1 Introduction

1.1 The global burden of infectious disease

Despite increasing availability of therapeutics and direct targeting by healthcare systems, infectious disease remains a major global challenge [1, 2]. The spectrum of infections differs geographically according to climate and environmental factors, and is closely related to economic development [2, 3]. Common infections in economically developed countries are often associated with healthcare settings, where there are higher concentrations of compromised individuals [4]. In contrast, many classical infectious diseases such as typhoid and cholera remain common in less economically developed regions [5, 6]. Infectious diseases can transmit directly between humans, from the environment, or from zoonotic sources, and the contribution of different transmission routes varying according to region.

Infection-related illness represents a dynamic problem. In an age of globalisation, with local and international travel greater than ever before, there are a growing number of infectious agents moving readily between healthcare institutions, countries and even continents, presenting an integrated global threat [7, 8]. Microbes can evolve rapidly, in part due to their short generation time, with selective pressures such as human therapeutic interventions (including antibiotics) driving change. The emergence of antibiotic resistant microbes and pathogen evasion of current vaccines further compound the challenge of preventing and treating infectious disease, and there is increasing recognition that new approaches are required to combat such threats [9].

Fortunately we live in an era in which new technologies and research themes are generating deeper insight into the molecular basis of infection and the epidemiology of disease, providing understanding, which we hope will lead to future effective disease interventions. Epidemiological work has helped to understand the makeup of the human (and zoonotic) population exposed to the infection threat, and guides policy decisions, such as prioritising between therapies and infrastructure. We are beginning to discover how colonisation by microbes (the microbiota) can influence health in a multitude of ways, from protection against infection, to involvement in diseases previously classified as non-communicable. For example, conditions such as inflammatory bowel disease and certain

allergies may be associated with dysbiosis, involving host colonisation by 'unhealthy' microbial communities which drive pathogenic immune responses [10]. Indeed, pathogenic microbes may be evolving from such communities [11].

1.1.1 Incidence, morbidity and mortality of diarrhoeal disease

Diarrhoeal diseases remain a major cause of illness and mortality. Diarrhoea can be caused by many classes of pathogen, including viruses (e.g. *Rotavirus*), bacteria (e.g. certain strains of *Escherichia coli* and *Salmonella*) and parasites (e.g. *Giardia* spp. and *Cryptosporidium* spp.); by the microbes themselves, and through intoxication by the toxins they produce. Thus the epidemiology of diarrhoea is complex, although there may be common underlying mechanisms associated with this syndromic event. Despite the large number of causes of diarrhoea, paediatric cases at healthcare centres in the developing world can be described by a just a few clinical syndromes. Simple gastroenteritis, accounting for around 80% of episodes, is characterised by loose watery stool, low grade fever, occasional vomiting, anorexia, weakness and discomfort. A further 5 - 15% of cases are described as overt dysentery, with obvious bloody diarrhoea indicating extensive damage to the intestinal mucosa, accompanied by fever, sometimes high. More rare are cases of profuse watery diarrhoea leading rapidly to severe dehydration, persistent diarrhoea, and diarrhoea accompanying extensive vomiting [12].

Young children are most severely affected; in 2011 diarrhoeal disease was estimated to account for 10% of the 6.9 million deaths in children under five, and of these 72% of deaths occurred in infants under two years of age [13, 14]. In developing countries classical childhood diarrhoea is extremely common; in 2010 there were an estimated 1.7 billion episodes of diarrhoea globally, with the highest incidence of disease in Africa and Southeast Asia [14]. Just 15 countries, including some of the poorest nations of the world, account for over half of the known global cases of diarrhoea. Progression to severe episodes of disease and case-fatality rates are much higher in low-income regions; Southeast Asia and Africa each contributed 26% to the total number of severe cases of diarrhoea in 2011 [14]. The intimate relationship between under-nutrition and diarrhoeal disease is a major factor in determining the distribution of severe disease. Chronic malnutrition predisposes children to severe diarrhoeal disease, which in effect often causes injury to the gut, leading to impaired nutrient absorption and slower growth. Consequently diarrhoea presents a major obstacle to child development in poorer nations [15].

Though a continuing obstacle to human health, reductions in diarrhoeal disease seen in recent years give cause for optimism. In the first 10 years of the millennium deaths in children under five decreased by 2 million globally, with reductions in diarrhoea, measles and pneumonia contributing most significantly to this decrease, diarrhoea individually contributing 18% [16]. The advent of the new millennium brought several initiatives with commitments to reduce global diarrhoeal disease including the UN Millennium Declaration and Development Goal 4 to reduce the under-five mortality rate, and the launch of the Bill and Melinda Gates Foundation, and Global Alliance for Vaccines and Immunization (GAVI). Improvements in hygiene and sanitation infrastructure are helping to cut transmission of enteric pathogens, and the improved availability of vaccines is contributing also [17]. Many significant challenges remain. The need to increase coverage of positive interventions is holding up progress. Vaccines against enteric pathogens with high efficacy in developed countries have in several cases failed to recreate the same levels of protection in developing nations [18].

A lack of detailed investigations into the burden and aetiology of diarrhoeal disease has until recently presented a barrier to implementation of effective interventions to combat it. The Global Enteric Multicentre Study (GEMS) was funded in 2006 to address this need, enrolling over 9,000 children with moderate diarrhoea and over 13,000 control children without diarrhoea across four sites in Africa and three in Asia [12, 19]. The study presented many interesting findings regarding the distribution and prevalence of enteric pathogens. Most cases of moderate to severe diarrhoea in children aged zero to five attributable to a specific pathogen were caused by *Rotavirus*, *Cryptosporidium* spp., Enterotoxigenic *Escherichia coli* (ETEC) producing heat-stable toxin, and *Shigella* spp. Some pathogens were strongly associated with the presence of diarrhoea but many were weakly associated, resulting in difficulties in determining the aetiological agent behind many diarrhoeal episodes. Clear patterns emerged in pathogen distribution; certain pathogens such as *Rotavirus* and *Cryptosporidium* spp. were found to be a common cause of diarrhoea at all test sites, whilst others, including the genus *Salmonella,* showed a more restricted distribution.

In developed countries, infectious diarrhoea is often zoonotic in origin and transmitted via food contaminated with animal waste, or improperly cooked meat products [20, 21]. Diarrhoea is also associated with antibiotic treatment and hospitalisation, with *Norovirus* and *Clostridium difficile* major causes of hospital diarrhoea. The economic impact of these is sizeable; in 2002 it was estimated that the cost of *C. difficile* disease in the United States

exceeds $1.1 billion per year [22]. Mortality from these infections is largely restricted to the elderly and patients with comorbidities [23, 24].

1.2 Biology of the gastrointestinal tract

1.2.1 The gastrointestinal mucosa

The human gastrointestinal tract runs from the mouth to the anus with an approximate length of nine metres, including multiple organs and regions with specialised structure and function in the absorption of nutrients from the diet. The innermost region surrounding the lumen, the mucosa, comprises three layers; at the centre the epithelium, surrounded by lamina propria followed by the muscularis mucosae. The following sections describe the mucosa of the small and large intestine.

1.2.1.1 The epithelium

The epithelium comprises a single layer of polarized cells arranged in crypt and villus structures that forms a physical barrier separating host tissues from the external environment. With a surface area of ~ 400 m^2 the intestinal mucosa is the largest mucosal surface of the body. The majority of intestinal epithelial cells are absorptive enterocytes, the major function of which is the transport of dietary nutrients such as glucose and amino acids across the epithelial barrier. In addition, enterocytes perform important functions in sensing and responding to microbial stimuli to trigger appropriate immune responses; both tolerance to dietary antigens and immune activation for pathogen responses [25]. Positioned between the absorptive enterocytes are epithelial cell types of specialised function, including intestinal epithelial stem cells and secretory cells including paneth, goblet, and enteroendocrine cells.

Intestinal epithelial stem cells reside at the base of the mucosal crypts and divide indefinitely to replace the huge number of epithelial cells which die and are sloughed off the surface of the epithelium. The environment of the intestinal lumen is harsh. Toxic molecules originating from both the diet and the microbial community are in continual contact with cells, and consequently the turnover time of the gastrointestinal epithelium is as little as ≤ 3 days [26]. Division of intestinal adult stem cells produces highly proliferative progenitor cells. As these migrate upwards from the crypt base they differentiate to form all the different epithelial cell lineages, with the exception of paneth cells which develop through a separate dedicated pathway [27].

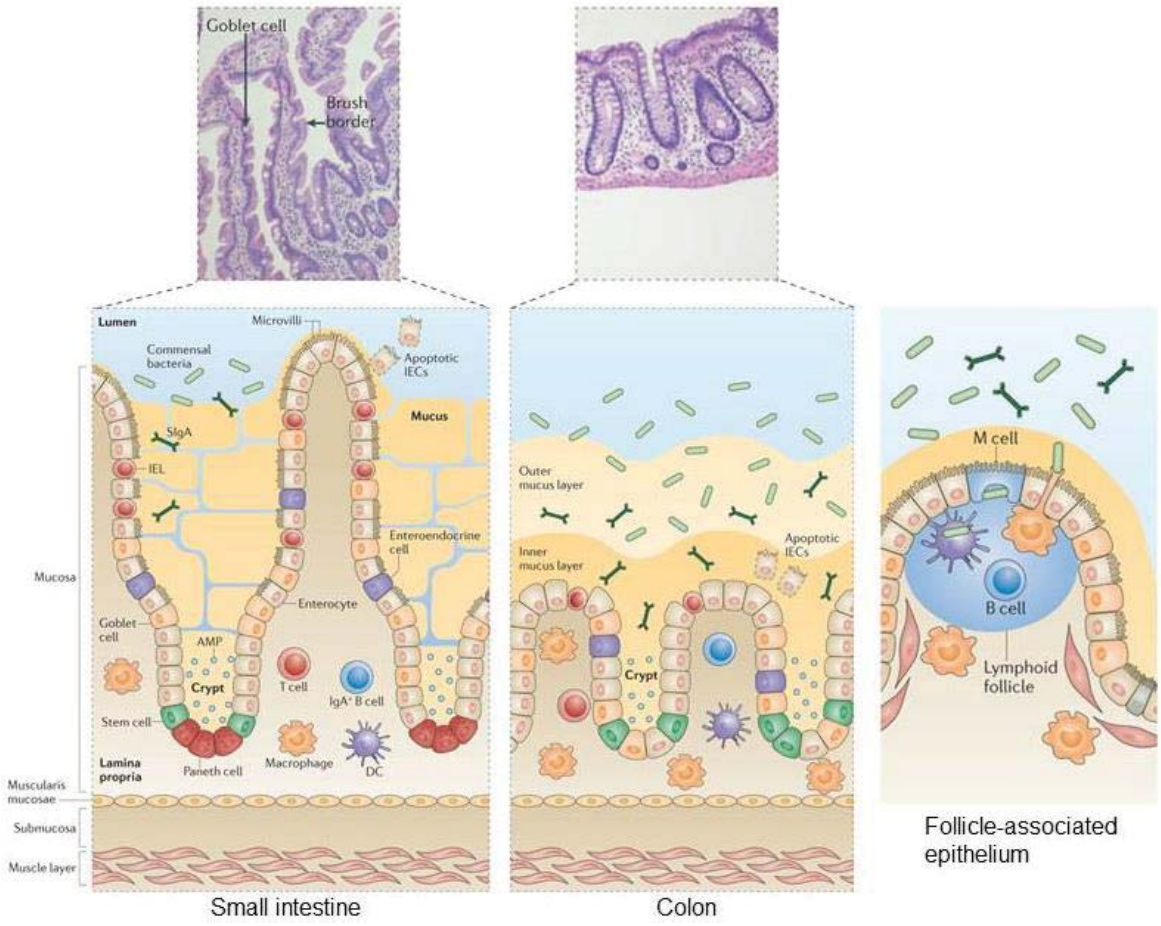

Figure 1.1. Structures and cell types of the gastrointestinal mucosa. Upper - Hematoxylin and eosin stained histological sections of human jejunum and colon. Lower - Schematic representations of the mucosa in the small intestine and colon, and the follicle-associated epithelium. Small intestine is characterised by deep crypts and villi while colonic crypts are much shorter. The intestinal epithelium in both organs consists of absorptive enterocytes and specialised cell types including adult stem cells, enteroendocrine cells and intestinal epithelial lymphocytes. Beneath the epithelial barrier the lamina propria contains an assortment of immune cells positioned to respond rapidly to microbes which breach the barrier and enter this region. A thick coating of mucus on the epithelial surface limits contact between intestinal content and the epithelial cells and contains secretory IgA antibodies which can bind and aggregate bacteria to prevent epithelial translocation. Follicle-associated epithelium are highly specialised regions containing microfold cells for sampling luminal antigens and closely associated phagocytes for uptake of these antigens and their presentation to adaptive immune cells. Adapted from [28] and [25].

Paneth cells remain localised to the crypts where they reside in small clusters, and are restricted to the small intestine. They play an important role in restriction of bacteria to the lumen through intensive secretion of antimicrobial peptides; both constitutively to prevent bacterial invasion of the crypt and epithelial mucus layer, and in response to specific microbial triggers. The presence of antimicrobial peptides influences the composition of the

microbiota at the mucosal surface, an important aspect of homeostasis to prevent excessive pro-inflammatory responses [29].

Goblet cells are located throughout the small and large intestine, increasing in density from proximal to distal colon. Goblet cells synthesise and secrete high molecular weight glycoproteins called mucins which form a thick protective glycocalyx over the surface of the epithelium. Mucins possess a net negative charge as a result of their acidic carbohydrate content and upon secretion form a network of ionic interactions. They can directly bind and trap certain bacteria through the carbohydrate moieties, some of which mimic epithelial cell surface molecules targeted by bacterial lectins. Pathogens may also become trapped in the mucus layer through binding by secretory immunoglobulin A (sIgA) within the mucus [30].

A third secretory cell type, enteroendocrine cells, comprise < 1% of the epithelial cell layer and these can be divided into multiple subtypes classified by the hormone and peptide content of their secretory vesicles. Molecules secreted by the enteroendocrine cells are important for regulation of a wide range of activities from gut motility to lipid absorption [31, 32].

In addition to the secretions from the cells within, the structure of the epithelium itself is adapted to prevent bacteria from accessing the tissue beneath. Sealing structures called tight junctions formed from integral membrane proteins directly below the apical surface encircle polarized epithelial cells. These connect adjacent cells to form a barrier relatively impermeable to small molecules and larger objects such as bacteria.

1.2.1.2 The lamina propria and muscularis mucosae

Surrounding the epithelial cell layer are multiple layers of connective tissue and muscle; first the lamina propria and the muscularis mucosae of the mucosa, followed by the submucosa, muscularis externa and the outermost layer of adventitia or serosa. The connective tissue of the lamina propria provides a structural base for the crypt and villus structures above, and contains the blood supply, local nervous system, and lymph drainage for the mucosa and immune cells. The muscle layers of the muscularis mucosae and muscularis externa are important to aid agitation and passage of lumen contents through peristalsis.

1.2.2 The gut-associated immune system

The intestinal immune system is challenged with the complex task of permitting commensal microbiota to safely inhabit the gut, avoiding the triggering of detrimental inflammation, yet providing appropriate protective immune responses against pathogens. A system of multiple specialised immune compartments with different immune cell compositions and functions has evolved to meet this demand [33]. The intestinal immune system comprises the mesenteric lymph nodes (mLN), and organised secondary and tertiary lymphoid tissues within the mucosa and submucosa. Together, these are referred to as gut-associated lymphoid tissue (GALT), with immune effector cells diffusely localised throughout the lamina propria and epithelium.

1.2.2.1 mLN and secondary lymphoid tissue

The mLN and organised structures of the GALT are the main sites of immune induction and regulation in the intestine. The formation of mLN and the patches of the small intestine (Peyer's patches), caecum and colon, begins early in embryonic development [34]. Like lymph nodes elsewhere in the body, the mLN sample antigens delivered via an afferent lymph vessel. The intestinal patches are located within the mucosa and submucosa, and in contrast to mLN, antigens are sampled from the intestinal lumen through a region of specialised columnar epithelium, the follicle-associated epithelium (FAE). FAE contains large numbers of highly specialised epithelial cells called microfold (M) cells. An opening in the glycocalyx assists M cells in endocytic uptake of luminal antigen at the apical surface, followed by transfer to a pocket at the basal side of the cell contacting dendritic cells, macrophages and lymphocytes in the subepithelial dome region beneath [35, 36]. Here dendritic cells process antigen and present it to lymphocytes located within the patch [37].

Intestinal patches consist of multiple B cell follicles in germinal centres surrounded by a smaller T cell zone. Germinal centres facilitate the replication, differentiation and mutation of antibody encoding genes in B cells in response to antigen stimulation within the patch. IgA-producing antigen-specific plasma cells differentiate following stimulation and are the main immune effector cell type. The total number of Peyer's patches and colonic and caecal patches varies between species but the pattern of fewer colonic and caecal patches compared with the small intestine is conserved. In mice there are typically < 10 colonic and caecal patches while small intestinal patches in humans number around 240 [38, 39].

1.2.2.2 Tertiary lymphoid tissue

Smaller tertiary intestinal lymphoid tissues, referred to collectively as solitary isolated lymphoid tissues (SILTs), perform similar functions to the secondary lymphoid structures; however these develop only after birth, and intestinal colonisation by the microbiota is required for normal development. Several types of follicle of varying size and cell composition have been described. Recent work has questioned the validity of strict definitions, suggesting that a changeable continuum modified in response to environmental signals better describes these structures compared with classical categories of cryptopatch and innate lymphoid follicle (ILF) [40]. SILTs are more numerous than the larger secondary lymphoid structures; the mouse small intestine is estimated to contain ~ 1,000 evenly distributed follicles [40]. Colonic SILTs are concentrated in the distal colon where the bacterial load is greater [38]. Tertiary intestinal lymphoid tissue growth and expansion is induced in response to microbes, resulting in increased production of antigen-specific IgA, potentially keeping microbial growth in check; this relationship acts as a feedback loop maintaining homeostasis [41]. Imbalance results in drastic effects, as seen in mice lacking these structures which show a 10-fold expansion in the microflora [42].

1.2.2.3 Individual immune cells

The epithelium and the lamina propria contain large numbers of individual immune effector cells, collectively the largest population of T cells, plasma cells and macrophages in the body [28]. Intraepithelial lymphocytes (IELs) sit within the epithelium with a frequency of around 10 - 15 per 100 epithelial cells, and these are primarily T cells [43]. Unlike most healthy tissues the lamina propria contains large numbers of plasma cells, of which 75% in the duodenum, and 90% in the colon, are IgA-producing. In addition the lamina propria contains T cells and plentiful innate cells including dendritic cells, macrophages, eosinophils, mast cells, and the recently discovered innate lymphoid cells (ILCs).

1.2.3 The intestinal microbiota

The human intestinal microbiota is a largely stable collection of at least 500 - 1,000 microbial species including bacteria, viruses, and eukaryotes [44, 45]. The relationship between the host and the microbial community is normally mutually beneficial. The host provides an environment favourable for bacterial survival; nutrient rich and at a constant

temperature. In return the microbiota performs a wide range of valuable functions. Commensal microbes metabolise dietary nutrients the host is otherwise unable to digest, and produce essential vitamins. Collectively their presence can resist the overgrowth of pathogenic bacteria; a phenomenon known as colonisation resistance. Bacterial population density and diversity in the GI tract increases from the stomach to the large intestine, with ~ 10^3 organisms per gram of content in the duodenum increasing to ~ 10^{12} per gram in the distal colon. Total bacterial cells outnumber those in the human host 10:1 [46]. The vast majority of bacterial species are strictly anaerobic; there are 100 - 1,000-fold fewer facultative anaerobes; and belong to one of a relatively small number of bacterial phyla. Most abundant by far are species of the phyla Bacteroidetes and Firmicutes, accompanied by smaller numbers belonging to the Proteobacteria, Verrumicrobia, Actinobacteria, Fusobacteria and Cyanobacteria [45, 47].

Until recently the significance of the microbiota in development and homeostasis in the healthy gut was poorly understood, however the past decade has uncovered many processes where the microbiota are crucial, and a much wider role for the microbiota than previously imagined [48].

1.2.3.1 Colonisation resistance

Colonisation resistance is an essential protective mechanism provided by the microbiota. Studies of *Salmonella* Typhimurium infection in mice have determined that the microbiota composition strongly dictates the degree of colonisation resistance conferred; in germ-free mice and mice with a defined microbiota of 4 - 20 species, colonisation resistance is absent. Co-housing of these mice with conventional mice, allowing the acquisition of a normal microbiota, restores resistance. Further study of mice with varying degrees of colonisation resistance determined that the presence of closely related species increases the chance of colonisation by an incoming species [49]. Clearance of *S*. Typhimurium from the gut after infection relies on the re-establishment of a complex microbiota, and similar to the loss of colonisation resistance, is deficient in mice with a defined low complexity microbiota [50]. Whilst these examples show that certain important aspects of colonisation resistance are being exposed, more work is needed to uncover exactly how resistance to colonisation is achieved and to determine the relative importance of individual species, phyla, and overall complexity in protection.

Antibiotics are essential for treatment of infections the body fails to resolve independently, however antibiotic therapy frequently impacts the species which make up the microbiota in addition to the pathogen target, reducing microbial diversity and shifting the composition at the levels of species and phyla [51, 52]. These changes collapse colonisation resistance and can render patients more susceptible to gastrointestinal infection with opportunistic pathogens. *C. difficile* is carried asymptomatically in the microbiota of some healthy individuals yet it is the most important nosocomial cause of diarrhoea in adults [53]. Disruption of the microbiota can allow rapid growth of the bacterium resulting in disease ranging from diarrhoea to pseudomembranous colitis, and in a small proportion of patients more serious complications including death [54]. Treatment of *C. difficile* infection involves cessation of treatment with the inciting antibiotic and employs antimicrobials targeting *C. difficile*, but whilst these can kill the *C. difficile* bacteria, indigenous microbial species are afforded little chance of recovery and relapsing infection is common. Utilising the natural protection in colonisation resistance, faecal transplantation is a promising therapeutic intervention for the treatment of *C. difficile* infection. This approach reintroduces species which comprise a healthy microbiota to the gastrointestinal tract, leading to suppression of pathogen growth [55].

1.2.3.2 The microbiota in intestinal development and maintenance

Studies in germ-free mice have vastly increased our understanding of the important functions of the microbiota in development of the gastrointestinal mucosa, in particular the full development of lymphoid tissues and production of mucus. Germ-free mice develop an enlarged caecum and defective small intestinal brush border, with a reduced total intestinal surface area relative to conventionally housed mice [56, 57]. As mentioned earlier, differentiation of secondary lymphoid tissues of the intestinal mucosa begins in the embryo, however microbial colonisation is required after birth for the increase in size and development of germinal centres during maturation [58]. The microbiota is critical for the generation and development of ILFs; the peptidoglycan of Gram-negative bacteria is needed to induce ILF genesis, and further maturation requires the detection of bacteria by toll-like receptors (TLRs) [42]. Specifically the differentiation of B cells to sIgA-producing plasma cells requires detection of Pathogen-Associated Molecular Patterns (PAMPs) such as bacterial flagellin by TLR5 on lamina propria dendritic cells [59]. sIgA targeting commensal antigens is essential

to maintain the balance in the microbiota and physical separation between the microbiota and host [60].

The microbiota is necessary to induce and maintain a strong epithelial barrier, affecting multiple components involved in integrity. Compared with conventionally housed mice, germ-free mice possess fewer RORγt^+ NKp46$^+$ ILCs, a key cell type for production of IL22 which signals to induce production of antimicrobial peptides, epithelial repair and barrier function [61]. In conventionally-housed mice the mucus which lines the intestinal mucosa is several hundred μm thick in the colon. This generous blanket physically limits bacterial contact with host cells at the epithelial surface. Germ-free mice possess fewer goblet cells resulting in a thinner mucus layer, and the mucus composition is skewed towards a higher proportion of neutral mucin molecules [62]. Finally many bacterial species of the microbiota have been found to impact barrier integrity through effects on tight junctions, for example several *Lactobacillus* rigidify tight junctions via signalling through pattern recognition receptors (PRR) [63].

Certain groups and even individual species of bacteria have been linked with specific aspects of intestinal immune development and function. For example tryptophan metabolism by *Lactobacillus* spp. triggers pathways leading to Th17 cell production of IL22, and *Clostridia* spp. promote a TGFβ-rich environment which leads to the preferential generation of regulatory T cells (Tregs) in the colon [64, 65].

1.2.3.3 Wider impact of the microbiota

Recent studies have begun to uncover important roles for the microbiota beyond development and maintenance of the gastrointestinal tract and immune compartment. Changes in the microbiota have been associated with tissue homeostasis at remote sites, including changes in bone density [66]. The metabolic potential of the microbiota is far greater than that of the host and it is therefore unsurprising that the microbiota is associated with metabolic disease and adiposity. The microbiota of obese mice was found to possess greater potential for metabolism of polysaccharides and synthesis of short chain fatty acids, and hence a greater ability to unlock the energy potential within food compared with mice of normal weight [67]. Future work in this area is likely to further broaden the range of host biological processes we know to be influenced by the intestinal microbial community.

1.2.3.4 The microbiota in diarrhoeal disease

The relationship of the microbiota with diarrhoeal disease is complex. Perturbation of the microbiota can lead to diarrhoeal disease, and in turn diarrhoea has large effects on microbiota composition, hence in many cases disambiguating cause and effect is a difficult task [68]. Pathogenic species significantly impact the population structure of the resident microbiota, creating a state of 'dysbiosis'. Similar changes are observed in inflammatory bowel disease (IBD) and other autoimmune diseases, indicating these changes are a characteristic of inflammation occurring in the gut rather than the result of pathogen-specific mechanisms. Studies of gastrointestinal infection with diverse pathogens and in IBD have shown outgrowth of the class Gammaproteobacteria, and in particular the *Enterobacteriaceae* family is a common feature [69-71]. Conversely fermentative bacteria of the phylum Firmicutes and in particular the class Clostridia are lost during inflammation [71]. The shift in composition reflects differences in adaptation of commensal species to the hostile environment of the inflamed gut. Species which thrive are often closely related to obligate pathogens and are able to express genes which help them to take advantage of the environment, for example to allow them to use metabolites such as nitrates [72]. Inflammation leads to dysbiosis, and in turn this shapes the immune system, creating positive feedback. Many of the species seen to overgrow or 'bloom' in the inflammatory conditions are inflammatory themselves, particularly in the environment of the damaged mucosa, and contribute to the immune activation taking place. Compounding problems further is the loss of normal inflammation dampening mechanisms. Fermenting bacteria reduced in inflammation are often important immunoregulators, for example Treg-inducing *Clostridia* spp. are amongst those whose numbers are diminished [65].

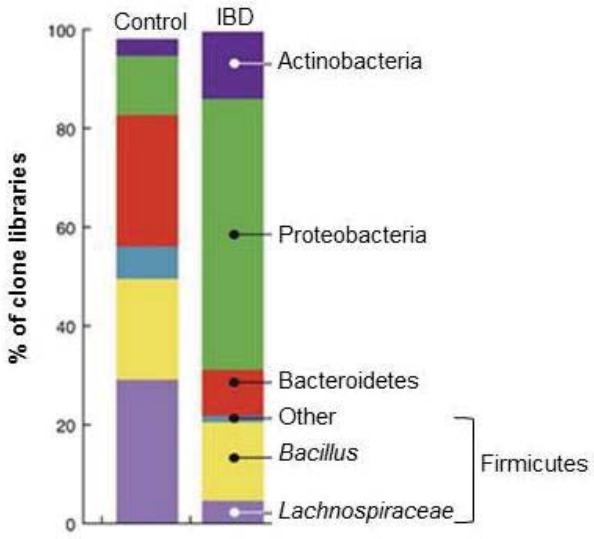

Figure 1.2. Altered microbiota composition in intestinal inflammation. Representative microbiota composition data for IBD patients and healthy controls. Bar shading represents bacterial groups and bar height the proportion of cloned sequences in the sample belonging to the group. Expansion of the phylum Proteobacteria and a decline in the phylum Firmicutes is typical in inflamed intestine. Adapted from [73].

1.3 *Salmonella*

1.3.1 Classification and phylogeny

The salmonellae are Gram-negative bacteria belonging to the class Gammaproteobacteria and the family *Enterobacteriaceae*. They are facultative intracellular bacteria and a model organism for the study of bacterial genetics and pathogenesis. The *Salmonella* genus diverged from closely related *E. coli* in the region of 100 - 140 million years ago, and diverged again between 71 and 100 million years ago to form two lineages which define the *Salmonella* species *S. bongori* and *S. enterica*. Two key genetic elements associated with virulence, *Salmonella* pathogenicity islands 1 & 2 (SPI-1 and SPI-2), were acquired by horizontal gene transfer after divergence from *E. coli,* in the case of SPI-1 prior to the split between *S. bongori* and *S. enterica*, whilst SPI-2 was acquired by the *S. enterica* lineage exclusively [74].

S. enterica is divided into seven subspecies, the first six originally defined by biochemical traits and the seventh distinguished by multi-locus enzyme electrophoresis (MLEE), though DNA-sequence based analyses confirms these subspecies are indeed evolving independently [75-77]. The salmonellae are further divided into serotypes based upon the O-antigen of LPS and flagellar or H- antigen. Combining the seven subspecies with

the 46 O-antigen groups and 114 H-antigen groups defines all recognised serotypes of *Salmonella*. *S. bongori* and *S. enterica* subspecies II, IIIa, IIIb, IV, and VI are mainly associated with cold-blooded vertebrates whilst members of *S. enterica* subspecies I often infect mammals and birds. Greater than 99% of *Salmonella* isolates from human and mammalian hosts belong to subspecies I [78, 79].

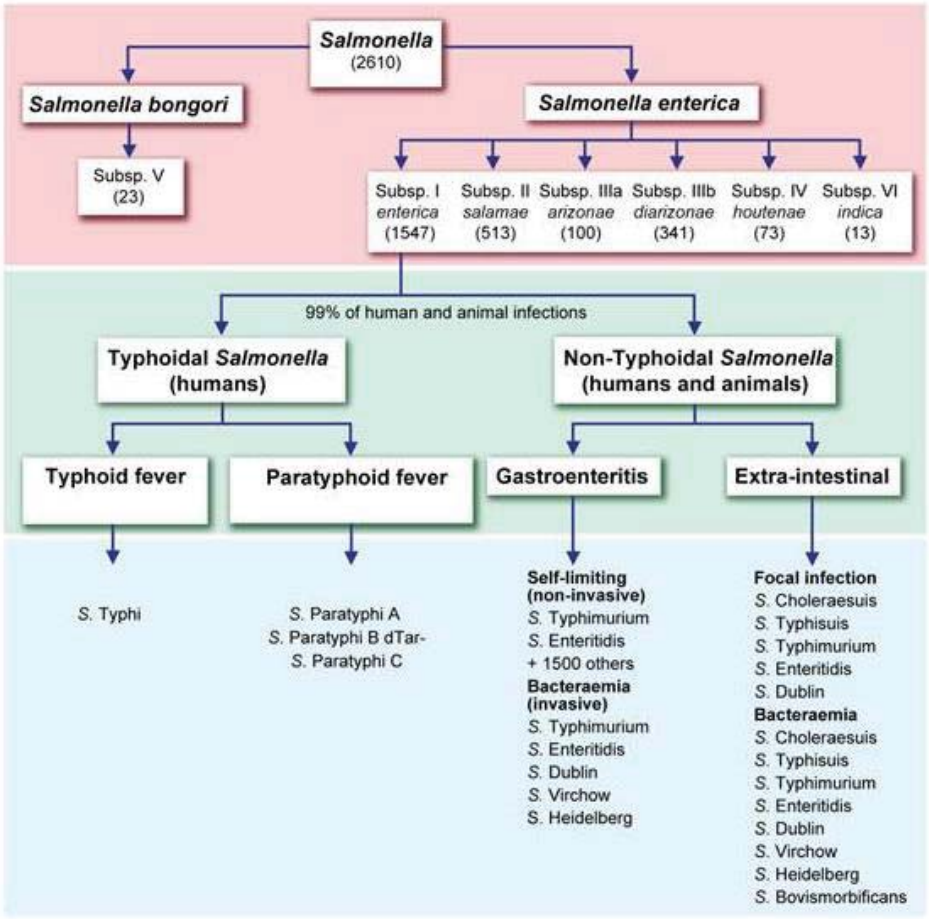

Figure 1.3. Classification of the genus *Salmonella*. Two species form the *Salmonella* genus, of these the majority of isolates belong to the species *S. enterica*. *S. enterica* is divided into subspecies according to biochemical and serological characteristics. *S. enterica* subspecies I accounts for the vast majority of human infections and is further divided into typhoidal and non-typhoidal salmonellae by the disease symptoms these bacteria generate. Within these two groups are around 1,500 serovars, characterised by the O- and H- antigens of the bacterial surface. Taken from [80].

1.3.2 Global distribution of *Salmonella* serotypes

Data collected through the World Health Organisation (WHO) Global Salm-Surv describe the distribution of *Salmonella* serotypes isolated by its members. 41 countries entered results for human isolates contributing to the report for 2000 - 2002. Of the > 350,000 isolates, *S.* Enteritidis was most common, accounting for 65% of all isolates, followed by *S.* Typhimurium at 12% and *S.* Newport at 4%. The vast majority of contributing isolates were North American and European and unfortunately the results are highly biased toward the industrialised world. Regional statistics show that serotypes vary greatly in proportion but more data is needed for developing nations. Serotypes *S.* Enteritidis, *S.* Typhimurium, *S.* Infantis, *S.* Montevideo, and *S.* Typhi displayed a broad geographical distribution whilst serotypes *S.* Rissen and *S.* Weltevreden were region specific. Overall the majority of human cases are caused by a small number of serovars [81, 82].

1.3.3 Typhoidal and non-typhoidal *Salmonella* (NTS)

Salmonella serotypes responsible for human infection are categorized by the clinical manifestations of disease and host range, into invasive (typhoidal) and non-invasive (non-typhoidal) strains.

1.3.3.1 Clinical features and burden of typhoid fever

Typhoidal salmonellae cause life-threatening systemic infection and are adapted to a human host. Infection is characterised by high fever and potentially complicated by sepsis and shock, gastrointestinal bleeding or perforation, encephalopathy and enlargement of the mLN, liver and spleen accompanied by granulomatous lesions [83]. *Salmonella enterica* serovar Typhi causes the largest proportion of typhoidal disease, with serotypes Paratyphi A, B and C also responsible for significant numbers of infections. Historically typhoidal *Salmonella* infections were a worldwide problem but now have been largely eradicated in developed countries as a result of improved sanitation. In the developing world infections remain a significant cause of morbidity and mortality. In 2000 there were an estimated 21 million cases of typhoid fever resulting in around 200,000 deaths, and over 5 million cases of paratyphoid fever [6]. Multiple typhoid vaccines are available but the most effective licensed vaccines achieve incomplete protection at around 70%, and immunization coverage in developing nations is poor [84].

1.3.3.2 Clinical features and burden of NTS disease

NTS serovars are more common than *S.* Typhi and are globally distributed; *Salmonella* gastroenteritis is a significant burden in both developed and developing countries. Globally there are an estimated 93.8 million cases of *Salmonella* gastroenteritis resulting in 155,000 deaths annually [85]. 1.4 million cases occur in the United States [86]. Non-typhoidal salmonellae infect mammals and birds in addition to humans, and the majority of human infections are thought to be the result of zoonotic transmission. Typical NTS infections are cases of self-limiting gastrointestinal disease, typified by diarrhoea and intestinal inflammation. Symptoms also include fever, and abdominal cramps. Following ingestion bacteria pass through the stomach to colonise the terminal ileum and colon; typically generating symptoms of gastroenteritis within 24 h. Early inflammatory signals are amplified to promote continued exudative inflammation in the mucosa. Vascular and epithelial permeability increases to allow neutrophils to enter the tissue and attack *Salmonella* that has invaded beyond the epithelial layer, and further leave the tissue to act on luminal *Salmonella*. Fluid leakage results from the increased permeability and contributes to diarrhoea, clearing out the contents of the gut and leading to dehydration.

1.3.3.3 Invasive non-typhoidal *Salmonella* (iNTS)

In developed nations bacteremia in NTS infection is a relatively rare occurrence, largely limited to individuals with inherited immunodeficiencies, or immunosuppression as a result of other factors such as steroid use or diabetes. In contrast NTS are a common cause of bloodstream infections in adults and children presenting with fever in many parts of sub-Saharan Africa, with a case fatality rate of 20 - 25%. iNTS is closely associated with specific risk factors. 95% of adult cases are in people with HIV, whilst in children HIV, malaria, and malnutrition are all closely associated with this type of infection [87]. The major feature of typical NTS infection, enterocolitis, is often absent and replaced by diverse clinical presentations including respiratory symptoms, fever and hepatosplenomegaly. Most cases of iNTS are caused by the serotypes *S.* Enteritidis and *S.* Typhimurium but there is a general lack of data describing whether the same strains responsible for NTS are also causing iNTS disease. Recent evidence points to the emergence of new strains in sub-Saharan Africa, which may be adapted to the niche provided by immunocompromised individuals [88]. The dominant regional genotype of iNTS in Malawi and Kenya is *S.* Typhimurium ST313 and

recent work has identified signatures of genomic degradation and convergence with phylogenetically distinct *S.* Typhi (see section 1.3.5.2.2) [89].

The association between HIV and iNTS may be explained by the viral destruction of $CD4^+$ T cells. In particular the loss of Th17 cells results in decreased cytokines critical for epithelial barrier function, such as IL22, and those for the recruitment, activation and survival of neutrophils, for example TNFα and GM-CSF. These weaknesses in host defence combine to enable *Salmonella* to disseminate more effectively, as has been demonstrated in an SIV rhesus macaque model. The diminished enterocolitis in iNTS infection is thought in part due to the failure to recruit neutrophils to the mucosa [90].

1.3.4 *Salmonella* transmission

Salmonella is predominantly transmitted by the faecal-oral route in contaminated food and water, and through consumption of animal products from species that harbour the bacteria. The relative importance of food- and water-borne transmission is thought to vary with regional economic development. In developing nations a lack of basic sanitation significantly aids transmission, providing increased opportunity for bacteria from the faeces of infected animals or individuals to contaminate water used for drinking and food preparation. In contrast the most common sources of *Salmonella* in developed nations are meat and egg products, and processed food contaminated with faeces from animal sources.

In the majority of cases of NTS *Salmonella* is completely eliminated from the gastrointestinal tract in recovery from infection, though antibiotic treatment may increase the chance of persistence [91]. On the other hand *S*. Typhi has a human reservoir in the form of asymptomatic carriers. Approximately 2 - 5% of typhoid patients fail to fully clear the bacterium within one year, asymptomatically carrying *Salmonella* in the gall bladder and biliary tract [92, 93]. *S.* Typhi is thought to enter the gall bladder directly from the liver during the systemic phase of infection. Persistence is closely associated with gall stones; ~ 90% of chronic carriers of *S.* Typhi can have them; and it is thought that formation of biofilms on gall stones is a key survival strategy employed by the bacteria [94, 95].

1.3.5 Molecular phylogeny of *S. enterica*

1.3.5.1 Serological and DNA-based classification

For almost a century, isolates of *Salmonella* have been classified according to serological and nutritional characteristics into serovars (see section 1.3.1). This globally accepted system, known as the Kauffmann-White scheme, has served to facilitate the epidemiological analysis of *Salmonella* infections. The scheme exploits diversity in the O (lipopolysaccharide) and H (flagella) antigens of *Salmonella*. However serological analysis lacks the ability to discriminate between closely related isolates and provides limited phylogenetic information compared to new approaches such as whole genome DNA-based typing systems. Further, although they have been tremendously useful, serotyping methods are comparatively slow, costly and low throughput.

Multi-locus Sequence Typing (MLST) was proposed to replace traditional serological classification of *Salmonella* in 2012 [80]. Based on sequences at sites within normally seven housekeeping genes, MLST uses neutral markers to identify genetically related isolates of *Salmonella* and classifies them into 'sequence types' (ST). Clustering algorithms group sequence types at a higher level into evolutionarily related groups. The use of this approach to study over 4,000 isolates from more than 500 serovars showed that the population structure of *S. enterica* is described by many discrete clusters of STs. Within these clusters a central node can represent the most recent common ancestor of the groups of isolates which radiate from it.

The relationships between clusters of STs and traditional serovars are largely but not entirely consistent. For some serovars, such as *S*. Typhi, all members of a particular serovar are found within one cluster, and the cluster contains isolates of this serovar exclusively. In contrast *S*. Typhimurium isolates are positioned within multiple clusters, and the major cluster containing the central sequence type ST19 also contains isolates of other serovars. In some cases there is very little match between serotype and ST cluster, for example *S*. Paratyphi B isolates are located within multiple distinct clusters [80]. MLST is relatively amenable to high-throughput techniques; requiring PCR to amplify regions within housekeeping genes, followed by sequencing. Despite its greater efficiency and the more comprehensive information it provides, the change from established serological typing methods to MLST will require a significant shift in global thinking.

MLST provides information for an outline population structure, which can be further investigated with whole genome sequencing (WGS). In light of the advances in sequencing technology the application of WGS to study *Salmonella* is increasing. As mentioned earlier, sequence analysis of *S*. Typhi has shown that all isolates form a single tree radiating from a common ancestor, indicating a recently (several thousand years) expanded clonal population [96]. The global population of *S*. Typhi has been estimated to be relatively small; unusually most of the links between sequential haplotypes consist of single SNPs and recombination with other salmonellae is uncommon. 85 haplotypes currently describe the genetic variation within *S*. Typhi but new sequencing efforts are likely to expand this number. The population generally shows a weak evidence of strong selection, potentially linked to the existence of the asymptomatic carrier state [96, 97]. However recent work has identified that antibiotic usage is contributing to the global selection of a single multi-drug resistant haplotype, H58, frequently found with resistance to quinolones through mutations in *gyrA* [98]. WGS studies such as this one are important to identify emerging strains and investigate causes of selection.

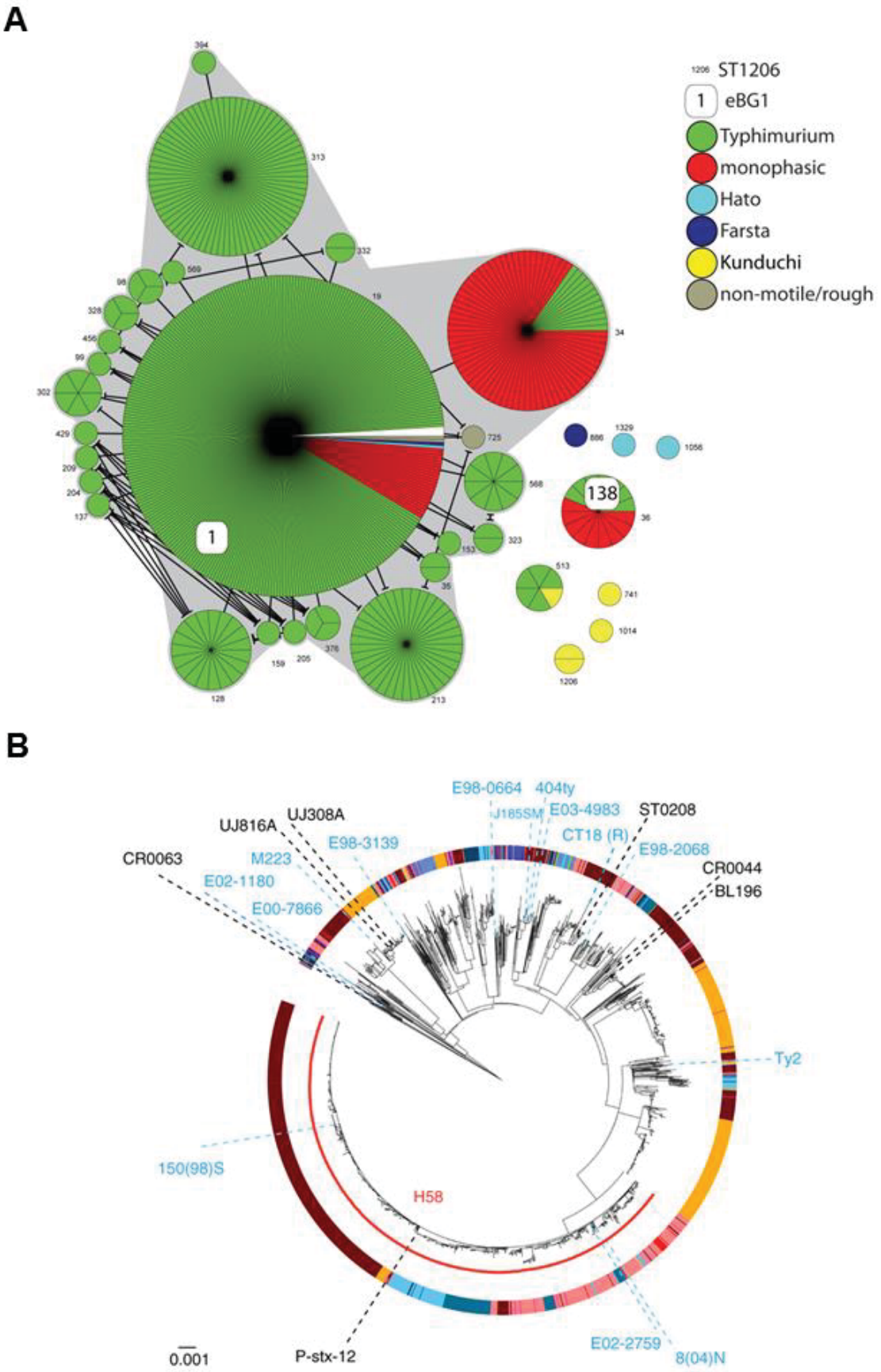
A
ST1206
eBG1
Typhimurium
monophasic
Hato
Farsta
Kunduchi
non-motile/rough
1
138
B
E98-0664
404ty
J185SM
E03-4983
ST0208
UJ816A
UJ308A
CT18 (R)
E98-3139
E98-2068
CR0063
M223
CR0044
E02-1180
BL196
E00-7866
Ty2
150(98)S
H58
0.001
P-stx-12
E02-2759
8(04)N

Figure 1.4. Phylogenetic relationships within serovars *S.* Typhimurium and *S.* Typhi. (A) Minimal spanning tree of *S.* Typhimurium and its serological variants based upon MLST for a collection of > 4,000 *Salmonella* isolates. Each circle represents one sequence type and segments represent individual isolates. Connecting lines indicate relationships between sequence types. White boxes indicate related clusters. The tree demonstrates that the serovar *S.* Typhimurium contains groups of relatively phylogenetically distant *Salmonella* and that isolates of other *Salmonella* serovars cluster with *S.* Typhimurium. (B) Rooted maximum-likelihood tree of > 1,800 isolates of *S.* Typhi inferred from 22,145 SNPs. The coloured ring indicates the geographical source of the isolate, and the red arc isolates of the haplotype H58. The tree demonstrates that the *S.* Typhi serovar is clonal. The H58 haplotype accounts for almost half of this global collection of isolates. A and B taken from [80] and [98] respectively.

1.3.5.2 The genome of *S. enterica*

As members of the *Enterobacteriaceae*, *Salmonella* genomes approach 5 Mb in size and contain sets of core genes encoding functions conserved throughout the family, such as intestinal persistence. The core genes are largely syntenic between individual members of the family, and are interspersed with species- and strain-specific regions, some encoding pathogenicity determinants. In addition to the chromosome many strains of *Salmonella* contain one or more plasmids, mobile genetic elements that typically encode virulence genes and genes which confer antibiotic resistance.

1.3.5.2.1 The genome of *S.* Typhimurium

The first *S.* Typhimurium to be sequenced completely was the common laboratory strain LT2, with a 4,857 kb chromosome and 94 kb virulence plasmid. The genome of LT2 serves to represent that of a promiscuous *Salmonella* gastroenteritis isolate. Analysis of the genome determined ~ 4,500 coding sequences, with just over 100 genes encoded on the plasmid. Comparisons were made between the genome of LT2, other *Salmonella* within subspecies I (*S.* Typhi, *S.* Paratyphi A, and *S.* Paratyphi B), and *Salmonella* within other subspecies. ~ 8% of genes in LT2 were found to have a homologue in at least one other member of subspecies I, but were not in *Salmonella* of other subspecies. As the only subspecies specialised to colonise warm blooded hosts, such genes may be important in conferring this adaptation [99].

1.3.5.2.2 The genome of *S.* Typhi

The complete sequencing of two isolates of *S.* Typhi facilitated a comparison between the genomes of the *S.* Typhimurium and *S.* Typhi serovars [100]. A major finding was the

extensive genome degradation which has occurred during the evolution of *S*. Typhi. Whilst the *S*. Typhimurium genome contains around 40 predicted pseudogenes, *S*. Typhi has ~ 200, corresponding to the inactivation of ~ 3 - 5% of all genes [100]. The nature of these pseudogenes has been investigated in order to understand how the functional loss in *S*. Typhi might relate to the characteristics of this serovar. 7 of the 12 fimbrial systems and other fimbrial-like genes are inactivated, and the loss of these structures important for directing attachment to surfaces such as host cells may contribute to the restriction of this serovar to human hosts. *ShdA* and *ratB*, genes, associated with intestinal persistence in *S*. Typhimurium, are also inactivated in *S*. Typhi. The loss of function might help to explain the lower numbers of *S*. Typhi in the gastrointestinal tract in the period following initial exposure, instead favouring rapid systemic dissemination [97].

Loss of gene function in the invasive serotype *S*. Typhi extends beyond this serovar to other salmonellae responsible for systemic disease, including *S*. Paratyphi A. The emerging sequence type of *S*. Typhimurium responsible for iNTS in sub-Saharan Africa, ST313, has accumulated inactivating mutations in some genes also inactivated in *S*. Typhi. In particular many metabolic genes are inactivated, for example Tartrate dehydratase (*ttdA)* is also inactivated or absent in *S*. Typhi, *S*. Paratyphi A and *S*. Paratyphi B. It has been suggested that differences in metabolic capacity might influence *Salmonella* fitness in different environments, and thereby impact both upon the niche within the body, and the host range of the bacteria [89]. Restriction of host range by the loss of gene capacity in an ancestor with a broad host range has been reported in other human-restricted pathogens such as *Mycobacterium leprae* and *Yersinia pestis* [101, 102].

As well as functional gene loss, during its evolution *S*. Typhi has gained genes absent in gastroenteritis-causing *Salmonella*. For example a major characteristic of *S*. Typhi is the expression of the Vi capsular polysaccharide, encoded in a genetic region designated SPI-7 containing many features associated with the horizontal acquisition of DNA. *S*. Paratyphi C and some isolates of *S*. Dublin have also acquired a SPI-7-like region [103]. The Vi capsule has been shown to modulate the recruitment of neutrophils to the gut, thought to reduce diarrhoea thereby adapting *S*. Typhi to a systemic lifestyle [104].

1.3.6 Virulence mechanisms of *S.* Typhimurium

While numerous *Salmonella* serotypes contribute to the burden of human disease, the majority of research has been carried out in just two: *S.* Typhi and *S.* Typhimurium. In specific experimental animal models *S.* Typhimurium behaves as a prototypical intestinal pathogen. Both serotypes are models for the study of host-pathogen interactions in the context of infection with intracellular pathogens, the cause of many major human diseases [105].

S. Typhimurium utilises a large assortment of virulence strategies in order to take advantage of the host environment, both in the intestinal lumen and intracellularly. Many of these highly effective strategies are shared by other *S. enterica* and in some instances by other bacterial pathogens, though the specific mechanisms behind their execution are often distinct. Chemotaxis towards and attachment to the host epithelial surface bring bacteria to a location which facilitates their entry into host cells [106]. Once inside virulence strategies are employed to avoid onslaught of the host defence mechanisms, and to control and replicate within the host cell.

The many individual pathogenic adaptations of *S.* Typhimurium described in the following section combine to implement a relatively simple, yet highly effective overall strategy for survival and transmission. *S.* Typhimurium virulence factors induce the host innate immune system to trigger rapid inflammation of the intestinal tissue. Dramatic changes in the environment within in the intestinal lumen result, and alter the ability of the bacterial species residing here to survive [107]. For many key beneficial species of the microbiota these changes convert the intestinal lumen from a hospitable to a hostile environment, and they fail to survive in the inflammatory environment. Conversely the pathogenic adaptations of *S.* Typhimurium enable it to thrive in the altered environment and its population 'blooms' [108]. Neutrophil chemotaxis to the gut and extravasation induced by inflammation lead to an increase in vascular permeability and fluid build-up in the tissue, and the passage of neutrophils and dendritic cells into the intestinal lumen damages the integrity of the epithelial barrier. The combination of these effects contributes to diarrhoea, which aids the faecal-oral transmission of *S.* Typhimurium to fresh hosts [109]. Induction of inflammation makes an important contribution to lowering colonisation resistance in other intestinal infections, such as *E. coli*, also [110].

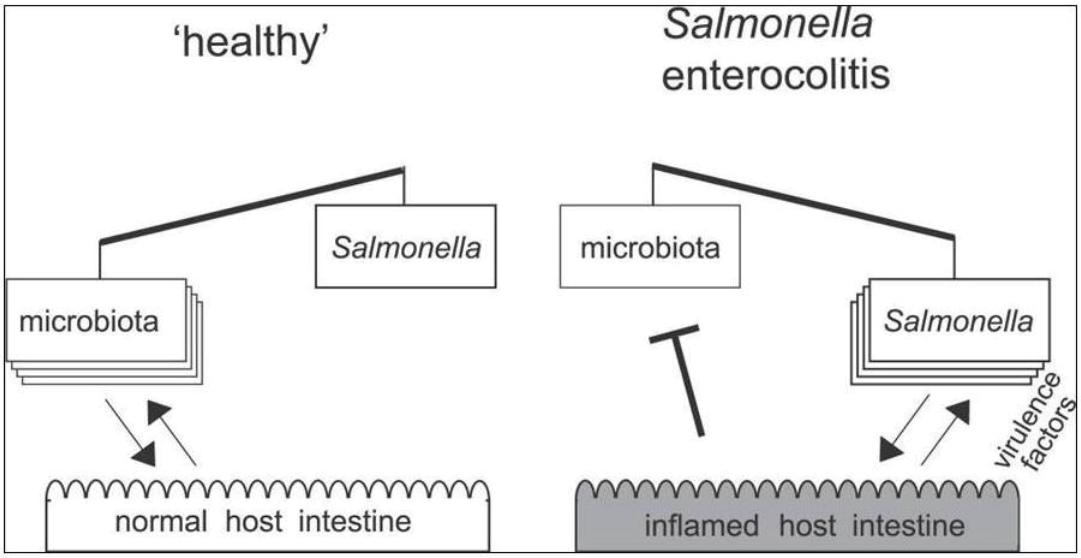

Figure 1.5. Interactions between *S.* Typhimurium, the microbiota and the intestinal mucosa. In the healthy gut mutually beneficial interactions between the microbiota and the host support the microbiota, and a diverse microbiota holds back *Salmonella* through colonisation resistance. *S.* Typhimurium targets the host with virulence factors resulting in inflammation. Inflammation alters the gut environment to favour *S.* Typhimurium growth and triggers host response pathways which directly inhibit the microbiota. Modified from [108].

1.3.6.1 Type 3 secretion systems (T3SS)

Key virulence-associated determinants of *Salmonella* are two horizontally acquired regions absent in related commensal *E. coli* species, SPI-1 and SPI-2 (see section 1.3.1) [111]. T3SS are complex bacterial membrane-associated protein export systems assembled from > 20 proteins. A needle-like structure spanning the envelope delivers specific bacterial proteins, known as 'effectors', into the cytosol of a host cell [112]. The SPI-1 and SPI-2 T3SS deliver different collections of effectors and function consecutively during bacterial interaction with a host cell. SPI-1 is activated in extracellular bacteria by stimuli present in the lumen whilst SPI-2 is actively transcribed when the bacteria are within a host cell, although there may be temporal overlap in the activities and differences between serotypes [113].

Salmonella SPI-1 encodes a T3SS pivotal for the directed invasion of non-phagocytic host cells [114]. At least 13 effectors encoded both within and separately to SPI-1 are translocated into the host cytoplasm by this system. Although the catalogue of functions these effectors perform *in vivo* is incomplete, their key role lies in remodelling of the cytoskeleton. Host actin binding proteins maintain a fine balance between monomeric and polymeric

filamentous forms of actin to control the structure of the cytoskeleton, and carry out specific functions such as vesicular transport. By interfering with signalling pathways controlling this dynamic process, and by directly binding actin, *Salmonella* effectors hijack and remodel the cytoskeleton [115, 116]. For example, actin binding proteins SipA and SipC decrease the critical concentration needed for actin polymerisation and nucleate and bundle actin [117, 118]. It is thought that these processes contribute to drive the growth of membrane ruffles and filopodia; protrusions of the host cell membrane which allow the cell to engulf and internalise bacteria [119]. Many more of the effectors transported by the SPI-1 T3SS contribute to modify the cytoskeleton for uptake and actively recover the normal state after internalisation. Co-ordination of these opposing processes requires strict temporal control [120].

The T3SS encoded by SPI-2 is essential for the intracellular survival and replication of *Salmonella* following uptake by phagocytes such as macrophages and dendritic cells, and after invasion of epithelial cells [121, 122]. In the absence of adaptations for intracellular survival, phagosome-lysosome fusion occurs after bacterial uptake and the compartment becomes unfit for survival. The vacuole is acidified, and antimicrobial peptides and hydrolytic enzymes are released. Under such conditions bacteria can be digested within 30 minutes of fusion [123]. Phagocytosis of *Salmonella* proceeds differently; as a result of T3SS effectors both the uptake mechanism and the morphology of the phagosome formed are unconventional. Uptake occurs by a process likened to macropinocytosis, creating a large compartment referred to as the 'spacious phagosome'. This contrasts with the close-fitting phagosomes generated by the zipper-like uptake mechanism for other bacteria [124, 125]. Effector proteins delivered to the host cytoplasm by the SPI-2 T3SS act to change the interaction of the *Salmonella*-containing compartment with the endosomal system, avoiding the fusion of lysosomes and creating a specialised '*Salmonella*-containing vacuole' (SCV) [122, 126]. SPI-2 effector proteins act to control the cellular location of the SCV and vesicle fusion through interference with microtubule-dependent trafficking events [127]. *Salmonella* exit from infected host cells has been shown to require SPI-2 effectors suggesting this T3SS is important for bacteria to spread to new infection foci [128]. They are implicated in a wide variety of processes, for example disruption of dendritic cell antigen presenting, though the biological functions of many remain unclear [129].

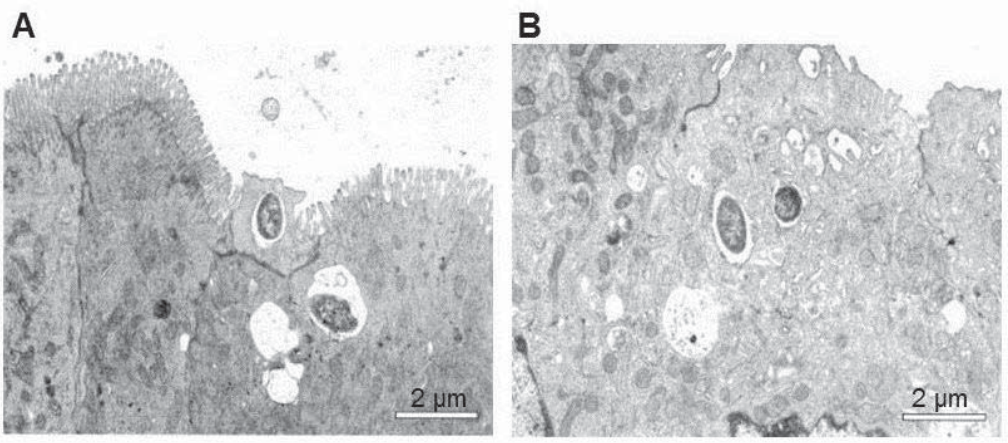

Figure 1.6. Epithelial cell invasion by *S.* Typhimurium. Transmission electron micrographs showing invasion of epithelial cells in the calf intestinal mucosa by *S.* Typhimurium. (A) Membrane ruffling is induced at the apical surface of the epithelial cell during invasion. (B) *S.* Typhimurium within vacuoles inside an M cell. Modified from [83].

The SPI-1 and SPI-2 T3SS can trigger pathogen-favourable host inflammatory responses by independent mechanisms. The host signalling pathways which mediate these responses are covered in detail in section 1.5.1. Briefly, SPI-1-induced colitis occurs independently of a key host signalling adaptor molecule, Myd88. SPI-1 pathways involve direct injection of proinflammatory molecules into epithelial cells, facilitating *Salmonella* invasion, which exposes receptors in the epithelial cell cytosol to PAMPs. The SPI-2 T3SS-mediated mechanism of triggering inflammation involves enhanced intracellular bacterial replication, increasing the load of bacteria to which the innate immune system is exposed [126, 130].

1.3.6.2 Nutrition and energy sources

A significant selective advantage of *S.* Typhimurium residing in the inflamed intestine is thought to arise from the ability to utilise the limited available nutrients [131]. Nutrients which support the growth of the resident microbiota are depleted by loss of luminal content in diarrhoea. *S.* Typhimurium can exploit chemotaxis up D-galactose gradients of the mucus layer and attachment to terminal mucus carbohydrates to position it to make use of mucus as an energy source. The absence of a selective advantage for flagella when alternative nutrient sources are available implicates chemotaxis as an adaptation to the inflamed intestinal environment [132].

Further, *S.* Typhimurium produces energy more efficiently than competing microbes. Most species of the microbiota are strictly anaerobic and rely on fermentation to supply energy for survival and growth. Conversely *S.* Typhimurium is able to support growth by anaerobic respiration using a molecule abundant in the inflamed gut, tetrathionate, as a terminal electron acceptor. Tetrathionate ($S_4O_6^{2-}$) is produced from oxidation of thiosulphate ($S_2O_3^{2-}$) on release of reactive oxygen species (ROS) by infiltrating neutrophils in inflammation [133].

1.3.6.3 Resistance to host antimicrobial peptides

S. Typhimurium is equipped with protective defences against the antimicrobial peptides produced constitutively by epithelial cells, and induced in the inflammatory response. Many bacterial species secrete a low molecular weight iron chelating molecule (siderophore), enterobactin, to enable acquisition of the essential element iron. The antimicrobial peptide lipocalin-2, released in the lumen in response to IL22 signalling, binds to enterobactin to inhibit growth of species which rely on this siderophore [134]. By producing a glycosylated derivative of enterobactin not bound by lipocalin-2, salmochelin, *S.* Typhimurium ensures a plentiful supply of iron [135]. In another strategy for protection against antimicrobial peptides *S.* Typhimurium modifies the lipid A moiety of its LPS, avoiding cationic peptides which target bacterial surfaces through electrostatic interactions. To increase resistance to the antimicrobial peptide polymixin the lipid A of LPS is modified by addition of aminoarabinose [136]. The response requires activation of a crucial two-component signalling pathway called PhoQ-PhoP by antimicrobial peptides, mediating the expression or repression of over 40 genes. In addition to modification of LPS these transcriptional changes generate a variety of responses including survival inside macrophages and altered antigen presentation [137, 138].

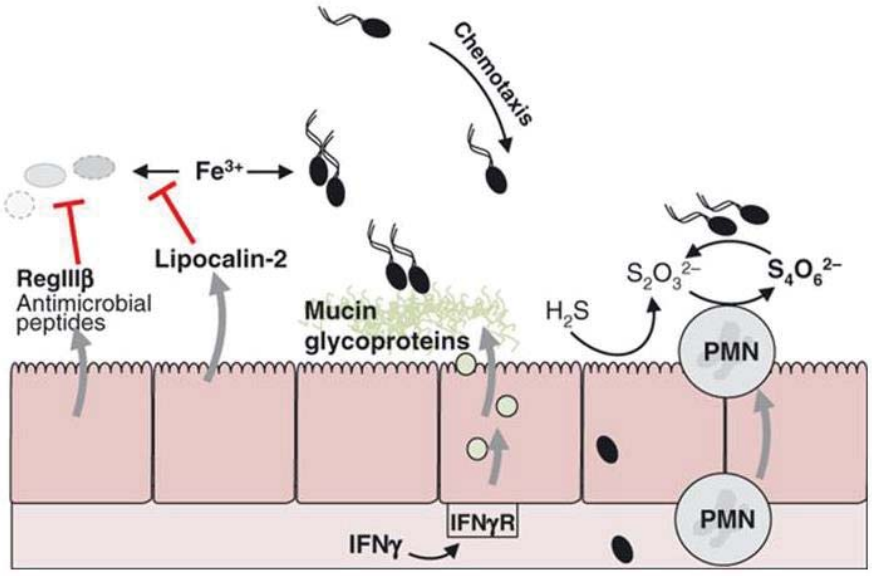

Figure 1.7. Adaptations of *S.* Typhimurium to the inflamed intestinal mucosa. Black cells represent *S*. Typhimurium and grey cells species of the microbiota. *S*. Typhimurium is resistant to harmful effects of many host antimicrobial peptides, for example it avoids shortages in iron induced by the host peptide lipocalin-2 by production of an iron chelating-protein absent in species of the microbiota. It uses chemotaxis to move toward the glycocalyx to take advantage of mucus glycoproteins as a nutrient source. Tetrathionate produced by infiltrating neutrophils in the inflammatory environment is adopted by *S*. Typhimurium as an electron acceptor giving a metabolic advantage over species of the microbiota which rely on fermentation. Taken from [139]

1.3.7 Animal models of human *S.* Typhimurium infection

Animal models are invaluable for studying the pathogenesis of *Salmonella* infection. Cell-based studies have helped identify many virulence factors and elucidate their mechanism of action but the relevance of these factors in the progression of a human infection is challenging to study in such a simplified system. Unlike cell systems, animal models of *S.* Typhimurium infection replicate aspects of the complex network of interactions occurring in human infection between the gut tissue, immune system, microbiota and pathogen. As discussed below calves are a model for human NTS infection, but due to considerations such as cost are used to a lesser extent than murine models. As humans and some species of primate are the only organisms naturally susceptible to *S.* Typhi this presents a barrier to the study of this serotype *in vivo*. However in mice *S.* Typhimurium infection gives rise to typhoid-like symptoms, and this mouse typhoid model has been studied extensively to gain understanding of typhoid fever pathogenesis. A primate model of *Salmonella* in rhesus macaques infected with Simian Immunodeficiency Virus (SIV) has been used to study the interaction between HIV and *Salmonella* [90].

Murine models of infection are attractive for several reasons, including relatively low costs of housing animals and ease of handling compared to larger organisms, the availability of a large number of genetically modified inbred lines, and well established techniques for genetic and immunological manipulation. *S.* Typhimurium is a natural pathogen of rodents and was discovered as the causative agent of murine typhoid in 1892. In contrast to the self-limiting gastroenteritis of human *S.* Typhimurium infection, in mice bacteria fail to invade epithelial cells, and *Salmonella* pass through the mucosa via M cells without triggering extensive inflammation. Consequently, translocated bacteria disseminate systemically causing a typhoid-like infection in susceptible mouse strains. Upon oral delivery of *S.* Typhimurium mice develop signs of infection at 4 - 8 days post-infection (PI) including fever, enlarged Peyer's patches and diffuse enteritis, similar to human typhoid patients. Bacterial replication in the spleen and liver results in enlargement of these organs, and formation of granulomas. Ultimately infected mice die, likely due to the liver lesions caused by lipid A-elicited cytokine and inducible nitric oxide synthase (iNOS) responses [140].

The failure of *S.* Typhimurium to thrive in the mouse gut indicates the microbiota here is more effective than that of humans in mediating colonisation resistance against invading *Salmonella*. However pre-treatment of mice with antibiotic prior to infection has proved successful in depleting the microbiota so that *Salmonella* may colonise the intestinal niche and trigger inflammation, creating a mouse model of *Salmonella* gastroenteritis. The pathological and histological characteristics of infection following antibiotic treatment of mice closely mirror infection in humans and calves.

Of importance in the study of *Salmonella* infection in mice is the differing susceptibility of mouse strains to this bacterium. Strains are divided into distinct groups of resistant, e.g. 129SvEv, and susceptible strains such as C57BL/6 and BALB/c. Following antibiotic pre-treatment, resistant and susceptible strains both develop acute intestinal inflammation; however while systemic dissemination in susceptible strains leads to death, resistant strains develop chronic colitis including crypt abscesses, ulceration, overshooting regeneration of the epithelium, and also inflammation of the gall duct epithelium (cholangitis) [141]. In the typhoid model of infection susceptible strains succumb to infection as described earlier in this section, whereas resistant strains control infection to become chronic carriers with *Salmonella* persisting in mLN over a year after infection [142]. A gene identified as responsible for the difference in susceptibility to *Salmonella*, and also other intracellular pathogens is Natural Resistance-Associated Macrophage Protein 1 (*Nramp1*), also called

Slc11a1 [143, 144]. NRAMP1 is produced selectively in macrophages and monocytes and targets the phagosome membrane, altering the environment within to assist in control of microbes here [145].

1.3.7.1 Bovine models

Cattle are natural hosts of *S.* Typhimurium and they can display clinical and histological manifestations strikingly similar to those of infection in humans. The outcome of infection depends on the dose of bacteria; an oral dose of 10^4 - 10^7 colony forming units (CFU) leading to transient diarrhoea followed by recovery, and a dose of > 10^8 resulting in lethality, though as in humans severity of infection reduces with age in early life. Clinical symptoms appearing within 12 - 48 h of infection include diarrhoea leading to dehydration and fever. Inflammation of the ileum is severe, with polymorphonuclear leukocyte (PMN) influx into the inflamed mucosa resulting in an initial drop in circulating immune cells and necrosis of the upper mucosa. Calf ligated ileal loops inoculated with *Salmonella* have provided a model to study the early stages of infection. With oral dosing, tissue along the intestine becomes occupied by *Salmonella* with different timings and densities of bacteria, whereas inoculation of ligated ileal loops resolves difficulties for studying temporal aspects of infection [146, 147]. Despite the closeness of the disease caused by *S.* Typhimurium in calves and humans, the costs of bovine models and lack of genetic homogeneity prevent their extensive use.

1.3.7.2 The streptomycin mouse model

Following the delivery of a single 20 mg dose of the antibiotic streptomycin, the natural colonisation resistance against *S.* Typhimurium afforded by the microbiota is transiently lost. An estimated > 10-fold reduction in the density and complexity of the microbiota has been observed [108]. Recovery of protection takes a few days and may be accelerated by co-housing with untreated mice [148]. Though antibiotic delivery has reported effects on intestinal tissue homeostasis beyond depletion of the microbiota, it is thought that these effects are of minor if any significance, relative to the effect on colonisation resistance.

Oral delivery of *S.* Typhimurium bacteria as few as 100 results in high levels of colonisation; 10^8 - 10^{10} CFU/g intestinal tissue, though larger doses are used experimentally. Inflammation occurs rapidly; within 6 - 8 h the mucosa is invaded by bacteria and pronounced

mucosal inflammation can be detected. Inflammation is localised to the proximal colon and in particular the caecum, the blind-ended sac located at the junction between the ileum and the colon which in herbivorous animals plays an important role in the digestion of their cellulose-rich diet. In humans the small intestine is generally regarded as the worst affected region and is the predominant site of inflammation in calves also. However in mice small intestine is rarely inflamed. Despite the difference in the tissue affected the histopathology of the inflammation is comparable [149]. Similarly studies of *Salmonella* virulence mechanisms suggest that similar factors are required for the colonisation of the mouse caecum and human/bovine small intestine.

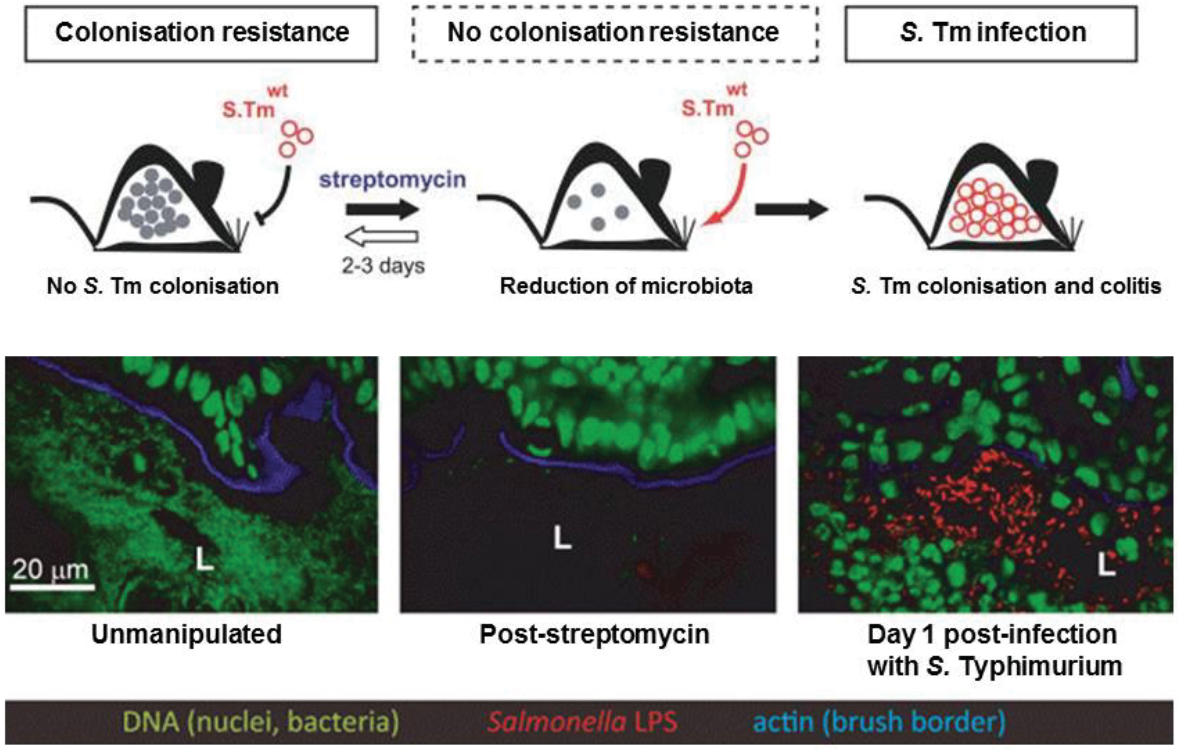

Figure 1.8. Antibiotic treatment in the streptomycin mouse model overcomes colonisation resistance. Upper - Schematic showing the relationship between the microbiota and colonisation of the mouse intestinal tract by *S.* Typhimurium. A substantial reduction in the microbiota occurs within 24 h of an oral dose of streptomycin enabling orally delivered *S.* Typhimurium to take hold in the caecum and colon. Without infection recovery of the microbiota is sufficient 2 - 3 days after streptomycin treatment to restore colonisation resistance. Lower - Confocal microscopy images of fluorescently stained caecum from an untreated mouse, 24 h after streptomycin treatment, and day 1 PI after streptomycin treatment. 'L' indicates the gut lumen. Luminal bacteria are dramatically reduced following streptomycin treatment. Within 24 h of infection *S.* Typhimurium is clearly visible in the lumen. Modified from [108] and [139].

Invasion of the mucosa occurs through multiple routes. As in the murine typhoid model *Salmonella* invade the intestinal patches of the GALT, but in the streptomycin model bacteria also invade the absorptive intestinal epithelial cells. Acute intestinal inflammation occurs regardless of the absence of the patch structures, ILFs and lymph nodes in mice lacking a receptor for immune system development factors called lymphotoxins ($LT\beta R^{-/-}$), indicating that the induction of inflammation occurs through the interactions of *Salmonella* with the enterocytes [149].

A major advantage of the model is that it is highly robust; mice are consistently colonised with high numbers of bacteria and quickly develop acute intestinal inflammation. Regardless of the numbers of bacteria in the inoculum the final density and the pathological characteristics are similar.

The pronounced inflammation of the caecum and proximal colon has been described in detail. In streptomycin-treated infected mice the caecum is shrunken, pale and pus-filled, and the proximal colon pale and swollen. At the microscopic level changes in the caecum include pronounced oedema in the submucosa and oedamatous changes in the lamina propria. Crypts are elongated and display disrupted architecture, there are fewer goblet cells, and the epithelium is eroded and/or ulcerated. There is pronounced PMN infiltration of the submucosa, lamina propria and epithelial layer, and PMN migration into the intestinal lumen. Changes in the colon are similar but less severe, as described in [149].

In most aspects *S.* Typhimurium infection in streptomycin pre-treated mice closely mirrors calves and humans however a notable difference is the absence of diarrhoea in mice. Murine infection results in increased stool water content and mucus, but such effects are incomparable with the extensive diarrhoea which defines human NTS. The difference is thought to result from the adaptation of the mouse gastrointestinal tract to maximise fluid absorption. At the microscopic level the changes that occur in the infected mucosa are similar in calves and in the streptomycin mouse model.

The use of genetically modified mice and bacteria in the streptomycin model has helped to uncover the pathways by which *S.* Typhimurium elicits inflammation in the intestinal tract. Two independent inflammation triggering pathways, one of which requires the T3SS of SPI-1 and the other requiring SPI-2 contribute in parallel, and through infections with *S.* Typhimurium derivatives harbouring mutations in these loci, the mechanisms have

been disentangled. Bacteria which are deficient in SipA, SopE and SopE2 effector proteins delivered into host cells by the T3SS of SPI-1 display attenuated inflammation in the streptomycin mouse model [150]. The induction of inflammation by active injection of inflammatory effectors is referred to as the 'classical pathway'. *Salmonella* mutants lacking a functional SPI-1 T3SS are able to induce inflammation via the 'alternative pathway' [149]. Penetration of the epithelium by routes other than through invasion of epithelial cells, such as transport via dendritic cell extensions and in M cells, bypasses the need for SPI-1 activity and the SPI-2-dependent occupation of macrophages leads to inflammation. The exact mechanism is not understood but it is thought detection of high bacterial loads through MyD88 signalling might be responsible.

Knockout and other genetically manipulated mice have produced insight into host response pathways, both protective and detrimental. The cytokine IFNγ induced early in response to infection was shown to be required for full inflammation and control of bacterial loads through knockout studies [151]. Infection of *IL22*$^{-/-}$ mice showed that despite the important role of IL22 signalling for maintenance of the mucosal barrier, IL22 increases the severity of infection with *S.* Typhimurium by induction of responses at the epithelial surface and in the lumen which favour survival of *Salmonella* over commensals [152].

1.4 Murine models of intestinal inflammation

S. Typhimurium infection in streptomycin pre-treated mice is one of many mouse models of intestinal inflammation. While diseases involving intestinal inflammation have disease-specific mechanisms they also share common pathways, and findings from one disease model can generate greater understanding of the pathogenesis of multiple conditions. Two clinically distinct diseases characterised by intestinal inflammation, ulcerative colitis and Crohn's disease (known collectively as IBD), are relatively common autoimmune disorders [153]. Symptoms of IBD include diarrhoea, abdominal pain, rectal bleeding and weight loss. A combination of environmental and genetic factors give rise to disease and the causes are not well understood. An involvement of bacteria has been demonstrated but there are multiple theories as to how, for example whether dysbiosis is a cause or a consequence of IBD, and whether intestinal inflammation induced by pathogens plays a role [73]. Studying systems such as the streptomycin mouse model can help to uncover mechanisms by which bacteria induce inflammation that may be relevant to IBD. Enteric bacterial pathogens share many

common virulence strategies and through the study of multiple infection models these conserved approaches can be understood and used to identify targets for drugs and vaccines.

Gut inflammation models include chemical, genetic, immunological and bacterial systems, each suited to the study of different aspects or stages of inflammation. As mentioned earlier mutant mice are important for the study of the innate and adaptive immune system in disease development. Immunological models which involve the adoptive transfer of specific immune cell subsets also help to determine the contributions of specific cell types. The following two examples are chosen to demonstrate the utility of chemical models for study of inflammatory processes, and to introduce a model of bacterial diarrhoeal disease with similarities to *S.* Typhimurium infection.

1.4.1 *Citrobacter rodentium*

C. rodentium is a Gram-negative bacterial pathogen of mice which colonises the intestine through specialised attaching and effacing (A/E) lesions. Closely related human A/E pathogens Enteropathogenic and Enterohaemorrhagic *E. coli* (EPEC and EHEC) colonise mice poorly. Consequently *C. rodentium* provides a useful model to study pathogenic mechanisms specific to A/E bacteria, and also those widespread amongst intestinal bacteria. Oral delivery of *C. rodentium* to mice results in colonisation 3 - 4 weeks in duration, initially occurring in the caecal patch and spreading to the distal colon. Concomitant with the peak of colonisation at 5 - 14 days PI is acute intestinal inflammation. *C. rodentium*-induced colitis is mild relative to *S.* Typhimurium-induced colitis in the streptomycin pre-treatment model, and characterised by crypt hyperplasia, loss of goblet cells and infiltration of mononuclear cells. In most strains of mice bacteria are cleared by 21 - 28 days PI, although very young mice are more susceptible and may die [154].

In contrast to *Salmonella*, *C. rodentium* is largely non-invasive, residing in the lumen attached to the surface of epithelial cells. Despite their different niches they share common virulence strategies. In common with the SPI-1 and SPI-2 T3SS of *S. enterica* the locus of enterocyte effacement (LEE) in *C. rodentium* encodes a T3SS for translocation of effectors, encoded both within the LEE genomic region and separately on prophages and elsewhere in the genome. The varied functions of the *C. rodentium* effector proteins have been explored through systematic mutagenesis and include permeabilisation of the mitochondrial membranes which triggers induction of cell death (EspF), and disruption of epithelial barrier

function (Map). In common with the SPI-1 T3SS effectors, which commandeer the host cell cytoskeleton to induce ruffling and ultimately bacterial uptake, several *C. rodentium* effectors interact with the host cytoskeleton. These direct cytoskeletal rearrangements leading to the formation of pedestal-like protruding structures in the host membrane with localised destruction of the microvilli [155].

The importance of the innate immune system in overcoming *C. rodentium* has been investigated. Epithelial cell secreted defensins may play a role, as has been indicated by increased susceptibility in *IL12*$^{-/-}$ mice which show reduced expression of β-defensin 3. iNOS is increased in epithelial cells during infection although this appears to have limited functional significance as *iNOS*$^{-/-}$ mice clear bacteria normally [156]. Mice lacking T and B cells are chronically infected, demonstrating adaptive immunity is essential for efficient *C. rodentium* clearance [157]. The restoration of protection in CD4^{+} T cell deficient mice by transfer of serum IgG and IgM from previously recovered mice indicates that the T helper-dependent serum antibody response is key [158]. The elimination of infection appears to require IgG in particular over IgM or IgA, which may access the gut lumen due to the increased epithelial permeability in the infected gut [159].

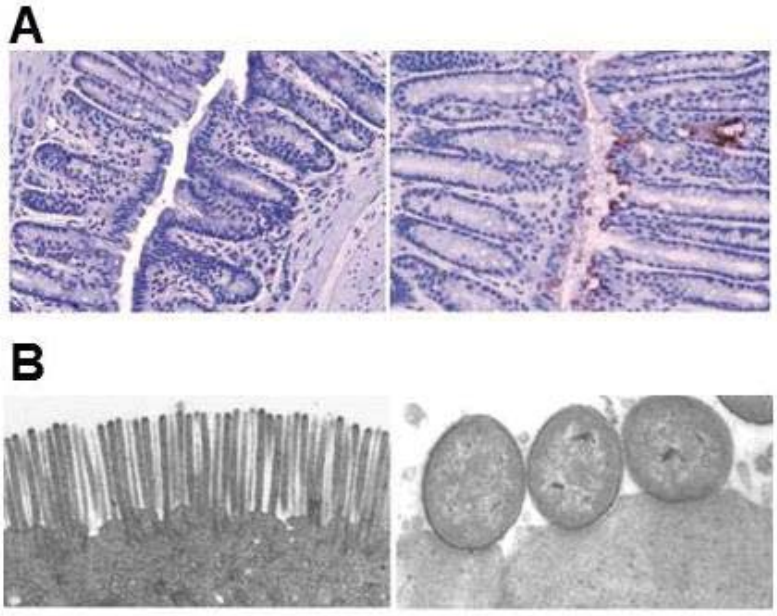

Figure 1.9. Microscopic changes in the mouse colon during infection with *C. rodentium*. (A) Hematoxylin and eosin staining combined with staining for the *C. rodentium* adhesin intimin in colonic tissue from an uninfected mouse (left) and a mouse colonised with *C. rodentium* (right). In infected tissue crypt hyperplasia transforms the mucosal architecture. (B) Transmission electron micrographs showing the normal brush border microvilli (left) and erosion of microvilli in A/E lesions associated with *C. rodentium* (right). Taken from [160].

1.4.2 Dextran sodium sulphate (DSS)-induced colitis

One model of chemically-induced inflammation involves the addition of the sulphated polysaccharide dextran sodium sulphate to drinking water. DSS is highly variable in molecular weight, ranging from 5 - 1400 kDa, and inflammatory responses induced vary according to size of the DSS and a range of other factors including concentration and frequency of administration, as well as mouse strain, intestinal flora composition and animal stress. DSS is toxic to epithelial cells and triggers cell death allowing DSS to penetrate the mucosal membrane.

The similarity between the clinical and histopathological features of DSS colitis and human IBD make this a valuable model. Addition of 2 - 5% DSS to drinking water for a period of around a week results in acute colitis and can involve weight loss, diarrhoea, blood in stools, anaemia, and eventually death. Histological changes include the depletion of the mucus barrier, and infiltration of neutrophils into the lamina propria and submucosa with their further migration to the lumen causing crypt abscesses. With continuous treatment of low doses mice develop chronic colitis within a few weeks. The changes resulting from longer term exposure include infiltration of mononuclear leukocytes, extensive disruption of crypt architecture, and widening of the gap between the bases of the crypts and the muscularis mucosa [161].

The microbiota has been shown to play an important role in the development of DSS colitis, as with human IBD. The delivery of antibiotics in acute DSS colitis results in improved histological parameters in treated mice compared with untreated controls showing that bacteria or their products are important to trigger inflammation [162]. Similarly germ-free mice fail to display the changes in intestinal morphology associated with DSS treatment in conventionally housed mice [163]. Microbes are not required for the destruction of the mucosa, however the loss of tight junction proteins and barrier integrity allows the luminal antigens and microorganisms entry to the mucosa triggering an overwhelming immune response. As early as one day after the commencement of DSS treatment inflammatory cytokines including TNFα, IL1β, IFNγ, IL10 and IL12 are induced, increasing further with continued treatment. IRF1 is a transcription factor downstream of TNFα and IFNγ signalling which induces major immune pathways including interferon signalling and iNOS production. *IRF1*$^{-/-}$ mice show increased susceptibility to DSS colitis indicating these responses are protective [164]. Similarly *TLR4*$^{-/-}$ and *MyD88*$^{-/-}$ mice display increased bacterial translocation

to lymph nodes and decreased epithelial proliferation showing the importance of TLR signalling for protection [165, 166].

1.5 Immune response to *S.* Typhimurium infection in the intestinal mucosa

Two arms of an immune response combine to confer protection against infectious microorganisms; an innate component is triggered initially and mediates rapid protection via antigen-independent mechanisms, and an adaptive response requiring several days to become active targets specific antigens. The innate component serves to highlight potential danger via immune receptors and downstream signalling pathways, resulting in production of cytokines. Cytokines mediate a plethora of functions acting to keep the threat under control, including recruitment and activation of phagocytes. The adaptive arm of the response mediates targeted killing through the action of specialised cells, and production of antibodies which bind and opsonize microbes to facilitate clearance by phagocytes. Upon re-exposure, the immunological memory conferred by the adaptive system controls the pathogen more rapidly and typically prevents re-infection.

1.5.1 Innate immune surveillance and signalling

The earliest interaction of the immune system with a microbe occurs via PRR, specialised receptors for interaction with conserved microbial molecules and structures (PAMPs). Several classes of PRR exist and together they possess a large number of binding specificities, across multiple locations. Three of the major families are the Toll-like receptors (TLRs), nucleotide-binding and oligomerization domain-like receptors (NLRs) and complement. Different TLRs are located at distinct sites within the cell membrane and membranes of intracellular compartments, both in epithelial cells and sentinel cells directly below, the compartmentalisation allowing a close watch over the microbes which invade the epithelium. NLRs are located in the cytosol where they typically detect the presence of virulence-associated factors such as T3SS components. In contrast to the two cellular receptors, complement is a humoral component of the PRR repertoire and is activated by cleavage after exposure to bacteria which have crossed the epithelial barrier. It has been argued that a distinction exists between pattern recognition and ‘pathogen-induced process recognition’, the former simply alerting the immune system to the presence of a microbe while the latter provides some indication of the pathogenic potential or level of threat [167].

Downstream of receptor activation, signalling pathways coordinate an immune response to the assault. Collections of TLRs recruit common adaptor proteins to signal by shared pathways to induce a similar response. For example TLRs 1, 2, 4 and 5 recruit the common adapter MyD88 and activate mitogen-activated protein (MAP) kinase signal transduction pathways. These activate transcription factor activator protein-1 (AP-1) and nuclear factor kappa-light chain enhancer of activated B cells (NFκB), which induce a collection of proinflammatory cytokines including IL23α, TNFα, IL12α, IL1β and IL18 in mononuclear and epithelial cells. Typically during infection a relatively small fraction of host cells contain bacteria, and therefore amplification pathways are required in order to generate a signal strong enough to induce effector functions. Two cytokines of the primary proinflammatory signal, IL18 and IL23 initiate major amplification pathways. T cells are important for amplification by the production of cytokines such as IFNγ, IL17 and IL22 [168]. This combined collection of cytokines mediates major antibacterial responses: macrophage activation, neutrophil recruitment, and epithelial release of antimicrobials.

Activation of C3 results in the production of the anaphylotoxins C3a and C5a, potent inducers of inflammation. These stimulate release of histamine and TNFα from basophils and mast cells, resulting in vasodilation and recruitment of neutrophils [169]. Reactive thioester groups of C3b produced in the proteolytic cleavage of C3 form esters with hydroxyl groups of bacterial surface carbohydrates, acting to opsonize bacteria for phagocytosis by neutrophils, macrophages and dendritic cells. Finally, activation of the alternative pathway for complement activation leads to the formation of the membrane attack complex (MAC), which kills gram negative bacteria by the formation of pores in the bacterial outer membrane.

S. Typhimurium activates a multitude of PRRs, including both humoral proteins and cellular receptors. The O-antigen of its LPS activates complement component 3 (C3) triggering the alternative pathway of complement activation. The lipid A moiety of LPS activates TLR4 in host cell vesicles, TLRs 1 & 2 are activated by the biofilm protein CsgA and TLR5 on the basolateral membrane of intestinal epithelial cells is activated by the major protein subunit of flagellin, FliC [170, 171]. Each of these TLR recruit Myd88 and activate signalling to induce a range of proinflammatory genes as earlier described. *S.* Typhimurium is responsible for triggering pathogen-induced process recognition as a result of T3SS-dependent delivery of proteins into the host cytosol. The needle complex protein PrgJ and the flagellar component flagellin are recognised by NOD-like receptor NLRC4. Further, cell wall fragments released into the cytosol as a result of host cell invasion are detected by the

receptors NOD1 and NOD2, which via protein kinase receptor interacting protein-2 (RIP2), mediate NFκB activation.

1.5.2 Three major innate effector responses to *S.* Typhimurium

1.5.2.1 Macrophage activation

The intracellular niche preferred by *S.* Typhimurium for survival and replication is the SCV inside tissue resident mononuclear phagocytes within the reticuloendothelial system. The host defends against *Salmonella* residing here by enhancing the ability of macrophages to kill bacteria within. In response to activation macrophages use iNOS to generate reactive nitrogen species for bacterial killing. Macrophage activation is enhanced by stimulation with the cytokine IFNγ, produced by CDαβ T cells, $CD4^+$ αβ memory type -1 T helper cells (Th1), and natural killer (NK) cells in response to IL12 and IL18. The importance of the IFNγ axis in maintaining barrier function in the mucosa is illustrated by increased susceptibility to disseminated infections with NTS serotypes in humans with defective components of the IFNγ axis [172].

In order to stay ahead of the increased killing action of the macrophage, *Salmonella* induces pathways which lead to host cell death and release of bacteria to infect new host cells [173]. After activation of the NOD-like receptor NLRC4 by the T3SS needle complex components PrgJ and flagellin, NLRC4 forms a complex with caspase-1 and ASC resulting in inflammasome formation and macrophage death by an inflammatory process called pyroptosis. Alternatively a T3SS-independent pathway via TRIF and receptor interacting proteins 1 & 3 (RIP1 & 3) induces programmed necrosis [174, 175].

Later in the course of the infection *Salmonella*-specific antibodies opsonize the bacteria to further assist macrophage killing. T cell-dependent processes contribute also [176].

1.5.2.2 Neutrophil recruitment

Compared with the macrophage, a second innate phagocytic cell type is a highly effective destroyer of *Salmonella*. Circulating neutrophils are recruited to the intestinal mucosa by chemoattractants produced by epithelial cells. Despite the largely intracellular lifestyle of *Salmonella* there exist small windows of opportunity for neutrophil attack in the short extracellular transition from an infected cell to a new host. The cytokines IL23 and

IL1β, produced in the initial inflammatory response, act upon Th17 cells, γδ T cells, and NKT cells to induce the production of IL17 [177]. Stimulation by this cytokine along with IL1β and TNFα induces epithelial cell production of neutrophil-recruiting CXC chemokines. The anaphylotoxin C5a produced during the complement cascade also functions to recruit neutrophils.

Lessons from infections in which neutrophil function is somehow absent or impaired demonstrate the importance of this cell type in resolving infection. Neutropenic individuals fail to control *S.* Typhimurium in the gastrointestinal mucosa and develop life-threatening bacteremia [178]. In *S.* Typhi infection the presence of the capsular polysaccharide Vi has been shown to negatively impact upon on C3b fixation and neutrophil killing by opsonophagocytosis. Greater resistance to killing by neutrophils likely aids the ability of *S.* Typhi to cross the intestinal barrier of the GI tract and disseminate systemically. Though essential, neutrophil recruitment comes with a cost; extravasation of neutrophils and passage into the gut lumen causes tissue damage [179].

1.5.2.3 Antimicrobial peptides

IL23 produced by *Salmonella*-infected macrophages and dendritic cells induces production of IL22 by $CD4^+$ αβ memory type-17 cells (Th17 cells), γδ T cells, and NKT cells. IL22 acts upon intestinal epithelial cells to stimulate the production of antimicrobial molecules which are secreted into the intestinal lumen to attack the bacteria. These molecules include the eneterobactin-binding protein lipocalin-2 and the bactericidal C-type lectin, regenerating islet-derived 3 gamma (Reg3γ) [180].

Depletion of the cells which initiate signalling to induce antimicrobial peptide production results in a compromised epithelial barrier as demonstrated in a mouse model and in SIV infection in rhesus macaques [90, 181].

Unlike the activation of macrophages and recruitment of neutrophils which target bacteria following their departure from the lumen into mucosal tissue, the release of antimicrobial peptides acts in the lumen where many beneficial bacterial species reside. Consequently these can have severe impacts on both enteric pathogens and normal gut bacteria, though as described in (section 1.3.6.3) pathogenic bacteria including *S.* Typhimurium have evolved mechanisms to overcome the effects of the antimicrobial peptides.

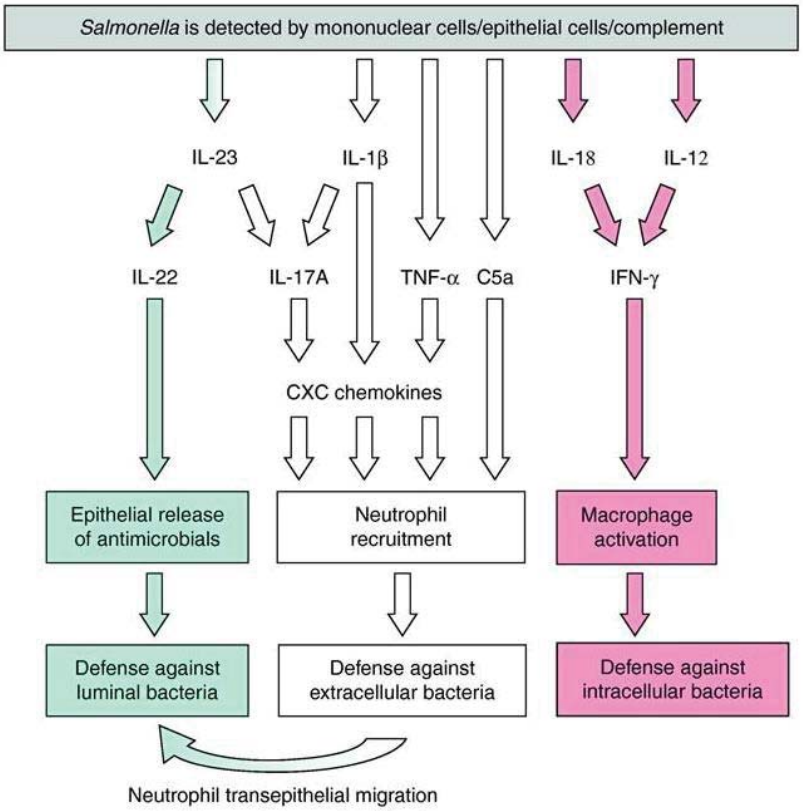

Figure 1.10. The major pathways in the mucosal response to *S.* Typhimurium. Following detection of *Salmonella* by epithelial or mononuclear cells, cytokine production is induced to bring about immune effector functions. Epithelial release of antimicrobial molecules occurs in response to activation by IL22. Multiple cytokines trigger the production of CXC cytokines which recruit circulating neutrophils to the site of infection, further aided by the anaphylotoxin C5a produced in complement activation. Th1 cytokines IL-18 and IL-12 act to induce IFNγ which stimulates macrophage killing of ingested bacteria. Through these three effector responses the immune system targets bacteria in multiple sites; in the lumen, and both extracellular and intracellular bacteria. Taken from [182].

1.5.3 The adaptive immune response

The role of the adaptive immune system in resolving infection and preventing re-infection has been relatively well studied in the murine *S.* Typhimurium model of typhoid fever. However, comparatively little research has investigated the involvement of the adaptive immune system in the gastrointestinal mucosa during gastroenteritis. Studies in the murine typhoid model have demonstrated that the importance of specific lymphocyte subsets is highly dependent on the infection method employed, and the particular *S.* Typhimurium and mouse strains involved [176]. However the consensus describes a significant role for T cells in both infection clearance and resistance to re-infection, with a role for B cells largely

restricted to the latter of these. T cell depletion studies and adoptive transfer of T cell-enriched cell fractions have shown that $CD4^+$ T cells play a more significant role than $CD8^+$ T cells in the murine model [183]. $CD4^+$ T cells undergo polarisation to Th1 cells in the cytokine environment created by infection [184]. The Th1 effector function of IFNγ production is key to the host response. Experiments show that in the absence of certain IFNγ-producing cell subsets responsibility for production of the cytokine is assumed by other cell types [185].

Systemic infection with *S.* Typhimurium results in a strong antibody response to both protein and non-protein antigens, though these may not be essential for resolving the primary infection [186]. However the transfer of serum from vaccinated to naïve mice can confer protection, demonstrating the importance of antibodies in the response to re-challenge [187]. The mechanism by which protection is achieved is only partially understood. Antibodies may bind *Salmonella* in the brief extracellular period following exit from an apoptotic phagocyte, preventing entry to a new cell. Opsonization of *Salmonella* for Fc-receptor mediated uptake by phagocytes and triggering the classical pathway of complement activation leading to complement fixation on the bacterial surface may also be important. Also *Salmonella*-specific IgA antibodies in the mucosa have been shown to play an important role; cells producing sIgA specific to the O-antigen are one of the best correlates of protection following vaccination in humans [188].

To study the role of the adaptive immune system in non-typhoidal *Salmonella* infection the streptomycin mouse model described in section 1.3.7.2 has been studied [50]. *S.* Typhimurium with a mutation in the SPI-2 secreted effector protein *sseD* generates infection which is confined to the intestinal mucosa and GALT and cleared naturally. In this model mice lacking either T cells ($TCR\beta^{-/-}\delta^{-/-}$) or B cells ($J_H^{-/-}$) demonstrate normal clearance and recovery from infection, indicating T or B cells are not essential for these processes.

Salmonella-specific sIgA are detected in the intestinal lumen within two weeks of infection with *S.* Typhimurium *sseD*, and are proposed to play an important role. sIgA was shown to agglutinate *S.* Typhimurium in the gut lumen, reducing access to the mucosal surface for invasion into tissue. However the presence of sIgA did not affect the process of clearance during an initial infection with *S.* Typhimurium in this model, instead *Salmonella*-specific luminal IgA was found to protect against re-infection. The use of an invasion deficient strain of *S.* Typhimurium confirmed previous work finding invasion is essential to

trigger production of *Salmonella*-specific sIgA [189]. Many of the sIgA antibodies produced during infection were specific for the O-antigen; the highly exposed and polymeric nature of this molecule making it a strong target. The O-antigen-specific nature of sIgA protection was demonstrated by following an initial infection with *S.* Typhimurium with a secondary *S.* Enteritidis challenge; mice were not protected against infection with the heterologous serovar. Although sIgA protects against re-infection, clearance of *S.* Typhimurium in the primary infection was shown to rely on the presence of a diverse microbiota. Future research is needed to identify how the adaptive immune system and microbiota may interact to deliver protection against intestinal pathogens [50].

1.5.4 Genetic susceptibility to intestinal inflammation in humans

Experimental animal models have provided many insights into pathways involved in inflammatory responses to enteric pathogens, however human studies are also an important approach to understanding inflammatory pathways and host defence mechanisms. Rare genetic deficiencies resulting in increased susceptibility to infection inform about the roles of the proteins they encode in a normal protective response.

1.5.4.1 Infection

Several genetic defects in individuals highly susceptible to severe or even fatal infections with otherwise weakly pathogenic strains of *Mycobacteria* or *Salmonella* have been identified. Mutations were discovered in genes of the pathway by which Th1 cells stimulate macrophage killing of intracellular bacteria, showing the central importance of this pathway in controlling intracellular bacteria. Mutations in IL12-p40 and IL12Rβ1 affect the stimulation of Th1 and NK cells while mutations in IFNγR1 and IFNγR2 impact upon IFNγ detection by macrophages. Mutations in signalling molecules downstream of the IFNγ receptor such as STAT1 similarly prevent the translation of activating signals to effector functions in the macrophage [190, 191]. Such defects in the host immune response have been seen to provide a niche for *Salmonella* within the host and facilitate evolution to adapt the bacteria to the systemic environment (Klemm, unpublished).

1.5.4.2 Inflammatory bowel disease

Genome-wide association studies (GWAS) involving sequencing or genotyping many individuals with or without a particular disease have been used to identify genetic loci associated with the disease condition. In inflammatory bowel disease, GWAS has identified over 160 loci, many of which are associated with both Crohn's disease and ulcerative colitis [192]. A potentially close relationship between the autoimmune disease-associated loci and bacterial defences was clear for some of these genes. Genes important for processing intracellular bacteria are highly overrepresented in the GWAS loci for Crohn's disease, for example NOD2, IRGM and ATG16L1 [193]. NOD2 is an intracellular sensor for bacteria which together with NOD1 recruits the autophagy protein ATG16L1 to the membrane at the site of bacterial entry [194]. The failure of this autophagy pathway leads to altered bacterial handling and ultimately inflammation. Many of the associations identified by GWAS require further study to determine their potential role in bacterial infection and inflammation.

1.6 Aims of the thesis

The Wellcome Trust Sanger Institute (WTSI) has established a phenotypic screening platform using novel mutant mice that incorporates a pathogen challenge component (http://www.immunophenotyping.org/). This screen includes a systemic but not an oral *Salmonella* challenge. Therefore we decided to explore the potential of the murine *Salmonella* oral streptomycin treatment model as a secondary phenotyping component of the screen. To this end we used combined functional genomic approaches, including RNAseq and proteomics, to analyse wild type and selected mutant mice in depth. We anticipated this work would lead to the identification of key signatures and drive hypotheses for investigation in further experiments. Examples of such signatures are included in the work described herein.