations in *foxA* and in *fepA iron cir* are recovered in higher numbers from within human DCs compared to their respective parental strains. This difference is not a result of differences in growth rates as determined in the previous chapter. *S. enterica* Typhimurium invade DCs but can also be phagocytosed therefore it is possible that the mutations have an affect on these processes resulting in different numbers within the DCs. There is also some debate as to whether DCs kill *Salmonella* or vice versa, in either case the mutations could be having an affect on this process resulting in a higher bacterial recovery. Determining the cause of the difference in recovery rates would provide information about the roles of the genes mutated and also about the interaction of *S. enterica* Typhimurium and DCs.

5.2.1 Production of GFP strains

To allow analysis of the changes in numbers of bacteria associated with DCs during infection using flow cytometry a plasmid containing the GFP gene (pGFP) was transformed into the strains previously investigated; SL1344/nr (wild type), SL2MK (*foxA*), CSL2MK (SL2MK, pWRSM), C2 (SL2MK, pRXH2), ATCC 14028 (wild type), WR1727 (*fepA*), WR1727 (*fepA* *iroN*), WR1728 (*fepA* *iroN cir*) and WR1729 (*fepA* *iroN iroBC*).

5.2.1.1 Transferring pGFP from SL1344 pGFP

The wild type strain SL1344 carrying pGFP was already available (courtesy of P. Everest, Glasgow Veterinary School) and from this strain, pGFP was isolated using the QIAprep Miniprep kit™ and quantified by electrophoresis. Electroporation was then used to transfer the plasmid DNA into competent cells of the previously mentioned *S. enterica* Typhimurium strains (sections 2.13.10 and 2.13.11). Transformed cells were selected by plating on ampicillin and then by identification of fluorescent colonies.
using a transilluminator. Positive colonies were re-cultured on LUA containing suitable antibiotics.

After a number of attempts, using this method the only strains that were successfully transformed were SL2MK and ATCC 14028. These two new strains were named SLJW1 (SL2MK, pGFP) and SLJW2 (ATCC 14028, pGFP).

5.2.1.2 Transferring an alternative plasmid carrying GFP

An alternative plasmid was used in an attempt to transfer GFP into the strains of interest. The plasmid chosen was kanamycin resistant pTR1-ompE GFP expressing plasmid extracted from E. coli PTRI-pMEK91-5 DHα5E. Competent cells were again produced for the strains and electroporation was used to introduce the plasmid. Transformants were selected using LUA plates containing 100 µg/ml of kanamycin. After a number of attempts using this method no transformants were produced for any of the strains.

5.2.1.3 Transforming SLJW1 with pWRSM or pRXH2

The previous two methods were unsuccessful at transferring plasmids carrying the gene for expression of GFP into the complemented foxA strains CSL2MK and C2, as well as into the IROMP mutants WR1726, WR1727, WR18728 and WR1729. To create the complemented GFP strains attempts were made to transfer pRXH2 (from C2) and pWRSM (from CSL2MK) into SLJW1 (foxA⁺, pGFP). Transformation of the other strains would be considered at a later date.

The purified plasmids pRXH2 and pWRSM were already available from previous work during this study. Competent cells of strain SLJW1 (foxA⁺, pGFP) were prepared and transformed by electroporation with either pWRSM or pRXH2. Transformants were selected for by blue/white selection on LUA plates containing X-
GAL and IPTG. Unfortunately, after repeated attempts no positive colonies were recovered.

5.2.2 Infection of DCs with GFP strains

Although the previous experiments failed to produce GFP-expressing mutants of every strain under investigation, the mutant foxA strain SL2MK was transformed and the wild type strain SL1344 carrying GFP was also available (SL1344 pGFP provided by P. Everest). As previous complementation studies showed that differences in recovery numbers from DCs were results of the foxA mutation, then comparisons of numbers of bacteria per cell could be made without the GFP expressing, foxA complemented strains.

5.2.2.1. Comparison of GFP strains to their parental strains

In order to confirm that the strains expressing GFP behave in the same way as their parental strains with respect to their recovery from DCs, gentamicin killing assays (section 2.15.2) were carried out over 2 hours and 24 hours using the strains SL1344 (wild type), SL1344 pGFP, SL2MK (foxA') and SLJW1 (foxA', pGFP), as well as CSL2MK (SL2MK, pWRSM) and C2 (SL2MK, pRXH2). The results show that GFP expressing strains were recovered at lower numbers than their parental strains at both time points. At 2 hours (Fig. 5.1a) recovery for SL1344 pGFP was 21% less than SL1344/nr and for SLJW1 was 22 % less than SL2MK. At 24 hours (Fig. 5.1b) recovery of SL1344 pGFP was 14% less than SL1344/nr and SLJW1 was 13% less than SL2MK.

The results also show that despite the difference in numbers between the GFP and their parental strains they still followed the same pattern of recovery when comparing the foxA mutants with their parental strains. At 2 hours (Fig. 5.1a) SL2MK was recovered in higher numbers than SL1344/nr (200%) and SLJW1 was recovered in
higher numbers than SL1344 pGFP (197%). At 24 hours (Fig. 5.1b) SL2MK was again recovered in higher numbers than SL1344/nr (155%) and SLJW1 in higher numbers than SL1344 pGFP (153%). At both time points the complemented mutants showed a similar pattern of recovery to the parental strain SL1344/nr, as recorded in previous experiments (Chapter 4), with slightly lower recovery rates but these differences are not considered significantly different. Results are the mean of six experiments and standard deviations were also calculated to provide an indication of variance. These experiments confirm that despite being recovered in smaller numbers the GFP mutants behaved in the same way as their parental strains with respect to recovery from DCs and therefore they can be used in future experiments to analyse differences between bacterial uptake into DCs.
Fig. 5.1 Recovery of *S. enterica* Typhimurium strains from within DCs after 2 hour (a) or 24 (b) hour exposure. DCs were exposed to the following strains of *S. enterica* Typhimurium for 2 hours or 24 hours; SL1344/nr, SL1344 pGFP, SL2MK, SLJW1, CSL2MK and C2. After incubation DCs were lysed and the surviving intracellular bacteria quantified after plating. The results are the mean of six experiments and show that the *foxA* mutant strains SL2MK and SLJW1 were recovered at a significantly higher number than their respective parental strains SL1344/nr and SL1344 pGFP, and the complemented strains CSL2MK and C2.
5.2.2.2 Time course of DC infection with GFP strains

Flow cytometry and the GFP strains were used to investigate whether differences in bacterial uptake into DCs could be the cause of the differences in numbers of bacteria recovered. The GFP producing *S. enterica* Typhimurium strains, SL1344 pGFP and SLJW1 were exposed to DCs for 24 hours. At time points during the infection samples were taken, washed in PBS and then analysed using flow cytometry. Using MFI (to compare differences in bacterial concentrations associated with DCs) and percentages of cells positive (to compare percentages of DCs infected with bacteria) a comparison between the *foxA* mutant and its parental strain was used to determine whether there were any differences in the concentrations of bacteria within DCs at 2 hours and 24 hours exposure times.

The results using the percentage of cells positive show a difference between the two strains at 2 hours; SL1344 pGFP (32%) and SLJW1 (29%); at 24 hours there was a greater difference; SL1344 pGFP (74%) and SLJW1 (67%) (Fig. 5.2a). The results using MFI also showed differences between the strains at 2 hours, SL1344 pGFP (93) and SLJW1 (86), and again at 24 hours there was a greater difference, SL1344 pGFP (203) and SLJW1 (241) (Fig. 5.2b). Using paired t tests with a 95% confidence limit, only the difference at 2 hours for percentage cells positive is considered significant. It should also be noted that at 10 hours the MFI results showed a significant difference between the two strains; SL1344 pGFP (356) and SLJW1 (509). Experiments were repeated four times and the mean of these values was used to calculate the changes in expression; standard deviations were also calculated to provide an estimate of variance. These results show that the increased recovery of *S. enterica* Typhimurium from DCs as a result of the mutation in *foxA* cannot be directly correlated to an increase in numbers of bacteria entering DCs.
Fig. 5.2. Percentage positive (a) and MFI (b) of DCs for bacterial infection with SL1344 pGFP (green) and SLJW1 (SL2MK pGFP) (pink) over 24 hour infection. DCs were exposed to bacterial strains for 24 hours; at time points during infection DCs were washed and analysed by flow cytometry. The markers were set using the DCs not exposed to bacteria so that less than 1% of cells were positive. The results include standard deviations and are the means of four experiments.
5.2.3 Maturation of DCs exposed to *S. enterica* Typhimurium

The maturation of DCs is a process with an important role in the development of an immune response to bacterial infection. As previously described it can be determined by changes in cell surface marker expression (Chapter 3). Exposure to bacteria has been shown to result in DC maturation in a number of studies (Hu *et al.*, 2006; Baba *et al.*, 2008) and previous studies with *Salmonella* have shown that degree of maturation can be affected by mutations that affect pathogenicity of that strain (Rimoldi *et al.*, 2005).

The aim of the following experiments was to confirm whether exposure to strains of *S. enterica* Typhimurium used in this study caused maturation of DCs equally. Comparisons would also be made between the effects on maturation of DCs of the strains with mutations resulting in increased recovery from DCs and their respective wild type and complemented strains. Therefore the cell surface phenotype of DCs was determined after exposure to the strains; SL1344/nr, SL2MK (*foxA*), CSL2MK (SL2MK pWRSM), C2 (SL2MK pRXH2) and to ATCC 14028 and WR1728 (*fepA*–*iroN*–*cir*). DCs were exposed to bacteria and controls as described in section 2.15.3 and analysis of CD marker expression compared by flow cytometry to determine DC phenotypes.

### 5.2.3.1 Maturation of DCs exposed to *foxA* strains

Comparison of the percentage of DCs expressing each cell surface marker (Fig. 5.3a) showed that upon exposure to the *foxA* mutant, its wild type parental strain and complemented strains DCs had a lower expression of CD1a than unstimulated DCs (a decrease of 16-19%). The DCs exposed to these strains had a higher expression of a number of markers compared to unstimulated DCs: CD80 (increase of 29-33%), CD86 (increase of 28-33%) and HLA-DQ (increase of 35-45%). Compared to LPS-stimulated
DCs the DCs exposed to these strains had similar expressions of CD1a (differences <7%), CD80 (differences <4%), CD86 (differences <4%) and HLA-DQ (differences <10%). The expression of CD14 (differences <2%) and HLA-DR (differences <10%) was similar for all DCs.

MFI (Fig. 5.3b) showed that compared to unstimulated DCs, those exposed to the foxA mutant, its wild type parental strain and complemented strains had a lower expression of CD1a (decrease of 0-24). The DCs exposed to these strains had a higher expression of a number of markers compared to unstimulated DCs; CD80 (increase of 228-265), CD86 (increase of 242-263), HLA-DQ (increase of 429-465) and HLA-DR (increase of 419-641). Compared to LPS-stimulated DCs the DCs exposed to these strains had similar expressions of CD1a (differences <24), CD80 (differences <30), CD86 (differences <21), HLA-DQ (differences <18) and HLA-DR (differences <186). Expression of CD14 was similar for all DCs (differences <27). Experiments were repeated three times and the means of the values obtained used to calculate the changes in expression, standard deviations were also calculated to provide an estimate of variance. Comparisons between cell surface marker expression of DCs exposed to SL1344/nr compared to SL2MK, CSL2MK and C2 showed no significant differences using a paired t test with 95% confidence.

These results show that exposure of DCs to the foxA mutant, its wild type parental strain and complemented strains results in DCs with a phenotype which has more similarities to those exposed to LPS than to unstimulated DCs. This suggests that DCs exposed to S. enterica Typhimurium strains SL1344/nr, SL2MK, CSL2MK and C2 undergo maturation. Furthermore, it appears that the mutation of foxA has no significant effect on DC maturation.
Fig. 5.3. Percentage (a) and MFI (b) of cells expressing cell surface markers on gated populations of DCs; left unstimulated (red) or exposed for 48 hours to LPS (blue), SL1344/nr (yellow), SL2MK (turquoise), CSL2MK (purple) or C2 (pink). Cell surface marker expression on DCs exposed to LPS or bacteria or left unstimulated were determined by flow cytometry. The results shown are the means of three experiments and indicate that DCs exposed to these strains of *S. enterica* Typhimurium undergo maturation.
5.2.3.2 Maturation of DCs exposed to WR1728 (*fepA* `iroN` `cir`*) and to ATCC 14028.

Comparisons of the percentages of DCs expressing cell surface markers (Fig. 5.4a) showed that upon exposure to WR1728 and its parental strain (ATCC 14028) DCs had a lower expression of CD1a compared to unstimulated DCs (decreases of 17% and 16% respectively). The DCs exposed to these strains had a higher expression of a number of markers compared to unstimulated DCs; CD80 (+35% - WR1728 and +39% - ATCC 14028), CD86 (+32% - WR1728 and +35% - ATCC 14028) and HLA-DQ (+43% WR1728 and +37% ATCC 14028). Compared to LPS-stimulated DCs the DCs exposed to these strains had similar expressions of CD1a (differences <5%), CD80 (differences <6%), CD86 (differences <3%) and HLA-DQ (differences <8%). Expression of CD14 (differences <1%) and HLA-DR (differences <7%) were similar for all DCs.

MFI (Fig. 5.4b) showed that compared to unstimulated DCs, DCs exposed to WR1728 and its parental strain (ATCC 14028) had a lower expression of CD1a (decreases of 33 and 26 respectively). The DCs exposed to these strains had a higher expression of a number of markers compared to unstimulated DCs; CD80 (+229 - WR1728 and +233 - ATCC 14028), CD86 (+249 - WR1728 and +307 - ATCC 14028), HLA-DQ (+387 - WR1728 and +422 ATCC 14028) and HLA-DR (+645 – WR1728 and +715 – ATCC 14028). Compared to LPS-stimulated DCs the DCs exposed to these strains had similar expressions of CD1a (differences <10), CD80 (differences <29), CD86 (differences <44), HLA-DQ (differences <60) and HLA-DR (differences <260). Expression of CD14 (differences <6) was similar for all DCs. Experiments were repeated three times and the means of the values obtained used to calculate the changes in expression, standard deviations were also calculated to provide an estimate of
variance. Comparisons between cell surface marker expression of DCs exposed to ATCC 14028 compared to WR1728 showed no significant differences using a paired t test with 95% confidence.

These results show that exposure of DCs to ATCC 14028 and WR1728 results in DCs with a phenotype which has more similarities to those exposed to LPS than to unstimulated DCs. This suggests that DCs exposed to *S. enterica* Typhimurium strains ATCC 14028 and WR1728 undergo maturation. Furthermore, it appears that the combined mutations of *fepA, iroN* and *cir* have no significant effect on DC maturation.
Fig. 5.4. Percentage (a) and MFI (b) of cells expressing cell surface markers on gated populations of DCs; left unstimulated (red) or exposed for 48 hours to LPS (blue), ATCC 14028 (green) or WR1728 (yellow). Cell surface marker expression on DCs exposed to LPS or bacteria or left unstimulated were determined by flow cytometry. The results shown are the means of three experiments and indicate that DCs exposed to these strains of *S. enterica* Typhimurium undergo maturation.
5.3 Discussion

To further our understanding of the roles of FoxA and the ability to utilise of 2,3-DHBS, enterobactin and salmochelin as siderophores on the survival of *S. enterica* Typhimurium within DCs attempts were made to transfer a plasmid expressing GFP into all strains used during this study (listed in Table 4.1). After attempts using a number of strategies, only SL2MK (*foxA*') and ATCC 14028 (wild type) could be transformed, producing the strains designated SLJW1 and SLJW2 respectively. It is possible that the strains CSL2MK and C2 could not be transformed due to an incompatibility between pGFP and the plasmids used to confer complementation of the *foxA* mutation (pWRSM and pRXH2 respectively). The other iron uptake mutants produced by Rabsch *et al.* (2000) – WR1726, WR1727, WR1728 and WR1729 were also not able to be transformed. To ensure the insertion of the plasmid carrying pGFP had not affected the pattern of recovery of these strains from DCs, gentamicin killing assays were carried out with the GFP strains. These showed that compared to their parental strains, the GFP mutants were recovered in lower number from within DCs. This can be explained by evidence that GFP expression can place a noticeable metabolic burden on bacteria (Wendland and Bumann, 2002; Tombolini *et al.*, 1999). However, it was also shown that SLJW1 (SL2MK, pGFP) was recovered from DCs in higher number than its parental strain (SL1344 pGFP). This confirms that the GFP strains follow the same pattern of recovery from DCs.

A number of studies have used GFP strains and flow cytometry to analyse bacterial infection of mammalian cells; including macrophage infection by *S. enterica* Typhimurium, *Yersinia pestis* and *Mycobacterium marinum* (Valdivia *et al.*, 1996) and murine liver DCs infection by *S. enterica* Typhimurium (Johanssen and Wick, 2005). Analysis of DCs exposed to SL1344 pGFP and SLJW1(*foxA*, pGFP) showed that the
percentages of DCs infected after 2 hours were statistically different but at 24 hours percentages were similar for both strains. At the 24 hour time point there were far fewer events being recorded by the flow cytometer, suggesting a decrease in total DCs. The difference in bacteria associated with each DC, as shown by MFI, between the strains were not statistically different at either 2 hours or 24 hours, The only statistical difference between the strains was the percentage of cells positive after 2 hours; the wild type result was higher. So at this time point if there were more wild type bacteria associated with the DCs how can a greater number of the foxA strain be recovered using the gentamicin assays? One possible explanation is that foxA is more effective at invasion of DCs and so a small number are within the DCs whereas the wild type are all associated with the exterior of the DCs. However, if this is the case then the difference should also be reflected in the MFI. It therefore appears that the results from this study cannot easily be used to explain the increased recovery of foxA mutants from DCs.

The development of alternative methods to investigate differences in the invasiveness of S. enterica Typhimurium strains during infection of DCs and the ability of DCs to phagocytose these strains could lead to more informative results. Fluorescence microscopy could allow differences in number of bacteria associated with DCs to be determined; the ability of murine DCs to transport bacteria across monolayers was determined using confocal fluorescence microscopy analysis of GFP-Salmonella (Resigno et al, 2001) and the susceptibility of human DCs to invasion by Brucella spp. was visualised using GFP bacterial strains and videomicroscopy (Billard et al, 2005).

Dextrans are hydrophilic polysaccharides produced by Leuconostoc bacteria (Panov et al, 1977) with properties that make them effective water-soluble carriers for dyes, indicators, and reactive groups in a wide variety of applications. DCs were shown to phagocytose Dextran-FITC (Xia and Kao, 2002). Exposure of DCs to GFP-
Salmonella in the presence of Dextran Texas Red and visualisation using fluorescence microscopy should determine whether or not DCs phagocytose Salmonella enterica Typhimurium.

The ability of DCs to present antigen to T cells and modulate the development of an immune response is reliant on the maturation of DCs. A number of phenotypic and morphological changes occur during maturation but it is the changes in cell surface marker expression which are usually used to determine maturation in response to stimuli. Studies by Yrlid et al (2001) and Dreher et al (2001) showed that DCs undergo maturation in response to Salmonella exposure. Any effects that the foxA and fepA, iroN, cir mutants have on maturation could be important in determining whether these genes could be considered as potential targets for the development of vaccines.

Exposure of DCs to the foxA and the fepA iroN cir mutants resulted in upregulation of the co-stimulatory molecules CD80, CD86, HLA-DQ and HLA-DR to levels comparable to those seen on DCs exposed to LPS confirming that they cause maturation of DCs. There were no statistically significant differences between cell marker expression on DCs exposed to the mutant strains compared to their parental strains confirming that the mutation in foxA and the triple mutations fepA, iroN, cir have no effect on DC maturation induced by S. enterica Typhimurium.

Results from this chapter provide limited information about the reasons for the differences between recovery of the mutant and their parent strains, differences were noted between infection of DCs by foxA mutant strain and the wild type but these could not easily be associated with the gentamicin killing assay results. However, a foxA mutant strain and its parental strain that express GFP were produced and are available for use in future studies. The foxA and the fepA iroN cir mutants were also shown to cause DC maturation comparable to their parental strains.
CHAPTER SIX

Discussion

6.1 General Discussion

The main aim of this study has been concerned with the assessment of the S. enterica Typhimurium ferrioxamine receptor gene foxA and of other iron uptake mutants described by Rabsch et al (2000) and their involvement during infection of human DCs.

Bacteria of the genus Salmonella are the causative agent of a number of foodborne diseases which affect a broad spectrum of host species. In man, these illnesses include gastroenteritis, septicaemia and typhoid fever. The many non-typhoid strains (including Typhimurium and Enteritidis) are capable of infecting a wide range of other animal hosts including mice, chickens, cattle and pigs. However, infection of certain inbred mice with S. enterica Typhimurium results in an enteric fever often used as a model for typhoid fever in humans.

In environments where iron is limited bacteria utilise iron-complexing agents called siderophores to satisfy their need for iron. Bacteria can produce these siderophores themselves (endogenous); enterobactin by E. coli and yersiniabactin by Yersinia pestis; or can utilise siderophores synthesised by other species; Campylobacter species can utilise ferrichrome (Baig et al, 1986) produced by many fungal species including Ustilago sphaerogena and Penicillium species and H. parainfluenzae can utilise enterobactin produced by bacterial species such as E. coli (Williams et al, 1990). The ability to recognise ferric siderophore complexes is dependent on the presence of specific iron-regulated outer membrane proteins (IROMPs); FyuA is the IROMP for yersiniabactin (Rakin et al, 1994), FepA the receptor for enterobactin (Hollifield et al,
IutA the receptor for aerobactin (Carbonetti and Williams, 1984). Despite the requirement of iron, experiments involving mutations of single genes involved in the production of siderophores or in IRMPS have generally been shown to have little effect on bacterial virulence which is likely to be the result of redundant systems for iron uptake. However, a number of studies have reported exceptions; S. enterica Typhi mutants defective in enterobactin synthesis or transport exhibit attenuation of virulence in mice (Furman et al., 1994) and the aerobactin system encoded on the ColV plasmid enhances the virulence of strains of E. coli in a mouse model of infection (Williams et al., 1979).

Dendritic cells (DCs) are antigen presenting cells with a major role in the activation and proliferation of T cells resulting in an immune response to antigen. The development of culture techniques using progenitors in bone marrow (Young et al., 1995) and peripheral blood (Romani et al., 1996) has enabled studies that have increased our understanding of these cells during immune responses to viruses (including HIV (Blauvelt et al., 1997; Cougnett et al., 1999) and bacteria as well as during autoimmune responses (Akbari and Umetsu, 2005). The response of DCs to infection by Salmonella has mainly involved the study of murine DCs and serovar Typhimurium (Rescigno et al., 2006; Guo et al., 2007; Grassl and Finlay, 2008).

6.2 Methods of culturing human DCs

The first part of this study described the analysis of methods used to produce cells with a DC phenotype from peripheral blood. A plastic adherence method was used to isolate DC precursors from peripheral blood before differentiation into DCs using the cytokines GM-GSF and IL-4. DCs do not carry a single marker that can be used for identification, and so changes in a range of cell surface marker expression were used to confirm differentiation of precursors into DCs. These cells were also exposed to LPS,
which is known to induce DC maturation (Granucci et al, 1999). Results confirmed that DCs prepared using this method have a phenotype comparable to that described in the literature (Sallusto and Lanzavecchia, 1994). Upon exposure to LPS DCs cultured by this method, upregulated expression of costimulatory molecules confirming they had undergone maturation.

A second method was also investigated using lower cytokine levels described by Zou et al (2000). The cells produced by this method (DC_{low}) were found not to have the same phenotype as DCs nor did they undergo maturation upon exposure to LPS. Upon further analysis it was found that the DC_{low} phenotype differed from that of both adherent cells (undifferentiated DC precursors) and cultured macrophages (these cells can be cultured in vitro from peripheral blood using only GM-CSF (Komuro et al, (2001). However, DC_{low} were similar to DC precursors on day 6 of their culture with cytokines suggesting that decreasing the cytokine concentration delays differentiation of precursors into DCs. As DC_{low} did not mature upon exposure to LPS it also confirms the necessity of the seven day culture to produce functional DCs. Changes in cell surface marker expression during the seven day culture period occurred gradually with the exception of CD86 which remained low until day 6 when it rapidly increased to a level similar to that seen on DCs. The reasons for this sudden change in expression however, were not investigated further.

6.3 The role of FoxA during infection of human DCs

The utilisation of ferrioxamines as sole sources of iron by S. enterica Typhimurium and Enteritidis distinguishes them from related species, including E. coli. Kingsley et al (1999) reported that transport of ferrioxamines B, E and G are dependent on the FoxA outer membrane receptor encoded by the Fur repressible foxA gene, with transport of ferrioxamine G also dependent on the energy-transducing protein TonB.
Mutations in *foxA* have also been reported to cause attenuation of virulence in mouse models of *S. enterica* Typhimurium infection (Kingsley *et al*, 1999).

To investigate the role of *foxA* during infections of human DCs a *foxA* mutant strain carrying a complementing plasmid was produced designated C2 (*foxA<sup>-</sup>* pRXH2). The cloned fragment containing *foxA* contained far less flanking sequence than the fragment used by Makki (2003) to produce the complemented strain, CSL2MK, ensuring that complementation of polar effects of the *foxA* mutation on neighbouring genes could not occur.

Gentamicin killing assays determined that the *foxA* mutant was recovered at a significantly higher percentage from within DCs than the wild type and the mutants complemented with *foxA<sup>+</sup>* were both recovered at percentages similar to the wild type. Makki (2003) suggested that the results were due to an attenuated ability of *foxA* mutants to kill DCs; however, no work was carried out to confirm this. As ferrioxamine is not present in DCs or the culture media it does appear that FoxA may have another role besides that of the receptor for ferrioxamine.

*S. enterica* Typhimurium exposure to DCs has been reported to have different outcomes; van der Valden *et al* (2003) reported the rapid killing of DCs by Typhimurium whereas Nagl *et al* (2002) reported the killing of ingested bacterial strains by human DCs. It is perhaps the method of entry of *Salmonella* into DCs that causes these differences in outcomes as entry can occur by either invasion or by phagocytosis, and it has been reported in macrophages that apoptosis is induced by *S. enterica* Typhimurium invasion but entry by receptor-mediated phagocytosis protects macrophages from apoptosis (Forsberg *et al*, 2003).

Using a wild type strain expressing GFP (SL1344 pGFP) and SLJW1, a GFP-expressing *foxA* mutant strain produced during this study, it was possible to analyse the
effect of the foxA mutation on infection of DCs. Results showed little difference between the strains upon comparison of the numbers of DCs infected or the number of bacteria associated with each infected DC during 24 hours exposure except at the 2 hours time point. A higher percentage of DCs were infected with the wild type than infected with the foxA mutant strain. If the increase in recovery of S. enterica Typhimurium from DCs as a result of the foxA mutation was caused by an increased ability to infect DCs then a higher percentage of DCs should be infected with the mutant strain. Therefore, this result is difficult to reconcile with the results from the gentamicin assay.

6.4 The role of other iron uptake mutations during infection of human DCs

The outer membrane proteins FepA, IroN and Cir are catecholate receptors. FepA and IroN are receptors for the siderophore enterobactin and all three proteins act as receptors for its breakdown product 2,3-dihydroxybenzoylserine (DHBS). Rabsch et al (2003) reported that upon intragastric infection of mice with S. enterica Typhimurium strains carrying mutations in these genes, the strains with a fepA mutation and a fepA iroN double mutation were similarly virulent to the parental strain however; a triple mutant fepA iroN cir strain was significantly attenuated. These reports suggest that enterobactin uptake is not essential for Typhimurium virulence but that the inability to uptake DHBS by any of these three receptors appears to play an important role.

The Typhimurium mutant strains used in that study, ATCC 14028 (wild type); WR1726 (fepA); WR1727 (fepA iroN); WR1728 (fepA iroN cir); WR1719 (fepA iroN iroBC), a kind gift from W. Rabsch, Robert Koch-Institut, Wernigerode, were used in gentamicin assays. Only the triple fepA iroN cir mutant WR1728 was recovered from DCs in significantly different numbers to the wild type strain and the it appears that the mutations have different effects upon initial exposure to those seen after prolonged
exposure. Is it possible that like FoxA, Cir could have another role as yet unidentified that affects recovery from DCs? Does the triple mutation cause an initial decrease in uptake in DCs but once inside they have an attenuated ability to kill DCs or are able to better avoid killing by the DCs?

6.5 Maturation of DCs exposed to *S. enterica* Typhimurium

Maturation of DCs is an important stage in the development of an immune response to antigen and has been shown to occur upon exposure to strains of *S. enterica Typhimurium* (Yrlid *et al.*, 2001; Dreher *et al.*, 2001; Biedzka-Sarak and El Skurnik, 2006). Analysis of cell surface marker expression of DCs showed that infection by strains of *S. enterica Typhimurium* causes DCs to mature. There were no significant differences in the degree of maturation caused by the *foxA* and *fepA iroN cir* mutant and their parental strains suggesting these mutations do not affect the ability of DCs to mature in response to infection by *S. enterica* Typhimurium.

6.6 Further work

The production of the strain, SLJW1 (SL2MK, pGFP) and the availability of the its parental strain SL1344 pGFP opens up the possibility of analysing the interactions of these strains with DCs using fluorescence microscopy.

A complemented strain of the triple *fepA iroN cir* mutant was not available to confirm that the effects seen were the result of mutations in these genes; neither was a strain carrying only mutations in *iroN or cir*. Also, only the wild type ATCC 14028 could be transformed with pGFP. Production of all these strains and their use in gentamicin assays and with fluorescence microscope techniques may further our understanding of the cause of the differences in recovery of the *fepA iroN cir* mutant strain and its parental strain from human DCs.
Attempts were made to investigate the DC death (not included in results) as a result of exposure to these strains using analysis by flow cytometry of DCs stained using Annexin V-FITC to detect apoptosis and propidium iodide to detect necrosis of DCs. Unfortunately, no reproducible results were obtained and time constraints prevented further study. Determining differences in DC death as a result of exposure to the different strains would be key in confirming whether or not the increase in recovery from DCs of the foxA mutant strain and the triple fep iroN cir mutant (after prolonged exposure) is a result of an attenuated ability to kill DCs.
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