7: Future directions

The work presented in this thesis generates some interesting observations on the interactions between enteric pathogens and the intestinal epithelium in the form of the hiPSC-derived iHO model. This work does however lead to additional questions and highlights aspects of this area which would benefit from further investigation or use of alternative techniques.

7.1 More detailed transcriptional profiling of the iHO model

Firstly, the data presented in Chapter 4 on the single cell sequencing of IL-22 stimulated iHO using Smartseq2 proved that sample size is key if one wishes to be able to make meaningful observations about the differences between single cell responses on the transcriptional level. Whilst Smartseq2 allows a great depth of sequencing, the work intensity and cost of the protocol meant that the size of any effect seen between IL-22 stimulated and unstimulated groups was masked by technical noise from the data. However, given the now widespread use of droplet-based single cell RNA-Seq,[1] a repeat experiment with a much larger sample size would likely facilitate the identification of any differences between the two groups, identify with confidence the different cell types contained within the epithelium and define their individual reactions to IL-22 treatment.

For example, a recent paper by Fujii *et al* (2018),[2] (authors of this paper produced the first report on production of organoids derived from murine intestinal crypts) suggested trial of a new combination of growth factors for primary iHO which may favour the emergence of secretory cells whilst maintaining the pluripotency of the intestinal stem cells; something which has been a difficult balance for those producing primary and hiPSC-derived iHO via standard methods. To demonstrate the utility of this protocol, the group reported the enhancement in secretory cell types via the use of droplet-based single cell RNAseq, examining >2500 cells per condition. They were able to discern four different subtypes of enteroendocrine cells, alongside goblet and Paneth cells. They were also able to denote tuft cells, M cells and transit amplifying cells, demonstrating the power of large data sets in this context. Similarly, researchers looking at mouse small intestine and organoids profiled

>50,000 single cells, allowing them to characterise novel subsets of cells which hadn't been defined at this resolution previously.[3]

In addition, single cell RNA-Seq was carried out on murine small intestinal cells after infection with *S.* Typhimurium SL1344 or the helminth *Heligmosomoides polygyrus.* It was possible to discern cell-intrinsic changes to the transcriptome displaying an inflammatory response to infection, and excitingly, altered cellular functions, such as induction of RegIIIα and RegIIIγ in all cell types, rather than just enterocytes during *Salmonella* infection. In addition, both organisms were able to change the cellular composition in the intestine, with *H. polygyrus* inducing an expansion of goblet and tuft cell populations, and *S*. Typhimurium causing increased abundance of mature Paneth cells (1.1% to 2.3%) and enterocytes (13.1% to 21.7%), but a marked decrease in transit-amplifying (52.9% to 18.3%) and intestinal stem cells (20.7% to 6.4%). Being able to study cell populations in this level of detail goes some way to resolving the question on the mechanism of increased proliferation of enterocytes when markers of ISC had been noted to be downregulated in murine organoids during infection.[4]

It would be attractive to complete these types of assay in human organoids to determine whether this picture is recapitulated in a model of human infection. In addition to using this model to establish the response of individual cell types to *Salmonella*, it would be fascinating to study infected cells in the context of IL-22 stimulation, both to learn more about the individual cellular response to stimulation and to seek further confirmation for our hypothesis of enhanced phagolysosomal fusion. This type of experiment would also provide more information on the antimicrobial peptides which are upregulated, and perhaps define these changes at various timepoints following infection.

In particular, detailed single cell response data from *S*. Typhi and *S*. Paratyphi A infected organoid-derived cells would be valuable for comparison with non-typhoidal *Salmonella* data, in order to define differences in cellular response to each pathogen and perhaps help guide the search for alternative treatment methods for drug-resistant infections. To undertake this type of work would require a FACS sorter in a CL3 facility, which is difficult to organise but will potentially be available to my group soon.

Another application of transcriptomic technology would be the use of dual RNA-Seq to assay both infected organoids and *Salmonella* to interrogate bacterial transcriptomic response during early interactions with the epithelium or macrophage. It would be fascinating to learn which pathogenicity factors are being upregulated in particular by the H58 *S*. Typhi used in this study which appear to have increased invasion and replication capacities within the macrophage and an undefined ability to subvert the immune response without reliance on AMR genes to make them treatment resistant. Further data could be gathered on the genes activated during events we witnessed on TEM images of infection, such as the novel finding of production of pili by *S*. Paratyphi A. This type of data could also help in the identification of targets for future vaccines. This would be especially valuable if commonalities between key clades of *Salmonella* were identified and efforts could be directed towards a multivalent vaccine.

It would be sensible to trial these types of assays at a range of different multiplicities of infection (MOIs), along with concurrent TEM imaging and RNA-Seq, in order to investigate whether MOI alters the behaviour or transcriptome of the bacteria and the factors it expresses when interacting with the epithelium. These findings could be relevant for clinical infections, as MOI will frequently differ here. Further data on genes employed by *Salmonella* to evade phagolysosomal fusion could be obtained by studies over different timepoints following infection. Selection of these timepoints could perhaps be guided by assays such as live confocal imaging during infection (requiring confocal facilities within a CL3 laboratory) with labelling of bacteria and relevant host proteins such as Rab7 or Lamp1. Use of the Perkin Elmer Opera Phenix™ system could provide this. In addition, the high throughput capabilities of such a system could allow study of aspects such as bacterial invasion and replication under stress conditions, such as the presence of a range of antibiotic concentrations in the cell media, to discover more about what is happening in individuals treated with inappropriate antibiotics.

7.2 Luminal studies

As described above, information on AMPs released into the lumen at timepoints following infection could be garnered from transcriptional data. In addition, proteomic data on the AMP concentrations within the organoid lumen and factors such as the luminal pH and

osmotic gradient would be fascinating, in order to build a picture of the environment that bacteria are exposed to during infection assays. This may prove tricky given the difficulties with accessing the luminal compartment of the organoids, but perhaps harvesting post-infection and extracting protein may be a method of achieving some measure of this, with trial of a more rapid dissolution of Matrigel with ice cold PBS rather than the 45 minutes usually required when using Cell Recovery Solution. This would however provide information on whole iHO protein expression rather than just luminal content. Another option would be development of a system whereby microinjection needles could be used to extract rather than inject contents into the lumen. One study reported directly aspirating iHO from their extracellular matrix (e.g. Matrigel) using a 30 gauge needle to disrupt the iHO and extract luminal bacterial contents, but this would again lead to the likelihood of some iHO material rather than just luminal contents being harvested, which would not be optimal.[5]

Another luminal issue requiring further investigation is the question of whether gentamicin is penetrating the mucus layer lining the iHO lumen and killing bacteria contained within, or whether less invasive bacteria such as *S*. Typhi are surviving in the mucus and being erroneously considered to be intracellular once harvested. Recent commercial release of fluorescently tagged antibiotics such as gentamicin could help to resolve this; using live imaging with fluorescent antibiotics and live/dead staining of bacteria could clarify whether antibiotics are co-localising with bacteria within the mucus and the outcome of these interactions.[6]

7.3 Alteration of iHO to closer resemble *in vivo* scenarios

Whilst one advantage of the iHO system is its reductionist nature, this is also a potential drawback to replicating conditions experienced by *Salmonella* in the intestinal lumen *in vivo*. Having shown that iHO can successfully be colonised by commensal *E. coli* [7] and more recently that murine intestinal organoids supported growth of human transplanted intestinal microbiota for up to 4 days,[5] the next step would be to trial microinjection of pathogens into iHO that harbour an established commensal community. This would more closely recapitulate the colonisation resistance conditions experienced by *Salmonella* in the

intestine, and would introduce infection into an epithelium primed for contact with microbes.

Another manner in which iHO cultures could be adapted to closer replicate innate immune response *in vivo* would be the growth of iHO on a monolayer (using the Transwell® support system for example), or perhaps released from Matrigel and grown freely in media for a brief period. In this way, bacteria could be added to the apical aspect of the epithelium, and macrophages to the basal aspect, in order to observe whether the iHO epithelial response to infection is enhanced by communication with and response to cytokines secreted by macrophages. Simultaneously, phagocytosis and microbial killing by macrophages may also take place.[8] Previous research has demonstrated that cell surface protein expression is altered when cells are co-cultured with macrophages in 2-D[9] and 3-D[10] cellular models; another factor which ought to be considered when translating findings from *in vitro* assays to *in vivo* infection.

Typhoid, paratyphoid disease and iNTS predominantly occur in populations experiencing additional factors which may alter their intestinal environment. Malnutrition is a risk factor for iNTS, and can effect structural changes on the gut epithelium such as decreased villus height and reduced proliferation of enterocytes.[11] In addition, there can be decreased bile secretion, meaning loss of another protective factor against infections with enteric pathogens.[12] Alongside *Salmonella* disease, prevalence of environmental enteropathy (EE) is also potentially increased in areas with limited access to clean water and sanitation facilities. EE can be a driver of malnutrition, and its clinical features include: malabsorption, growth restriction, increased intestinal permeability and impaired gut immune function.[13] The increased permeability of the gut epithelium seen in individuals with this condition can lead to increased translocation of bacteria into the lamina propria and local and systemic inflammation.[14] Importantly, EE has been linked to failure of oral vaccines against polio, cholera and rotavirus.[15] There is also some evidence that environmental insults can be inherited epigenetically,[16,17] suggesting that there would be value in producing either hiPSC-derived or primary organoids from individuals with EE, in order to recreate a gut architecture and environment, which may more closely resemble that seen in children experiencing *Salmonella* infection in endemic areas.

7.4 Neglected pathogens

Finally, whilst the burden of typhoid disease worldwide remains high and treatment becomes more complicated by the emergence of AMR, it is promising that interventions such as effective vaccines are starting to become more widely available to prevent cases of the disease. It has become clear during the course of this project that this is not the case for paratyphoid disease. Our understanding of this pathogen is very limited in comparison to what we have established about *S*. Typhi and NTS, largely due (until now) to the lack of an animal proxy or representative human disease model. Although it causes a similar clinical picture to typhoid disease, clearly *S*. Paratyphi A does not behave in the same way at an epithelial level, therefore efforts ought to be focused on learning more about this pathogen in order to advance attempts to create vaccines and contingencies for the likely continued increase in its prevalence worldwide if we do not intervene.

It should also be noted that I have suggested experiments specific to *Salmonella* in these ideas on future directions for iHO technology, but these techniques could equally be used with other human restricted or neglected pathogens in order to generate a cohesive understanding of disease and guide drug or vaccine development for these pathogens too. If *in vitro* disease modelling using organoids progresses as rapidly over the next 5 years as it has over the past 5, these ambitions are eminently achievable.

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