# 7 Final discussion

The aim of the work presented in this thesis was to produce a detailed description of the streptomycin mouse model of *S.* Typhimurium gastroenteritis, and investigate the potential of this model for the identification of host genes involved in infection susceptibility. Characterisation of the model involved classical phenotyping approaches, such as enumeration of bacterial counts and flow cytometric analysis of immune cell populations, in addition to 'omics'-based approaches to profile changes in the microbiota, and regulation of the caecal tissue transcriptome and proteome during *S.* Typhimurium infection.

## 7.1 New opportunities presented by high throughput technologies

The arrival of high throughput 'omics' technologies for biological research has provided new approaches to understanding large scale biological effects, such as the response of a host to infection, at the levels of molecules and pathways. High throughput analyses provide an unbiased approach to investigating a system; rather than using a pre-selected focus, the measurable repertoire of molecules present in the materials is interrogated. Signatures of known processes serve to validate the quality of the data, and novel processes for which a role in the system under investigation was not previously appreciated can be identified.

Whilst the advantages of high throughput research are plentiful there are also problems demanding attention. The challenge of extracting biological meaning from large volumes of data has been tackled by the development of analysis tools for identifying pathways and processes enriched in molecular profiles. However the coverage of pathway annotations, and accuracy with which models reflect true biology, limits the insight these tools generate. In addition, established tools for the integration of multiple data types are not widely available, yet integration will be central to the future understanding whole systems, and the multiple levels of regulation within.

An area of rapidly-paced progress, proteomics has advanced far beyond the identification of individual proteins to quantitative profiling of thousands of proteins present in complex mixtures. Although the sensitivity limit of MS precludes the quantification of low abundance proteins the captured abundant fraction can be used to validate pathways identified as regulated in more comprehensive transcriptomic profiles. In this work we selected the overlap of regulated genes at the transcript and protein level as 'functionally validated

regulated transcripts' and used these to identify pathways regulated in *S.* Typhimurium-infected caecal tissue at the stage where *Salmonella* numbers are high and inflammation extensive. An alternative approach might use changes in the transcriptome for identification of pathways which are regulated and accept that a set degree of support at the protein level (for example a minimum of two proteins within the pathway regulated in the direction supported by the transcriptome) confirms the regulation of these pathways; thereby reducing the bias toward greater significance for pathways which contain more abundant proteins.

Unfortunately work to profile changes in the metabolome of caecal tissue and serum in response to *S.* Typhimurium infection was not completed soon enough for inclusion in this report. Metabolomics data will provide a further level of information on the effects of *S.* Typhimurium infection on the host, complementary to the transcriptome and proteome. A study of the host metabolic response to *Salmonella* infection in the murine typhoid fever model reported infection had 'a profound impact' on host metabolism [228]. Suggesting that dramatic changes in metabolism are also a feature of gastrointestinal infection, a large proportion of transcripts and proteins which were downregulated in infection of the caecum in our work are annotated to metabolic pathways. The metabolomics data will allow us to observe how changes in the regulation of metabolic genes are realised in the metabolic state of the caecum during *S.* Typhimurium-induced inflammation. This data is currently being collected ready for analysis through collaboration.

The transcriptomic and proteomic analyses reported in this thesis were performed upon whole caecal tissue, the samples analysed consisting of a large number of different sub-regions and cell types. By averaging all signals across the whole tissue information is lost; abundance changes in rare cell types are diluted to insignificance and where a gene is up- or down-regulated in different cell types these effects are combined in one overall fold change. Applying a multi-'omic' approach on a more tissue or cell-focussed level would allow us to dissect the overall changes reported here in order to uncover their contributions. Approaches might include dissociation of the epithelium from the mouse caecum, or potentially infection of mouse intestinal organoids; three-dimensional crypt structures which contain a well-defined collection of cell types [362].

## 7.2 A role for complement in gastrointestinal *S.* Typhimurium infection

In Chapter 5 we outlined results which implicate the complement protein cascade in the host response to mucosal *S.* Typhimurium infection. These results suggest that during *S.* Typhimurium infection complement production and activation might occur locally in intestinal tissue. However further work is needed to dissect the contributions of hepatic and locally-produced complement in the caecum. Previous studies have reported that complement activity is important for protection against *C. rodentium* though these reports made no attempt to describe the source of protective complement proteins [309, 310]. It would therefore be interesting to compare both the extent of regulation of complement proteins and their sources in infections with luminal-resident bacterial pathogens such as *C. rodentium*, and invasive intestinal bacteria including *Salmonella.*

Future experiments should also characterise the roles of complement in the intestinal mucosa during *S.* Typhimurium infection. Mice deficient in the central complement protein C3 could be used to investigate both the sources of C3 and functional importance of complement activation. Comparison of the relative levels of C3 in the blood and the intestinal mucosa of wild type and $C3^{-/-}$ mice with systemic C3 replacement by serum transfer during *S.* Typhimurium infection will provide insight into the source of C3 in the mucosa. The functional role of systemic and locally-derived complement could be examined by testing the susceptibility of $C3^{-/-}$ mice to infection in the streptomycin mouse model, and determining whether transfer of serum from wild type mice to $C3^{-/-}$ mice has an effect on infection outcome. Oral delivery of a targeted inhibitor of complement activation has been shown to ameliorate intestinal injury during DSS-induced colitis, presenting an interesting potential approach to investigate the effects of intestinal complement activation in our model [363].

The processes of local complement activation in the intestinal mucosa and the infiltration of products from systemic complement activation may have overlapping roles and outcomes in infection. It would be interesting to determine the specific effector pathways of the complement cascade to which these processes contribute. Previous work has suggested that MAC-mediated killing of *Salmonella* is of minor functional relevance compared with opsonisation of *Salmonella* by complement activation fragments in mouse serum [287]. It would therefore be of interest to investigate whether this is also true of complement-mediated effects on *Salmonella* within the intestinal mucosa.

In this work we detected a dramatic increase in the complement activation product C3d in the plasma of mice at day 4 PI with *S.* Typhimurium in the streptomycin mouse model. At this time point numbers of systemic *Salmonella* are considerable, and it is therefore possible that much of the C3d detected was generated outside of the gut. However, it would be interesting to determine whether complement activation fragments can be detected in the blood during self-limiting gastrointestinal infection with *S.* Typhimurium in humans, and whether these fragments might potentially serve to differentiate gastroenteritis caused by *Salmonella* from diarrheal disease resulting from other intestinal pathogens. We are planning to perform such experiments through collaboration.

## 7.3 Insight into genes involved in the host defence to infection from the streptomycin mouse model

The work reported in Chapter 6 demonstrates that characterisation of responses of mutant mice to infection in the streptomycin mouse model can provide valuable insight into the involvement of host genes in defence against *Salmonella*. For example in the case of *BC017643* our data suggested that defects in this gene affect host susceptibility in both the mucosal and systemic stages of *Salmonella* infection. *IL22ra1* mutant mice displayed a contrasting susceptibility profile with our data suggesting a reduction in the cytokine receptor component this gene encodes has little influence on mucosal infection, yet a protective effect upon systemic infection. The outcome of infection in *BC017643* mutant mice is consistent with the recently discovered role of the encoded protein in the generation of phagocyte reactive oxygen species, and the major role of phagocytes, in particular neutrophils recruited to the intestine, in *Salmonella* killing. The observations in *IL22ra1* mutants however call for deeper investigation of the mechanisms involved.

Amongst the findings described in Chapter 6 the apparent detection of ‘leaky’ gene expression from the targeted gene in two tm1a mutant mouse lines tested suggests caution may be required in interpretation of results from experiments using tm1a mutants. Regrettably we failed to determine the difference between mutant and wild type mice at the level of the target protein, the functional molecule. Ideally immunoprecipitation followed by Western blotting would be used in order to determine whether mutant mice produce the protein of interest, and if applicable whether the amino acid sequence of the target protein is identical in mutant and wild type mice.

Though likely resulting in an underestimation of the number of 'hits', incomplete knock down of target gene expression is not hugely problematic for primary phenotyping, where the aim is simply to establish a link between a gene and a particular process. However variable levels of target gene expression in hypomorphs prevent solid conclusions on the involvement of the target gene in particular pathways being drawn, representing a major barrier to secondary phenotyping efforts. For genes of particular interest, or where a phenotype was observed in the primary pipeline, tm1b lines should automatically be generated to facilitate secondary phenotyping.

RNAseq analysis of *Salmonella*-infected caecal tissue from *IL22ra1* and *IL10rb* mutant mice identified collections of genes whose expression was affected, either directly or indirectly, in response to reduced expression of just these single target genes. The sizeable overlap in genes with altered expression in these two mutants is in line with the known relationship of IL22ra1 and IL10rb in IL22 cytokine signalling. The observation of a T cell signature in genes more highly expressed in *IL10rb* mutants relative to wild type controls is broadly consistent with the immunomodulatory effects of IL10, and further study of T cell activation in the caecum during *Salmonella* infection and the effects of *IL10rb* and *IL10* mutations would be interesting.

Whilst the value of the streptomycin model in uncovering aspects of the host response to infection is evident, there are a number of features of the model which require more extensive consideration and testing in order to maximise the information which might be gained in these experiments, as follows.

In the murine typhoid model differences in *Salmonella* counts recovered from the liver and spleen of wild type and mutant animals are thought to be determined predominantly by the involvement of the gene of interest in systemic host defences. However in the streptomycin model it seems likely that systemic *Salmonella* counts also depend upon the control of bacteria in intestinal tissue, especially the passage of *Salmonella* from the intestinal mucosa both into the circulation and lymphatic system. Therefore whilst differences in intestinal counts between wild type mice and a mutant line inarguably indicate a defect in intestinal defence mechanisms, differences in counts in the liver and spleen need further investigation. The complementary murine typhoid model might be used to investigate these mechanisms. In the case of *IL22ra1*mutant mice no difference in liver and spleen *Salmonella* CFU following IP delivery of *S.* Typhimurium in the MGP phenotyping pipeline was detected

in comparison with wild type controls. Though the number of mice tested in the MGP pipeline was small this result might indicate that the systemic protection observed in the streptomycin model has a founding at the level of intestinal control.

Of the three mutant lines examined in this work none displayed a strong phenotype with respect to intestinal bacterial counts; in *BC017643* mutant mice a difference in intestinal counts relative to wild type mice was observed, but compared with the liver and spleen the effect here was much smaller. The failure to detect a strong phenotype in intestinal counts in this work despite reproducible phenotypes in *Salmonella* numbers in the liver and spleen is likely due to the small number of lines tested. Nonetheless it would be valuable to confirm that the challenge as it was carried out in this work finds a strong difference in intestinal counts in a mutant mouse with a known phenotype in susceptibility to intestinal colonisation. As alluded to above, and potentially supported by the findings in *IL22ra1* mutant mice, the possibility exists that despite the absence of *Salmonella* colonisation of liver and spleen in the majority of human NTS infections, the extent of colonisation of these sites may act as better indicators of defects in the intestinal immune response to *Salmonella* in the streptomycin mouse model. The large range in intestinal *Salmonella* CFU reported here may have been a barrier to the detection of a phenotype in intestinal colonisation. Further experimentation should be carried out to determine whether this range truly reflects naturally occurring variability, or whether variability might be a consequence of faecal contamination or methodological problems such as insufficient homogenisation of intestinal tissue.

The infection time point selected as the major focus of analysis in this work was day four, at which stage intestinal tissues are extensively colonised by *Salmonella*, intestinal inflammation is severe, and considerable systemic spread of *Salmonella* has occurred. Many published studies which investigated effects of host and bacterial genotype on the outcomes of infection in the streptomycin mouse model focused their attention or at least included analysis of earlier time points [130, 152]. Whilst the results described in this thesis demonstrate it is possible to detect phenotypic differences in infections with both bacterial and host mutants (Chapters 3 and 6 respectively) at day 4 PI, it is possible that differences may be more significant at earlier time points. In addition the use of earlier time points where systemic infection is limited might be useful in distinguishing intestinal and systemic processes, as discussed in the case of complement activation. A more detailed characterisation of earlier time points is warranted to investigate variability in infection parameters throughout the

course of infection, and how the interval between infection and experimental characterisation impacts upon effects of host and bacterial genotype.