1: Introduction

1.1 Classification of Salmonellae and global burden of disease

Salmonellae are Gram-negative, predominantly flagellated, facultative intracellular bacteria that are an important cause of enteric disease in humans and animal hosts worldwide. They are members of the Enterobacteriaceae family, thought to have diverged from *Escherichia coli* between 100-150 million years ago[1] and are genetically diverse, having adapted to colonise numerous animal hosts and are even able to exist freely in the environment.[2] Transmission is largely via the faeco-oral route. Classification and nomenclature methods have led to some confusion about the number of species of the genus *Salmonella*, but molecular work determined that there are two *Salmonella* species which have the ability to infect humans: *Salmonella enterica* and *Salmonella bongori*.[3,4] Isolates from the *S. enterica* species are the predominant cause of disease in humans, therefore this species will be the main focus of the remainder of this introduction. *S. enterica* is subdivided into six subspecies: *S. enterica* subsp. *enterica*, *S. enterica* subsp. *salamae*, *S. enterica* subsp. *arizonae*, *S. enterica* subsp. *diarizonae*, *S. enterica* subsp. *houtenae* and *S. enterica* subsp. *indica*.[5] These subspecies are separated by a number of differential characteristics, modified from the scheme proposed by Kauffmann in 1973,[6] including physiological characteristics and serological identification of: O (lipopolysaccharide - LPS), H (flagella), and K (capsular) surface antigens. For the purposes of this study, we will focus solely on *S. enterica* subsp. *enterica,* as serovars from this subspecies are the predominant cause of human salmonellosis, both typhoidal and non-typhoidal. *S. enterica* subsp. *enterica* itself is antigenically diverse in terms of major surface antigens, incorporating over 1500 serovars of the ~2500 that have been identified as belonging to the *S. enterica* species.[5]

Medically relevant *S. enterica* serovars can be arbitrarily classified into typhoidal and non-typhoidal (NTS) types based on their pathogenicity in a particular host. *S. enterica* serovar Typhi (*S.* Typhi) is a human-restricted serovar which causes typhoid fever, and is responsible for ~21 million cases and 222,000 deaths per year,[7] with peak incidence in the paediatric and elderly populations. Cases are predominantly concentrated in low-income settings

where sanitation facilities and access to clean water are limited. Other risk factors for the development of typhoid fever are influenced by setting but include: consumption of food or drink from street vendors,[8] a close contact or relative with typhoid fever[9] and recent antimicrobial use.[8]

Parts of South Asia, Southeast Asia and sub-Saharan Africa have traditionally had the highest incidence of typhoid disease, followed by China and Oceania (excluding Australia/New Zealand).[10] However, the incidence of typhoid can vary over time and fluctuations in the levels of disease are common. In addition, many endemic countries do not have a well-established national surveillance system for typhoid fever, so incidence may be inaccurately estimated.[11] Similarly, passive surveillance relies on clinical diagnosis with blood culture facilities not being readily available, so areas where health care infrastructure is weak (and disease burden potentially high) may not be able to record incidence definitively, which can certainly limit available data on circulating strains and antibiotic sensitivities. In recent years, initiatives such as the STRATAA study[12] have produced detailed datasets from areas of high incidence, and obtained important anthropological data on population dynamics and healthcare seeking behaviours, which could prove vital to controlling disease.

The estimated burden of paratyphoid fever, (caused by *Salmonella enterica* serovars Paratyphi A, B and C) suggested that there were ~3.75 million cases of paratyphoid fever in 2016,[13] with 25000 deaths from the condition.[14] Paratyphoid fever may generally have a less severe disease course but these data indicate that it also constitutes a significant global health burden. In fact, in some regions, such as Thailand[10] and China,[15] paratyphoid fever incidence appears to be increasing, which is of especial concern given that there is no effective vaccine available for this pathogen, and cross-protection from typhoid vaccines is limited.[16] **Figure 1.1** demonstrates the global incidence of typhoid and paratyphoid fevers.

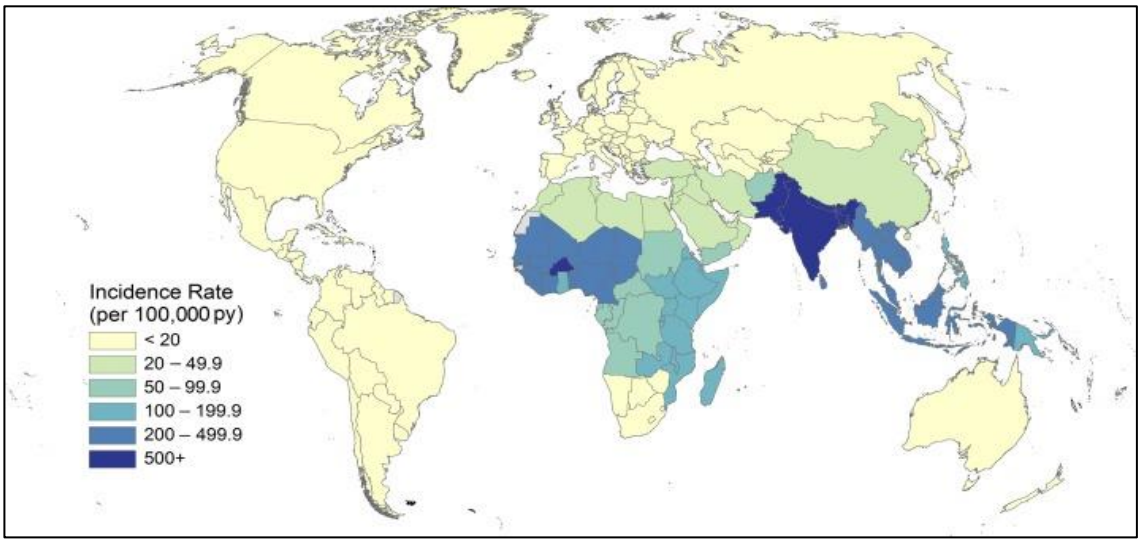

Figure 1.1: Estimated incidence of typhoid and paratyphoid fevers by country per 100,000 population, 2015. (Figure taken from Radhakrishnan, A. *et al*[10])

Non-typhoidal *Salmonella* (NTS) serovars (e.g. *S. enterica* serovar Typhimurium (*S.* Typhimurium), *S. enterica* serovar Enteritidis (*S*. Enteritidis)) are responsible for over 93 million infections and ~155,000 deaths worldwide per year,[17] the majority of which are thought to be food-borne infections. NTS infections impact a range of vertebrate hosts in addition to humans[18] and cause varying disease phenotypes. These pathogens typically cause a self-limiting gastroenteritis in patients in high-income countries, with incidences of complications generally limited to certain patients, such as the elderly or immunocompromised.[19] However, in low-income settings, NTS infections are a common cause of invasive disease (iNTS) involving bacteraemia, with a mortality rate as high as 25%.[20] In adults in these settings, *Salmonella*-associated invasive disease is found predominantly in those co-infected with HIV.[21] In children, malaria,[22] HIV,[23] malnutrition[24] and sickle cell disease[25] are frequently associated with the invasive phenotype. Symptoms of iNTS may be non-specific, but can include persistent fever, pneumonia, enterocolitis and hepatosplenomegaly.[18] Treatment of iNTS infection has also been complicated by the emergence of epidemic-causing MDR strains with distinct genotypes, such as the *S*. Typhimurium ST313 serovar isolated in Sub-Saharan Africa,[26] and the spread of disease is exacerbated by a lack of vaccines directed against iNTS strains.

Given the faeco-oral nature of *Salmonella* transmission, WaSH strategies such as improvements in water supply and sanitation infrastructure and case identification and

treatment have made incidence of typhoidal disease rare in parts of the world where these practices have been put into place. Cases in these areas occur mostly via travellers returning from endemic countries.[27] Much remains to be achieved in countries where disease incidence of typhoid, paratyphoid and iNTS remain high, with antimicrobial resistance becoming an increasing threat to current treatment options.[28]

1.2 Pathogenesis of and host response to *Salmonella* infection

1.2.1 Initial host-epithelial interactions

As implied by the differing disease courses they follow, there are some similarities and some differences between the interactions of typhoidal and non-typhoidal Salmonellae with the host epithelium. This section will explain the generally accepted mechanisms of non-typhoidal *Salmonella* infection and highlight serovar-specific mechanisms where appropriate.

1.2.1.1 From ingestion to the mucosal barrier

Following ingestion in contaminated water or food, *Salmonella* need to reach the small intestine in order to penetrate the intestinal mucosa. This requires the ability to survive the hostile acidic environment experienced within the stomach, with pH reaching as low as 1-2.[29] *Salmonella* have developed acid stress responses, such as the sigma(E) pathway[30] and the PhoPQ regulatory system[31] which enable them to do this. Factors increasing the stomach's pH can increase susceptibility to infection, such as use of proton pump inhibitors,[32] and the infective dose of *Salmonella* is thought to drop if bacteria are ingested with food, due to the temporary increase in stomach pH that food can produce.[29] Other methods by which *Salmonella* can temporarily adapt to a low pH environment, whilst maintaining a constant intracellular pH, include: use of innate proton pumps to extrude protons from the cytoplasm, intracellular conversion of lysine to cadaverine and arginine into agmatine
(reactions which lead to consumption of protons) and alteration in membrane content to increase levels of cyclic fatty acids.[33] Following their journey through the stomach,

Salmonella are further challenged in the duodenum by contact with bile, secreted from the gallbladder. Bile plays an important role in the digestion of lipids and its detergent-like properties make it inherently antimicrobial, allowing it to damage bacterial cell membranes, bacterial DNA and even alter membrane protein composition.[34] Factors affecting susceptibility to iNTS such as malnutrition can decrease the amount of bile produced, increasing the likelihood of bacterial survival in the intestine.[35] *Salmonella* have a number of mechanisms in place to counteract the effects of contact with bile, including bile efflux pumps, LPS expression (with O antigen providing a barrier to entry of external compounds) and again, the PhoPQ transcriptional regulatory system; overexpression of which enhances bile resistance.[36] This ability to tolerate high levels of bile salts is of particular importance for *S*. Typhi, given that chronic carriage of the pathogen is thought to occur within the gallbladder.[37] The PhoPQ system is also involved in controlling genes of the type III secretory systems (T3SS). High levels of bile salts induce *Salmonella* to transcriptionally repress genes of their Salmonella Pathogenicity Island 1 (SPI-1) T3SS, reducing epithelial invasion; suggesting that Salmonella are able to sense bile concentration, allowing them to determine their location in the intestinal lumen and utilise appropriate gene sets. The SPI-1 T3SS is likely then upregulated once *Salmonella* pass through the mucus layer towards the epithelium, as exposure to bile salts is diminished and invasion-related proteins are required.[38]

Having reached the small intestine, the next obstacle to *Salmonella* invasion is the colonisation resistance presented by immunological (e.g. T cell profile), microbiotal and metabolic (e.g. short chain fatty acid predominance) factors in the intestinal lumen,[39] alongside secretory Immunoglobulin A (sIgA), which is able to reduce adhesion and invasion of *Salmonella* into epithelial cells. Non-typhoidal Salmonella are able to overcome colonisation resistance by inducing an inflammatory response from the intestinal epithelium, which they are able to survive and then outcompete the host microbiota.[40,41]

Lastly, in order to adhere to and invade the intestinal epithelium, *Salmonella* need to penetrate the mucus layer lining the gut. Goblet cells in the intestinal epithelium secrete glycosylated proteins called mucins at their apical surface, which form a gelatinous layer preventing contact between the epithelial surface and inflammatory particles such as bacteria.[42] This layer is essential for keeping the intestinal epithelium in a quiescent state, with mice deficient in MUC2 (the major secretory mucin in mice and humans) developing

spontaneous colitis[43] and being significantly more susceptible to *S*. Typhimurium infection with a higher mortality rate.[44] It is not clear exactly how *Salmonella* penetrate the mucus layer; a recent study demonstrated 'near surface swimming' of *S*. Typhimurium in the colonic mucous layer, apparently sensing for sites of mucus heterogeneity which may provide an easier path down to the epithelium.[45] Other research suggests that non-fimbrial adhesins such as SiiE play an important role in invasion as they allow *Salmonella* to bind to glycosylated structures on the apical surface of the epithelial cells.[46,47] Indeed for *S*. Typhi, which has genes encoding a number of fimbrial operons not present in the *S*. Typhimurium genome, fimbrial structures also appear to be important for adhesion to and invasion of host cells.[48] Additionally, flagella are important both for chemotaxis towards the epithelium and subsequent colonisation of cells and induction of inflammation, but it is a matter of debate as to whether these structures facilitate invasion or merely provide proximity to the target epithelium to allow the SPI-1 T3SS to act.[49]

1.2.1.2 Penetration of the intestinal mucosa

If they survive the range of challenging conditions they encounter prior to reaching the small intestinal mucosa, *Salmonella* have the potential to penetrate the epithelial layer via enterocytes, microfold cells (M cells) or migrating dendritic cells. Some of these processes remain obscure given that much of what we know about *Salmonella* interactions with the epithelium, particularly those of the human-specific typhoid-causing serovars, have been extrapolated from mouse or 2-D cell culture models. Entry to enterocytes will be discussed below, but alternate routes of entry may play an important role in *Salmonella* invasion.
M cells are the specialised epithelial cells overlying Peyer's patches in the intestine, which sample antigens from the epithelial surface, initiating immune responses where required. However, many enteric bacteria actively interact with these cells as part of their invasion strategy, as M cells could represent a direct route to the gut-associated lymphoid tissue and potentially into the systemic circulation. Both *S*. Typhi[50] and *S*. Typhimurium[51] appear to selectively invade M cells in mouse models of infection, although *S*. Typhi do this at a lower frequency than *S*. Typhimurium.[52] It may be that this is the case in human disease also, with hyperplasia and ulceration noted at Peyer's patches during typhoid infection.[53] However, *in vitro* studies using both explanted intestinal biopsies and organoid-derived monolayers,

which contained M cells, were only able to demonstrate invasion into enterocytes.[54] In mouse models, it has been established that *S*. Typhimurium is efficient both at entering M cells and causing large structural changes of the cell (membrane ruffles) shortly after invasion. Within 30-60 minutes of *S*. Typhimurium entry, M cells can become necrotic and die.[55] *S*. Typhi are also able to induce large changes in M cell membrane structure, but do not induce cell death as efficiently and are cleared from Peyer's patches, rather than replicating inside them as does *S*. Typhimurium.[52] Interestingly, the T3SS is not required for entry of M cells, with SPI-1 mutants still able to invade M cells and colonise Peyer's patches.[56]

Another SPI-1-independent mechanism of entry for *Salmonella* to the lamina propria is via uptake by CD18+ dendritic cells (DCs), which are migratory phagocytic cells that sample antigens in mucosal tissues; performing a sentinel function similar to M cells. DCs also express some tight junction proteins, allowing them to open tight junctions between epithelial cells, send dendrites to the epithelium to sample bacteria and then re-instate the intestinal barrier's integrity.[57,58] Lastly, the T3SS of SPI-1 and SPI-2 have been shown to contribute to colitis and disruption of tight junctions in mouse models, allowing luminal *S.* Typhimurium to enter the lamina propria between rather than through the epithelial cells.[59]

1.2.1.3 Entry into epithelial cells

Intracellular pathogens such as *Salmonella* enter enterocytes via at least two mechanisms which are differentiated by the morphology induced by membrane re-modelling. The 'trigger' mechanism involves radical cytoskeletal arrangements called membrane ruffles (as described above for uptake by M cells), whereas the 'zipper' mechanism (also known as receptor-mediated entry) requires only limited cytoskeletal change, as the invading bacteria are avidly bound to the host cell membrane.[60] The trigger mechanism requires injection of bacterial effector proteins via T3SSs, whereas the zipper mechanism is induced by activation of host cell receptors and bacterial ligands such as invasin. *Salmonella* are unusual in being able to invade cells via both of these mechanisms,[61] although invasion is primarily thought to occur via the SPI-1 T3SS with the zipper mechanism being less well defined (**Figure 1.2**).

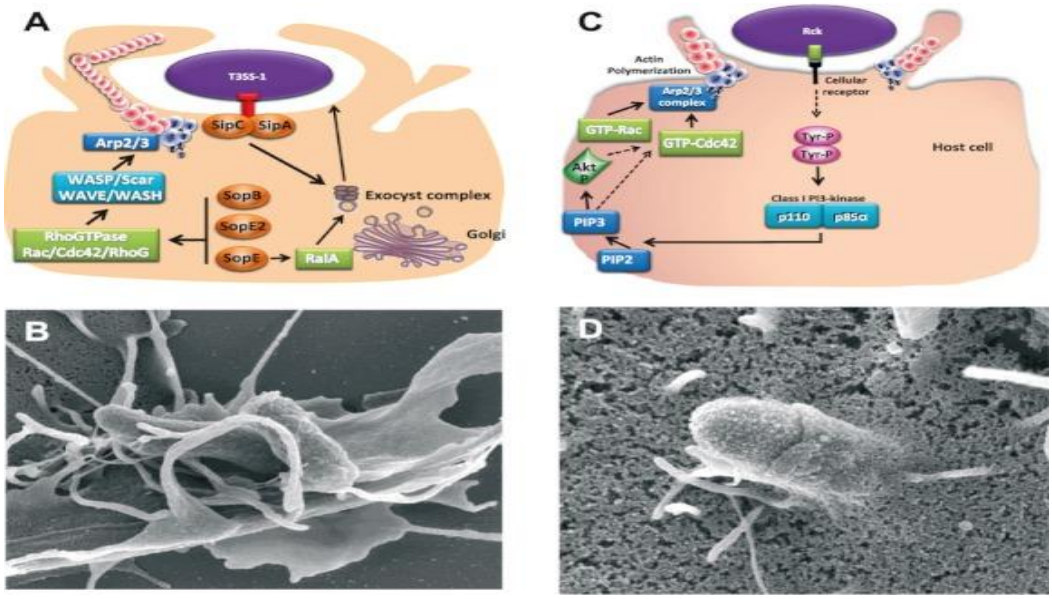

Figure 1.2: Schematic of 'trigger' and 'zipper' mechanisms of *Salmonella* cell entry. (**A**) Trigger mechanism – using T3SS; effector proteins (sipA, SipC, SopB, SopE, SopE2) are injected into host cells. SipA and SipC bind directly to actin. SopB, SopE, SopE2 active RhoGTPases to allow actin remodelling via WASP/Scar/WAVE/WASH proteins which activate Arp2/3 complex. SipC and SopE act on Ras-related protein RalA to recruit exocyst complex and allow bacterial internalisation. (**B**) Scanning EM image of *Salmonella* entering cell via the trigger mechanism with large membrane ruffles at bacterial entry site. (**C**) Zipper mechanism – the Rck invasins expressed on the *Salmonella* membrane interact with host cell membrane receptor, leading to tyrosine kinase phosphorylation. This activates class I PI 3-kinase, inducing PI (3,4,5)P3 formation, activating Akt. This leads to activation of GTPases Rac and Cdc42, triggering actin polymerisation via the Arp2/3 complex. This pathway is less well defined, with dotted lines indicating possible signalling events. (**D**) Scanning EM image of *Salmonella* entering cell via the zipper mechanism, with less defined membrane alterations. (Figure taken from Velge et al 2012[60])

Up to 21 different pathogenicity islands have been annotated in the genome of *S*. enterica,[62] but only 12 have been identified as having a clear role in *Salmonella* pathogenesis.[63] The T3SS encoded by the SPI-1 locus is one of the best characterised *Salmonella* virulence-associated factors, inducing *Salmonella* entry into eukaryotic cells. The T3SS is a protein complex sometimes described as a 'molecular syringe' as it is able to inject effector proteins directly from the bacterial cytoplasm into the host cell cytosol. These effector proteins modify cellular processes in a manner which benefits the bacterium injecting them.[60] Expression of T3SS genes and construction of the T3SS apparatus at the bacterial membrane is modified by environmental factors such as osmolarity, pH, Ca^{2+} availability and growth phase of the bacteria.[64] In a similar manner, genes encoding the Vi capsule in *S*. Typhi are affected by osmolarity; for example, the gene *TviA* which positively regulates the Vi capsule is repressed in high-osmolarity environments and induced in low-osmolarity environments.[65]

As *Salmonella* binds to the host cell surface, bacterial proteins are injected into the host cell via the T3SS needle complex in a specific order,[66] beginning with translocase proteins such as SipB, SipC and SipD. SipA and SipC induce cytoskeletal actin rearrangement, causing membrane ruffles and micropinocytosis of *Salmonella* into the cell; internalising bacteria inside membranous vacuoles (*Salmonella*-containing vacuoles, SCVs).[67] Effector proteins SopE, SopE2 and SopB target and activate Rho GTPases CDC42 and Rac, triggering signal transduction events, which also lead to actin rearrangement and release of pro-inflammatory cytokines via activation of MAP kinases JNK and p38.[68] These effectors can induce activation of the WASP/Scar/WAVE/WASH proteins, which activate Arp2/Arp3 complexes, triggering actin remodelling.[69] Membrane ruffles are transient and tend to occur within 10-30 minutes of contact with bacteria; rearrangements are usually reversed by 2-3 hours after bacterial entry. This reversal is mediated by SptP, an effector protein with a longer half-life than the other effectors, which downregulates CDC42 and Rac1, allowing cell membranes to return to a normal appearance.[70] It was also noted that SipC and SopE-dependent activation of RalA both induce exocyst recruitment, with the exocyst delivering vesicles to sites of bacterial entry to provide extra membrane material to enable ruffling and invasion to occur.[71]

Alternative *Salmonella* genes have also been identified as having a role in invasiveness, for example, *invA*. *S*. Typhimurium with mutations in this gene were less able to invade epithelial cells, in spite of being able to attach to them. The exact mechanisms underlying this invasion deficiency are ill-defined, but mutants appeared unable to alter the distribution of actin microfilaments in infected cells.[72]

1.2.1.4 Intracellular survival

Bacterial internalisation induces changes in host cell signalling, influencing numerous cellular processes, such as cell division, apoptosis, cytokine production, membrane trafficking and antigen presentation.[73] SPI-1 T3SS effectors can play a role in influencing these factors; for example SopB plays a role in SCV genesis and trafficking,[60]

but once invasion into the cell has occurred, *Salmonella* pathogenicity island 2 (SPI-2) T3SS effectors are upregulated and take on the task of promoting *Salmonella* survival within the cell. The SCV initially acquires early endosome markers, which are sequentially replaced by

late endosome and lysosome markers, such as the lysosomal glycoprotein Lamp1.[74] The SCV migrates from the cell periphery towards the nucleus within 1-2 hours of invasion,[75] under control of SPI-1 T3SS (SopB, SopA) and SPI-2 T3SS (SseF, SseG, SifA).[73]

As well as being involved in the early stages of SCV formation and movement, SopB is able to induce dissociation of a number of Rab proteins from the SCV, which delays SCV-lysosome fusion and prolongs bacterial survival.[76] In addition, this protein induces sorting nexin 3 (SNX3) activity, delivering Lamp1 and Rab7 to the SCV, allowing maturation.[77]

Salmonella replication normally occurs 4-6 hours post-invasion and coincides with extension of Lamp1 containing membrane tubules (Sifs) from the surface of the SCV,[74] under the influence of SPI-2 T3SS effectors, which allow the SCV both to remain in its juxtanuclear position and extend tubules towards the cell peripheries.[78] SCVs and Sifs are enriched in cholesterol, due to action of the SPI-2 T3SS effector SseJ.[79] Interestingly, in *S*. Typhi, SseJ is a pseudogene, and when *S*. Typhi are complemented with the *S*. Typhimurium SseJ gene, *S*. Typhi are significantly less toxic to epithelial cells, perhaps suggesting a mechanism by which *S*. Typhi are adapted to causing systemic disease.[80]

SifA, acting in conjunction with SipA, induces actin accumulation around the SCV, essential for intravacuolar replication.[81] One challenge that the SCV does face is a progressive acidification, caused by fusion with endolysosomes. *Salmonella* use mechanisms such as the PhoPQ regulatory system, which is activated by low pH to modify intravacuolar pH.[31] In addition, SifA and PipB2 proteins can manipulate the course of phagosomal maturation, preventing vacuolar lysis and ensuring replication of *Salmonella* within the vacuole.[82]

Having replicated within cells, *Salmonella* are able to induce host cell apoptosis, usually occurring 12-18 hours after bacterial invasion and mediated by TNFα and nitric oxide. Apoptosis may function to remove damaged and infected cells, in order to restore epithelial integrity to the host, but this delay in onset of apoptosis also has the benefit of allowing *Salmonella* to adapt to the intracellular environment before moving deeper into the mucosa.[83] In addition to apoptosis, inflammatory cell death (pyroptosis) can also be induced by the presence of *Salmonella* within the cell. Flagellin, SipB and SopE can all induce activation of caspase-1 and inflammasome construction, which in turn activates IL-1β and IL-18.[84,85] Additionally, LPS is a potent agonist of TLR4, triggering DCs to produce IL-23 in response to *Salmonella* infection.[86] Induction of pyroptosis may be advantageous for

Salmonella, with destruction of the epithelial barrier allowing further bacterial invasion into the lamina propria and exposing cells directly to intestinal luminal contents. This inflammation can also induce host immune response and recruitment of immune cells to the infection site.[87]

Interestingly, although persistence within the macrophage cytosol is potentially fatal for *Salmonella*, they are capable of surviving and replicating within the epithelial cell cytoplasm, at an even higher rate than when inside of the SCV. Once *Salmonella* escape into the cytoplasm (controlled in part by host cell autophagy[88]), they upregulate SPI-1 T3SS and flagellar genes, and as the host cell triggers inflammatory cell death and is extruded from the epithelial layer; this allows the release of numerous invasive and motile *Salmonella* to infect further cells.[89]

1.2.1.5 Invasion factors specific to typhoidal strains

The above pathogenic mechanisms for cellular entry and replication are active largely for non-typhoidal *Salmonella* strains lacking the Vi capsule expressed by *S*. Typhi, which can significantly modify interactions with host cells. Invasion (e.g. via SPI-1 T3SS), survival and replication in the epithelium (e.g. via SPI-2 T3SS) and recognition of bacterial pathogen-associated molecular patterns (PAMPs) by the mucosa all contribute to the inflammatory picture and neutrophil influx induced by non-typhoidal *Salmonella* infection. This neutrophil influx is largely absent in *S*. Typhi infection. *S*. Typhi is able to use SPI-1 T3SS to invade epithelial cells, SPI-2 T3SS to survive intracellularly, expresses PAMPs such as flagellin and LPS, and yet this serovar stealthily evades the inflammatory immune response.[90] Until recently, we lacked the ability to study this human restricted serovar in 3-D cellular models, therefore 2-D tissue culture models were used in attempts to clarify these differences between the serovars. Macrophage stimulation by *S*. Typhi induces much less IL-8 production compared with *S*. Typhimurium.[91] *S*. Typhimurium, but not *S*. Typhi, is able to induce migration of neutrophils across a monolayer of polarised colonic epithelial cells,[92] and *S*. Typhi does not induce a similarly pro-inflammatory transcriptional profile in epithelial cells.[93] These differences could be due, at least in part, to the shielding of flagellin and LPS by the Vi capsule, as *S*. Typhimurium mutants deficient in flagellin produced a similar inflammatory picture to *S*. Typhi, yet with flagellin intact and knocking out of the SPI-1 T3SS,

S. Typhimurium remained strongly pro-inflammatory.[93] Similarly, the presence of the Vi capsular antigen could explain the ability of *S*. Typhi to downregulate the Toll-like receptor (TLR)-mediated host response reducing inflammation and neutrophil influx into the gut epithelium.[94] Transcriptomic studies of *S*. Typhimurium have shown that SPI-1 and flagellin genes were upregulated for longer within epithelial cells than macrophages, potentially contributing to this inflammatory picture.[95] The outcome of differences in these interactions is the differing clinical picture associated with typhoidal and non-typhoidal *Salmonella* disease. The inflammatory response induced by S. Typhimurium for example, induces a diarrhoeal illness, whereas the early stages of *S*. Typhi infection are relatively undetectable, with symptoms only occurring once systemic bacteraemia occurs.

The Vi antigen does have a role in virulence of *S*. Typhi, with isolates lacking in Vi being 10,000 times less virulent than encapsulated *S*. Typhi in intraperitoneal murine infections.[96] Although unencapsulated *S*. Typhi were still able to cause typhoid disease in human challenge studies, they were associated with half the number of cases of Vi encapsulated *S*. Typhi, unless a 100-fold higher inoculum was used.[97] Interestingly, loss of the typhoid toxin gene does not appear to cause any attenuation in ability to induce disease in volunteers, although it did lead to an altered cytokine response suggesting that whilst not necessary for disease induction, toxin is able to modify host responses.[98]

The genes required to produce the Vi capsular antigen are encoded by the *viaB* locus, located on SPI-7, a genetic element which is lacking in *S*. Typhimurium.[99] We know that this antigen is expressed in human infection given the low but significant levels of Vi antigen in recovering typhoid patients and the immunogenicity of typhoid vaccines incorporating the Vi antigen.[100] The SPI-7 element is inherently unstable and Vi expression can be lost during laboratory passage over time. It is also influenced by factors such as osmolarity. For example, TviA (a gene necessary for capsular expression) positively regulates the Vi capsule genes whilst negatively regulating flagellar and SPI-1 T3SS genes.[101] At high osmolarity (as in the intestinal lumen), TviA is repressed, allowing *S*. Typhi to be non-encapsulated and flagellated to increase invasiveness, but then at low osmolarity (in the intestinal mucosa) TviA is induced, allowing *S*. Typhi to be encapsulated with downregulated flagellar and SPI-1 T3SS proteins to reduce host inflammatory response.[102] Supporting this hypothesis is a study of calf ileal loop infection, showing that TviB (another gene necessary for capsular

production) was upregulated after cells entered the epithelium, with Vi capsule being visualised on fluorescence microscopy within cells.[103]

S. Paratyphi A and B cause a similar clinical picture to *S*. Typhi, yet in the absence of SPI-7 and a capsule (in comparison to *S*. Paratyphi C, which harbours SPI-7). *S*. Paratyphi A has been shown to express lower levels of SPI-1 effector proteins compared to *S*. Typhimurium, especially when grown aerobically (as would be the case inside intestinal epithelial cells). When *HilA*, an SPI-1 activator was overexpressed in *S*. Paratyphi A, increases in host cell invasion, pro-inflammatory cytokine release and disruption of epithelial integrity were reported, suggesting that suppression of the SPI-1 components at higher oxygen tension may be a mechanism employed by *S*. Paratyphi A to reduce inflammatory response and evade detection.[65]

There are many gaps in our knowledge of the mechanisms behind the ability of *S*. Typhi and *S*. Paratyphi A to cause invasive disease with a dampening of host immune response. Novel 3-D human cell culture technologies such as the intestinal organoid system may help us to address this.

1.2.2 Innate immune response to *Salmonella* infection

Following epithelial invasion, *Salmonella* reach the lamina propria, where bacteria are phagocytosed by neutrophils or mononuclear cells such as macrophages. The host immune system is able to differentiate luminal commensal bacteria from pathogens such as *Salmonella* by expression of pathogen recognition receptor TLR5 on their basolateral surface.[104] Phagocytes within the lamina propria express numerous pathogen recognition receptors (TLRs 1, 2 and 4-6) on their surface, which are specialised in detecting PAMPs such as flagellin (detected by TLR5)[105] and LPS (detected by TLR4).[106] Detection of *Salmonella* in the lamina propria through TLRs on macrophages and epithelial cells induces a pro-inflammatory transcriptomic change in these cells, inducing expression of neutrophil chemoattractants such as interleukin 8 (IL-8) via NFκB activation[107] and leading to the neutrophil influx into the intestinal mucosa observed in *Salmonella*-induced gastroenteritis. **Figure 1.3** outlines elements of the innate immune response to *S*. Typhimurium infection.

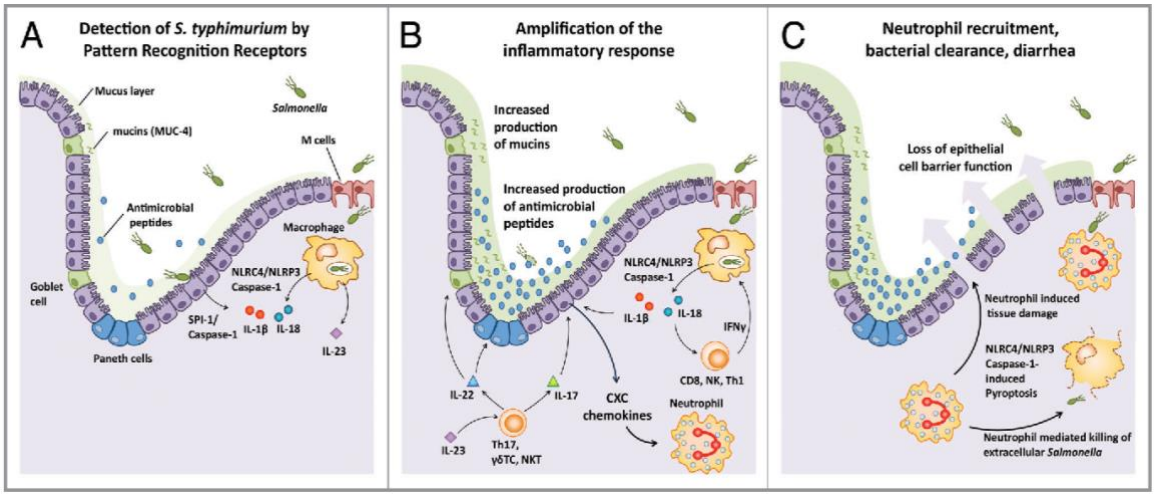

Figure 1.3: Illustration of the innate immune response to *S.* Typhimurium infection. (**A**) After invading the mucosa, *Salmonella* is detected by pattern recognition receptors, or in the case of extracellular *Salmonella*, TLRs, inducing a transcriptional response causing production of pro-inflammatory cytokines such as IL-23. Intracellular *Salmonella* activate NOD-like receptors that also promote IL-23 secretion, and assemble the NLRC4/NLRP3 inflammasomes that activate caspase-1, inducing secretion of IL-1β and IL-18. SPI-1 mediated activation of caspase-1 also contributes to IL-18 secretion. (**B**) IL-18 and IL-23 amplify the inflammatory response via paracrine signalling, causing induction of IFNγ and IL-22/IL-17 respectively. These cytokines increase production and secretion of AMP, mucins, and promote release of CXC cytokines (e.g. IL-8), causing an influx of neutrophils into the mucosa. (**C**) Infiltrating neutrophils kill extracellular *Salmonella*. *Salmonella* may be extracellular following transcytosis via M cells or pyroptosis and host cell lysis. Neutrophils can also cause inflammatory damage to intestinal tissue, causing loss of epithelial barrier integrity and inducing diarrhoea. (Figure taken from Broz et al, 2012[108])

As outlined in the previous segment, interaction of the host epithelium with *Salmonella* induces activation of the inflammasome and production of pro-inflammatory cytokines such as IL-8 and immune cell influx. In addition, antimicrobial peptides (AMPs) are produced by epithelial cells, as are factors such as Lipocalin2, which is an iron sequesterer, thought to limit bacterial growth. Contact between *Salmonella* and the host epithelium also induces expression of the IL-23/IL-22 axis, leading to secretion of AMPs, such as the c-type lectins RegIIIβ and RegIIIγ by Paneth cells.[109] Counterintuitively this may be of benefit to *Salmonella,* as RegIIIβ is able to kill a number of Gram positive and Gram negative pathogens in vitro, but not *Salmonella,* suggesting that this is one way which *Salmonella* overcomes colonisation resistance.[110] *Salmonella* also have defences against other AMPs, expressed via influence of the PhoPQ system, which is able to bind and inactivate cationic AMPs and reduce the immunogenicity of LPS by modifying its lipid A portion.[111]

Another factor *Salmonella* needs to overcome in order to survive in the inflamed gut is limited iron availability. Bacteria produce siderophores; iron chelating compounds, which

transport available iron into the pathogen. Lipocalin2, secreted into the gut lumen, is able to block this iron acquisition by binding a siderophore called enterochelin, produced by *Salmonella.*[112] However, most *Salmonella* can express the *iroBCDEN* gene cluster, which encodes production of salmochelin (a derivative of enterochelin) which is not bound by lipocalin2, allowing *Salmonella* to continue to scavenge iron and resist the action of this AMP.[113]

Production by the epithelium of reactive oxygen species is another defensive mechanism; NADPH oxidase (Nox) and Dual oxidase (Duox) have a role to play in gene expression, apoptosis and the respiratory burst. Production of superoxide (O^-_2) by Nox1 is known to activate NFκB, TNFα and IL-8 production, enhancing the pro-inflammatory response.[114] Paneth cells (producers of AMPs) are thought to proliferate following *Salmonella* infection in order to enhance antimicrobial response.[115] This proliferative response appears to come solely from the transit-amplifying cells, as the study noted no increase in LGR5, and murine organoids have been observed to downregulate iPSC markers (LGR5 and Bmi1) during *Salmonella* infection.[116] Lastly, increased mucus production by goblet cells in the epithelium is a defensive mechanism to increase barrier function, but may also benefit *Salmonella*, as it has been demonstrated that *S*. Typhimurium adjust to lack of nutrients in the inflamed gut by using mucus carbohydrates as a source of energy.[117]

As described above, there are two distinct clinical phenotypes for non-typhoidal *Salmonella* infection, with an inflammatory gastroenteritis caused in most cases, but with invasive systemic infection being possible in those with impaired immunity.[18] iNTS disease is also different to the clinical picture of typhoid disease, with much earlier onset of fever and systemic illness versus the 8-14 day wait normally observed between ingestion of *S*. Typhi and symptom onset. Host factors increasing the likelihood of invasive disease would include mutations in genes involved in the IL-23 axis, which is activated in response to *Salmonella* detection by DCs. IL-23 in turn acts on T cells to induce the IL-22 and IL-17A responses, which are required for maintenance of T_H17 cells, important in the immune response to *Salmonella*.[118] Similarly, the IFNγ response is key to preventing disseminated disease. IL-12 is produced by antigen presenting cells in response to *Salmonella* antigens and stimulates T cells to produce IFNγ, which activates the STAT-1 system in macrophages in order to

eliminate intracellular pathogens. These responses, along with activation of the inflammasome, are key to restricting the spread of NTS infection, with mutations in any pathway increasing susceptibility to iNTS.[119,120] Similarly, individuals with chronic granulomatous disease, characterised by an inability to produce NADPH oxidase and thus reduced bacterial killing within phagocytes, are more susceptible to systemic *Salmonella* infection,[121] as are children with *Plasmodium falciparum* malaria; with haemoglobin breakdown inhibiting phagocytosis.[122] Macrophage defects in those with sickle cell disease may also predispose to invasive disease.[123]

1.2.3 *Salmonella* within the macrophage

Having reached the lamina propria or Peyer's patches, *Salmonella* can be phagocytosed by macrophages, dendritic cells or neutrophils. Uptake by DCs and neutrophils is generally disadvantageous to the bacteria, however, *Salmonella* have developed the ability to survive and replicate inside of macrophages. In macrophages, much like within epithelial cells, *Salmonella* are contained within an SCV, which progressively acidifies, as it sequentially fuses with endosomes. Again SPI-2 T3SS come into play in delaying maturation of the SCV and optimising intravacuolar conditions.[124,125] Originally thought only to occur in epithelial cells, Sifs have also been noted in *Salmonella*-infected macrophages.[120] The PhoPQ system and T3SS effector protein SpiC play key roles in limiting endosome/lysosome fusion with the SCV.[126,127] SPI-2 T3SS are also key in avoiding damage from the NADPH oxidase dependent respiratory burst within the macrophages.[128,129]

In murine studies, the cation transporter natural resistance-associated macrophage protein 1 (Nramp1), located in the phagolysosomal membrane of macrophages, also appears to play a role in host cell resistance to *Salmonella,* both by withholding the availability of cations such as Fe^{2+} and Mg^{2+},[130] and increasing expression of lipocalin2.[131] SPI-2 also acts to exclude damaging reactive nitrogen intermediates, produced by inducible nitric oxide synthase (iNOS) from co-localising with SCVs in the macrophage.[132]

Salmonella are able to induce cell death in macrophages; in comparison to the predominant form of cell death induced in epithelial cells, this is via pyroptosis rather than apoptosis. Pyroptosis is caused by two mechanisms, either by early SPI-1 T3SS induced killing, via SipB mediated caspase-1 activation,[133] or by SPI-2 T3SS mechanisms, involving the spv and PhoPQ systems.[134,135]

S. Typhimurium behaviour inside the macrophage is relatively well studied, but there are a number of unknowns about the actions of *S*. Typhi within the macrophage. Transcriptomic studies of *S*. Typhi within macrophages demonstrated that SPI-1 and SPI-2-encoded T3SS were down- and up-regulated respectively, as would be expected. *S*. Typhi inside macrophages demonstrated upregulation of genes for resistance to AMPs and fatty acid utilisation and did not induce SOS or oxidative stress responses (whereas *S*. Typhimurium within macrophages invoked the expression of SOS response genes). Flagellar expression, iron transport and chemotaxis-related genes were downregulated, as were pili and Vi capsule related genes on SPI-7. However, a number of *S*. Typhi genes with unknown functions were upregulated, suggesting there is more to be learnt about the interactions of *S*. Typhi with the macrophage.[136] Fascinatingly, *S*. Typhi with knockouts for various components of the SPI-2 T3SS [a translocon mutant (*sseB*), an apparatus mutant (*ssaR*) and a transcriptional regulator mutant (*ssrB*)], all known to be required for *S*. Typhimurium survival within macrophages, were not defective in uptake and survival within macrophages compared to wild type equivalent isolates. Rather than suggesting that SPI-2 is not necessary for *S*. Typhi survival in macrophages, these data suggest some form of host adaptation, as a number of SPI-2 effectors expressed by *S*. Typhimurium and required for long term survival in the mouse, have proven to be pseudogenes in *S*. Typhi.[137]

Other mechanisms demonstrated by the Vi encapsulated *S*. Typhi for modifying the immune response within macrophages include dampening inflammasome activation and decreasing IL-1β secretion by repression of flagellin expression (controlled by TviA). This decreased incidence of inflammatory cell death in *S*. Typhi-infected macrophages and was reproduced when the Vi locus was introduced into *S*. Typhimurium prior to macrophage infection.[138] Another capsular-related resistance mechanism is that Vi may be able to prevent innate immune recognition of *S*. Typhi by complement. Compared with an unencapsulated mutant, a Vi encapsulated *S*. Typhi isolate was able to interfere with complement component 3 (C3) deposition, leading to reduced bacterial binding to complement receptor 3 (CR-3) on the surface of murine macrophages and decreased CR-3 dependent clearance of *S*. Typhi from murine livers and spleens post-infection.[139] An additional defence factor employed by S. Typhi for survival and replication within the macrophage appears to be the use of the SPI-1 T3SS to

block the RAB32-associated pathway, given that knockdown of RAB32 or its nucleotide exchange factor BLOC-3 (biogenesis of lysosome-related organelle complex-3) increased S. Typhi replication in human macrophages.[140]

S. Paratyphi A is relatively poorly studied inside the macrophage. One study looking at bacterial transcripts of *S*. Paratyphi A in blood from three bacteraemic patients noted enhanced expression of transcripts of PhoP and the transcriptional regulator SlyA that influences SPI-1 and SPI-2 expression. However, in this study, the largest category of dysregulated transcripts were associated with proteins of unknown function.[141] Additional work on proteins expressed in *S*. Paratyphi A cultured from bacteraemic patients versus laboratory grown *S*. Paratyphi A noted increased expression of proteins involved in cell adhesion, fimbrial structure, antimicrobial resistance, ion transport, proteolysis and oxidoreductase activity in bacteria isolated from blood.[145]

Analysis of the core proteome of laboratory cultured *S*. Paratyphi A versus *S*. Typhi demonstrated differential enrichment of proteins involved in carbohydrate and polysaccharide synthesis and metabolism between the serovars. Proteomes for these serovars were compared to those for *S*. Typhimurium and *S*. Enteritidis. Typhoidal and non-typhoidal serovars readily separated from each other on analysis. This suggests that other than the influence of the Vi capsule, *S*. Paratyphi A and *S*. Typhi behave relatively similarly in culture, but this does not answer the question of what is happening *in vivo* during infection.[146]

Gene expression analysis of blood from patients infected with *S*. Paratyphi A demonstrated elevations in IFNγ, TNFα, IL-6, 8, 10 and 15 in response to infection, but no increase in IL-12 (which induces IFNγ release in Th1 and NK cells and is elevated in NTS infection[142]).[143] The elevation in this study of IFNγ levels was striking (around 75 times baseline levels), which fits with the IFN overexpression observed in patients with acute typhoid fever in human challenge studies.[144]

The data presently available suggest a differing host response to typhoidal versus NTS serovars. However, it is hard to draw conclusions based on limited evidence, and it is clear that further work is required to define the interactions between both *S*. Typhi and *S*. Paratyphi A and the macrophage.

1.2.4 Systemic spread of *Salmonella*

Having been taken up by and replicated within macrophages, and now residing in Peyer's patches (PP) or the lamina propria, typhoidal *Salmonella* are able to spread to the mesenteric lymph nodes (MLN), where they can enter the lymphatic system, eventually reaching the thoracic duct and bloodstream.[147] CD18+ cells such as monocytes, macrophages or DCs are able to traffic *Salmonella* directly from PP or the lamina propria to these organs via haematogenous spread, and DCs may assist in trafficking *Salmonella* to the MLN either directly from the intestinal lumen or from PP / the lamina propria. After prolonged infection, *Salmonella* can travel from the liver and spleen to other organs via the blood, and in the case of *S*. Typhi, excretion from the liver in bile can result in colonisation of the gall bladder, with bacteria from the gall bladder periodically shedding back into the intestine.[87,148] Thus, after passing through the epithelium, primary bacteraemia occurs with little symptomatology or evidence of intestinal inflammation (certainly in comparison to non-typhoidal infection) (**Figure 1.4**).[148] *S*. Typhi bacteria can reside in the reticuloendothelial system for an extended incubation period (usually 8-14 days); thereafter clinical illness may emerge, frequently linked to a secondary bacteraemia. During this bacteraemia, bacterial counts in the blood are low, averaging 1 cfu/mL,[149] versus 10 cfu/mL in bone marrow.[150] Symptoms of typhoid disease include: prolonged fever, fatigue, abdominal pain, nausea, rash, diarrhoea or constipation and headache. Complications that can occur with extended or untreated disease include intestinal perforation and haemorrhage, hepatitis and cholecystitis. Significantly, up to 4% of those who have had typhoid infection can go on to become chronic carriers, shedding *S*. Typhi in their stool sporadically over long periods (from months to years), putting contacts at risk of infection.[151]

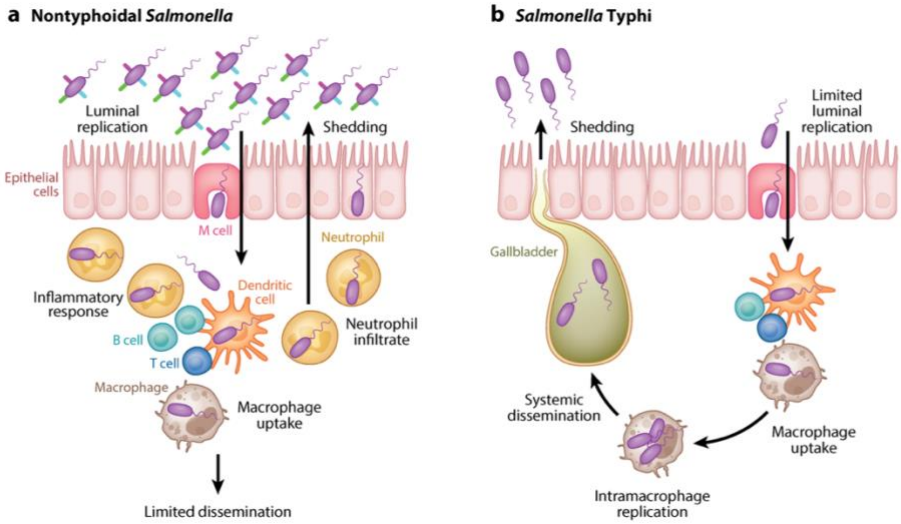

Figure 1.4: Systemic differences between nontyphoidal and *S*. Typhi infections: (**a**) Demonstrates the propensity of nontyphoidal strains to express surface molecules undisguised by Vi capsule, to invade the intestinal epithelium in high numbers, causing an inflammatory response and neutrophil infiltrate, followed by fairly rapid clearance and limited dissemination. (**b**) Demonstrates *S*. Typhi invading in more limited numbers, but uptake, replication and dissemination inside macrophages with a limited inflammatory response. (Figure taken from Dougan & Baker, 2014[148])

In nontyphoidal *Salmonella* infection in healthy individuals, there is limited dissemination of the pathogen, with numerous bacteria being killed by the inflammatory neutrophil response in the gut. Infection is usually restricted to the intestine and mesenteric lymph nodes.[152] Phagocytes in the liver and spleen can rapidly clear *Salmonella* from the blood, should it have disseminated this far. Thus, the clinical picture is one of an inflammatory enterocolitis, with profuse watery diarrhoea, vomiting and abdominal pain, and spontaneous resolution.[153] There is a low rate of secondary bacteraemia (<5%) and this has a mortality rate of 1-5%.[18]

In those with immunodeficiencies as discussed above, iNTS infection can occur, with *Salmonella* able to cause a rapid-onset bacteraemia with fairly diverse symptoms including: fever, enterocolitis (but only in up to 50% cases), pneumonia (may be due to co-infections) and hepatosplenomegaly.[18] Symptoms are difficult to distinguish from other febrile pathologies, such as malaria or lower respiratory tract infection, and even with microbiological confirmation and appropriate treatment, in sub-Saharan Africa this illness has a mortality rate of 22-47%.[20,154]

1.2.5 Adaptive immune response to *Salmonella*

The innate immune response is very effective at controlling the initial aspects of *Salmonella* infection, but is insufficient for achieving protective immunity. Models used in this study will largely focus on cell-mediated (innate) immunity, but here I will briefly discuss the adaptive immune response to *Salmonella* infection. Control and eradication of bacteria during a primary infection and protection from subsequent infections requires the development of a *Salmonella*-specific T-lymphocyte response, in order to recruit these cells to sites of infection,[1] and allow clearance of bacteria.[155] This appears to be in the form of CD4+ TCR-alpha beta cells and associated IFNγ response, with CD8+ cells playing an auxiliary role.[156] Th1 cells also mediate the regulation of *Salmonella*-specific B cell activation and maturation, producing antibodies against bacterial polysaccharide and protein antigens.[157] In mice given oral attenuated vaccines, CD4+, CD8+ and anti-*Salmonella* antibodies all had a role to play in infection resistance.[158] CD4+ cells had a role in cytokine production, particularly IFNγ release, and importantly, mice with deficiencies in CD4+ T cells or IFNγ production experience uncontrolled *Salmonella* growth.[159] This runs in parallel with IFNγ production in the innate response, stimulated by IL-18 release after caspase-1 cleavage in response to flagellins as described earlier in this chapter. CD8+ cells differentiate into cytotoxic T lymphocytes, removing *Salmonella* from infected macrophages.[1]

The expression of certain major histocompatibility complex class II alleles conferred resistance to enteric fever in one genome-wide association study, suggesting that CD4+ T cells have a role both in control of typhoidal and non-typhoidal serovars in humans.[160] Studies on T cells from participants in the *S*. Typhi human challenge model were able to identify antigen-specific T cell responses to three particular antigens: Hlye (a haemolysin with an as yet undetermined role in *Salmonella* pathogenicity), CdtB (a component of the typhoid toxin proposed as a virulence factor for *S*. Typhi and *S*. Paratyphi A[161]) and PhoN (an acid phosphatase induced after PhoPQ system activation). CdtB was able to elicit T cell responses targeting infected cells, and antibody responses neutralising toxin activity. *S*. Typhi CdtB CD4+ responses were not cross-reactive against *S*. Paratyphi A and vice versa, however PhoN-specific T cell responses were active against both typhoidal and

nontyphoidal *Salmonella* strains.[162] Other findings of interest were that *Salmonella* CD4+ responses targeted both constitutively expressed proteins as well as those only expressed after infection, showing that specific T cell repertoire is shaped by the plasticity of the *Salmonella* transcriptome and that T cell response can be tissue specific to the location of the bacteria of interest. In addition, the CD4+ cells studied here (CD4+ CD38+ CCR7- cells) displayed gut homing markers, suggesting that these circulating cells may be able to migrate to the site of bacterial invasion and join tissue resident CD4+ cells in the gut mucosa to prevent re-infection.[162]

Th17 cells are also thought to have a role in control of *Salmonella* infections. These cells express IL-17A, IL-17F, IL-22 and IL-26, with receptors for IL-22 and IL-26 being located on epithelial surfaces;[163] IL-22 receptor complexes are found on the basal surface of intestinal epithelial cells, suggesting a role in the local infection response.[164] IL-17 and IL-22 activate mucosal immune responses, inducing AMP release and chemokine expression. This process will be discussed in more detail later on in this chapter.

Despite their limited role in primary infection, B cells do provide some protection against secondary infection, with sera from Malawian children containing anti-*Salmonella* antibodies able to kill NTS strains,[165] and recent findings in human participants challenged, then re-challenged with *S*. Paratyphi A or *S*. Typhi demonstrating some degree of protection, with baseline anti-O:2 IgG being higher in *S*. Paratyphi A re-challenged patients than in naïve controls.[166]

1.3 Treatment and prevention of *Salmonella* infections

1.3.1 Treatment of *Salmonella* infections and concerns about MDR organisms

Untreated, typhoid fever historically had a mortality rate of up to 15%,[147] but this declined with the introduction of chloramphenicol in the 1940's. With appropriate antimicrobial therapy, mortality can be as low as 1%,[167] although other factors such as age, length of illness before appropriate treatment and ingested dose of the organism also affect disease severity.[147] Other agents used for treatment include trimethoprim/sulphamethoxazole and

ampicillin; however, since the 1990's, multi-drug resistant (MDR) isolates of *S.* Typhi (defined as resistance to chloramphenicol, trimethoprim/sulphamethoxazole and ampicillin) have been isolated with varying frequencies, leading to a higher incidence of severe disease in those infected with drug resistant or intermediately resistant isolates.[167] Current treatment options include fluoroquinolones (e.g. ciprofloxacin), 3rd generation cephalosporins (e.g. ceftriaxone) and azithromycin, but worryingly, resistance to some of these antibiotics has also been recorded, especially as fluoroquinolones have become the predominant treatment used for MDR infections. Whole genome sequencing data allows us to draw associations between antibiotic resistance and *S*. Typhi lineage. For example, in a Vietnamese study, severe typhoid disease was associated with organisms intermediately resistant to ciprofloxacin.[168] This intermediate response has been associated with the H58 MDR haplotype,[169] although such resistance has become common in other *S*. Typhi lineages. A recent study based on the whole genome sequences of over 1800 isolates demonstrated that the H58 lineage has disseminated throughout Asia and into Africa, displacing antibiotic-susceptible lineages and driving disease epidemics.[170] Numerous local typhoid outbreaks have been linked to various H58 sublineages.[171-174]

AMR gene transfer is often facilitated by transposon or plasmid exchange; in the case of *S*. Typhi H58 clades, these genes were initially associated with an IncHI1 plasmid. This type of plasmid has a transposon able to carry multiple resistance genes, including: *dfrA7*, *sul1*, *sul2* (trimethoprim-sulfamethoxazole resistance), $bla_{\text{TEM-1}}$ (ampicillin resistance), *strAB* (streptomycin resistance) and *catA1* (chloramphenicol resistance).[169]
However, this transposon has been integrated into the *S*. Typhi chromosome in some recent H58 lineages and the plasmid has been lost.[170,172] Fluoroquinolone resistance is associated both with acquisition of AMR genes and chromosomal mutations. In H58 clades, mutations in the chromosomal quinolone resistance-determining region (QRDR), which is composed of topoisomerase IV (*parC* and *parE* genes) and DNA gyrase (*gyrA* and *gyrB*) genes are increasingly widespread. Plasmid-mediated resistance (PMQR) genes including *qnr, oqxAB* and *aac(6')Ibcr* can also be acquired and contribute to fluoroquinolone resistance. Ceftriaxone resistance is associated with extended-spectrum β-lactamase (ESBL) gene acquisition.[28] More recently, there has been an outbreak of extensively drug resistant (XDR) *S*. Typhi in Pakistan,[175] resistant to chloramphenicol, trimethoprim/sulphamethoxazole,

ampicillin, fluoroquinolones and 3rd generation cephalosporins. The clade responsible is a H58 clone, with an additional plasmid encoding the *bla*$_{CTX-M-15}$ extended-spectrum β-lactamase and *qnrS* fluoroquinolone resistance gene.[28] Treatment options for such strains are very limited, especially in settings where access to specialist intravenous antibiotics is scarce. These concerning developments have highlighted the need for focused efforts to control typhoid. These efforts could include improved sanitation measures and the licensing and distribution of effective vaccines.

Although less common than *S*. Typhi infection, *S*. Paratyphi A causes up to 40% of cases of enteric fever in certain areas of Asia.[176,177] Fluoroquinolone resistance is the commonest mechanism of drug resistance in *S*. Paratyphi A isolates, recorded in up to 90% of isolates in some studies.[178-180] There is a growing body of evidence on MDR *S*. Paratyphi A infections, as they are becoming an increasingly significant problem across Asia,[176,181-183] although the genetic basis of MDR in many cases is not yet clearly defined.[184]
Studies describing antimicrobial resistance in *S*. Paratyphi A point to plasmids such as IncHI1 as a possible mediator of resistance, although molecular studies are limited thus far.[185-187] Plasmids of varying sizes have been reported as encoding MDR in studies from China, Bangladesh and Calcutta.[184-186] Interestingly, sequencing of an IncHI1 plasmid (pAKU_1), which encoded MDR in an *S*. Paratyphi A isolate from Pakistan, demonstrated that the pAKU_1 plasmid shares a common backbone with the *S*. Typhi plasmid pHCM1 and an S. Typhimurium plasmid pR27; the backbone being thought to have originated from an ancestral IncHI1 replicon. pAKU_1 and pHCM1 share a composite transposon comprising 14 antibiotic resistance genes within mobile elements. The transposons are located in different places on the backbone of each plasmid, suggesting these genes were independently acquired via horizontal transmission. Worryingly, two IncHI1 plasmid types from Vietnamese *S*. Typhi contained features of the pAKU_1 backbone sequence, with the transposon located in exactly the same place as in the pAKU_1 *S*. Paratyphi A plasmid.[184] This is very unlikely to have happened by chance, raising the likelihood that plasmids have been interchanged between these serovars at some point; whether directly or via another pathogen. Other studies have proposed the likelihood of chromosomal recombination between *S*. Typhi and *S*. Paratyphi A, allowing them to adapt to their niche in the human host.[188] These similarities between the pathogens and their mechanisms of antimicrobial

resistance suggest that care needs to be taken when making policy for antimicrobial choices for *S*. Typhi, as this may well simultaneously affect the resistance patterns we see in *S*. Paratyphi A too.

S. Typhimurium is responsible for the majority of cases of iNTS disease in Sub-Saharan Africa, although *S*. Enteritidis is also responsible for many of these infections.[20,21,189] Until recently, extensive iNTS disease had largely been restricted to the African continent, but reports have emerged of iNTS in cohorts of patients in parts of Asia, including: India,[190] Taiwan[191] and Thailand.[192] iNTS in Vietnam has also been documented, with cases associated with the HIV epidemic, as was the case when the disease emerged in sub-Saharan Africa, with the emergence of ST313 lineages I and II each associated with periods of HIV expansion in the early 1980s and 1990s respectively.[193] Acquisition of chloramphenicol resistance has also been associated with increased transmission of this pathogen in Kenya[26] and Malawi[20]

Antimicrobial resistance was common in *S*. Typhimurium associated with iNTS in Vietnam, with over 50% isolates being resistant to ampicillin, amoxicillin, chloramphenicol, trimethoprim/sulfamethoxazole, ciprofloxacin and gentamicin. Multilocus sequence typing on these isolates demonstrated that *S*. Typhimurium STs 34, 19, 1544 and *S*. Enteritidis ST11 were responsible for the majority of cases in this study; of which, all but ST1544 were also resident sequence types seen in African isolates. However, in Africa in recent years, many circulating isolates have been replaced with a newer multidrug resistant *S*. Typhimurium ST313 clone.[26] In this study, sequencing of representative isolates of ST313 lineage I (D23580) and II (A130), showed a unique prophage repertoire and composite genetic element encoding MDR genes, which was situated on a virulence-associated plasmid. Genome degradation had occurred, with a number of invasion related pseudogenes and deleted genes identified which are either absent or known to be pseudogenes in *S*. Typhi and *S*. Paratyphi A. This suggests that ST313 has become adapted to a particular clinical niche or to systemic disease, and continues to microevolve to better suit this environment.

Sequencing has also been performed on another iNTS-causing ST34 clade currently causing the pandemic of iNTS in HIV-infected individuals in Vietnam.[194] In contrast to the ST313 clone, ST34 does not exhibit evidence of genome degradation, and is able to produce both

invasive disease and enterocolitis, unlike ST313 which was primarily associated with invasive disease. Fascinatingly, The Vietnamese ST34 variant derives from the European clone of the monophasic ST34 *S*. Typhimurium variant, *S*. I:4,[5],12:i:−. At some point, these Vietnamese ST34 have re-acquired a phase 2 flagellum, potentially conferring an invasiveness advantage, which would appear to be borne out by studies in murine macrophages (S. Baker, unpublished data). *S*. I:4,[5],12:i:− has also acquired an extensive MDR plasmid, encoding: *oqxAB, blmS, sul1, ⊿aadA2, dfrA12, aph3, sul3, aadA1a, cmlA2, aadA2, floR, sul2, hph, aac(3')-Iva, aac(6')-Ib-cr, blaOXA-1, catB3* and *arr3*. These genes, cause predicted resistance to: fluoroquinolones, bleomycin, sulphonamides, trimethoprim, kanamycin, streptomycin, chloramphenicol, spectinomycin, florfenicol, hygromycin B, apramycin, beta-lactams, and rifampin.[194] It is therefore feasible that this clone would have occupied a niche in HIV-infected individuals, given the frequent use of broad-spectrum antibiotics likely in this population.

Given the very high mortality rates observed with iNTS and their frequent possession of numerous MDR genes, it is important to focus efforts on preventative strategies against these types of pathogen as well as implementing WaSH measures for control of disease.

1.3.2 Status of vaccine development against typhoid, paratyphoid and NTS disease

Given the concerns about the spread of increasingly MDR *S*. Typhi, strategies for vaccination and prevention of cases have been the subject of intensive investigation in recent years. WaSH interventions, such as improved water supply and waste disposal could do much to eradicate infection, as has been the case in Europe and North America. It is clear that the infrastructure required to effect these changes is unlikely to be realised in the short to medium term, therefore, efforts have focused on case prevention and outbreak control. Any vaccine effort ought to be focused on younger children, given that they shoulder a large burden of typhoid disease.[195] However, until recently, available licenced vaccines did not protect this population. The oral live attenuated typhoid vaccine, Ty21a is unsuitable for children under 5, given that it is formulated in large capsules which would be difficult for children to swallow[196] and the parenteral Vi capsular polysaccharide vaccine is not immunogenic in early childhood. Excitingly, there have been recent developments in producing vaccines that would be suitable for use in the paediatric population. Typhoid

conjugate vaccines (TCVs) have been constructed, which combine the Vi polysaccharide capsule with a protein carrier. TCVs can induce enhanced immune responses and appear to be both safe and effective from infancy.[197-200] Rapidly obtained efficacy data for this type of vaccine came from a human challenge study, wherein participants were randomised either to receive a Vi conjugate (Vi-TT; in this case the conjugate was tetanus toxoid), Vi polysaccharide (Vi-PS) or meningococcal vaccine as a control, prior to receiving an oral inoculum of *S*. Typhi sufficient to cause disease.[100] 77% of control participants were diagnosed with typhoid disease, versus 35% in each of the Vi-PS and Vi-TT groups, giving vaccine efficacies of 54.6% for Vi-TT and 53.0% for Vi-PS. The criteria for diagnosis of typhoid disease in this study were rather broad, with a typhoid case being defined as fever of $\geq$ 38 °C for $\geq$ 12 hours, or *S*. Typhi bacteraemia. If a definition of fever of $\geq$ 38 °C followed by *S*. Typhi bacteraemia is used, this vaccine prevented 87% infections versus 52.3% prevented by the Vi-PS vaccine. This latter definition is probably a more realistic representation of diagnostic criteria for reported cases in the field, and has previously been used in field vaccine studies.[201] In addition, protection levels in endemic settings may be higher, as the vaccine is being used in a pre-exposed population, rather than a naïve population as in the challenge study, and in children as well as adults. For example, efficacy of Vi-PS was calculated at 69% within the first year after vaccination during field trials as opposed to the 52.3% found in this study.[196] Seroconversion in the Vi-TT group was 100% and 88.6% in Vi-PS group. One month after vaccination, Vi-TT group participants had significantly higher mean anti-Vi IgG titres.

In addition to the ability to directly prevent cases of typhoid fever, vaccination may also reduce the spread of the disease via reduction of stool shedding during infection. Human challenge studies showed that Vi-PS and Vi-TT both significantly decreased incidence of stool shedding versus unvaccinated controls during typhoid challenge. *S*. Typhi-exposed participants were twice as likely to have stool shedding versus those exposed to *S*. Paratyphi A, with overall shedding rates of 14.5% vs 7.5% including unvaccinated and vaccinated cases.[202] This study, together with evidence from other clinical and immunological studies of TCV vaccines, led in 2017, to the World Health Organisation's (WHO) Strategic Advisory Group of Experts (SAGE) recommending the introduction of TCVs for infants and children > 6 months of age in endemic countries, with priority given to those countries with the highest

disease burden or levels of AMR.[203] It was also recommended to have catch-up campaigns for children up to 15 years of age where feasible / necessary, and that TCVs be used in response to confirmed outbreaks of typhoid fever, as has been the case during the current XDR typhoid outbreak in Pakistan.[204] In January 2018, the WHO pre-qualified its first TCV, Typbar-TCV®, meaning that the vaccine can be procured by UN agencies; also that lower income countries may apply to Gavi, the Vaccine Alliance, for funding assistance to implement vaccine programmes. Initial data from post-licensure phase IV trials, presented at the International Conference on Typhoid and Other Invasive Salmonelloses in April 2019 demonstrated Typbar-TCV® to be both safe and effective at disease prevention, but these studies are not yet published. Overall, there is the potential for TCV vaccines to make a big public health impact on the prevention of typhoid fever over the coming years.

S. Paratyphi A vaccine development is some way behind that of *S*. Typhi. Challenges include the lack of an approved serological correlate of protection, and the host restriction of this pathogen, making the utility of animal models limited. Unfortunately, little cross-protection is seen between *S*. Paratyphi A and *S*. Typhi, either following challenge after previous infection with the other serovar,[166] or for *S*. Paratyphi A following vaccination with either Vi-PS or the oral Ty21a vaccines.[16,205] Some efficacy of Ty21a vaccines against *S*. Paratyphi B has been reported in trials in Chile, with predicted efficacy of 49%.[206] The mechanism for this protection is not entirely clear, with the authors postulating that it may be secondary to some sharing of epitopes amongst the O antigens on the bacteria (although epitopes are shared also with *S*. Paratyphi A, for which there is no cross-protection), or on observing strong T cell responses to the vaccine, a cell-mediated immunity, with as yet undefined shared antigens being a target for the immune response.

To produce safe and effective protection against *S* Paratyphi A, attention is again focused on the development of conjugate vaccines, with phase 1 and 2 immunogenicity trials in Vietnam on O antigen conjugated to tetanus toxoid showing significant increases in mean anti-*S*. Paratyphi A LPS IgG and IgM in both adults and children. These conjugates produced a > 4 fold rise in anti-LPS IgG in $\geq$ 80% participants.[207] The O:2 antigen of *S*. Paratyphi A is known to play a role in virulence and act as an antigen, stimulating the host immune response.[208] Other potential vaccine candidates have been undergoing trials in mice, with

O:2 conjugated to a carrier protein CRM_{197} (a component of diphtheria toxin) also showing promising immugenicity and eliciting a bactericidal serum response.[209] Similarly, *S*. Paratyphi A flagellar protein, FliC has also been shown to enhance phagocytosis and clearance of *S*. Paratyphi A in mice immunised with live attenuated *S*. Paratyphi A strains prior to intraperitoneal challenge.[210] Recent work has improved the O-linked glycosylation method for producing conjugate vaccines, making it more rapid and less expensive.[211]

Efforts have also been made to produce a bivalent vaccine that would protect against *S*. Typhi in addition to *S*. Paratyphi A, by the cloning of the *S*. Typhi *viaB* locus (responsible for Vi capsule biosynthesis) and its insertion into an attenuated *S*. Paratyphi A to produce a candidate oral vaccine. In mice, nasal immunisation with this vaccine induced high levels of *S*. Paratyphi A and Vi-specific antibodies in sera, and total sIgA in the intestine. In addition, the vaccine was significantly protective against *S*. Paratyphi A and *S*. Typhi challenge.[212] Clearly there is some way to go before a clinically implementable vaccine is produced, but given the increase in prevalence of *S*. Paratyphi A across parts of Asia, it is important that these efforts continue, alongside attempts to control spread of disease in endemic areas.

Producing a vaccine for iNTS may prove slightly simpler in principle, given that although these pathogens do not have a published correlate of protection, it is possible to quantify serum bactericidal activity against *S*. Typhimurium and *S*. Enteritidis. Additionally, murine models are very helpful for early work, given that *S*. Typhimurium can cause systemic infection in mice, as well as enterocolitis (following pre-treatment with streptomycin), although differences obviously remain between the human and murine responses to these pathogens *in vivo*. Vaccines for iNTS would need to be safe and immunogenic for infants, as peak disease incidence occurs at 12 months of age, and would need to be safe to use in HIV-infected populations, as these patients are at increased risk of iNTS.

It would make sense to design antibody-inducing vaccines against iNTS serovars, as epidemiological work has shown that incidence of disease decreases with increasing age and acquisition of antibodies. Serum antibodies have also been shown to have *in vitro* bactericidal activity and mediate oxidative killing of iNTS serovars intracellularly.[165,213]

Proposed vaccine targets have included outer membrane proteins (OmpD) purified from whole bacteria, with the idea that if conserved protein antigens, such as OmpC, F or D or flagellin were targeted, the resulting vaccine would achieve broad coverage of clinically relevant serovars.[214] It has been proposed that a multivalent vaccine comprised of 5-6 conjugates could protect against the most prevalent forms of iNTS and gastroenteritis-causing *Salmonella* worldwide.[165,215,216] A bivalent conjugate vaccine linking core and O polysaccharide (COPS) components of *S*. Typhimurium and *S*. Enteritidis LPS to their phase 1 flagellin subunits is under development. Instead of linking the antigens to a protein, such as tetanus toxoid or CRM_{197}, it is hoped that efficacy will be enhanced as both elements of the vaccine will be antibody targets. *S*. Enteritidis COPS-FliC (a flagellin protein) conjugates elicited protective antibody responses in murine trials prior to intraperitoneal challenge.[215] Ongoing work on this project is being done by Bharat Biotech, who produce the recently pre-qualified Typbar-TCV®.

One group has attempted to produce a live attenuated NTS vaccine, derived from a gastroenteritis-associated *S*. Typhimurium strain, with deletions induced in *aroC* and *ssaV* genes. Testing for this vaccine did not go beyond phase 1 trials, as stool shedding occurred in volunteers for up to 23 days post-immunisation.[217] Work on a bivalent live attenuated NTS vaccine is ongoing at the University of Maryland, where attenuated strains of *S*. Typhimurium (CVD 1931, which is derived from an ST313 isolate) and *S*. Enteritidis (CVD 1944, derived from an invasive *S*. Enteritidis) have elicited significant seroconversion in the form of anti-LPS and anti-flagellin antibodies. At the same institute, vaccination with CVD1921, an attenuated ST19 iNTS derivative has proven adequately safe and well tolerated, with limited shedding in simian immunodeficiency virus-infected rhesus macaques.[218]

Other institutes are using the Generalised Modules for Membrane Antigens (GMMA) technique to generate bivalent vaccines for *S*. Typhimurium and *S*. Enteritidis. This involves the introduction of mutations that moderate LPS toxicity into production strains, which also induces the strains to increase production of membrane blebs of immunogenic particles ~50-90nm in diameter. This technology has been used to produce a *Shigella sonnei* vaccine which is currently in phase 1 trials,[219] and immunisation with GMMA has been

demonstrated to be at least as effective as *S*. Enteritidis and *S*. Typhimurium O-antigen-CRM_{197} glycoconjugate vaccines at inducing immunogenicity and reducing bacterial burden in head to head trials in mice.[220]

There are a number of promising avenues then for iNTS vaccines, however at present, there is less political will and funding available for tackling this form of salmonellosis, despite its increasing prevalence and high mortality rates.[221] This needs to be addressed going forwards to accelerate interventions to reduce the global impact of iNTS disease.

1.4 Models for study of host-pathogen interactions and reasons for their use

1.4.1 Current methods of studying host-pathogen interactions for *S*. Typhi, *S*. Paratyphi A and NTS strains

As outlined above, there are numerous gaps in our knowledge about the detailed interactions between host and epithelium for *Salmonella* serovars causing enteric fever, simply because these pathogens have adapted to cause disease in the human host, and are now restricted to this niche. A proxy for a lot of what we know about *S*. Typhi infection comes from studies of *S*. Typhimurium in murine models, since this pathogen causes an invasive disease phenotype in susceptible mice with superficial similarities to that caused by *S*. Typhi in humans. Susceptible target species would include mice that have mutations in genes important for intracellular immunity such as Nramp1. Nramp1 is an intracellular protein recruited to the endosome, where it acts as an Fe^{2+} and Mg^{2+} transporter. Much has been learnt from murine study, such as the actions of the SPI-1 and SPI-2 T3SS during infection,[222] however significant differences remain in the immune response between human and murine hosts and the pathogens it is possible to use in these models. For example, the presence of the Vi capsule of *S*. Typhi, which is absent in *S*. Typhimurium, would cause host-pathogen interactions to differ in humans versus in the murine model. One attempt to bridge this gap has been the study of an *S*. Typhimurium/*S*. Typhi chimera to learn more about the function of the Vi capsule in the murine host.[223] This chimeric derivative is *S*. Typhimurium C5.507 Vi^+, which harbours SPI-7, encoding the genes responsible for producing the Vi capsule. Infection of mice with C5.507 Vi^+, resulted in a decreased recruitment of NK and polymorphonuclear neutrophils, leading to a blunted pro-

inflammatory cytokine response, affecting TNF-α, MIP-2 and perforin, but a large increase in the anti-inflammatory cytokine IL-10. This cytokine was expressed in DCs, macrophages and NK cells in the spleen, and neutralisation of the IL-10 response led to increased migration and activation of splenocytes.

A murine model thought to better mimic the human response to *S*. Typhi involves production of immunodeficient Rag2$^{-/-}$ γc(-/-) mice which have been engrafted with human foetal liver stem and progenitor cells. These mice are able to partially support *S*. Typhi infection, and an *S*. Typhi with a mutation in the PhoPQ system (a gene required for virulence), was unable to replicate in these mice.[224] Human-like innate and adaptive responses were produced by the mice, with *S*. Typhi-specific antibody production occurring and elevated levels of TNFα, IL-8, IL-10, IL-12, IFNγ, MIP-1α and IP-10 being recorded. Similarly, another group used non-obese diabetic (NOD)-scid IL-2Rγ(null) mice engrafted with human haematopoietic stem cells to model typhoid disease, finding that *S*. Typhi were able to replicate and cause lethal infection in these mice, who also produced a cytokine picture similar to that seen in human disease.[225]

Modelling of *S*. Paratyphi A has proven incredibly difficult, given its lack of a proxy for mouse studies, such as *S*. Typhimurium for *S*. Typhi. Some mouse work has been undertaken using attenuated strains and focusing on response to a particular component of the bacterium, such as flagellar proteins.[210]

Models for the study of gastroenteritis-causing NTS strains again include murine hosts; in this case animals are treated with streptomycin prior to *Salmonella* infection in order to deplete the resident microbiota and allow rapid colonisation and expansion of *Salmonella*, which can invade the mucosa and induce an inflammatory colitis as seen in human infections.[226] *S*. Typhimurium, *S*. Enteritidis, *S*. Dublin, *S*. Gallinarium and *S*. Pullorum have all been studied in this fashion.[227] Calf models have also been used for the study of NTS strains, as cows are natural hosts for a number of *Salmonella* serovars, such as *S*. Typhimurium,[228] which causes a gastroenteritis with a secretory and inflammatory response similar to that in humans.[229] Ligated ileal loops from calves have also been used, cells from which display apical membrane ruffling in response to *S*. Typhimurium infection, with bacteria invading M

cells or enterocytes, as may occur in humans.[230,231] Whilst we have learnt a lot from these models, interspecies differences are present in virulence factors, for example, the *spv* operon is required for systemic infection in mice, but not in calves.[232]

Alternatives to animal models have therefore been explored for detailed study of these pathogens. Use of 2-D cell culture models (e.g. HeLa or Caco-2 cells) has enabled us to learn a lot about interactions of numerous serovars of *Salmonella* with the epithelium. Additionally, intestinal samples obtained via biopsy have provided data from differentiated intestinal epithelium.[233,234] Methods used to study these samples are advancing, with tissue explants being maintained in culture, and structurally supported in a way that allows access both to the apical and basal side of the tissue layer.[235] In addition, replication of the intraluminal microbiotal environment has been attempted with the colonic fermentation model of cell culture to recreate colonisation resistance to invasive pathogens.[236] One study co-cultured Caco2 cells with Raji B cells to produce a model containing M-like cells, in order to observe the transcriptional processes of *Salmonella* translocation across the epithelium.[237]

Cell culture models are becoming increasingly more complex, with 3-D organotypic models being developed, such as the use of a rotating wall vessel (RWV) to propagate colonic cell cultures on microbeads; allowing them to create an organised intestinal epithelium more representative of that seen *in vivo*. This also allows reconstitution of some of the chemical and molecular gradients in all dimensions that would occur in the intestine (i.e. apical, basal and lateral interactions).[238] Recently, groups have attempted to take this further and produce models which investigate both the epithelial and immune response to pathogens by setting up organotypic cultures derived from colonic epithelial cells, grown in a RWV, and adding macrophages into the basal aspect of the culture medium to try to recapitulate what would occur in the lamina propria. Macrophages in this study did exhibit phagocytosis and reduce adherence, invasion and survival of a number of *Salmonella* strains versus an epithelial model alone.[239]

Discrete 3-D organoid models which contain an organised, polarised epithelium have been developed from a number of tissues, including mouse intestinal crypts.[240,241] Clearly work

with this particular type of organoid would have some of the same caveats as using a live mouse model, but they are a much more efficient way of looking at the mouse epithelium, as organoids from one animal will self-perpetuate, rather than requiring sacrifice of numerous mice for a set of assays. Organoids have also been generated from human embryonic stem cells (hESC),[233] human intestinal tissue from biopsies (primary organoids),[242] minced intestinal tissue[243] and human induced pluripotent stem cells (hiPSCs) as demonstrated in **Figure 1.5**.[244] As well as self-renewal, organoids are capable of self-organisation, during growth on an extracellular matrix scaffold, and demonstrate similar organ functionality as their tissue of origin.[245] One benefit of the organoid model is the lack of stromal tissue, allowing a reductionist approach for studying the tissue of interest (the epithelium) without confounding influences from the local environment. It is also much more feasible to manipulate signalling pathways or create organoids from different genetic backgrounds than in animal models, particularly in the case of hiPSC-derived organoids (iHO), as CRISPR/Cas9 editing can be used to produce knockout lines alongside the isogenic control line. Some researchers have used organoids as a starting point to produce a more complex model of the gut in vitro, with studies combining differentiating iHO with neural crest cells in culture, and transplanting this into murine kidney capsules, to form an intestinal model featuring epithelium, mesenchyme and neuroglial structures which showed some neuronal activity.[246]

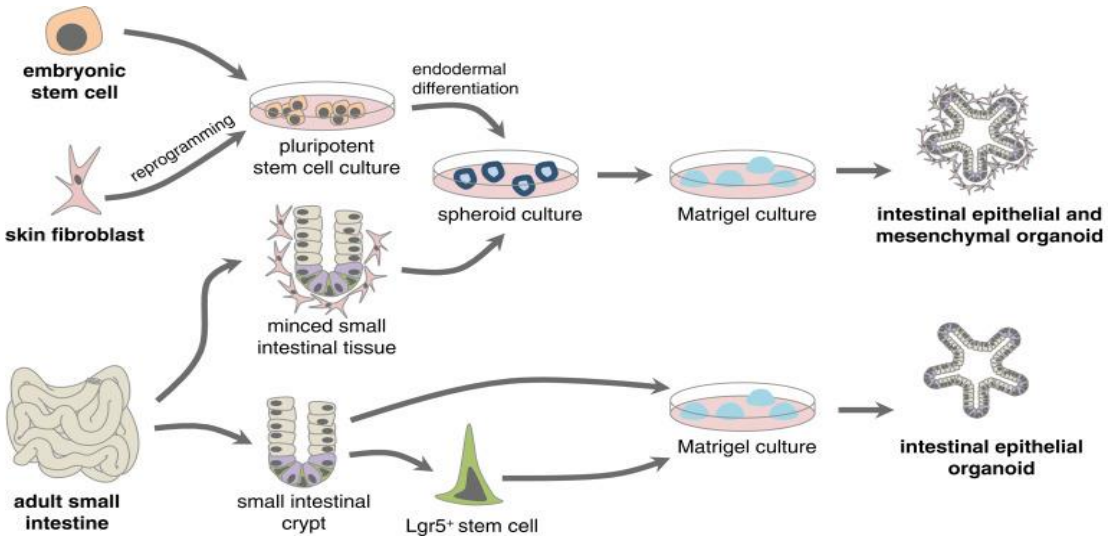

Figure 1.5: Sources of tissue for production of intestinal organoids. (Figure taken from Kretzschmar & Clevers, 2016[247])

Studies of *Salmonella* in human blood, rather than the intestine are rather simpler, given that there is the ability both to make human macrophage-like cells from easily available cell lines, such as THP-1, and the possibility to directly complete assays on blood or serum collected both from healthy volunteers and those who have been exposed to the infection. Nonetheless, hiPSC-derived macrophages are an important development in the study of disease pathogenesis. Not only do these cells phenotypically resemble human macrophages, they also show a more robust killing and cytokine production response versus their THP-1 derived counterparts.[248] In addition; as with the iHO, they can be produced either from iPSC from individuals with disease-causing mutations of interest, or CRISPR/Cas9 could be used to knockout genes of interest. It is possible therefore, to study the host response to *Salmonella* in two different compartments (epithelial and blood) if iHO and macrophages are differentiated from the same donor iPSC, as is the case in this project.

Lastly, the human challenge model, originally used to investigate *S*. Typhi in the 1950-60's,[97] has been revived by the Oxford Vaccine Group to investigate multiple facets of *S*. Typhi and *S*. Paratyphi A infection. Alongside detailed clinical information and blood cytokine, transcriptional and metabolic data, this model has contributed evidence for vaccine efficacy that assisted in the recommendation by Gavi for pre-qualification of the TCV vaccine.[100] This group has since developed a model for *S*. Paratyphi A challenge,[249] and studies are underway to help us discover more about this pathogen in its natural host. One thing that this model cannot offer however, is detailed data on the epithelial response to infection, as gathering these samples would be incredibly invasive and potentially dangerous. Stool samples provide some information by proxy but nothing at the level possible with direct epithelial studies using the iHO model.

1.4.2 Advantages of using the hiPSC-derived iHO model

hiPSC can be generated via reprogramming of cells from somatic tissues such as fibroblasts from skin biopsies, using Sendai vectors (protein factors *OCT4*, *KLF4*, *SOX2* and *MYC*).[250] These hiPSC are then forward programmed using a sequential cocktail of cytokines over 10 days to produce iHO, which are embedded into extracellular matrix and cultured until they reach maturity a few weeks later.[251] The processes for this and embryological rationale behind them will be discussed in more detail later in this chapter. An alternative source of

pluripotent cells, which can be differentiated in this way, are embryonic stem cells (ESC), however, these are much more difficult to obtain and given that their harvest requires the destruction of early embryos, there is ethical debate about use of these cell types. In fact, hiPSC and ESC have been shown to be very similar in gene expression and DNA methylation,[252] with a study looking at hiPSC derived from three different tissue types from individuals and ESCs, finding that inter-individual transcriptional variation in hiPSCs was greater both than variation from somatic tissue of origin or between ESC and hiPSC.[253] There was little evidence of epigenetic 'memory' of previous tissue type, and cell phenotypes within individuals were very reproducible (with three cell lines produced from each tissue). This study suggested that hiPSC should be taken from numerous individuals for experiments, rather than an increasing number of cell lines taken from one individual for replicates, and that hiPSC are a robust and powerful platform for large-scale studies of genetic differences between individuals. Ease of access to numerous cell lines is certainly one advantage that iHO derived from hiSPC have over primary iHO.

Generation of primary iHO from intestinal biopsies containing crypts, requires the availability of donor tissue, usually taken from an individual undergoing investigation for a condition such as inflammatory bowel disease. Biopsies from those who do not have disease are treated as being from 'healthy' samples, however this must be treated with caution if the individual was displaying gastrointestinal symptoms severe enough to warrant a biopsy. Primary iHO are more rapid to manufacture than hiPSC derived iHO, as following isolation and embedding of crypts, LGR5+ cells rapidly divide and reproduce the organoid structure within a matter of days. However, primary cultures require a number of additional growth factors and Wnt conditioned medium, which is tricky to produce consistently. In addition, a number of these growth factors are removed from the culture medium to induce terminal differentiation prior to experimentation, whereas growth conditions for hiPSC-derived iHO remain consistent.[242] Primary iHO are smaller than hiPSC-derived iHO, with their lumen being much more difficult to access via microinjection technology. Response to *Salmonella* infection and rhIL-22 stimulation was demonstrated to be consistent between primary and hiPSC-derived iHO during this project,[254] reinforcing the decision to investigate host-pathogen interactions for *Salmonella* using the hiPSC-derived iHO model.

Finally, it is possible to investigate genetic mutations of interest in primary iHO, using samples from patients with a disease / mutation of interest. In order to have a control line to compare the diseased line to, one could use transcription activator-like effector nucleases (TALENs) or CRISPR/Cas9 to target and repair the mutation in order to restore function and produce a complemented line. This requires time and precision, and could mean that each mutation of interest would be studied in a line with a different genetic background. An efficient way of studying SNPs of interest would be to use CRISPR/Cas9 with single-stranded donor oligonucleotides[255] to induce mutations in hiPSC, then use the original hiPSC line as an isogenic control. Advantages of this would include being able to produce multiple different mutants in the same genetic background, meaning that the control line used would be well characterised, and optimisation of experimental design would not be required every time a new mutant line is produced. It may also be the case that a particular mutation affects more than one target organ. hiPSC derived organoids could be produced for a number of different tissues in order to investigate effects of disease in different compartments, both in organs, and in blood cells such as macrophages, perhaps even simultaneously if it were possible to produce monolayers from iHO and culture them with macrophages from the same iPSC line for infection assays. The beginnings of this type of assay have been trialled by one author with enteroids and PBMCs,[256] and another with murine organoids and intraepithelial lymphocytes.[257] This plasticity of hiSPCs and their ability to differentiate into numerous tissues is one of their advantages as a model for investigating host-pathogen interactions.

1.5 Host defences against enteric pathogens

1.5.1 The role of the intestinal epithelium in defence against enteric pathogens

This chapter has discussed the specific host response to *Salmonella*, but this is better understood by looking at the general role of the intestinal epithelium in defence against enteric pathogens. *In vivo,* intestinal epithelial cells (IECs) play a key role in regulating homeostasis between the epithelial barrier, overlying microbiota and the gastrointestinal immune system. If this homeostasis and the continuity of the epithelium is threatened by attack from a pathogen, the innate immune system rapidly activates; initially with a

generalised response, prior to a slightly more delayed pathogen-specific adaptive immune response.[258] Prior to reaching the epithelial barrier, potential pathogens have to deal with competition for nutrients with the microbiota, or even avoid products which may inhibit pathogen growth, such as bactericidal organic acids produced by *lactobacilli* and *bifidobacteria* which suppress growth of *S*. Typhimurium in vitro.[259]

Secondly, pathogens have to breach the intestinal mucus layer, largely composed of mucin 2. The mucin layer consists of a thinner outer layer which contains components of the microbiota, and a dense inner layer which normally does not contain bacteria, functioning to prevent microbiotal translocation and excessive immune activation.[260] Goblet cells are epithelial cells which produce the mucins making up the mucus layer and also produce trefoil factors, which can increase the viscosity of mucus to increase protection from pathogens. Additionally, trefoil factors enhance mucosal restitution and prevent apoptosis, aiding epithelial repair after damage.[261] Many pathogens have had to acquire virulence-associated factors to overcome this mucus barrier, such as production of flagella and chemotaxis in *S*. Typhimurium and secretion of proteases by Enteropathogenic *Escherichia Coli* (EPEC) once they have adhered to the mucus layer.[262] Goblet cells increase mucus production after stimulation by pathogens, which can expel some pathogens from the lumen, but unfortunately others, such as *Salmonella* can colonise the mucus layer and take advantage of the nutrients and carbohydrates contained within it.[263]

The epithelial layer itself is composed of a number of different cell types, each of which has a role to play in the defence against enteric pathogens. These include enterocytes, M cells, Paneth cells, enteroendocrine cells and goblet cells; the role of which has been outlined above. Enterocytes act as a barrier by forming tight junctions, composed of claudins and zonula occluden proteins, preventing microbes translocating between cells into the lamina propria. Attaching and effacing pathogens (e.g. EPEC) work by inducing tight junction alteration to disrupt the epithelium. Tight junction proteins are partly regulated by cytokines; inflammatory cytokines such as IFNγ and TNFα downregulate junctional proteins.[264] Enterocytes are also able to secrete a number of cytokines and antimicrobial peptides, playing a vital role in the immune response, as illustrated in **Figure 1.6**.

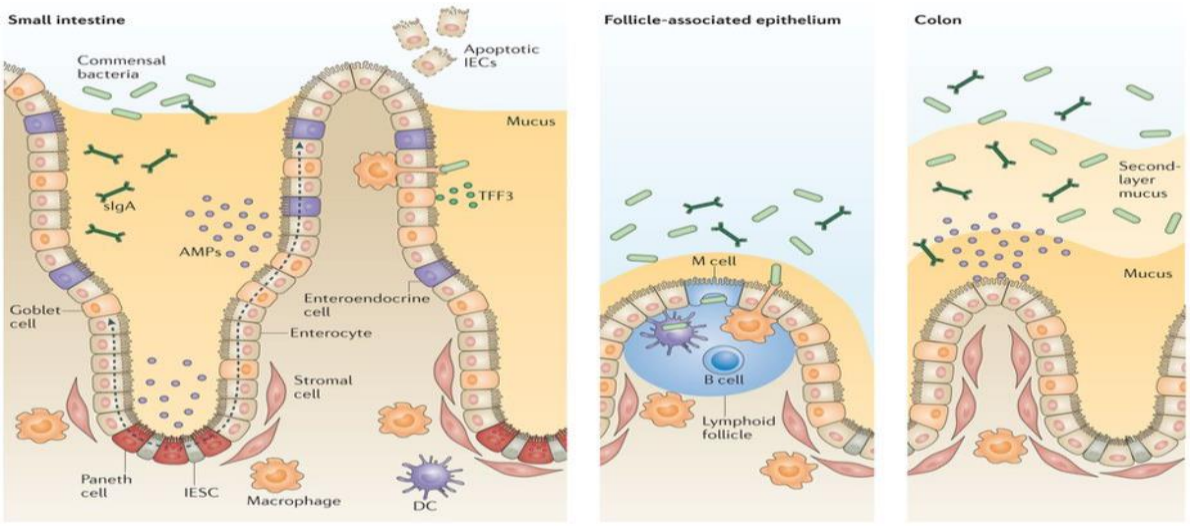

Figure 1.6: Schematic of intestinal epithelial defence mechanisms. The different epithelial cell types and their secretory products are demonstrated here. Paneth cells remain in the crypt, but all other cell types migrate up towards the tip of the villi after production by intestinal epithelial stem cells (IESCs), as shown by dashed arrows. Antimicrobial peptides (AMPs) are secreted by Paneth cells and enterocytes, with mucus and trefoil factor 3 (TFF3) secreted by goblet cells. Secretory IgA (SIgA) is produced by plasma cells in the lamina propria and transcytoses to the lumen. M cells mediate transport of antigens and bacteria across the epithelium to DCs and macrophages. DCs are also able to sample the luminal contents by sending dendrites through epithelial tight junctions. (Figure taken from Peterson & Artis, 2014).[265]

M cells are a part of the follicle-associated epithelium which is located over Peyer's patches. These cells sample and transport pathogens from the lumen to the underlying immune cells, in order for bacteria to be recognised by their antigens and adaptive immune responses initiated.[266] M cells do not have microvilli as do enterocytes, instead having a 'microfold' appearance from whence they get their name. They also largely lack a mucus layer, allowing them to detect antigens and engulf pathogens. On their basolateral surface, M cells have specific invaginations which work as docking sites for DCs, T cells, B cells and macrophages.[267] Whilst M cells are able to initiate the adaptive immune response, they can also be exploited by enteric pathogens, such as *S*. Typhi, *S*. Typhimurium, *Vibrio cholerae* and *Shigella flexneri*, as a method of direct transcytosis into the lamina propria.[268]

Paneth cells are located in the crypts of the small intestine, adjacent to epithelial stem cells. They produce and secrete antimicrobial peptides in response to myeloid differentiation factor (MyD88)-dependent TLR activation on sensing bacteria.[269] These AMPs include α- and β-defensins, which target bacterial membranes that do not contain cholesterol, producing transient pores in these membranes to disrupt their integrity. Defensins also have a chemoattractant property for DCs and T cells. Other compounds secreted by Paneth cells

include lysozyme and phospholipase A2 (sPLA2), which target bacterial cell walls. Paneth cells can also be induced to secrete C-type lectins (RegIIIβ/RegIIIγ), cathelicidins and angiogenin4 after detection of PAMPs.[108] Epithelial cells can also secrete RegIIIγ, calprotectin, β-defensins and RELM-b when PAMP detection occurs.

Lastly, enteroendocrine cells secrete numerous hormones with roles in gastrointestinal motility and digestion. These include: cholecystokinin (gall bladder contraction), somatostatin (an inhibitory hormone for digestive endocrine and exocrine function), glucagon-like peptide 1 (satiety) and serotonin (intestinal motility, secretion and appetite). These cells do not have any direct antimicrobial action, but hormones such as glicentin and glucagon-like peptide 2 promote mucosal enterocyte proliferation, which may be important for repair after pathogen-mediated damage to the epithelial barrier.[270]

Plasma cells in the lamina propria are able to produce secretory IgA (sIgA),[265] which can be antigen specific or non-specific, and is transcytosed across enterocytes to the intestinal lumen. Here, it can bind to surface isotopes on the surface of pathogens to prevent them binding to epithelial cells.[271] In addition, sIgA can cause pathogens to agglutinate in the lumen by binding to antigens on bacterial and viral surfaces.[272] Specific sIgA can be produced as part of the adaptive immune response and has been shown to protect against *Salmonella* infection in mice,[273] alongside reducing bacterial ability to deal with oxidative bursts.[274]

The epithelium also contains a number of pattern recognition receptors (PRRs) which are able to detect PAMPs; for example, LPS or endotoxins expressed by potential invasive pathogens, and are able to activate the innate immune response to try and control this threat. PRRs include Toll-like receptors (TLRs) which are located on the cell surface or inside of lysosomes and endosomes. There are numerous TLRs, but the ones pertinent to the gut are: TLRs 2, 3, 4, 5 and 9. After recognising a pathogen, TLRs recruit adaptor molecules which contain MyD88 and Toll-interleukin 1 receptor to the cytoplasm, initiating transcription of pro-inflammatory genes via activation of NFκB and MAPK.[258] TLRs can also

be triggered by danger-associated molecular patterns (DAMPs) or alarmins, which are released by the host cell in response to tissue injury, stress and necrotic cell death.[108] Other PRRs; nucleotide oligomerization domain (NOD)-like receptors (NLRs) in the cytoplasm detect intracellular microbial molecules. NOD1 and NOD2 are the best studied of this family and are noted to recognise components of peptidoglycans and in response, activate NFκB and induce IL-12 secretion. NLRs are also involved in inflammasome assembly; with the inflammasome composed of pro-caspase-1, an NLR protein and an adaptor molecule such as ASC. The inflammasome induces maturation of pro-caspase-1 into caspase-1 which activates IL-1β and IL-18; cytokines involved in the inflammatory response to infection.[275]

C-type lectins are PRRs which recognise specific carbohydrate structures on pathogen surfaces, and are largely found on macrophages and DCs. An example of one of these proteins would be mannose-binding lectin (MBL), which is able to bind to bacteria, viruses, fungi and protozoa. On binding, MBL changes shape and triggers phagocytosis of the pathogen by immune cells and activation of complement. Lastly, retinoic acid-inducible gene-1 (RIG-1) like receptors (RLRs) are involved in the response to viral pathogens, detecting double stranded RNA in the cytoplasm and inducing IFNα, IFNβ and inflammatory cytokines via activation of NFκB, MAPK and interferon regulatory factors.[276]

IEC have a number of other defence mechanisms to guard against infection, such as the expression of intestinal alkaline phosphatase (IAP) in their apical brush border. IAP removes the phosphate group from LPS, limiting its ability to activate TLR4, and preventing translocation of LPS into the systemic circulation, where it can induce inflammatory cytokine driven septic shock.[277] IEC are also able to produce reactive oxygen species (via expression of Nox and Duox proteins),[114] which have microbicidal effects and help epithelial repair.[278] Autophagy is also a defensive response, aimed at preventing further spread of intracellular bacteria. This process both degrades cellular cytoplasmic contents, but also recognises and degrades intracellular pathogens. Autophagy is mediated by MyD88, in conjunction with the proteins LC3, ATG16L and ATG5 and is activated under cellular stress conditions. *S*. Typhimurium is capable of inducing autophagy in the intestinal epithelium.[279] With increased apoptosis, there is also the need for replacement of apoptosed IEC, requiring an increase in

proliferation by the intestinal stem cells. One pathway which regulates this proliferation is the β-catenin pathway, which can be suppressed by LPS and other bacterial elements.[280] Clearly the intestinal epithelium has many complex regulatory and defence mechanisms that act to maintain homeostasis in both health and disease states. Important to consider also are the mechanisms of pathogen defence which happen once the pathogen has entered the cell.

1.5.2 Phagolysosomal fusion as a mechanism of pathogen destruction

1.5.2.1 Formation of the phagolysosome

Following phagocytosis and entry of the pathogen into the cell, the phagosome undergoes a process of maturation in order to form the microbicidal phagolysosome. Debate has occurred over the mechanism by which lysosomes transfer their contents to the endosomes involved in the maturation process, but imaging studies have shown that this occurs both via lysosomes repeatedly and transiently fusing with endosomes as well as incidents of complete fusion, following which the lysosome reforms from the membrane of the hybrid vesicle.[281] There are three reported stages of phagosome maturation: early, late and phagolysosome, each with different membrane proteins associated.
The early phagosome is defined by the presence of the GTPase Rab5, which regulates fusion events between the phagosome and early endosomes via the membrane recruitment of EEA1 (early endosome antigen 1).[282] Rab 5 is also able to recruit hvPS34 (human vacuolar protein-sorting 34), which is a class III phosphoinositide 3-kinase. This molecule generates phosphatidylinositol 3-phophate, which then recruits other phagosomal maturation proteins such as Rab7, a late endosomal marker.[283] Vacuolar ATPase (V-ATPase) accumulates on the phagosomal membrane and translocates H^+ ions into the phagosome, causing the pH inside the phagosome to acidify (pH 6.1-6.5).[284]

Eventually, Rab5 is lost and replaced by Rab7 on the phagosome membrane. This mediates fusion of the phagosome with late endosomes.[285] Recycling vesicles are formed, which remove proteins to be recycled from the phagosome. Meanwhile, intraluminal vesicles (ILVs) containing proteins for degradation are sent into the lumen of the late phagosome. Accumulation and action of further V-ATPase molecules means that the intraphagosomal pH

drops further (pH 5.5-6.0).[286] Rab7 recruits Rab-interacting lysosomal protein (RILP), which facilitates contact between the phagosome and microtubule and lysosomes. Lysosomal-associated membrane proteins (LAMPs) and proteases such as cathepsins and hydrolases are introduced into the phagosome after fusion with late endosomes. LAMPS regulate membrane fusions and are necessary for phagolysosomal fusion.[287]

Finally, phagolysosomal fusion occurs, producing a very hostile environment for any microbe within the phagolysosome. The pH for the phagolysosome is between 5.0-5.5 following further V-ATPase action,[284] and enzymes such as cathepsins, proteases, lysozymes and lipases are contained within. In addition, NADPH oxidase and other reactive oxygen species are present on the phagolysosomal membrane, and restriction of nutrients such as iron occurs via action of molecules like lactoferrin.[287] **Figure 1.7** demonstrates the phagosomal maturation process.

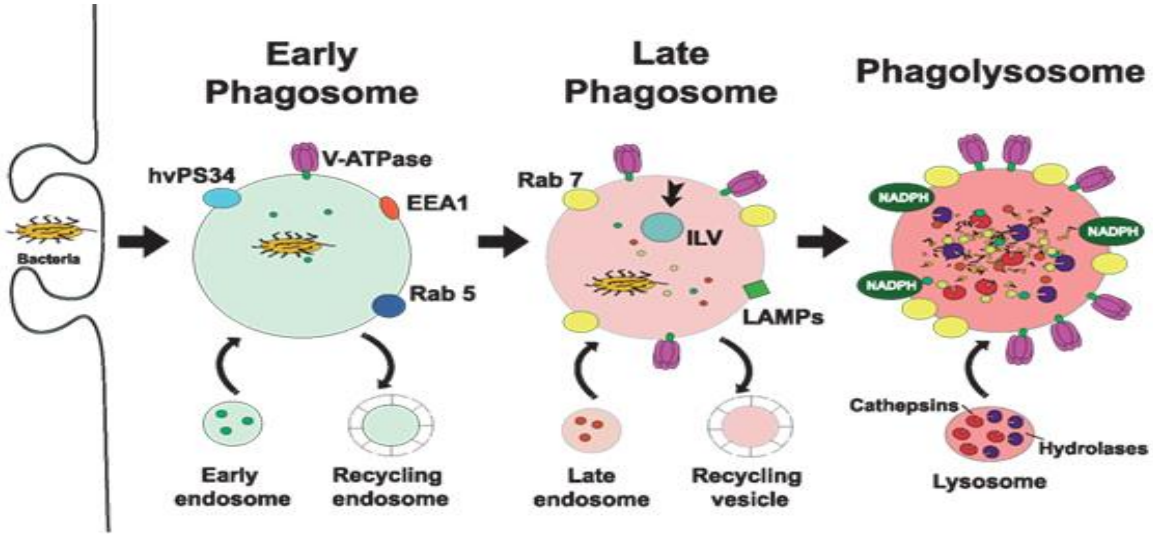

Figure 1.7: Markers involved in phagosomal maturation and phagolysosomal fusion. Presence of Rab5, hvPS34, EEA1 and accumulation of V-ATPase, alongside fusion events with early endosomes mark the early phagosomal stage. This is followed by expression of Rab7, LAMPS, further V-ATPase, fusion with late endosomes and the presence of recycling and intraluminal vesicles (ILVs). Highly acidic phagolysosomes form after fusion of late phagosomes with lysosomes. Enzymes and reactive oxygen species degrade the intraphagosomal pathogen. (Figure taken from Uribe-Querol & Rosales, 2017[287])

1.5.2.2 Avoidance of phagolysosomal fusion

Given the incredibly harsh environment produced inside of the phagolysosome, many intracellular bacteria use inhibition of phagolysosomal fusion as a survival strategy, such as

Mycobacterium tuberculosis, *Brucella* spp., *Legionella pneumophilia*, *Salmonella* and *Listeria monocytogenes*. Some pathogens, such as *Coxiella burnetii* have simply evolved to withstand the low pH inside of the phagolysosome.[288] Others are able to manipulate actions of the Rab GTPases to arrest phagosomal maturation at different stages; for example, *Mycobacteria*-containing phagosomes acquire Rab5a, but maintain their phagosomal compartment at the early endosomal stage, by blocking acquisition of Rab7. In addition, *M*. *tuberculosis* are able to reduce accumulation of vacuolar ATPase, meaning that the phagosome does not fully acidify. Treatment of macrophages with IFNγ restores these processes, allowing phagolysosomal fusion and killing of mycobacterium within the phagolysosome to occur.[289] The mechanisms by which phagolysosomal fusion is inhibited by *M*. *tuberculosis* are not fully established, but interestingly, IL-22 has been found to increase S100A8 and Rab7 expression in *Mycobacterium*-infected macrophages, leading to enhanced phagolysosomal fusion, suggesting that further investigation of the relationship between these effectors may hold clues as to what *Mycobacteria* are inactivating to reduce phagolysosomal fusion *in vivo.*[290]

Studies on *E. coli* K1, which is able to translocate the blood brain barrier after invasion of human brain microvascular endothelial cells (HBMEC) demonstrated that *E. coli*-containing vacuoles (ECV) acquire early endosomal markers in the form of EEA1 and transferrin receptor, along with the late endosomal/lysosomal markers Rab7 and Lamp-1, yet do not undergo phagolysosomal fusion; measured by the lack of cathepsin D, a lysosomal enzyme, and intravacuolar survival of bacteria. An isogenic mutant without the capsule was unable to arrest phagolysosomal fusion and was degraded within the vacuole. The mechanism of action is not yet understood, but it appears that the K1 capsule is somehow able to influence ECV trafficking, in order to avoid phagolysosomal fusion.[291]

Early endosomal markers EEA1 and Rab5a are acquired by phagosomes engulfing *Listeria*, *Legionella* and *Brucella*, but these pathogens have alternative escape mechanisms to avoid phagolysosomal fusion. *Brucella* and *Legionella* enter compartments composed of endoplasmic reticulum membranes, which resemble autophagosomes, in order to evade acquisition of further markers,[289] whereas vacuoles containing *Listeria* acquire late

endosomal markers, but the bacterium then perforates the late endosomal membrane and escapes into the cytosol to replicate therein.[292]

Formation and maturation of the *Salmonella*-containing vacuole (SCV) are discussed in more detail earlier in this chapter, but worth highlighting is the fact that *Salmonella* appear to have cultivated a number of strategies to either avoid phagolysosomal fusion or modify their phagosomal environment to allow improved survival and replication. One study which aimed to investigate methods of phagolysosomal fusion avoidance by *Salmonella* noted that SCVs divide along with *Salmonella*, resulting in many cases in one bacterium per SCV and thus an increased SCV load within the cell, overloading the capabilities of the cell to produce sufficient lysosomes to acidify and deliver enzymes to all of the SCVs.[293] *S*. Typhimurium was found to actively inhibit phagolysosomal fusion in murine macrophages and preferentially divided inside of unfused phagosomes.[294] The exact mechanism by which inhibition occurs is not defined, but one factor which some hosts have to overcome it is the expression of Nramp1. Nramp1 is expressed in lysosomal compartments within macrophages and facilitates killing of intracellular bacteria by increasing phagosomal fusion with lysosomal membrane proteins such as mannose 6-phosphate,[295] and withholding Fe^{2+} and Mg^{2+} from intraphagosomal bacteria.[130]

Similarly to findings with *E. coli*, *S*. Typhimurium within SCVs in HeLa cells acquire EEA1, transferrin receptor, Rab5, Rab7 and Lamp-1 but do not obtain cathepsin D or fuse with lysosomes.[296] This was thought to be due in *Salmonella* to the actions of the SpiC protein, an effector of the SPI-2 T3SS being exported into the cytosol, inhibiting interactions between SCVs and lysosomes, and disrupting vesicular transport, with a SpiC mutant derivative unable to prevent phagolysosomal fusion.[127] Additionally, SifA, an effector protein which is injected into host cells by the SPI-2 T3SS, is required for maintenance of SCV integrity and formation of Sifs in epithelial cells.[297] PipB2 is also thought to prevent vacuolar lysis.[82] The mechanism of action *SifA* is unknown; but thought to be via control of Rab7-dependent recruitment of additional endosomal membranes to the SCV during replication.[298] Lastly, *Salmonella* use determinants such as the PhoPQ regulatory system, which is activated by low pH change to modify intraphagosomal pH and create an optimal environment for replication.[31]

There are many questions still around the mechanisms both driving phagolysosomal fusion and its modification by intracellular pathogens. It would appear that there are numerous effectors exploited by different pathogens alongside multiple methods of avoidance of phagolysosomal fusion, either by breaking out of the vacuole, adapting to life within the phagolysosome or modifying the contents of the phagosome and its maturation to produce a more satisfactory intravacuolar environment. Further detailed study of intracellular bacteria in models such as the iHO model should help to elucidate some of these mechanisms.

1.5.3 The Interleukin-22 (IL-22) pathway

1.5.3.1 Components of the IL-22 pathway and its mechanism of action on the intestinal epithelium

IECs are able both to produce and respond to cytokines as part of their role in maintaining epithelial homeostasis. The cytokine IL-22 is also known to have a role in maintenance of the gut epithelial barrier,[299] is involved in the induction and secretion of antimicrobial peptides and chemokines,[300] epithelial cell proliferation and maintenance of tight junctions[301] in response to infection. It is a part of the IL-10 family of cytokines, made up of: IL-10, IL-19, IL-20, IL-22, IL-24 and IL-26, all of which have differing roles in inflammation and immunity.[302] All but IL-26 have a homolog in mice, meaning that a number of these cytokines are well characterised in part due to murine studies. Common to all of these cytokines is that they require a 2-part receptor complex, with one element of this complex being shared in a number of cases; for example, IL-10R2 (or IL-10Rβ) is a part of the complex for IL-10, IL-22 and IL-26 (**Figure 1.8**). IL-10R2 is fairly ubiquitously expressed across cell types. The other part of the receptor complex for IL-22, is IL-22R1, which is expressed only on epithelial cells lining barrier sites, such as the skin, intestine, liver, lung, kidney and pancreas.[303] IL-22 receptor complexes are located on the basal surface of the polarised intestinal epithelium. Mutations in IL-10R2 can lead to lack of sensitivity to IL-22 and are associated with early-onset inflammatory bowel disease.[304] Functional IL-22 and IL-22R1 protect against dissemination of bacterial infection following *Citrobacter rodentium* infection or DSS-induced colitis in mice.[305,306]

The IL-10 cytokine family are secreted by a number of different cell types, as shown in **Figure 1.8,** with IL-22 being produced by activated T cells, more specifically CD4+ Th17 cells and NK cells and acting on non-haematopoietic cells. More recently, IL-22 has also been discovered to be produced by innate lymphoid cell 3 (ILC3) cells, located in Peyers patches and GALT.[307] Similarly, the other cytokines in the IL-10 family act predominantly on non-haematopoietic cells, with only IL-10 (and possibly IL-19) thought to be able to exert their effects on haematopoietic cells.

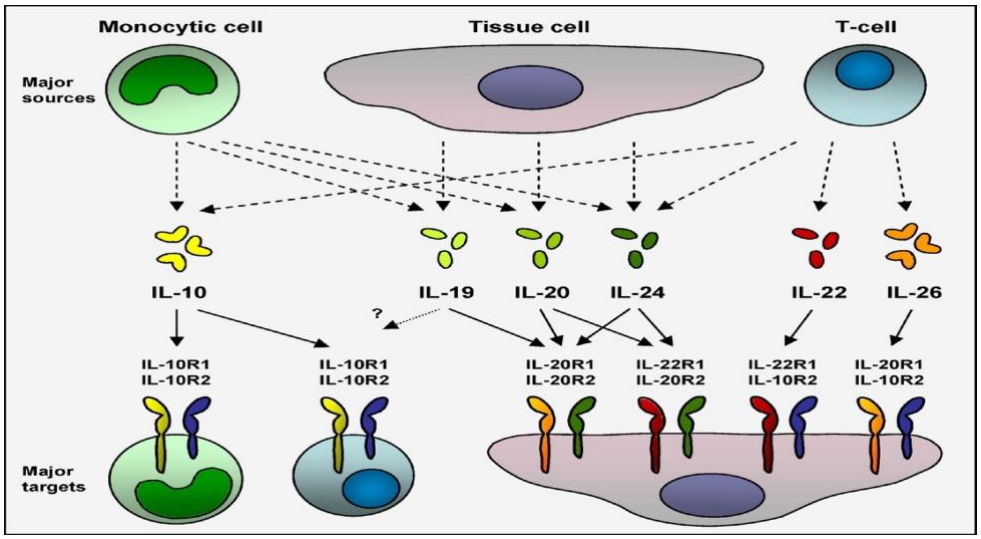

Figure 1.8: Receptor complexes and cells secreting / responding to IL-10 family cytokines. (Figure taken from Sabat, 2010)[302]

IL-22 enhances innate immune response in the intestinal epithelium, as it acts to increase chemokine expression, induce mucus secretion by goblet cells, increase epithelial cell proliferation and induce production of AMPs including RegIIIβ and RegIIIγ,[308] defensins and S100 proteins.[309] It is also able to induce secretion of acute phase reactants in response to liver injury. IL-22 also mediates intestinal epithelial fucosylation, which reduces expression of bacterial virulence genes.[310]

As well as its dimeric receptor complex, a soluble secreted version of the IL-22 receptor exists, IL-22 binding protein (IL-22BP). This is an inhibitory regulator of IL-22, and is highly expressed by DCs in the intestine. It is thought to be involved in maintenance of epithelial homeostasis by ensuring IL-22-induced inflammatory responses are not disproportionate to what is required, as can be seen in diseases associated with increased IL-22 production, such as

Crohn's disease, rheumatoid arthritis, psoriasis and interstitial lung disease.[311] Levels of IL-22 or its transcripts have been shown to correlate with the severity of disease in a number of the above conditions.[302] In addition, these are all T cell mediated diseases, which fits with the preferential production of IL-22 by T cells. IL-22BP appears to act only in response to a sustained increase in IL-22 levels, as the NLRP3 and 6 inflammasomes initially downregulate IL-22BP following acute intestinal epithelial damage[312]

Signalling induced by the binding of IL-22 to its receptor complex largely occurs via the JAK/STAT pathway. IL-22R1 is associated with JAK kinase 1 (JAK1) and IL-10R2 with tyrosine kinase 2 (Tyk2) in particular. JAK kinases phosphorylate tyrosines, and a STAT transcription factor binds to this complex and becomes phosphorylated. STAT molecules exist as dimers in the cytoplasm and change their structure following activation by JAK kinases. STAT3 is the molecule activated most ubiquitously by IL-10 family members, but STAT1 and STAT5 can also be phosphorylated at high IL-22 concentrations. (**Figure 1.9**) Phosphorylated STAT3 migrates into the cell nucleus and binds to promoters, upregulating transcription of certain genes such as suppressor of cytokine signalling (SOCS)3. SOCS3 binds to JAK molecules, inhibiting their activity and completing the feedback loop.[302] Alongside JAK/STAT activation, IL-22 has also been shown to induce phosphorylation and activation of the three major MAP kinase pathways of NFκB via MAPK1/MAPK3, JNK and p38 kinase [313]

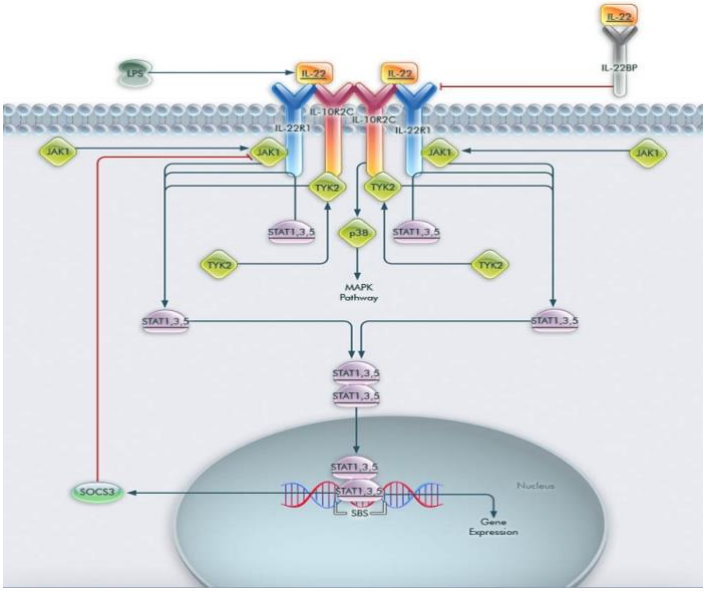

Figure 1.9: Intracellular mechanisms of IL-22 signalling. (Image taken from https://www.thermofisher.com/uk/en/home/life-science/antibodies/antibodies-learning-center/antibodies-resource-library/cell-signaling-pathways/il-22-pathway.html)

1.5.3.2 Sources of IL-22

IL-22 is produced by CD4+ T_H cells (predominantly Th17 cells) in response to IL-6 and TNFα during infection or inflammation. IL-23 is also an important inducer of IL-22 production, enhancing its expression in maturing Th17 cells.[314] This increase in IL-22 expression leads to increased expression of the IL-23 receptor also, and thus increased interactions between IL-23 and its receptor, further increasing IL-22 production. IL-23 itself is produced by DCs and macrophages in response to pathogen invasion of the epithelium. Aryl hydrocarbon receptor (AhR) is activated by cellular stress and Ca^{2+} influx and can induce IL-22 either via direct regulation of IL-22 transcription, or via regulating production and development of Th17 cells and Type 3 innate lymphoid cells (ILC3s). Lastly, IL-1β, which can be produced by macrophages, DCs, neutrophils, T and B cells and epithelial cells, is able to activate NK cells, ILC3s and Th17 cells to produce IL-22 and also promotes NK cell expansion. IL-22 secretion is inhibited by the actions of TGFβ, which is required for Th17 differentiation and is able to influence IL-23R expression in a number of tissues.[315] Sources and actions of IL-22 are illustrated in **Figure 1.10**.

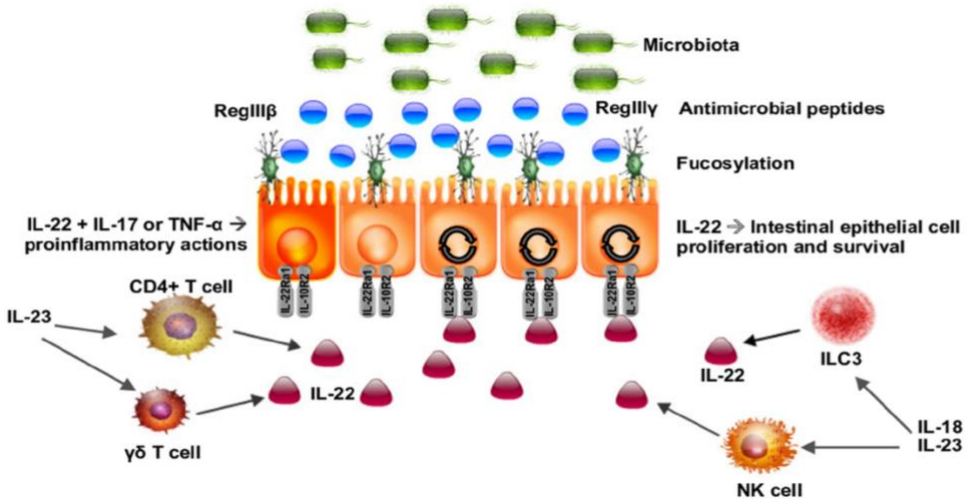

Figure 1.10: Interactions of IL-22 with the intestinal epithelium and immune system. IL-22 is produced by NK cells, T cells and ILC3s. Its actions include fucosylation of epithelial cells, increased mucus production, increased cellular proliferation and AMP release in order to maintain the epithelial barrier. Alongside IL-17 and TNFα, IL-22 can promote a pro-inflammatory response to pathogen invasion. (Figure taken from Parks et al, 2016[309])

The role of IL-22 in pathogen defence is under investigation. Murine intestinal organoids stimulated with IL-22 showed an inflammatory response to pathogens, improved antimicrobial defences and wound healing.[305] IL-22 has been shown to enhance murine survival after exposure to the attaching/effacing organism *C. rodentium* (a murine paralog of EHEC/EPEC),[306] *Klebsiella pneumoniae*[316], *S*. Enteritidis[317], *Candida albicans*[318] and increase the relative growth inhibition of *M. tuberculosis* in human cells.[319]

1.6 hiPSC-derived systems for recapitulating host response to pathogens in vitro

1.6.1 Production of hiPSCs

The simultaneous discovery in 2007 by Takahashi *et al.* and Yu *et al*. that somatic cells could be reprogrammed into a pluripotent state using 4 reprogramming factors (OCT3/4, SOX2, Klf4, c-Myc and OCT4, SOX2, NANOG, LIN28 respectively) brought about an explosion of interest in the possibilities of making patient and disease-specific stem cells and tissues.[320,298] Since that time, alternative means of delivery of these reprogramming factors have been developed, as original methods required retroviral vectors to deliver the factors into cells. Genomic instability and increased risk of tumourgenicity was a problem, as viral vectors integrated permanently into the cellular DNA; especially concerning in the case of c-Myc, which is a potent oncogene.[321] Reprogramming methods have now developed to use either non-viral methods (RNA based delivery or DNA plasmid delivery[322]), or non-integrating viruses such as Adenovirus or Sendai virus.[323] Sendai virus is one of the more widely used methods, as it has proven efficient in a number of different cell types, produces large quantities of protein and following around 10 passages of reprogrammed cells, no trace of viral RNA is detectable in cells. This method is favoured by the HipSci consortium (http://www.hipsci.org) who have produced a large and well-phenotyped bank of hiPSC from both healthy and diseased individuals. The workflow for reprogramming of cells using Sendai virus is depicted in **Figure 1.11**.

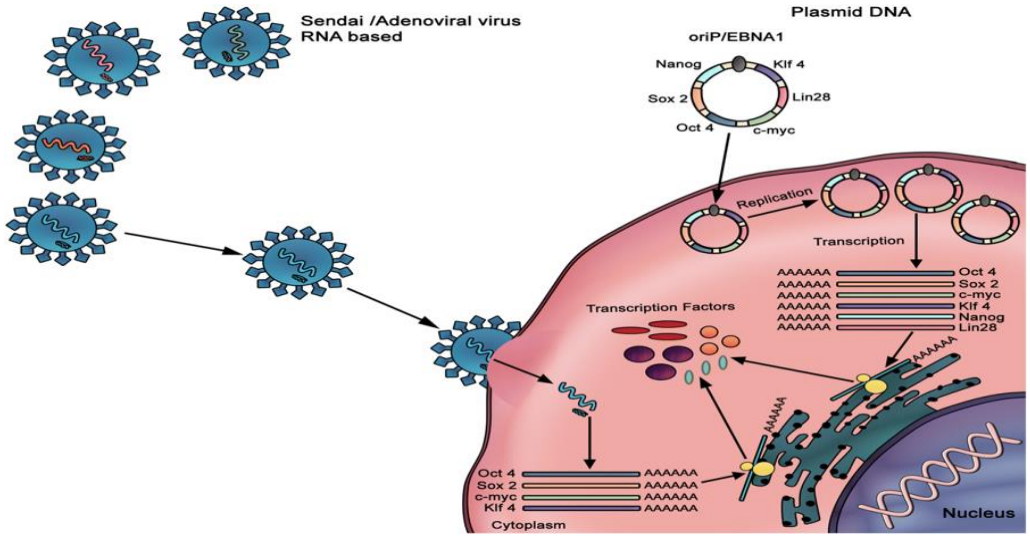

Figure 1.11: Non-integrative methods of delivering reprogramming factors. For RNA-based methods, such as Sendai viral delivery, reprogramming factor mRNA is delivered into the cell without reverse transcriptase and is translated directly into proteins. For plasmid-based methods, DNA is delivered to the cells as a self-replicating plasmid, which does not integrate into the host cell genome. The plasmid is transcribed to RNA and protein produced. (Figure taken from Abou-Saleh et al, 2018[322])

hiPSC have become a popular progenitor cell for the generation of different tissues, as they are easily obtainable, self-renewing and can be genetically manipulated with relative ease, given the recent advent of the CRISPR/Cas9 system. Briefly, this method works via adaptation of a genome editing system that occurs in bacteria. Bacteria capture DNA fragments from invading viruses and create clustered regularly interspaced short palindromic repeats (CRISPR) arrays; storing the viral DNA to allow future recognition of the same or similar pathogens by the bacteria. If this occurs, the relevant CRISPR RNA segment is used to target viral DNA; bacteria attach this RNA to a Cas9 enzyme, which is able to cut the viral DNA at the targeted site, disabling the pathogen. To use this technology for human genome editing, a short guide RNA is produced that targets the DNA sequence of interest. This is attached to a Cas9 enzyme, and the resulting molecule is able to cut the host cell DNA at the targeted region. DNA repair by the cell then takes place either via non-homologous end joining or homology directed repair, either inducing deletions in the targeted gene/sequence, or allowing the researcher to provide a template for DNA repair and make a modification to the existing DNA sequence; by repairing a mutation, for example.[324]

In this way, hiPSC could be produced from patients with disease and repaired to see if cellular functions are restored. Similarly, genes of interest could be knocked out, providing both a mutant cell line and an isogenic control. Wider hiPSC applications currently in progress include high throughput drug screening to reduce need for clinical trials, personalised drug

screening and production of differentiated tissue structures for disease modelling.[325] These applications are discussed in more detail below. Even gene therapy or autologous tissue transplantation could be possibilities, with reports of a patient receiving hiPSC-derived retinal pigment epithelial cells demonstrating arrest of macular degeneration and improved vision and another patient with severe heart failure receiving a scaffold of ESC-derived cardiac progenitor cells and showing improvement in cardiac function post-transplant.[326]

It is possible to differentiate hiPSCs into an increasing number of different cell types or tissues, with protocols being based on studies of embryological development, in order to deduce the correct combination of signals to drive hiPSC to differentiate into the tissue type of choice. These signals can take the form of recombinant growth factors, synthetic small molecules, spontaneous differentiation (e.g. production of embryoid bodies) or co-culture with supporting cell lines.[327] The different cell and tissue types produced using directed differentiation for this study will also be discussed below.

1.6.2 Generation of macrophages from hiPSC

Alongside production of complicated organotypic tissues, cells of haematopoietic lineages such as macrophages can be derived from reprogrammed hiPSC. This has been a particular benefit given that previously, research into infections in which pathogens replicate within macrophages, such as HIV-1, *M. tuberculosis* and *Salmonella* have hit difficulties in producing sufficient and relevant macrophage models for use in studies. Use of blood monocyte-derived macrophages has been the experimental model of choice, however, large amounts of blood are required to obtain sufficient cells to work with. Genetic differences between donors, and the physiological state of the donor on each donation episode will produce variation within data, leading to large amounts of donors and multiple sampling episodes being required to produce representative data.[328] Additionally, terminally differentiated macrophages are not amenable to genetic manipulation, meaning that patient or disease-specific mutations cannot be studied in comparison to isogenic controls. Animal models also cannot completely recapitulate what would be seen in human studies. The other most frequently used representation of macrophages are produced by treatment of THP-1 cells (a monocyte-like immortalised cell line) with Phorbol 12-Myristate 13-acetate

(PMA), however this cell line is karyotypically abnormal and it is not possible to fully differentiate these cells into macrophages. It has been possible to isolate CD34+ haematopoietic stem cells from umbilical cord blood or bone marrow for differentiation and use in experiments. It is possible to genetically manipulate these cells, but they do not self-renew in the way that hiPSC and ESC do.[328] Therefore hiPSC-derived macrophages offer a promising high-throughput, replicable and genetically modifiable system for investigating host-pathogen interactions,[248] as well as having possible applications such as use in cancer therapies,[329,330] modelling of genetic diseases[331,332] and drug screening.[333,334]

Producing terminally differentiated macrophages from hiPSC or ESC requires them to undergo three different steps; firstly, spontaneous differentiation of cultured hiPSC into embryoid bodies (EBs) over 3-4 days. EBs are made up of an ectoderm, mesoderm and endodermal layer. Following this, EBs undergo directed differentiation into myeloid cells, via addition of myelogenic cytokines IL-3 and macrophage colony stimulating factor (M-CSF) to the growth media. This process takes 21 days and produces a population of non-adherent monocytes which can be harvested weekly thereafter from the supernatant around the embryoid bodies. Monocytes are plated and further treated with a higher concentration of M-CSF for 6-7 days to undergo differentiation into macrophages.[328] These matured macrophages have been shown to be comparable to blood monocyte-derived macrophages both phenotypically and in terms of functionality.[335] The process of hiPSC-derived macrophage production is outlined in **Figure 1.12**.

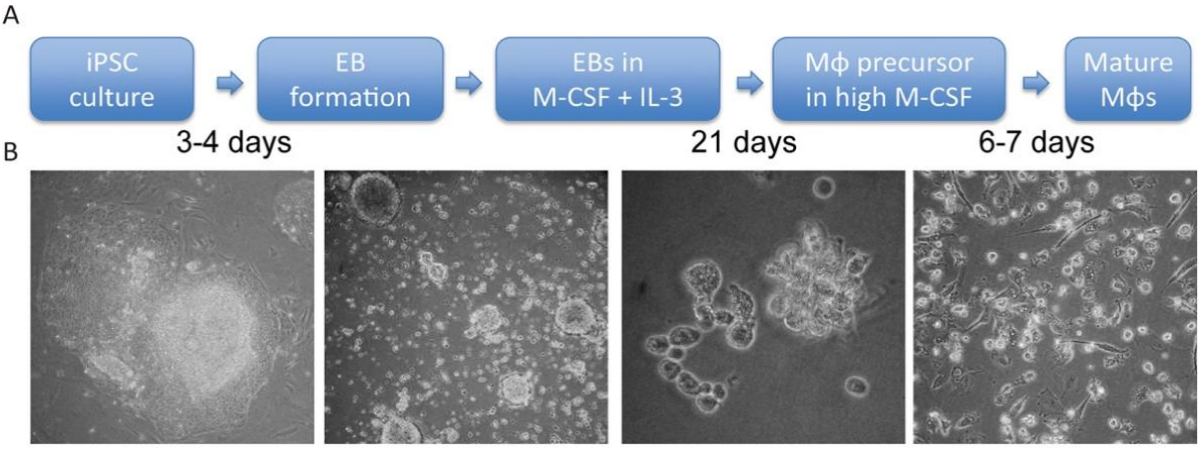

Figure 1.12: Differentiation of iPSC to macrophages. (**A**) Describes culture condition and length of time for each step and (**B**) shows phase contrast micrographs of (L-R): hiPSC, EB, monocytes and macrophages in culture. Mϕ = macrophages (Figure adapted from Hale et al, 2015[248])

1.6.3 Generation of intestinal organoids from hiPSC

One of the first tissue types to be derived from directed differentiation of hiPSCs was intestinal epithelium. This was a hugely exciting development, since as outlined earlier, previous models attempting to reproduce an organised, polarised intestinal epithelium consisting of differentiated cells had proven difficult, or required costly equipment, such as the rotating wall vessel. However, the ability to generate intestinal organoids (iHO) has offered a potential solution, with a consistent and reproducible method of generating self-renewing and expanding models of the intestinal epithelium *in vitro* for relatively long periods of time.[336] Each iHO is a discrete system, consisting of an epithelial monolayer, arranged around a luminal cavity. The monolayer is composed of cells from secretory and absorptive lineages, and these cells are arranged in the manner that they would be in the intestine, with mature organoids demonstrating a 'budded' structure, meaning that they have folds which represent the crypts and villi of the *in vivo* intestine. At the base of these crypts are intestinal stem cells (ISCs) which can be detected by their expression of LGR5, and are responsible for the self-renewing nature of the iHO.[241] iHO are able to expand, since new iHO can develop from each crypt domain when the iHO structure is broken up (**Figure 1.13**).

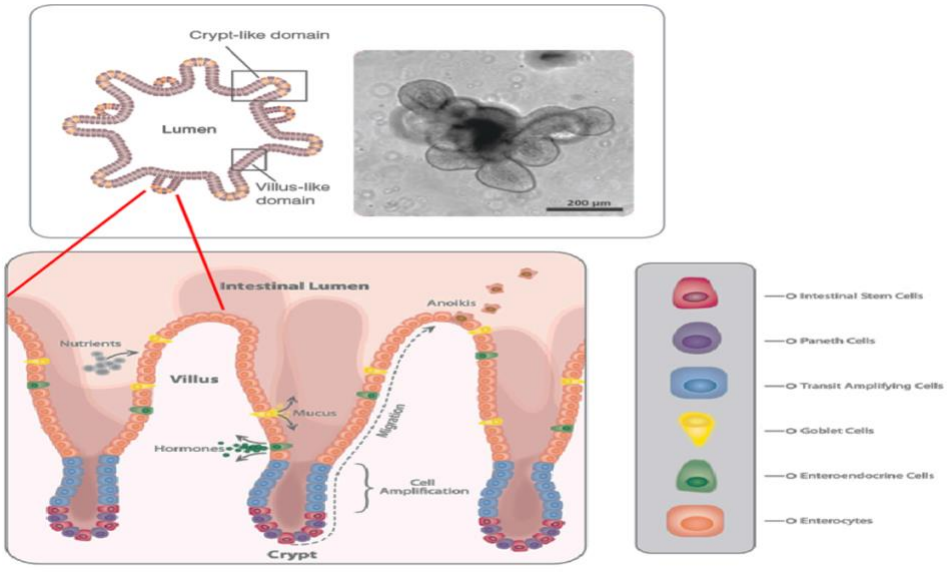

Figure 1.13: Architecture of the iHO and cell types within. iHO retain the crypt/villus structure seen in the intestinal epithelium *in vitro.* Within each crypt are contained: intestinal stem cells, Paneth cells, and after having undergone terminal differentiation, enterocytes, goblet cells and enteroendocrine cells migrate to the tips of villi and are exfoliated into the lumen. (Figures taken from: https://www.stemcell.com/intestinal-organoid-culture-lp.html, https://www.stemcell.com/technical-resources/area-of-interest/organoid-research/intestinal-research/overview.html)

Other cells found in the iHO crypts are Paneth cells, identifiable by their production of lysozyme. These Paneth cells are able to synthesise antimicrobial peptides and secrete them into the iHO lumen. Goblet cells are also present on the villi, detected by their expression of mucin 2. These cells secrete mucus into the lumen, which can be detected as a layer lining the organoid lumen via lectin staining. Enteroendocrine cells are also recapitulated in the iHO, recognisable by expression of chromogranin A and the secretory granules visible in their cytoplasm on TEM. Enterocytes make up the majority of the iHO monolayer, and are able to demonstrate polarisation of the iHO epithelium, with expression of villin on their apical brush border, projecting into the lumen. Having been produced in the crypts, terminally differentiated epithelial cells (with the exception of Paneth cells) migrate up the villous structures and slough off into the iHO lumen within 2-3 days.[336] This exfoliation and collection of cells in the lumen means that iHO have to be passaged via mechanical disruption every 5-7 days to prevent the lumen being filled with dead cells and subsequent death of the iHO. It is possible to passage iHO for long periods, but caution should be exercised with samples older than 6 months, as studies of reprogrammed hiPSC have showed some genetic deletions associated with tumour suppressor genes and duplications of oncogenes at late passage numbers (>12 months).[337]

The process of directed differentiation to produce iHO from hiPSC requires hiPSC to progress down the endodermal lineage to form definitive endoderm, which is then patterned into hindgut. These islands of hindgut are embedded into an extracellular matrix to provide a scaffold (such as Matrigel), that allows progression into iHO to occur.[244]

In order to produce definitive endoderm (DE), Nodal/TGFβ signalling is employed, in the form of Activin A, which is able to mimic the actions of Nodal.[244,338] Wnt signalling enhances endoderm production,[339] and PI3K, a signal transducer, inhibits it. PI3K inhibitors such as LY294002 are therefore used to maximise DE formation.[340] In addition, Wnt inhibitors such as GSK3β are suppressed by the use of CHIR99021.[341] DE can be recognised by elevated expression of the genes: *FOXA2, SOX17* and *CXCR4*. Once DE is produced, it is further differentiated by patterning into hindgut. Two different protocols exist, both leading to formation of islands of hindgut, recognised by expression of *CDX2.* One method uses Wnt3a + FGF[244] and another, (the method used in this study) CHIR99021 + retinoic acid (**Figure 1.14**).[338]

Islands of hindgut are embedded into a pro-intestinal culture system, such as Matrigel, which provides the structure of an extracellular membrane (ECM) to support iHO growth.[244] The presence of this ECM substitute is vital, as without attachment between the epithelium and basal membrane, isolated cells die due to a lack of integrin signalling. The islands of hindgut are overlaid with a growth medium containing supplements and growth factors which both support intestinal stem cell (ISC) development, and the differentiation and proliferation of the ISC into cells from the secretory and absorptive lineages.[245] Those growth factors include R-spondin 1, a Wnt agonist, required for ISC maintenance. Wnt production by Paneth cells following their differentiation is able to produce the budded structure of the iHO. Noggin; a BMP family antagonist and epidermal growth factor (EGF) are necessary in the culture medium to sustain cultures long term and promote iHO growth. Prostaglandin E2 activates the Wnt pathway, blocks anoikis and activates mitogenic signalling,[342] and ROCK inhibitor Y-27632 is added directly following passage to promote cell survival.[343]

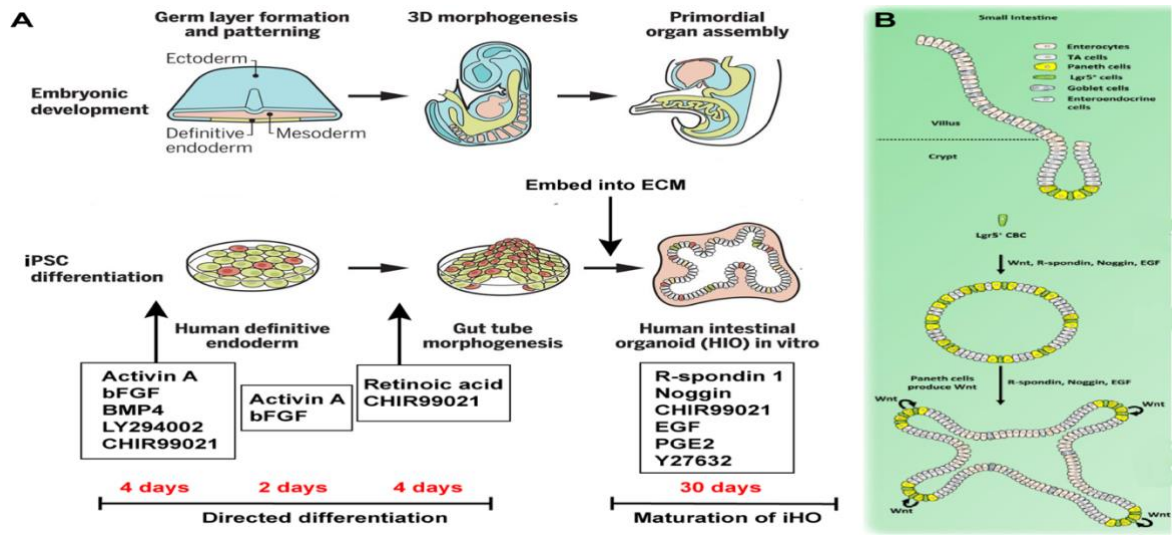

Figure 1.14: Differentiation of hiPSC to iHO. (**A**) Demonstrates growth factors required to drive differentiation from iPSC to definitive endoderm, hindgut and iHO formation/maturation after embedding into ECM (Matrigel). This mimics embryological development seen in the human foetus. (**B**) Demonstrates reformation, differentiation and budding of iHO following splitting, due to presence of LGR5+ ISC in crypts, and Wnt produced by Paneth cells alongside exogenous growth factors. (Figures adapted from: (**A**) Takebe & Wells, 2019[344], (**B**) Merker et al, 2016[345])

Initial investigations into transcriptomic profiles of hiPSC-derived iHO found them to have more similarities to foetal intestinal tissue than to adult intestinal tissue. Genes that are involved in development of the digestive tract were upregulated both in foetal intestinal tissue and iHO, compared to upregulation of genes related to Paneth cell action and digestive

function in adult tissue, and increased expression of *OLM4,* a marker of ISC maturity.[346] Interestingly, after transplantation into murine renal capsules, iHO were transcriptionally more mature and on microscopic examination had developed a more complex structure including the presence of a lamina propria, suggesting that *in vivo* biochemical or structural cues are required to complete maturation.

It has also been demonstrated that some of this maturation can occur *in vitro* for primary foetal iHO. A study comparing iHO derived from primary foetal, paediatric and adult intestinal tissues demonstrated stable epigenetic signatures specific to the gut region of derivation once iHO had been produced, which were retained over prolonged periods in culture. These signatures showed similarities with primary epithelium from the same gut region. Paediatric and adult organoids demonstrated little change in DNA methylation patterns over time, whereas foetal gut-derived organoids underwent dynamic DNA methylation and transcriptional changes, indicating that these cells were maturing *in vitro*.[347]

Other studies suggest that hiPSC-derived iHO are able to demonstrate features of mature tissue; with iHO most closely resembling mature colonic epithelium on transcriptional analysis, but displaying similarities both with mature small and large intestinal tissues, suggesting again that iHO had not reached full maturation and differentiation.[348] This is likely due to the mechanical requirements of the culture model to be dissociated on a regular basis, making continuous uninterrupted culture impossible. However, in spite of their apparent immaturity, hiPSC-derived iHO were able to support stable colonisation by a non-pathogenic strain of *E. coli,* and appeared to make maturational changes as a result of this symbiosis. Innate antimicrobial defence (including NFκB and TLR signalling, and cytokine production) and epithelial barrier function related genes were increased at 24 hours post-colonisation, and then went on to decrease by later time points. Gene sets related to tissue maturation, including those for organ morphogenesis, developmental maturation and regionalisation, differentiation of mesenchymal and muscle cells and nervous system were all upregulated following colonisation.[233]

1.6.4 Applications of organoid technology, including host-pathogen interactions

The use of organoids as models for different tissue types is a rapidly expanding field, with protocols having been developed to produce numerous different tissues from various cell

types with differing degrees of complexity and maturity. Current organs for which organoids have been produced and potential clinical applications are summarised in **Table 1.1**.

Tissue / organ:	**Cellular source:**	**Clinical applications:**
Optic cup / retina	Mouse PSC / Human PSC	Transplantation of retinal organoids for mouse / primate retinal degeneration
Cerebral structures (neocortex, olfactory bulb, hippocampus, hypothalamus, midbrain, choroid plexus, cerebellum)	Mouse PSC / Primate PSC / Human PSC	Model of microcephaly Model of Zika infection on forebrain organoids Drug screening for Zika using forebrain organoids
Stomach (gastric fundus, corpus, pyloric antrum), oesophagus	Mouse PSC / ASC / dissociated tissue / hiPSC / Human dissociated tissue	Gastric organoids from tumour cells to model human gastric cancer Model of Helicobacter pylori infection in gastric organoids to study pathogenesis
Small intestine	Mouse ASC / dissociated tissue Human PSC / Human dissociated tissue	Transplantation of mouse intestinal organoids onto damaged mouse colonic epithelium Modelling of congenital loss of enteroendocrine cells in humans CRISPR/Cas9 correction of *CFTR* in intestinal organoids from patients with cystic fibrosis Development of an *in vitro* readout to evaluate recovery of CFTR function
Colon	Mouse ASC / dissociated tissue / Human PSC / ASC / dissociated tissue	Transplantation of mouse colonic organoids onto damaged mouse colonic epithelium Human colon organoids from tumour cells to model colorectal cancer Use of human organoids with *PHOX2B* mutation to study colon development in Hirschsprung's disease
Liver	Mouse ASC / Human PSC / ASC	Transplantation of mouse liver organoids into mouse model of type I tyrosinaemia Use of patient-derived liver organoids to model α-1 antitrypsin deficiency and Alagille syndrome
Pancreas	Mouse ASC / dissociated tissue / Human dissociated tissue	Use of mouse pancreatic organoids from normal and neoplastic cells has highlighted genes involved in development of pancreatic ductal adenocarcinoma

Trachea / bronchi / alveoli	Mouse ASC / Human PSC / ASC	Use of patient derived bronchial organoids to trial cystic fibrosis drug screening
Thyroid	Mouse PSC	
Prostate	Mouse ASC / dissociated tissue / Human ASC	Genetically engineered murine prostate organoids have been used to model prostate cancer
Fallopian tube	Human ASC	
Kidney	Mouse PSC / Human PSC	Use of human kidney organoids to evaluate nephrotoxicity of compounds CRISPR/Cas9 modified human organoids to model polycystic kidney disease
Mammary gland	Mouse ASC / dissociated tissue / Human ASC	Production of human breast cancer organoid biobank
Salivary gland	Mouse ASC / Human ASC	Mouse organoids have been used to expand gland stem cells that restored salivary function in murine hyposalivation models
Embryonic organoids – pre and post implantation / gastruloids / neural tube	Mouse PSC / Human PSC	

Table 1.1: Tissues from which organoids have been derived and potential clinical applications

(Table adapted from Rossi et al, 2018 – individual studies for each cell type referenced in paper[349])

Key: *ASC = adult stem cell, PSC = pluripotent stem cell (hiPSC / primary tissue), CFTR = cystic fibrosis transmembrane conductance regulator*

It is clear there are many exciting possibilities for what can be done with organoid technology. Regardless of the tissue type generated, these applications can be split up into 5 main categories:[349]

1) Basic research – use of organoids to understand normal tissue development
2) Study of disease mechanisms – this could either be using cells from individuals with genetic diseases, tumour cells, or studying host/pathogen interactions in the tissue that is usually infected
3) Drug screening – use of organoid biobanks would allow large scale screening to identify drugs that are effective against particular disease phenotypes
4) Personalised medicine – organoids from patients could identify which drugs would have the most impact on their particular disease phenotype
5) Regenerative medicine – either using organoids derived from healthy donor cells, or from the patient themselves following correction of a genetic mutation could be transplanted into patients to alleviate disease phenotype

In addition, comparison of what we learn in human organoid models with what is known from animal models of disease may allow us to use organoids over animal models in future, if organoids can prove to be complex and realistic enough models of disease. This type of technology is especially valuable for human restricted diseases or pathogens, as disease can be studied in its natural tissue niche. One example of this would be the use of gastric organoids to study *Helicobacter pylori* infection, for which there is no appropriate animal model. Infection of hiPSC-derived gastric organoids recapitulated histological features of *in vivo* disease; particularly important given the need to understand how this pathogen is associated with development of gastric cancer.[350]

Intestinal organoids are proving a robust model to help us learn about host-pathogen interactions in the gut. Murine primary organoids were initially used for the study of *S*. Typhimurium, and were able to demonstrate bacterial invasion into cells, tight junction disruption, release of pro-inflammatory cytokines and activation of the NFκB pathway. It was also noted that markers of stem cells Lgr5 and Bmi1 were downregulated in infected organoids, which could be a protective mechanism given that *Salmonella* preferentially attacks mitotic cells due to their increased surface cholesterol.[351] Bacteria in this study were however delivered basally, as opposed to apically which would be a more realistic target *in vivo*. Studies have since progressed to the use of microinjection, in order to deliver pathogens directly into the organoid lumen. Studies on murine organoids demonstrated that α-defensins secreted by Paneth cells were able to restrict growth of *S*. Typhimurium in culture, and that *Mmp7*$^{-/-}$ mice, who lack matrix metalloproteinase 7, the enzyme which converts α-defensins into their active form were unable to restrict *S*. Typhimurium growth.[352] Moving into the human model, Forbester *et al* (2015) set up a microinjection infection model for *S*. Typhimurium in hiPSC-derived iHO,[353] which we use as the basis of our investigation into host-epithelial interactions in this project.

In addition to *H. pylori*, and *S*. Typhimurium, hiPSC-derived iHO have proven a useful culture system for other enteric pathogens, including *Cryptosporidium,* a protozoan which is an important cause of diarrhoeal disease and mortality in infants in developing countries. This pathogen is an obligate parasite, needing to complete its entire life cycle inside of its host, meaning that previous attempts to study this pathogen *in vitro* have been relatively

unsuccessful. Excitingly, *Cryptosporidium* were able to propagate and complete their life cycles inside of intestinal and lung organoids, creating opportunities to learn a lot more about the pathophysiology and direct potential drug development for this protozoan.[354]

Another important pathogen for which detailed study is now possible is human norovirus, which is the commonest cause of gastroenteritis worldwide. It was possible to cultivate norovirus in enterocytes in monolayers produced from primary organoids; in this case monolayers were used, given the requirement for bile to be delivered to the apical surface of the epithelial cells for growth of certain strains.[355] Robust studies of human rotavirus were also possible for the first time in human primary organoids; with much of what is previously known about rotavirus having come from use of animal strains in animal models. In this case iHO were disaggregated and rotavirus added to culture medium, before being allowed to re-seal; in this case both apical and basal exposure was occurring. Rotavirus infected enterocytes and enteroendocrine cells, inducing them to produce viroplasms and lipid droplets. Luminal swelling of iHO was also seen following infection, recapitulating the osmotic diarrhoea induced by rotavirus *in vivo*.[356]

In a more complicated model, embryonic stem cell-derived iHO were microinjected with Shiga toxin-producing *E. coli* O157:H7, which induced loss of actin and epithelial integrity. It was possible to show that iHO were demonstrating a defensive response to infection, with increased reactive oxygen species (ROS) production, and on microscopy, O157:H7 were seen growing as filaments, consistent with the bacterial SOS response induced by ROS. In addition, neutrophils were added to the culture medium following infection and were shown to be recruited into the iHO tissue or the lumen.[357]

Lastly, hiPSC-derived iHO were used to study the important nosocomial pathogen *Clostridium difficile*. The iHO epithelial barrier was disrupted following microinjection with a toxin-producing strain of *C. difficile.* This was not the case when a non-toxigenic derivative was used, therefore purified toxins (TcdA and TcdB) were injected into the iHO, demonstrating that TcdA was responsible for damage to the epithelium by the isolate used in this study.[358]

The demonstrated ability of intestinal organoid-derived infection systems to recapitulate *in vivo* features of enteric infection is very encouraging for the possibilities of learning more about direct host-epithelial interactions with these pathogens, and the potential applications of this knowledge to developing and screening treatments or vaccines. It is certainly the

reason for which we chose this model for our studies of the human-restricted pathogens *S*. Typhi and *S*. Paratyphi A which have not previously been closely studied in this fashion.

1.7 Summary

The past 10 years have been an incredibly exciting time for the study of host-pathogen interactions, with the key discoveries of the ability to reprogram somatic cells into hiPSCs, their forward differentiation into numerous tissue models and the ability to rapidly edit the genome of those from whom the tissue models can be made, all combining to allow host and pathogen-specific modelling of human disease in more detail than has ever been possible before. We have already garnered much information on the pathogenic and immunomodulatory qualities of *Salmonella* and other enteric pathogens in different tissues, but the use of iHO technology will allow direct study of the intestinal epithelial response to infection, particularly valuable for human-restricted or difficult to grow pathogens such as typhoidal strains of *Salmonella* and Cryptosporidium. This type of study is becoming increasingly important in the current climate of increasing dissemination of MDR *Salmonella* of multiple serovars, and may aid efforts to discover new vaccine targets or treatments to better prevent disease and control the spread of these pathogens.

1.8 Aims of the thesis

Use of the novel hiPSC-derived iHO system allows non-invasive modelling of the interactions between enteric pathogens and the gut epithelium. This project aims to use this model to investigate further the interactions between *Salmonellae* and the host; commencing by establishing mechanisms of restriction of *S*. Typhimurium invasion by IL-22 both intracellularly and in the iHO lumen. We exploit the ability of the hiPSC-derived iHO system to produce iHO from different genetic backgrounds, using iHO from cell lines with isogenic mutations to model *Salmonella* infections with genes of interest knocked out. This project also examines the possibilities of using the iHO model to study interactions with alternative pathogens and to assess competitiveness of epithelial invasion between different *Salmonella* serovars. Finally, we investigate the interactions of human-restricted pathogens, *S*. Typhi and *S*. Paratyphi A with both iHO and macrophages derived from the same hiPSC

line, learning about early interactions with the epithelium and immune system. This project uses a combination of techniques to investigate these questions, including: infection assays, confocal and electron microscopy imaging, cytokine analysis and transcriptomics, both at the single cell and bulk RNA-Seq levels.

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